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MOLECULAR SUBTYPES AND IMMUNOHISTOCHEMICAL PROFILES IN BREAST CANCER

For obtaining the degree of a Doctor of Medicine

Speciality – Pathology

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ANNOTATION

The doctoral thesis “Molecular subtypes and immunohistochemical profiles in breast cancer” is devoted to morphological and immunohistochemical research on breast cancer. Breast cancer is one of the most common malignant tumours in the European population and the most frequent malignancy in female. In Latvia, the incidence of breast cancer remains significant. Breast cancer is a heterogeneous disease including several entities with different clinical behaviour. The classics of breast cancer characteristics are represented in the classification of breast tumours by the World Health Organization. However, even tumours belonging to the same histologic type can have different clinical course. Additional information can be obtained from molecular subtyping of breast cancer thus disclosing subgroups with different biological properties and response to treatment. The molecular subtypes initially were discovered by gene expression profiling in high throughput microarray technologies. At present, immunohistochemistry is accepted as adequate surrogate marker. In the present work, 5 molecular subtypes of breast cancer (luminal A, luminal B (HER2 positive), luminal B (HER2 negative), HER2 positive and triple negative) are detected according to novel St. Gallen (2011) classification and characterised in detail. Further, new potential prognostic factors are analysed, targeting proteins that are involved in the cardinal tumour features as cell proliferation and cell cycle control (cyclin D1), evasion of apoptosis (BCL2 oncoprotein), expression of oncoproteins due to mutations in proto-oncogenes (p53) and angiogenesis (cyclooxygenase-2). The theoretical basis of the doctoral thesis employs 247 literature sources. The aim of the doctoral thesis was to classify breast cancer by molecular subtypes and to evaluate the above listed novel prognostic factors by immunohistochemistry. Within the research work, 383 patients with primary invasive breast cancer were enrolled. The tumour tissues were evaluated by morphological, immunohistochemical visualisation and *in situ* hybridisation technologies. In the result, 4 immunohistochemical technologies for the detection of p53, BCL2 protein, cyclooxygenase-2 and cyclin D1 have been developed, 5 molecular subtypes of breast cancer are characterised in detail and the molecular portraits of p53, BCL2, cyclooxygenase-2 and cyclin D1-positive breast cancers are obtained. The complex evaluation of the prognostic value of several factors, revealed that the local spread (pT) of breast cancer, regional lymph nodes status (pN) cancer grade and molecular subtype as well as expression of p53 and BCL2 influences the survival.

ANOTĀCIJA

Promocijas darbs "Krūts vēža molekulāro apakštipu un imūnhistoķīmiskā profila raksturojums" veltīts krūts vēža morfoloģiskajai un imūnhistoķīmiskajai izpētei. Krūts vēzis ir viens no biežākajiem ļaundabīgajiem audzējiem Rietumu valstu populācijās un visbiežākais ļaundabīgais audzējs sievietēm. Tā izplatība Latvijā saglabājas augsta. Krūts vēzim raksturīgās heterogenitātes dēļ Pasaules Veselības organizācijas apstiprinātā morfoloģiskā klasifikācija nespēj atklāt visus audzēja parametrus, kas raksturo audzēja bioloģisko potenciālu un ļauj izvēlēties personalizētu terapiju, tādēļ krūts audzēju raksturojumam izmantojama molekulārā klasifikācija. Molekulārie apakštipi sākotnēji tika noteikti, analizējot gēnu ekspresiju ar mikrokartēšanas tehnoloģiju. Šobrīd imūnhistoķīmija tiek pieņemta kā atbilstošākā aizvietojošā tehnoloģija. Promocijas darbā detalizēti raksturoti krūts vēža 5 molekulārie subtipi (luminmāls A, luminmāls B (HER2 negatīvs), luminmāls B (HER2 pozitīvs), HER2 pozitīvs un trīskārši negatīvs) atbilstoši St. Gallēnas klasifikācijai (2011), kas ir novatoriska pieeja arī pasaules praksē, kā arī veikti pētījumi jaunu prognostisku faktoru atklāšanai, analizējot molekulas, kas nosaka audzēja galvenās bioloģiskās īpašības – iesaistās šūnu proliferācijā un šūnu cikla kontrolē (ciklīns D1), ļauj audzēja šūnām izvairīties no apoptozes (BCL2 onkoproteīns), saistīti ar mutācijām proto-onkogēnos (p53) un angioģenēzi (ciklooksigenāze-2). Pētījuma teorētiskās bāzes izstrādei izmantoti 247 literatūras avoti. Promocijas darbā izvirzīts mērķis klasificēt krūts vēža gadījumus pēc molekulāriem apakštipiem un izvērtēt minētos jaunos potenciālos prognostiskos faktorus krūts vēža audos. Izmantojot morfoloģiskas, imūnhistoķīmiskās vizualizācijas un *in situ* hibridizācijas tehnoloģijas, izpētīti 383 secīgi invazīva krūts vēža gadījumi. Pētījuma rezultātā izveidotas 4 imūnhistoķīmiskās vizualizācijas tehnoloģijas p53 proteīna, BCL2 proteīna, ciklooksigenāzes-2 un ciklīna D1 noteikšanai, detalizēti raksturoti krūts vēža 5 molekulārie apakštipi, iegūts p53, BCL2, ciklooksigenāzes-2 un ciklīnu D1 ekspresējošu krūts vēžu molekulārais portrets un noteikts šo faktoru sastopamības biežums. Kompleksi izvērtējot daudzu parametru prognostisko nozīmi, kā dzīvildzi ietekmējoši faktori identificēti krūts vēža lokālā izplatība (pT) un reģionālo limfmezglu metastātisks bojājums (pN), audzēja diferenciācijas pakāpe, molekulārais subtips, kā arī p53 un BCL2 ekspresija.

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LIST OF ABBREVIATIONS

ABCSG – Austrian Breast and Colorectal Cancer Study Group

ASCO – American Society of Clinical Oncology

BCL2 – BCL2 oncoprotein

CAP – College of American Pathologists

CD – Cluster of differentiation

CDK – Cyclin dependent kinase

CI – Confidence interval

CISH – Chromogenic *in situ* hybridization

CK – Cytokeratin

COX – Cyclooxygenase

DCIS – Ductal carcinoma *in situ*

DFS – Disease free survival

DNA – Deoxyribonucleic acid

EGFR - Epidermal growth factor receptor

ER – Oestrogen receptor alpha

FISH – Fluorescent *in situ* hybridization

G - Grade

HE – Haematoxylin – eosin

HER – Human epidermal growth factor receptor

HR – Hazard ratio

IDC - Invasive ductal carcinoma

IHC – Immunohistochemistry

IP – Immunoperoxidase

kDa – Kilodalton

LCIS – Lobular carcinoma *in situ*

MDM2 - Murine double minute 2

NPI - Nottingham Prognostic Index

OM – Original magnification

OS – Overall survival

PCR – Polymerase chain reaction

PG – Prostaglandin

PR – Progesterone receptor

RFS – Relapse – free survival

RNA – Ribonucleic acid

RR – Relative risk

SD – Standard deviation

TNM – Classification of malignant tumours: T - size of the tumour, N – involvement of regional lymph nodes, M - distant metastasis

WHO – World Health Organization

INTRODUCTION

Breast cancer is one of the most common malignant tumours in the European population and the most frequent malignancy in female [Bombonati and Sgroi, 2011]. As the treatment of breast cancer is complex, wider understanding of breast cancer biology is necessary.

Breast cancer is a heterogeneous disease including several entities with different clinical behaviour. The classic of breast cancer characteristics is represented in the classification of breast tumours by the World Health Organization [Tavassoli and Devilee, 2003]. Even tumours belonging to the same histologic type can have different clinical course. Naturally, the largest group – ductal cancer – shows the highest heterogeneity. Additional information can be obtained from molecular subtyping of breast cancer. This approach is based on expression patterns of so called intrinsic genes showing higher variation of expression between tumours than within one tumour [Perou *et al*., 2000; Strehl *et al*., 2011]. The molecular subtyping discloses subgroups with different biological properties and response to treatment. The genes in breast cancer became up-regulated or down-regulated in larger groups, as will be described further for each molecular subtype. The molecular subtypes initially were discovered by gene expression profiling in high throughput microarray technologies [Perou *et al*., 2000]. At present, immunohistochemistry (IHC) is accepted as adequate surrogate marker [Nielsen *et al*., 2004; Carey *et al*., 2006] benefitting from higher economic effect and simpler technology despite less robust data in predictive sense [Sørlie, 2004].

The best-known molecular subtypes of breast cancer include luminal, human epidermal growth factor receptor (HER) 2 positive and triple negative tumours [Guarneri and Conte, 2009]. The division of luminal subtype into luminal A and luminal B is also well-accepted. The basal-like breast cancer is matter of active discussions as it overlaps with triple negative subtype but is not synonymous with it.

The luminal molecular subtype is characterised by oestrogen (ER) and progesterone (PR) receptor positivity [Strehl *et al*., 2011]. The prognostically worse luminal B subtype can be recognised by co-expression of HER2 in addition to ER and PR in contrast to HER2 negative luminal A subtype, or by higher proliferative activity [Cheang *et al*., 2009; Nielsen *et al*., 2010; Goldhirsch *et al.*, 2011; Strehl *et al*., 2011]. HER2 positive breast cancer lacks expression of ER and PR, but is defined by HER2

protein overexpression by IHC and/or *HER2/neu* gene amplification by *in situ* hybridisation [Strehl *et al*., 2011]. Breast cancer negative for ER, PR and HER2 protein expression is called triple negative. It partially overlaps with basal-like subtype showing expression of basal cytokeratins that normally are present in the basal cell of mammary ducts. High proliferative activity is typical in triple negative breast cancer.

The hot topics in breast cancer research include the epigenetic research [Huang *et al*., 2011], investigation of microenvironment and breast adipocytes [Place *et al*., 2011; Tan *et al*., 2011] and studies of additional immunohistochemical factors. Novel molecular factors that might play role in breast cancer development, reveal prognosis and potentially become target for treatment, include cyclooxygenase-2 [Kang *et al*., 2011], interleukins [Iliopoulos *et al*., 2011], p53 [Malhotra *et al*., 2010], p27 [Wander *et al*., 2011], cyclin D1 [Li *et al.*, 2011], cytokeratin 5/6 [Li *et al.*, 2011] and apoptosis-related factors including BCL2 oncoprotein [Zaha and Lazar, 2012]. Among the potential prognostic factors, the most promising targets represent proteins that are involved in the cardinal tumour features as cell proliferation and cell cycle control (cyclin D1), evasion of apoptosis (BCL2 oncoprotein), expression of oncoproteins due to mutations in proto-oncogenes (p53) and angiogenesis (cyclooxygenase-2).

Research aim: To classify breast cancer by molecular subtypes and evaluate novel prognostic factors by immunohistochemistry.

Research objectives:

1. Applying total test approach, to develop immunohistochemical visualisation technologies for detection of BCL2 oncoprotein, p53, cyclin D1 and cyclooxygenase-2 protein expression.
2. By the acquired technology, to determine immunohistochemical expression of BCL2 oncoprotein, p53, cyclin D1, cyclooxygenase-2 protein and cytokeratin 5/6 in breast cancer tissues.
3. To classify breast cancer cases by molecular subtypes (luminal A, luminal B (HER2 positive), luminal B (HER2 negative), HER2 positive, triple negative).
4. To analyze the associations between the novel immunohistochemical markers, molecular subtype and known prognostic factors (pT, pN and grade) as well as survival.

5. To establish the immunohistochemical markers that can be recommended as an adjunct to routinely detected markers.

Scientific assumptions or working hypotheses:

Proteins that are involved in the cardinal tumour features as cell proliferation and cell cycle control (cyclin D1), evasion of apoptosis (BCL2 oncoprotein), angiogenesis (cyclooxygenase-2) and expression of oncoproteins due to mutations in proto-oncogenes (p53) can have pathogenetic significance as reflected by association with certain morphological and molecular features. Molecular classification, as well as research-measurable immunohistochemical characteristics of breast cancer may have prognostic value. In addition, the findings can provide insight into breast cancer heterogeneity.

Scientific and practical diagnostic novelty

Within the frames of the present scientific work, five molecular markers with equivocal published diagnostic and prognostic value are evaluated in a large and well-characterised group of primary breast cancers. The findings will add evidence-based knowledge to the published research data. Regionally, the study represents the largest and widest morphological study of breast cancer. Regarding the recognised geographic differences in the breast cancer incidence and morphology, the data present novel findings.

The present work has facilitated the practical implementation of the molecular classification of breast cancer into the regular diagnostic practice. The molecular classification has been carried out in accordance with St. Gallen guidelines (2011) that represent novel approach even in world medical practice.

Personal contribution

The author has performed all stages of the study, including the study design and selection of the markers, the scientific measurements and statistical analysis. The author performed immunohistochemical visualisation and is the author of the demonstrated gross and microscopic photographs.

Ethical concerns

The study was approved by the Committee of Ethics, Riga Stradiņš University.

1. LITERATURE REVIEW

Breast cancer is the most frequent malignant tumour in female [Jemal *et al*., 2011]. The morbidity and mortality continue to increase, despite remarkable progression of early diagnosis and adjuvant therapy. In 2011, there were 1235 new cases of invasive breast cancer in women of Latvia, and mortality was 39.7 per 100 000 females [*spkc.gov.lv*, accessed 15.08.2012.]. Figure 1.1. shows the growing number of new cases of breast cancer in Latvia per year; the other most frequent malignant tumours are shown for comparison as well.

Similarly, breast cancer is among the most frequent malignant tumours in many developed countries. Breast cancer is one of the most common malignant tumours in the European population and the most frequent malignancy in female [Bombonati and Sgroi, 2011]. In USA, over 200 000 new cases of invasive breast cancer were reported in 2011 and approximately 40 000 women died of the disease during that time period [de Santis *et al*., 2011].

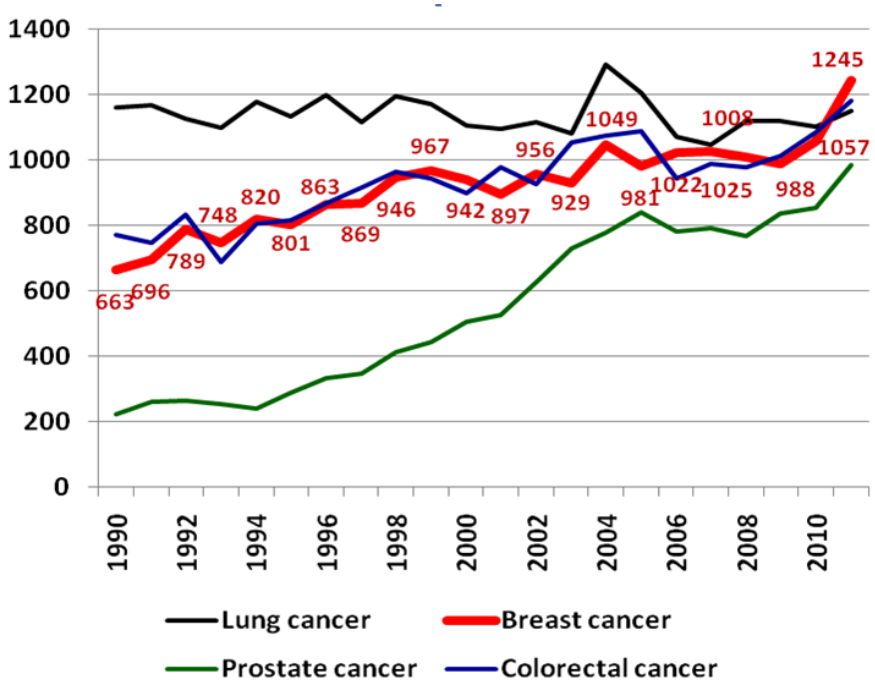


Figure 1.1. The number of new cases / per year of the most frequent malignant tumours in Latvia.

Previously, pathologic diagnosis was the "gold standard" in determination of the microscopic subtype and grading. Later it was found that breast tumours with similar

histopathological appearance (Figure 1.2.) can exhibit different clinical presentations, disease aggressiveness, response to treatment and outcome.

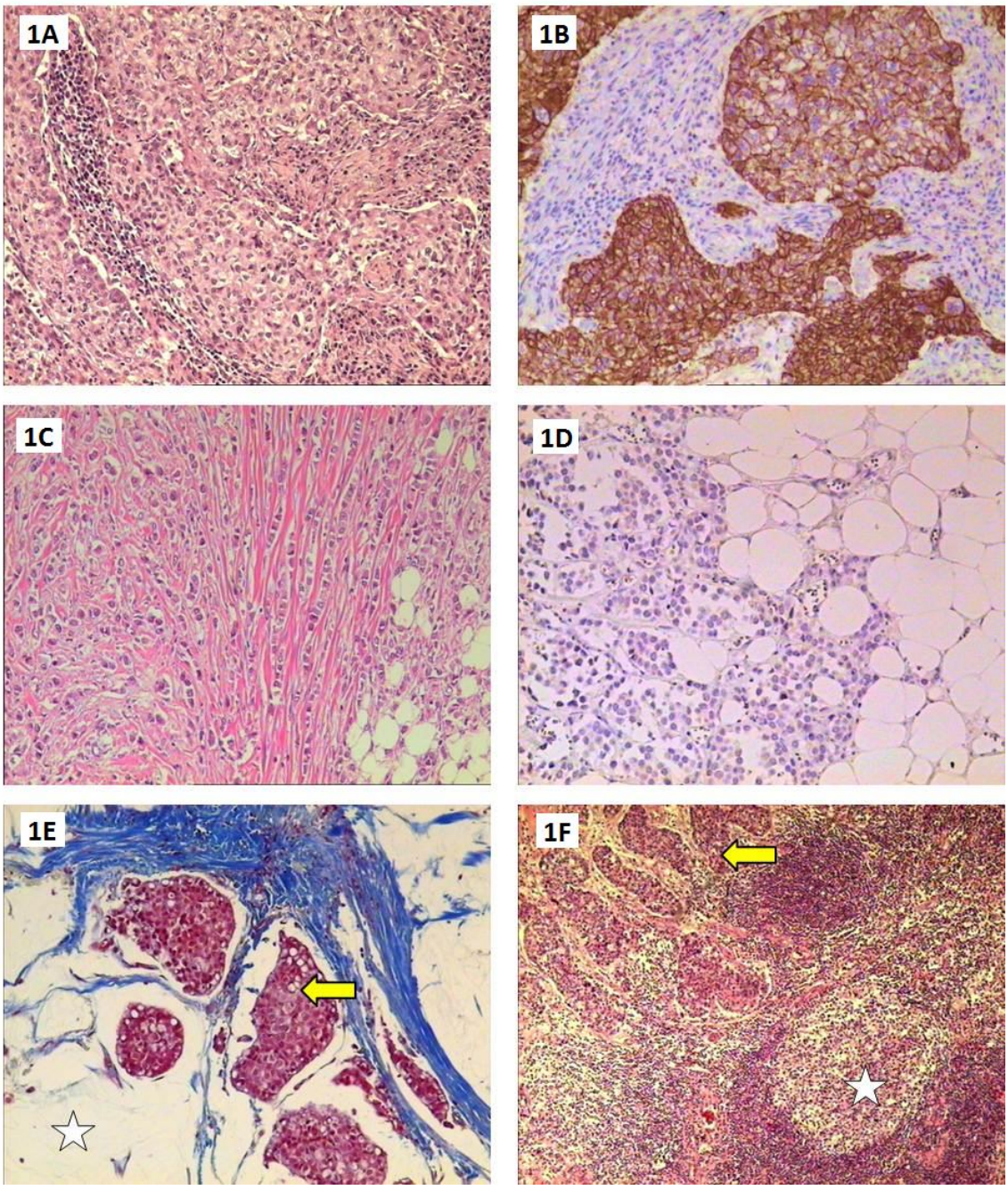


Figure 1.2. Histological types of breast cancer. A, High-grade ductal cancer. Haematoxylin-eosin (HE), original magnification (OM) 100x. B, Membranous expression of E-cadherin in ductal cancer confirming the histogenesis even in high-grade case. Immunoperoxidase (IP), anti-E-cadherin, OM 100x. C, Lobular carcinoma. HE, OM 100x. D, Lack of E-cadherin in lobular carcinoma. IP, anti-E-cadherin, OM 100x. E, Mucinous cancer. Note the abundance of mucus (star) and lower amount of neoplastic cells (arrow). Masson trichrome, OM 100x. F, Medullary cancer. Note the presence of lymphoid follicle (star) as well as neoplastic growth (arrow). HE, OM 50x. Microphotographs by A.Abolins.

Nowadays the routine morphological diagnostics of breast cancer involves immunohistochemical investigation to classify the cases into homogeneous groups by objective evidence. The loss of actin-positive myoepithelial cell layer is useful to distinguish invasive breast cancer from benign proliferations [Walker *et al*., 2012; Lee, 2013]. Expression of E-cadherin is present in ductal cancer and can be employed in the differential diagnostics with lobular breast cancer that is mostly negative for E-cadherin [Abdollahi *et al*., 2011; Walker *et al*., 2012; Arps *et al*., 2013; Rakha *et al*., 2013].

The differences in morphology had insufficient prognostic and predictive power for the current classification of breast cancer. Systematic investigation of gene expression patterns and their correlation with specific features of phenotypic diversity changed the way of classifying breast carcinoma at the molecular level. Analysis of gene expression profiling and immunophenotypic characteristics suggests that breast cancer is not a single entity but a heterogeneous disease, composed of a growing number of recognized biological subtypes. "Molecular portraits" of human breast tumours were recently developed through hierarchical clustering of genes on the basis of similarity in the expression pattern. Breast cancers were categorized into at least four main groups which differ markedly by incidence within distinct races/ ethnicities, distribution of risk factors, prognosis, therapeutic treatment responsiveness, clinical outcomes and both overall survival (OS) and relapse-free survival (RFS) as decribed by Spitale *et al.*, 2009. Some authors have classified the breast cancer in five groups [Voduc *et al.*, 2010; Irigoyen *et al.*, 2011]. The main breast cancer subtypes include luminal cell-like tumours, subdivided into luminal A and B (both expressing ER and showing similar molecular profiles to those of normal luminal cells of breast glands), basal cell-like or triple negative phenotype cancers (ER, PR and HER2 negative tumours with genes usually expressed by basal/ myoepithelial cells) and HER2 positive tumours (amplification of the *HER2/neu* gene). The fifth group was unclassified/ normal breast-like, described in several articles [Carey *et al*., 2007; Millikan *et al*., 2008; Raica *et al*., 2009].

Breast cancer subtypes can be defined by genetic array testing [Perou *et al*., 2000; Parker *et al*., 2009] or approximations to this classification using immunohistochemistry as shown in Figure 1.3 [Nielsen *et al*., 2004; Cheang *et al*., 2009; Blows *et al*., 2010]. These subtypes have different epidemiological risk factors [Millikan *et al*., 2008; Phipps, Chlebowski *et al*., 2011], different natural histories [Liedtke *et al*., 2008; Phipps, Buist *et al*., 2011], and different responses to systemic and

local therapies [Nguyen *et al*., 2008]. These differences imply that clinicians managing breast cancer should consider cases within the distinct subtypes in order to arrive at appropriate therapeutic conclusion. In general, the recommendations are intended to guide therapy considerations outside clinical trials in communities with reasonable levels of available resources. Remarkably, in appropriate situations it is wise to note the availability of alternatives, which might be only marginally less effective but less expensive [Goldhirsch *et al*., 2011].

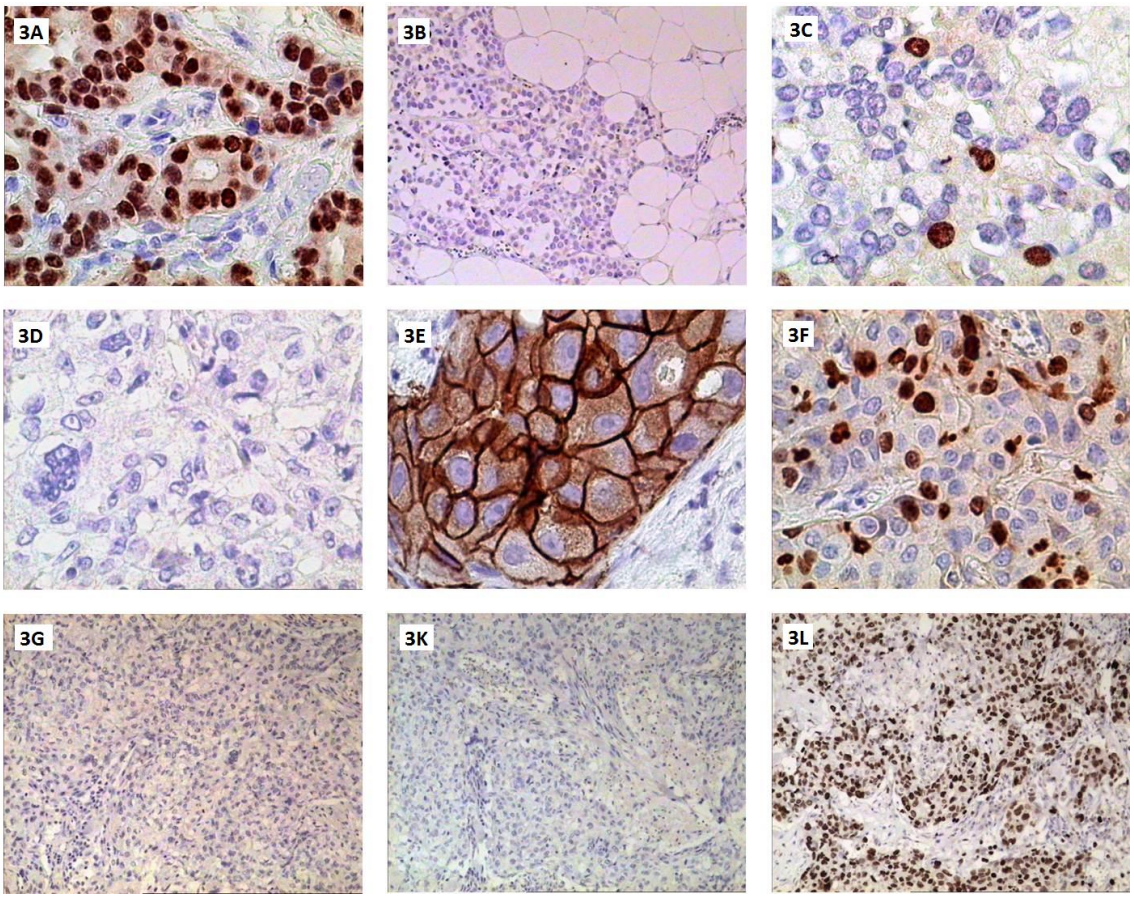


Figure 1.3. Molecular subtypes of breast cancer. A-C, Luminal breast cancer: A, Oestrogen receptor expression; B, Lack of HER2 protein; C, Low proliferation fraction. D-F, HER2 overexpressing breast cancer: D, Lack of oestrogen receptors; B, HER2 protein overexpression; C, Moderate proliferative fraction. G-L, Triple negative breast cancer: G, Lack of hormone receptors; K, Lack of HER2 protein; L, High proliferative fraction. Immunoperoxidase; A, D and G, Anti-oestrogen receptor alpha; B, E and K, HercepTest; C, F and L, Anti-Ki-67. OM 100x (B, G-L) and 400x (A, C-F). Microphotographs by A.Abolins.

1.1. Molecular subtypes of breast cancer

Analysis of gene expression arrays has resulted in the recognition of several fundamentally different subtypes of breast cancer [Perou *et al*., 2000]. Because it is not always feasible to obtain gene expression array information, mainly due to financial reasons, a simplified classification, closely following that proposed by Cheang *et al*. [Cheang *et al*., 2009], has been adopted as useful shorthand. Subtypes defined by clinico-pathological criteria are similar to but not identical to intrinsic subtypes and represent a convenient approximation. This approach uses immunohistochemical detection of ER and PR, the detection of overexpression of HER2 protein and/ or amplification of the corresponding gene – *HER2/neu* oncogene, and Ki-67 labelling index, a marker of cell proliferation, as the means of identifying tumour subtypes (Figure 1.3.). Ki-67 labelling index presents more substantial challenges, but important guidelines for this test are under development. Initially, Ki-67 was not included between markers by which breast cancer molecular subtypes were determined [Viale, Regan *et al*., 2008; Cheang *et al*., 2009; Raica *et al*., 2009; Dowsett *et al*., 2011].

1.1.1. Luminal-like breast carcinoma

Luminal breast cancer is characterized by the expression of ER and/or PR in the background of high, low or any Ki-67 and positive or negative HER2. Additional markers like GATA3, BCL2 oncoprotein (BCL2) and cytokeratin (CK) 8/18 were previously searched for in the luminal type. At present, the definition of the luminal subtype is independent on other markers like the CK 5/6 and epidermal growth factor receptor (EGFR), but the expression of these markers may be found in some cases.

According to positivity or negativity of HER2 and the degree of cellular proliferation, luminal breast cancers can be divided in two distinct groups: luminal A and luminal B.

Luminal A

The typical immunohistochemical profile of luminal type breast cancer is ER positive and/or PR positive, and HER2 negative. Based on the molecular profile, all cases with pure lobular carcinoma *in situ* represent luminal A tumours [Millikan *et al.*,

2008]. Consecutively, the large majority of invasive lobular carcinomas have a profile characteristic for luminal A. Depending on literature, luminal A subtype comprises 56-61% of cases and tend to have the most favourable long-term survival [Zaha *et al*., 2010]. Many of the genes found in luminal A breast carcinoma are typically expressed in the luminal epithelium that lines the ducts [Raica *et al*., 2009; Millikan *et al.*, 2008].

Luminal B

Previously, luminal B molecular subtype included all breast cancer cases, which immunohistochemically coexpressed hormone receptors (ER and/or PR) and HER2. This group comprises 9-16% of all cases and is associated with more aggressive nature than luminal A. Luminal B breast cancers include high grade tumours and are associated with lower long-term survival [Zaha *et al*., 2010]. Initially, Ki-67 was not included in the criteria defining this subtype [Spitale *et al*., 2009].

According to recent modifications in the surrogate classification of intrinsic breast cancer subtypes, luminal B group is divided in two parts: luminal B (HER2 negative) and luminal B (HER2 positive). Luminal B (HER2 negative) subtype includes all cases with ER and/or PR positivity, HER2 negativity and high Ki-67, but luminal B (HER2 positive) subtype includes breast cancer cases with positive ER and/or PR in connection with positive HER2 and any Ki-67 level [Goldhirsch *et al*., 2011].

1.1.2. HER2 positive type (non luminal)

The HER2 positive type is characterised by lack of ER and PR expression by immunohistochemistry in association with HER2 overexpression or *HER2/neu* gene amplification by fluorescent *in situ* hybridisation (FISH).

The frequency of HER2 positive subtype is 8-16%. The HER2 positive subtype includes two distinct subtypes based on the expression of ER: ER-negative that cluster near the basal-like tumours (HER2 positive ER negative subtype), and ER (may also express PR) positive as in luminal B subtype [Raica *et al*., 2009]. In the majority of the cases, p53 is not expressed, and the expression of CK 8/18 is heterogeneous and moderate. If positive, reaction for EGFR is focal and restricted to less than 5% of tumour cell population. HER2 type is frequently associated with ductal carcinoma *in*

situ (DCIS), many cases have high grade and are characterized by poor prognosis [Raica *et al*., 2009; Zaha *et al*., 2010].

1.1.3. Normal breast-like type/unclassified breast cancer

The frequency of normal breast-like type/unclassified breast cancer is 6-10%. Basal cells in the normal breast duct immunohistochemically stain with CK 5/6, but luminal cells express CK 8/18 [Millikan *et al.*, 2008]. Basal cells represent a mixture of different cell types with high proliferative potential, but luminal cells are more differentiated. Whether these cell types include a stem cell population capable of self-renewal is still unknown.

Normal breast-like cancer mainly is a triple negative tumour and is close to basal-like carcinoma in terms of the molecular profile. Regarding the immunohistochemical profile, outcome and survival, these tumours also are close to the basal-like breast cancer. Nuclear grade is higher than in luminal breast cancer types, as is the mitotic index. The unclassified type is negative for all five markers: ER, PR, HER2, CK 5 and EGFR. It has a slightly better prognosis than basal-like type, and does not respond to neoadjuvant therapy. It is important to point out that the term 'unclassified' within the frames of this classification is not synonymous with 'not otherwise specified' [Zaha *et al*., 2010].

1.1.4. Basal-like breast carcinoma

Basal-like breast cancer (8 to 20% of breast cancer cases) lacks ER, PR and HER2 expression, but express CK 5/6 and/or EGFR [Rakha *et al*., 2007] in gene microarray analyses or by immunohistochemistry. The term "basal-like cancer" describes a molecular phenotype initially defined using complementary deoxyribonucleic acid (DNA) microarrays, whereas "triple negative" is a term based on clinical assays for ER, PR, and HER2 [Perou *et al*., 2000, Sørlie *et al*., 2001]. Although most triple negative breast tumors cluster within the basal-like subgroup, these terms are not synonymous; there is up to 30% discordance between the two groups [Nielsen *et al*., 2004]. There are no specific hallmark features on routine histopathological slides that

help to identify these tumours, although some common morphological traits are described. The basal-like cancer is frequently associated with solid architecture, pushing borders, prominent lymphocyte infiltration, scant stroma, high grade, high nuclear/ cytoplasmic ratio, high mitotic index and presence of necrosis, especially *comedo* type necrosis [Winter, 2008; Popovska *et al*., 2010]. It is more frequent in premenopausal patients [Carey *et al*., 2006]. Basal-like cancer shows a high rate of p53 mutations and is common among *BRCA1* mutation carriers [Raica *et al*., 2009]. A high proportion (90.8%) of basal-like tumours presents with metaplastic features [Reis-Filho, Milanezi *et al*., 2006]. The metaplastic breast cancer shows positive reaction for EGFR, CK 5/6, CK 14, CK 17, and p63 in the majority of cases. By immunohistochemical panel, 93.8% metaplastic breast cancer can be classified as basal-like tumours [Vincent-Salomon *et al*., 2007].

Majority of medullary cancer cases fall into this subtype as well [Reis-Filho, Milanezi *et al*., 2006]. Based on genetic and immunohistochemical analysis, medullary carcinoma seems to be a subtype of basal-like type, based on the triple negative character and CK 5/6 expression [Vincent-Salomon *et al*., 2007].

Many but not all basal-like tumours stain for both CK 5/6 and CK 8/18. Almost half of basal-like tumours consist of a mixture of CK 5/14 positive and negative tumour cells [Raica *et al*., 2009]. Specific markers of the myoepithelial cells (smooth muscle actin, p63, cluster of differentiation (CD) 10) are not frequent and not substantial to characterize this subtype of tumour [Winter, 2008].

The DCIS associated to invasive basal-like carcinoma shows the same immunophenotype as the invasive tumour, and this provides evidence for early *in situ* precursor lesion [Raica *et al*., 2009]. In these cases, P-cadherin is expressed in 75%, and can be considered a good additional marker for basal-like DCIS. DCIS that is associated with basal-like cancer has solid, flat or micropapillary structure, high nuclear grade and necrosis. The absence of atypical ductal hyperplasia and small quantities of DCIS can be explained by rapid growth of tumour [Banerjee *et al*., 2006].

The basal-like cancers less frequently disseminate in axillary lymph nodes, liver and bones, and develop metastatic deposits in the brain and lungs [Hicks *et al*., 2006; Tischkowitz *et al*., 2007; Onitilo *et al*., 2009]. Basal-like carcinoma is associated with higher rate of recurrence and of cancer-related death, independently of lymph node status and tumour size [Tischkowitz *et al*., 2007]. Adjuvant anthracyclin based chemotherapy is less effective in case of basal-like carcinoma.

1.1.5. Triple negative breast cancer phenotype

Triple negative phenotype includes all breast cancers that lack ER, PR, HER2, CK 5/6 and EGFR expression by gene and immunohistochemical analyses. Triple negative breast cancer represents 10 to 17% of all breast cancers [Tan *et al*., 2008; Foulkes *et al*., 2010]. The prevalence of triple negative tumours is 15-23% in patients under the age of 40, 16-30% for patients aged 40-49, and 11-54% for patients over 50 years [Raica *et al*., 2009; Thike *et al*., 2010]. The evaluation of the molecular profile in large series has demonstrated that triple negative tumours fall into the basal-like and unclassified tumours. The diagnosis of these tumours has the advantage that these three stains (ER, PR and HER2) are already routinely used in immunohistochemistry to guide the therapeutic strategy. The aggressive character of this type of tumour is demonstrated by the recurrences that occur between 1 and 3 years, and the majority of deaths occur in the first 5 years, following therapy [Zaha *et al*., 2010]. The unfavourable prognosis is also supported by the fact that the majority of triple negative cases are predominantly of histological grade 3, up to 77%-96.8% of cases [Tan *et al*., 2008; Foulkes *et al*., 2010; Thike *et al*., 2010; Zaha *et al*., 2010]. Triple negative tumours form a heterogeneous group, and 56 to 84% of them express CK 5/6 and EGFR.

Approximately 80% overlap between triple negative and intrinsic basal-like subtype but triple negative breast cancer also includes some special histological types such as (typical) medullary and adenoid cystic carcinoma with low risk of distant recurrence [Goldhirsch *et al*., 2011].

1.2. Relation between molecular classification and adjuvant treatment

Considering the wide spectrum of treatment possibilities for breast cancer, it is important to choose appropriate options avoiding both over-treatment and under-treatment. However, treatment failure occurs in approximately 30% of patients [Andre and Pusztai, 2006]. ER positive cases are treated with hormone therapy, but respond poorly to chemotherapy. The response of ER negative patients to chemotherapy is not uniform necessitating more exact predictive subdivision [Raica *et al*., 2009]. In the HER2 positive cases, the treatment with trastuzumab significantly improves the prognosis and, combining with chemotherapy, it induces a remarkable reduction in the

relapse rate. However, not all HER2-positive cases respond to trastuzumab therapy; resistance may be induced by phosphatase and tensin homolog loss or CD184 up regulation.

Anthracycline-based chemotherapy has become a standard treatment. Data about the efficacy of preoperative chemotherapy in relation to the molecular classification are still controversial. It has been reported that the molecular subtype predicts the response to preoperative chemotherapy [Raica *et al*., 2009] but other researchers have found only ER status to be useful [Conforti *et al*., 2007]. Basal-like and HER2 tumours are more sensitive to neoadjuvant anthracycline based chemotherapy than luminal types [Carey *et al*., 2006]. Up to 45% of basal-like tumours show complete response after 12 weeks of paclitaxel followed by neoadjuvant chemotherapy with 5-fluorouracil, doxorubicin and cyclophosphamide. In the study by Sotiriou *et al.*, basal-like and HER2 types were associated with the highest rate of pathological complete response (45% for both), but luminal type showed a lower rate of complete response (6%). In the normal breast-like type, no complete response was noticed. Thus, the molecular profile as detected before surgery was useful in the prediction of response to chemotherapy [Sotiriou *et al*., 2002]. Unfortunately, none of the biomarkers is strongly predictive of chemotherapy response in cases with metastatic disease, but survival seems to be dependent on the hormone receptor and p53 status [Pusztai *et al*., 2006].

Targeted therapy became largely applied in the last decade. EGFR tyrosine kinase inhibitors might be a useful option and clinical trials have been initiated, based on gefitinib and erlotinib. A subset of basal-like carcinoma expresses CD117 and is associated with better prognosis, and therefore, targeted therapy could be initiated. Until now, only disappointing breast cancer response rates were reported. CD117 positive tumours usually fall into the category of basal-like carcinoma and patients were treated with imatinib or sunitinib. The efficacy of targeting CD117 will probably depend on its prevalence in the tumours and its role as predictive marker of response (both aspects are largely unknown). Recently, it was shown that anti-vascular endothelial growth factor, bevacizumab, improves survival in metastatic breast cancer when combined with paclitaxel [Raica *et al*., 2009]. More than 60% of cases in this study were hormone receptor positive and none was HER2-positive, suggesting that antiangiogenic strategies may be effective in the luminal type tumours. It becomes clear that there is a need to identify new specific therapeutic targets. Only by using the best of the old classic

approach and the best new possibilities, the maximal therapeutic benefit will be ensured with the least possible risk of adverse side effects.

1.3. Future directions and perspectives in breast cancer classification

The molecular classification brought new insights into the biology and behaviour of breast cancer. At present time, at least four types of breast cancers are characterized by gene analysis and five - based on immunohistochemical profile. Despite the fact that this classification correlates with prognosis, there are still many questions to be answered. The first of them is related to the possibility to replace the conventional morphologic classification. At present, the answer is probably negative, because some types are not fully characterized and it is very probable that some of them (e.g. basal-like and unclassified) include different subtypes. Moreover, such a change may create confusion, mainly because some types are not completely characterized. A good example is represented by the expression of EGFR and CD117 that could represent viable targets for therapy, both found in some but not all cases with basal-like carcinoma. Results on their immunohistochemical expression are still controversial.

1.4. Prognostic and predictive factors in breast cancer

Breast cancer is a heterogeneous disease with variable morphological appearances, molecular features, behaviour, and response to therapy. Current routine clinical management of breast cancer relies on the availability of robust clinical and pathological prognostic and predictive factors to support decision making regarding the choice between the growing ranges of potentially suitable treatment options [Weigel *et al.*, 2010]. A prognostic factor is any measurement available at the time of surgery that correlates with disease-free survival (DFS) or OS in the absence of systemic adjuvant therapy and, as a result, is able to correlate with the natural history of the disease. In contrast, a predictive factor is any measurement associated with response to a given therapy [Cianfrocca *et al.*, 2004]. Breast cancer treatment has experienced several changes in the past decades due to the discovery of specific prognostic and predictive biomarkers that enable the application of more individualized therapies to different

molecular subgroups. These subgroups show specific differences regarding biological clinical behaviour. In addition to the classical clinical prognostic factors of breast cancer, established molecular biomarkers such as ER and PR play a significant role in the selection of patients benefiting from endocrine therapy. These markers also are shown to have prognostic role [Rossner *et al*., 2009; Weigel and Dowsett, 2010; Jung *et al*., 2011; Gaur, 2013]. More recently, the HER2 has been validated to be not only a prognostic factor, but also a predictor of response to HER2 targeting therapy [Rakha *et al*., 2010; Weigel and Dowsett, 2010].

In the last few decades, proliferative markers have been evaluated as prognostic and predictive factors for early stage breast cancer patients. Several papers evaluating one or more markers have been published, often with contradictory results. As a consequence, there is still uncertainty about the value of these markers.

Colozza *et al*. critically reviewed the current knowledge about the following markers: thymidine labeling index, S phase fraction/flow cytometry, Ki-67, thymidine kinase, cyclins E, cyclin D, the cyclin inhibitors p27 and p21, and topoisomerase IIa. For each marker, the prognostic and predictive role was separately analysed. In addition, the prognostic and predictive role of the markers had to be assessed through multivariate analyses. One hundred and thirty-two papers fulfilled these criteria and 159516 patients were analysed. Unfortunately, several methodological problems prevented from including any one of these proliferative markers among the standard prognostic and predictive factors [Colozza *et al*., 2005]. Several recent reviews have summarised the present knowledge regarding biomarkers in breast cancer; however, controversies still are identified [Weigel and Dowsett, 2010; Gaur, 2013].

The shift towards an earlier diagnosis of breast cancer due to improved imaging methods and screening programs highlight the need for new factors and combinations of biomarkers to quantify the residual risk of patients and to indicate the potential value of additional treatment strategies. The role of proliferation marker Ki-67 has been recently emphasised due to several applications in neoadjuvant therapy in addition to its moderate prognostic value.

The Ki-67 antigen is used to evaluate the proliferative activity of breast cancer. In order to better define the prognostic value of Ki-67, de Azambuja *et al*. performed meta-analysis of studies that evaluated the impact of Ki-67 on DFS and/or on OS in early breast cancer. Sixty-eight studies were identified and 46 studies including 12 155 patients were included in the meta-analysis. Thirty-eight studies were suitable for the

aggregation of results for DFS, and 35 studies – for OS. Patients were considered to have Ki-67 positive tumours according to the cut-off points defined by the authors. Ki-67 positivity was associated with higher probability of relapse in all patients (hazard ratio (HR) =1.93; 95% confidence interval (CI) =1.74-2.14; $P<0.001$), in node-negative patients (HR=2.31; 95% CI=1.83-2.92; $P<0.001$) and in node-positive patients (HR=1.59; 95% CI=1.35-1.87; $P<0.001$). Furthermore, Ki-67 positivity was associated with worse survival in all patients (HR=1.95; 95% CI=1.70-2.24; $P<0.001$), node-negative patients (HR=2.54; 95% CI=1.65-3.91; $P<0.001$) and node-positive patients (HR=2.33; 95% CI=1.83-2.95; $P<0.001$). Meta-analysis suggested that Ki-67 positivity confers a higher risk of relapse and is associated with worse survival in patients with early breast cancer [de Azambuja *et al*., 2007].

Viale *et al*. evaluated the prognostic and predictive value of Ki-67 labeling index in a trial comparing letrozole with tamoxifen as adjuvant therapy in postmenopausal women with early breast cancer. Higher values of Ki-67 were associated with adverse prognostic factors and with worse DFS (hazard ratio [HR; high: low] = 1.8; 95% CI=1.4-2.3). The magnitude of the treatment benefit for letrozole versus tamoxifen was greater among patients with high tumour Ki-67 labeling index (HR [letrozole: tamoxifen] = 0.53; 95% CI=0.39-0.72) than among patients with low tumour Ki-67 (HR [letrozole: tamoxifen] = 0.81; 95% CI=0.57-1.15; interaction $P=0.09$). Thus, authors confirmed Ki-67 as a prognostic and predictive factor [Viale, Giobbie-Hurder *et al*., 2008].

In more recent studies, the role of Ki-67 has been reconfirmed [Ryu and Lee, 2012]. Recently, international panel of experts developed recommendations on preanalytical and analytical assessment as well as on interpretation and scoring of Ki-67 [Dowsett *et al*., 2011].

Thus, molecules that are used routinely to make treatment decisions in patients with breast cancer include hormone receptors, markers of proliferation (e.g. Ki-67) and the HER2. With the introduction of high-throughput technologies, numerous multigene signatures have been identified that outperform traditional markers: current prospective clinical trials are seeking evidence for their definitive role in breast cancer. There exist many more factors and approaches that have the potential to become relevant in the near future including the detection of single disseminating and circulating tumour cells in blood and bone marrow as well as of circulating cell-free micro ribonucleic acid (RNA) and DNA [Rakha *et al*., 2010].

By multidisciplinary group of clinicians, pathologists, and statisticians, different prognostic and predictive factors in breast cancer have been stratified into categories reflecting the strength of published evidence. Factors were ranked according to previously established College of American Pathologists (CAP) categorical rankings: category I, factors proven to be of prognostic value and useful in clinical patient management; category II, factors that had been extensively studied biologically and clinically, but whose importance remains to be validated in statistically robust studies; and category III, all other factors not sufficiently studied to demonstrate their prognostic value. Factors in categories I and II were considered with respect to variations in methods of analysis, interpretation of findings, reporting of data, and statistical evaluation. Category I included classification of malignant tumours (TNM) staging information, histologic grade (G), histologic type, mitotic figure counts, and hormone receptor status. Category II factors included HER2 (*HER2/neu* gene amplification), proliferation markers, lymphatic and vascular channel invasion and p53. Factors in category III included DNA ploidy analysis, microvessel density, EGFR, transforming growth factor-α, BCL2 oncoprotein, pS2, and cathepsin D [Fitzgibbons *et al*., 2000].

The scope of markers that have been analysed for hypothetic predictive and/or prognostic role in breast cancer include cyclooxygenase-2 [Kang *et al*., 2011; Kim et al., 2012], interleukins and chemokine receptors [Liu *et al*., 2010; Iliopoulos *et al*., 2011], p53 [Rossner *et al*., 2009; Malhotra *et al*., 2010; Jung *et al*., 2011; Millar *et al*., 2011], p27 [Wander *et al*., 2011], p16 [Kröger *et al*., 2006], cyclin A [Aaltonen *et al*., 2009; Boström *et al*., 2009], cyclin D1 [Esteva *et al*., 2004; Aaltonen *et al*., 2009; Boström *et al*., 2009; Li *et al.*, 2011; Yu *et al*., 2012], cyclin E [Esteva *et al*., 2004; Aaltonen *et al*., 2009; Boström *et al*., 2009], cytokeratin 5/6 [Li *et al.*, 2011], cathepsin D [Esteva *et al*., 2004; Bradley *et al*., 2007], maspin [Kröger *et al*., 2006], microvessel density [Bradley *et al*., 2007], urokinase-like plasminogen activator/ plasminogen activator inhibitor [Esteva *et al*., 2004] and apoptosis-related factors including BCL2 oncoprotein [Won *et al*., 2010; Hwang *et al*., 2012; Yu *et al*., 2012; Zaha and Lazar, 2012]. Several studies have been devoted to EGFR [Hadžisejdić *et al*., 2010; Liu *et al*., 2010; Rimavi *et al*., 2010]. Androgen receptors have been studied extensively [Gonzalez-Angulo *et al*., 2009; Park *et al*., 2010; Hu *et al*., 2011]. Caveolin has been evaluated in the tumour stroma [Koo *et al*., 2011]. Nestin and claudin-4 have been associated with poor prognosis [Lanigan *et al*., 2009; Piras *et al*., 2011]. Cullin-1 has recently been analysed as possible therapeutic target and marker of poor prognosis [Bai

et al., 2013]. Both E-cadherin [Querzoli *et al*., 2010] and actin [Yamashita *et al*., 2012] have been evaluated for prognostic role in addition to the routine diagnostic importance [Abdollahi *et al*., 2011; Walker *et al*., 2012].

Cyclin D1, BCL2 oncoprotein, p53 protein and COX-2 cause major scientific and practical interest as these proteins are involved in the cardinal tumour features: cell proliferation and cell cycle control, evasion of apoptosis, expression of oncoproteins due to mutations in proto-oncogenes and angiogenesis. These biomarkers will be discussed in more detail in the subsequent sections.

1.4.1. Expression of oncoprotein p53

p53 is a nuclear phosphoprotein with a molecular mass of 53 kilodaltons (kDa). Wild-type p53 protein is present in a wide variety of normal cells, but the protein has a very short half-life and thus is present in only minute amounts, generally below the detection level of immunohistochemical methods. Somatic mutation of the *TP53* gene is a very frequent event in the development of human neoplasia. Mutant p53 proteins often are more stable than wild-type p53 protein; therefore the mutant p53 protein accumulates to a high level. As examples, p53 protein accumulation was observed in 76% of 212 human malignant lesions, including breast, colon and stomach carcinomas, melanoma, embryonic carcinoma of the testis, transitional carcinoma of the urinary bladder, uterine carcinoma and soft tissue sarcomas. Wild-type p53 protein functions as a transcription factor, i.e., as a modulator which can turn crucial genes either on or off. It also inhibits DNA replication and is a check-point control molecule for progression of the cell cycle. Furthermore, p53 protein is involved in the regulation of apoptosis. The wild-type p53 protein protects cell from neoplastic transformation by multiple mechanisms. In short, if cell has experienced DNA damage, p53 protein undergoes post-transcriptional modification that releases it from the binding protein homolog murine double minute 2 (MDM2), a protein that normally stimulate the destruction of p53. The released p53 becomes a transcription factor and the cell cycle is arrested. If the DNA damage can be repaired, the cell cycle reverts to normal progress. Otherwise, the cell is subjected either to senescence or death by apoptosis [Wei *et al*., 2006; Riley *et al*., 2008]. At least part of the wide function spectrum of p53 is realized through microRNA34 [He *et al*., 2007]. In transfection assays, wild-type p53 behaves as a

tumour suppressor, while mutant p53 behaves as a dominant transforming oncogene. Cells labelled by the antibody generally display a nuclear staining pattern, but cytoplasmic staining has been reported in some cases [*Dako Denmark A/S, M7001*] and can be biologically true phenomenon explained by monoubiquitination induced by different MDM2 levels [Brooks *et al*., 2004].

Nearly one third of breast cancers have mutations of the tumour suppressor gene *TP53*, which are associated with high histologic grade and clinical aggressiveness. Since part of the mutations result in prolonged half-life and protein accumulation, immunohistochemical detection of p53 can be used as a surrogate for mutational analysis. Immunostaining should be considered a screening method for *TP53* mutation, as some cases have neither protein overexpression nor an increased half-life [Alsner *et al*., 2008]. The reported frequencies of p53 protein expression in breast cancer have been as high as 54% and as low as 20% [Göhring *et al*., 1995; Sjögren *et al*., 1996; Bidard *et al*., 2008]. However, several studies using immunohistochemistry and *TP53* gene analysis have found remarkably similar estimates of p53 protein overexpression or gene mutation: 29.0% (by IHC), 29.0% (by IHC), 29% (by sequencing) and 29.6% (by IHC), respectively [Yamashita *et al*., 2004; Rolland *et al.*, 2007; Alsner *et al*., 2008; Al-Joudi *et al*., 2008]. Marchetti *et al*. used polymerase chain reaction (PCR) single strand conformation polymorphism assay to assess *TP53* mutations in invasive breast carcinoma. A strong correlation ($P<0.001$) was observed between *TP53* mutations and nuclear accumulation of the p53 protein: 10 tumours were scored positive for both *TP53* mutation and overexpression. However, in 9 cases having a mutated *TP53* gene the researchers failed to find positive immunoreactions [Marchetti *et al*., 1993].

The frequency of *TP53* mutations in different publications clusters close to 15-17% of patients [Soontrapornchai *et al*., 2007; Rossner *et al*., 2009; Fernández-Cuesta *et al*., 2012]. The *TP53* gene mutations in breast cancers appear to cluster in exons 5 through 9. Studies of mutation based on genetic sequencing have been limited because of the molecular complexity of this large gene. Sequencing studies of breast cancer are often limited to the exon sequences 5 through 9 because of the mutational hot spots that have been identified there. Other methods to detect *TP53* abnormalities include PCR based amplification with screening for mutations using single strand conformational polymorphism assays. New high-throughput sequencing technologies are developing. Given the diverse functions of the *TP53* gene and the location and type of genetic

abnormalities, the specific genetic lesion may have prognostic importance [Alsner *et al*., 2008].

The p53 function can also be significantly influenced by expression of MDM2 protein as high levels of it induce p53 destruction [Onel and Cordon-Cardo, 2004; Shmueli and Oren, 2004]. The binding of p53 protein by exogenous factors like E6 protein of human papilloma virus can result in loss of function as well [Chen, Huang *et al*., 2012]. The levels of microRNA can be targeted by pathologic factors [He *et al*., 2007].

While most *TP53* abnormalities occur as somatic events, patients with germline *TP53* mutations (Li-Fraumeni syndrome) also have an increased incidence of breast cancer [Nichols *et al*., 2001]. The breast cancer in Li-Fraumeni syndrome patients represents invasive or *in situ* ductal cancer with virtual absence of other types. The tumours are characterised by early origin (median age, 32 years), positivity for ER / PR (84%), high grade (81%) and high rate of HER2 overexpression or gene amplification [Masciari *et al*., 2012].

p53 appears to have prognostic and/or predictive value. However, consensus as to the need for routine p53 immunostaining has not occurred. Some studies report antigenic degeneration with time; therefore storage and fixation issues may be relevant. Patients with p53-immunopositive cancers may develop autoantibodies against p53, which have been used to detect or follow cancers [Anderson *et al*., 2010; Kulic *et al*., 2010]. Array-based technologies that can screen for mutations in some regions of the gene have become commercially available, but have not been widely adopted [Fitzgibbons *et al*., 2000].

Several researchers have evaluated the immunohistochemical expression of p53 protein in relation to different principal features of breast cancer [Marchetti *et al*., 1993; Göhring *et al*., 1995; Sjögren *et al*., 1996; Dublin *et al*., 1997; Megha *et al*., 2002; Rolland *et al*., 2007; de Roos *et al*., 2007; Soontrapornchai *et al*., 2007; Al-Joudi *et al*., 2008; Alsner *et al*., 2008; Bidard *et al*., 2008; Kim *et al*., 2010; Millar *et al*., 2011; Dookeran *et al*., 2012; Fernández-Cuesta *et al*., 2012]. The structure of the most relevant studies along with the size of patient's group and short description of methods is represented in Table 1.1., but the main findings and conclusions will be analysed subsequently.

Table 1.1.

Logistic and technological characteristics of selected studies devoted to p53 analysis in breast cancer tissues

Author	Patients and materials	Methods	The analysed correlations
Marchetti *et al*., 1993	148 invasive breast carcinomas, selected by histotype (56 lobular, 47 ductal, 19 mucinous, 18 medullary, 8 papillary cancers).	PCR single strand conformation polymorphism assay to detect *TP53* mutations. IHC for p53 overexpression in 122 tumours by antibody PAb 1801.	Presence of *TP53* mutation; nuclear immunostaining for p53; histologic type; Ki-67.
Göhring *et al*., 1995	204 formalin-fixed, paraffin-embedded biopsies of primary breast carcinomas.	IHC for p53 protein by antibody PAb 1801. The influence of p53 expression on prognosis in 197 patients (T1-4 N0-2 M0, median observation time 72 months).	Tumour grade; patient's menopausal status; age; tumour size; axillary lymph node involvement; ER/ PR status; disease-free survival.
Sjögren *et al*., 1996	316 primary breast tumours.	IHC by mouse monoclonal antibody Pab 1801 (that recognizes both wild-type and mutant forms of p53). Screening of entire coding region of *p53* gene by reverse transcription, PCR and DNA sequencing. Kaplan-Meier method, log-rank test.	Overall survival; breast cancer-corrected survival; death from breast cancer; RFS.
Dublin *et al*., 1997	277 women with node-positive primary breast cancer.	The randomisation: either 6 cycles of cyclophosphamide/ methotrexate/ 5-fluorouracil or no treatment after tumour excision and axillary clearance. IHC for p53 protein. Follow-up: data available on all patients (median, 9 years).	Survival; treatment response.
Megha *et al*., 2002	37 cases of *in situ* and 27 cases of invasive ductal breast carcinoma.	IHC for the expression of the p53 and BCL2 proteins. Polymerase chain reaction single strand conformation polymorphism analysis for *p53* gene mutation.	Phenotypic characteristics and cellular kinetic parameters (mitotic and apoptotic indices).

Table 1.1. (continued)

Yamashita *et al*., 2004	506 patients with invasive ductal carcinoma (1981 – 1999).	IHC for HER2, p53, and Ki-67. Survival analysis with median follow-up 82 months.	Disease free and overall survival.
Rolland *et al*., 2007	819 cases of resected primary breast cancer (1986 – 1998).	Tissue microarray; IHC by anti-p53 (clone DO-7) and BCL2 (clone 124). Statistics by univariate and multivariate (Cox's regression) analyses.	Clinico-pathological data; disease specific survival; BCL2 expression.
de Roos *et al*., 2007	Consecutive patients (July 1996 – December 2001) treated for pure ductal carcinoma *in situ* (110) and invasive ductal carcinoma (243).	Tissue microarray application. IHC for ER, PR, *HER2/neu*, p53, and cyclin D1. Follow-up: median, 49.8 months.	Local recurrence. All analyses were stratified for diagnosis (*in situ* vs. invasive cancer) and pathological grade.
Soontraporn-chai *et al*., 2007	71 node-negative breast carcinomas.	IHC for p53 on formalin-fixed, paraffin-embedded sections.	Tumour size; ER expression; survival.
Al-Joudi *et al*., 2008	382 cases of invasive ductal breast carcinoma, treated in 3 major hospitals in Malaysia.	IHC for the detection of p53 protein.	Age; grade; lymph node status; tumour size; side of tumour, expression of ER / PR.
Alsner *et al*., 2008	630 breast cancer patients from the Danish Breast Cancer Cooperative Group, protocols DBCG82 and DBCG89.	IHC for p53; scoring based on staining intensity and percentage of invasive tumour cells with nuclear staining. Sequencing to identify *TP53* mutations.	Mutation; protein expression; disease-specific survival.

Table 1.1. (continued)

Bidard *et al.*, 2008	293 breast cancer samples from two different centres.	IHC for ER, PR, HER2 and p53. Logistic regression for multivariate analysis of predictors for pathological complete response.	Grade; ER / PR status; molecular subtype; efficacy of preoperative fluorouracil, anthracycline and cyclophosphamide treatment by p53 status and molecular classification.
Kim *et al.*, 2010	125 patients having radiotherapy after breast conserving surgery and axillary lymph node dissection; 87 patients had adjuvant chemotherapy and/or hormonal therapy.	IHC for p53 and BCL2 expression (100 patients).	Conventional clinicopathologic variables; treatment-related factors.
Millar *et al.*, 2011	498 patients with invasive breast cancer.	IHC for ER, PR, Ki-67, p53, HER2. Kaplan-Meier and Cox proportional hazards test.	Ipsilateral recurrence; locoregional recurrence; distant metastasis-free survival; breast cancer-specific survival.
Yang *et al.*, 2011	21 patients with inflammatory and locally advanced breast cancer receiving neoadjuvant bevacizumab and chemotherapy.	IHC for p53, ER, PR, HER2, Ki-67, tumour apoptosis, vascular endothelial growth factor, microvessel density Cox proportional hazard analysis.	Survival; progression-free survival.
Dookeran *et al.*, 2012	Consecutively treated 331 African American and 203 non-African American women affected by breast cancer.	IHC for p53 protein. Cox regression model.	Stage; grade; ER / PR status; occurrence of triple negative subtype; mortality due to all causes.

Table 1.1. (end)

Fernández-Cuesta *et al.*, 2012	BIG 02-98 randomized phase III trial: women with node-positive breast cancer, treated with adjuvant doxorubicin-based chemotherapy with or without docetaxel.	*TP53* gene status was determined for 18% (520 of 2887) of the women. *TP53* gene sequencing within exons 5 to 8. Patients were classified according to p53 protein status predicted from *TP53* gene sequence, as wild-type (no *TP53* variation or variations which were predicted not to modify p53 protein sequence) or mutant (*p53* non-synonymous mutations). Mutations were subcategorized as missense or truncating. Survival analyses by Kaplan-Meier method and log-rank test; Cox-regression analysis to identify independent predictors of outcome.	Age; morphology; grade and ER / PR status.

Abbreviations in the Table: PCR, polymerase chain reaction; IHC, immunohistochemistry; ER, oestrogen receptors; PR, progesterone receptors; DNA, deoxyribonucleic acid; RFS, relapse-free survival; HER, human epidermal growth factor receptor.

Regarding the characteristics of patients and tumours, the following findings have been reported. The expression of p53 showed statistically significant correlation with younger patient's age and higher histological tumour grade [Al-Joudi *et al*., 2008; Guarneri *et al*., 2010]. No correlation by p53 protein expression and patient's age or menopausal status was identified by Göhring *et al*., 1995. In contrast, non-synonymous *TP53* mutations, found in 16.3% of breast cancers, were associated with older age, but confirmed the correlation with higher grade [Fernández-Cuesta *et al*., 2012]. In another study, p53 immunostaining (54%) was associated with high cancer grade (P=0.002) as well [Bidard *et al*., 2008]. The statistically significant correlation (P=0.013) between p53 expression and loss of tumour differentiation has been described by Göhring *et al*. already earlier [Göhring *et al*., 1995] and confirmed by Le *et al*., 1999 reporting P=0.0001. The association between high grade and p53 expression was reported also by Guarneri *et al*., 2010.

The expression of p53 has been associated with ductal morphology [Fernández-Cuesta *et al*., 2012]. By PCR, the distribution of *TP53* mutations was significantly different (P=0.006) in the examined histotypes: mutations were frequent in medullary (39%) and ductal (26%), less common in lobular (12%), and absent in mucinous and papillary carcinomas. The frequency of mutations in the exon 5 of the *TP53* gene was significantly higher (P=0.012) in medullary carcinomas than in the other histotypes: 5 (63%) of the mutations found in exon 5 were observed in medullary carcinomas [Marchetti *et al*., 1993].

An interesting study has highlighted the role of p53 pathway damage as an early event in breast carcinogenesis specific for certain pathogenetic way. Megha *et al*. compared *TP53* mutations in breast cancer with phenotypic and differentiation markers. Both ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC) with a stem cell phenotype (expression of CK 8, CK 14, CK 18, vimentin, and EGFR) were p53 positive and BCL2 negative by immunohistochemistry. In IDC, p53 expression was associated with a reduction of both mitotic index and apoptotic index compared with DCIS. Most of the tumours showing a more differentiated luminal phenotype (CK 8 and CK 14, weak or negative expression of CK18, negativity for vimentin and EGFR) were p53 negative and BCL2 positive. In these cases, cell kinetic parameters increased from DCIS to IDC. These data suggest the existence of subsets of DCIS and IDC that could be derived from subpopulations of normal breast cells having different control mechanisms of cell proliferation and neoplastic progression [Megha *et al*., 2002].

Several studies have failed to identify correlation between the expression of p53 protein and the size of tumour [Göhring *et al*., 1995; Soontrapornchai *et al*., 2007; Al-Joudi *et al*., 2008; Alsner *et al*., 2008]. Naturally, there is no correlation between p53 expression and the side of breast cancer [Al-Joudi *et al*., 2008].

The association between p53 expression and lymph node status has been evaluated by several research groups. No significant statistical correlations were found regarding p53 expression and lymph node status by Göhring *et al*., 1995 and Al-Joudi *et al*., 2008. In contrast, p53 expression has recently been associated with higher number of lymph node metastases [Lialiaris *et al*., 2011]. The p53 expression along with other biological markers did not appear to be helpful predictors of non-sentinel lymph node metastasis in sentinel-node positive breast cancer patients [Kwon *et al*., 2011]. The concordance of p53 expression in the primary tumour and lymph node metastases of breast cancer is 85% [Sjöström-Matson *et al*., 2009].

The molecular portrait of p53 positive breast cancer shows association with high-risk features [Guarneri *et al*., 2010]. However, the published data are controversial. There was no correlation between the expression of ER and/or PR and p53 positivity [Göhring *et al*., 1995; Soontrapornchai *et al*., 2007; Al-Joudi *et al*., 2008]. Non-synonymous *TP53* mutations were associated with hormone-receptor negativity [Fernández-Cuesta *et al*., 2012]. The association between p53 immunostaining and ER negativity (P=0.04) was confirmed by Bidard *et al*., 2008 and Le *et al*., 1999 reporting P=0.0001 for the association. It has been suggested to apply p53 negativity as an additional criterion for prognostically beneficial luminal A molecular subtype [Millar *et al*., 2011]. A significant association (P=0.01) has been reported between mutations in the *TP53* gene and high proliferative activity of the tumours determined by immunohistochemistry with monoclonal antibody Ki-67 [Marchetti *et al*., 1993].

A significant association was found between p53 status and survival by Dublin *et al*. Patients with p53-positive tumours had a less favourable outcome than those with p53 negative disease [Dublin *et al*., 1997; Guarneri *et al*., 2010]. The correlation between p53 expression and survival was not confirmed by Soontrapornchai *et al*., 2007.

The prognostic value of p53 protein has also been analysed in association with other molecular markers and in specific groups of patients. The p53 status by multivariate analysis showed high prognostic value in luminal breast cancer

[Jacquemier *et al*., 2009]. It was less valuable in high-risk breast cancer [Somlo *et al*., 2008].

Knowing the ability of wild-type p53 to control the apoptosis, the relation between p53 and BCL2 protein expression has been evaluated. The purpose of Rolland *et al*. was to determine if the immunohistochemical p53 positive BCL2 negative phenotype predicts survival in breast cancer patients. Abnormal p53 expression was detected in 29% tumours. p53 expression correlated with the clinicopathological features of aggressive cancers and a reduction in survival (log rank 17.81; $P<0.001$). Nineteen percent of tumours displayed a p53+ / BCL2- phenotype. Kaplan-Meier analysis revealed a significant reduction in survival in these cases (log rank 34.01; $P<0.001$). Multivariate analysis showed that while neither p53 expression nor BCL2 expression alone had independent prognostic significance, the p53+ / BCL2- phenotype remained independently associated with a worse prognosis as revealed by HR=1.79; 95% CI=1.10-2.89, $P<0.018$ [Rolland *et al*., 2007].

According to Yamashita *et al*., accumulation of p53 protein significantly decreased disease free ($P=0.01$) and overall survival ($P=0.01$). Overexpression of HER2 also significantly reduced disease free survival ($P=0.02$) and overall survival ($P=0.005$). Patients with tumours that were positive for both HER2 and p53 relapsed and died within a significantly shorter period of time after surgery ($P=0.0001$ and $P<0.0001$, respectively). In multivariate analysis, patients with both HER2 and p53 positive tumours had considerably decreased overall survival ($P=0.04$), as did patients with larger tumour size and positive lymph node status. The findings indicated that the coexistence of HER2 over-expression and p53 protein accumulation is a strong prognostic molecular marker in breast cancer [Yamashita *et al*., 2004].

In a retrospective study showing p53 expression in 38% of breast cancers, detection of p53 protein was associated with a significantly longer disease-free survival in node-positive women ($P=0.03$). However, p53 protein did not prove to be a prognostic factor in node-negative patients [Göhring *et al*., 1995]. In contrast, correlation between p53 positivity and worse prognosis was observed in lymph node-positive breast cancer patients by Lara *et al*., 2011. Prognostic power of p53 expression in node-negative breast cancer has been reported as well [Jung *et al*., 2011]. However, contrary data are also available. Thus, p53 expression was not a significant prognostic factor for survival in node-negative breast carcinoma [Soontrapornchai *et al*., 2007].

Regarding specific patient groups, higher prognostic value of p53 status in women of African descent has been reported [Dookeran *et al*., 2012].

The value of p53 detection is retained in patients receiving treatment. Thus, prognostic value of p53 regarding survival after neoadjuvant bevacizumab and chemotherapy has been reported [Yang *et al*., 2011].

In 1000 high-risk breast cancer patients randomized to postmastectomy radiotherapy p53 accumulation was not significantly associated with increased overall mortality, distant metastases or locoregional recurrence in univariate or multivariate Cox regression analyses [Kyndi *et al*., 2008]. Kim *et al.* evaluated the prognostic significance of p53 and BCL2 expression in patients treated with breast conserving surgery and radiotherapy. The 5 year loco-regional relapse-free and distant metastasis-free survival rates were 91.7% and 90.9%, respectively. On univariate analysis, age, T parameter and the absence of BCL2 and ER expression were associated with loco-regional RFS. When incorporating these variables into Cox proportional hazard model, only BCL2- / ER- phenotype was an adverse prognostic factor (P=0.018). As for the distant metastasis-free survival, age, T stage, and p53 expression were significant on univariate analysis. However, p53 expression remained the only significant prognostic factor on multivariate analysis characterised by P=0.009 [Kim *et al*., 2010].

The p53 protein has also been evaluated as a predictive marker. Pre-clinical data suggest p53-dependent anthracycline-induced apoptosis and p53-independent taxane activity. Fernández-Cuesta *et al*. retrospectively explored the prognosis and predictive values of *TP53* somatic mutations. Non-synonymous *TP53* mutations were found in 16.3% patients and included missense (12.3%) and truncating (3.6%) mutations. Only truncating mutations showed significant independent prognostic value, with an increased recurrence risk compared to patients with non-modified p53 protein (HR=3.21, 95% CI=1.74-5.94, P=0.0002). p53 status had no significant predictive value for response to docetaxel [Fernández-Cuesta *et al*., 2012].

Bidard *et al*. hypothesized that, among molecular subclasses of breast cancer, p53 status may have a differential predictive value for the efficacy of anthracyclines/alkylating agents-based regimen. p53 immunostaining was detected in 54% of all cases and 59% of triple-negative tumours. In the general patient group, pathological complete response (9.6%) was independently predicted by high tumour grade (P=0.002) and triple negativity (P=0.0004), but not by p53 status (P=0.12). p53 immunostaining was associated with a trend for a higher rate of pathological complete

response in triple negative tumours [relative risk (RR) = 2.5, 95% CI=0.8–7.5, *P*=0.09], but not in non-triple negative tumours (RR=0.73, 95% CI=0.16–3.3, *P*=0.69). Thus, it was concluded that p53 status may have a different predictive value for efficacy of anthracycline/ alkylating agents-based regimen in each molecular subclass, a result which may explain the different results reported in literature [Bidard *et al*., 2008]. In contrast, adjuvant chemotherapy with cyclophosphamide/ methotrexate/ 5fluorouracil was associated with a survival benefit in women with node-positive breast cancer irrespectively of immunohistochemically determined p53 status [Dublin *et al*., 1997]. Guarneri et al. characterised p53 expression as significant predictor of pathologic complete response following anthracycline and taxane based treatment [Guarneri *et al*., 2010].

Contrary to Bidard *et al*., de Roos *et al*. evaluated biological markers that could predict local recurrence following treatment for all stages of primary operable ductal carcinoma of the breast. In univariate analyses, *HER2/neu* (HR=3.1, 95% CI=1.1-8.7, *P*=0.032) and p53 overexpression (HR=3.5, 95% CI=1.3-9.3, *P*=0.014) were associated with local recurrence both in patients treated for *in situ* and invasive ductal cancer. In multivariate analysis, p53 overexpression (HR=3.0, 95% CI=1.1-8.2, *P*=0.036 and HR=4.4, 95% CI=1.5-12.9, *P*=0.008) and adjuvant radiotherapy (HR=0.2, 95% CI=0.1-0.8, *P*=0.026) were independent predictors of local recurrence after treatment in both patient groups [de Roos *et al*., 2007].

After several retrospective studies that have suggested *TP53* gene mutation as an adverse prognostic indicator in breast cancer patients, a cohort of 90 Caucasian breast cancer patients was analyzed prospectively (60 months of follow-up) with a rigorous mutation detection methodology. The presence of a *TP53* gene mutation was the single most adverse prognostic indicator for recurrence (*P*=0.0032) and death (*P*=0.0001), and was associated with poor response to both adjuvant (*P*=0.0001) and palliative (*P*=0.006) therapy [Blaszyk *et al*., 2000].

Sjögren *et al*. compared a complementary DNA based sequencing method and an IHC method for their abilities to detect *TP53* mutations in breast cancer specimens and prognostic value of the obtained data. As a result, 22% of tumours had *TP53* gene mutations detected by the complementary DNA based sequencing method; only 45% of these mutations were located in evolutionary conserved portions of the *TP53* coding region. Sixty-four tumours (20% of the total) had elevated levels of p53 protein as detected by IHC, suggesting the presence of mutations. IHC failed to detect 33% of the

mutations. Furthermore, 19 of the IHC-positive tumours were sequencing negative (i.e., p53 wild-type), suggesting a 30% false-positive frequency with IHC. The 5-year estimates for RFS, breast cancer-corrected survival and OS were significantly shorter for patients with p53 sequencing-positive tumours (P=0.001, P=0.01, and P=0.0003, respectively). Patients with IHC-positive tumours showed reduced survival in all three categories when compared with those with IHC negative tumours, but the differences were not statistically significant [Sjögren *et al*., 1996].

Alsner *et al.* investigated interrelationship between p53 accumulation and *TP53* mutations as well as the importance of different *TP53* mutation types. *TP53* was mutated in 29% of the patients. The disease-specific survival at 15 years of follow-up for patients with missense mutations directly involved in DNA or zinc binding was 21±8%. Patients with the remaining missense mutations within the structural/conserved domains and patients with null mutations had a disease-specific survival of 36±6% and 31±17%, respectively. For patients without *TP53* mutations and patients with mutations affecting amino acids outside these domains, the 15 year disease-specific survival was 51+/-3% and 71±10%, respectively. p53 accumulation was successfully scored in 567 patients and categorized into three groups. Tumours with no p53 expression had a high frequency of null mutations (37% compared to 10% in the whole cohort), and tumours with high p53 expression contained 82% of the missense mutations inside structural/conserved domains including those directly involved in DNA or zinc binding. The clinical outcome for breast cancer patients was significantly different for different *TP53* mutation types. Most of the mutations that lead to mutant p53 protein accumulation can be detected by immunohistochemistry but the specificity is low. Samples showing lack of detectable p53 protein should be considered as an indication of a possible null mutation [Alsner *et al*., 2008].

The reported findings suggest several conclusions. Although *p53* gene alterations in breast cancer have been associated with poor prognosis, there is not yet consensus that p53 testing should be performed routinely. Utility as a predictive marker has been reported, but extensive validation studies have not yet been performed. Several methods can be used to screen for or define p53 alterations in human tissue samples, but consensus regarding optimal methodology or reagents does not exist for either molecular or immunohistochemical assays.

1.4.2. Role of BCL2 in breast cancer

BCL2 oncoprotein is a blocker of apoptotic cell death. Gene transfer experiments have shown that elevated levels of this protein can protect a wide variety of cells from diverse cell death stimuli ranging from growth factor withdrawal and cytotoxic lymphokines to virus infection and DNA-damaging anticancer drugs and radiation. BCL2 oncoprotein resides on the cytoplasmic side of the mitochondrial outer membrane, endoplasmic reticulum and nuclear envelope, and has a molecular mass of 26 kDa. The *BCL2* gene is involved in the t(14;18) chromosomal translocation found in 85% of human follicular lymphomas and 20% of diffuse B cell lymphomas. In this translocation, the *BCL2* gene at chromosome segment 18q21 is juxtaposed with the immunoglobulin heavy chain locus at 14q32, resulting in deregulated expression of BCL2 oncoprotein. The cells labelled by the antibody display a cytoplasmic staining.

In normal tissues, the antibody labels almost all peripheral blood lymphocytes. In lymphoid tissue, small lymphocytes in the mantle zones and T-cell areas are positive whereas very few cells in germinal centres are labelled. In the spleen, many cells in both T- and B-cell areas and the red pulp stain by the antibody. In the thymus, many cells in the medulla are labelled, while most cells in the cortex show weak or negative staining [*Dako Denmark A/S, M0887*]. BCL2 is physiologically expressed in ductal epithelia of the normal breast [Binder *et al*., 1995].

Considering the expression of BCL2 in pathology, the antibody labels many neoplastic cells including lymphoproliferative disorders of low and high grade, such as chronic lymphocytic leukaemia, hairy cell leukaemia, T-cell lymphoma, B- and T-cell large cell type and anaplastic large cell Ki-1 lymphoma, and follicular lymphoma. Expression of BCL2 oncoprotein is also detected in synovial sarcomas, and in muscle-derived tumours [*Dako Denmark A/S, M0887*].

Since BCL2 inhibits most kinds of programmed cell death and provides a selective survival advantage to various cell types the biological significance of BCL2 overexpression for the development and progression of breast cancer has to be evaluated. Several authors have analysed the expression of BCL-2 protein in breast cancer [Binder *et al*., 1995; Le *et al*., 1999; Callagy *et al*., 2006; Tsutsui *et al*., 2006; Lee, Im *et al*., 2007; Martinez-Arribas *et al*., 2007; Trere *et al*., 2007; Alireza *et al*., 2008; Callagy, Webber *et al*., 2008; Talley *et al*., 2008; von Minckwitz *et al*., 2008; Dawson *et al*., 2010; Chen, Wu *et al*., 2012; Zaha and Lazar, 2012]. The logistic and

technological characteristics of selected studies devoted to BCL2 analysis in breast cancer tissues is shown in the Table 1.2. but the main findings will be discussed in short further.

Table 1.2.

Logistic and technological characteristics of selected studies devoted to BCL2 analysis in breast cancer tissues

Author	Patients and materials	Methods	The analysed correlations
Binder *et al*., 1995	133 primary breast cancers.	IHC for BCL2 protein.	Grade; status of ER/ PR; HER1; HER2; HER3.
Le *et al*., 1999	175 operable breast tumour patients.	IHC for BCL2 and p53 proteins. Northern blot for the expression of the *c-myc* gene. Follow-up: mean, 9.5 years.	Tumour size and grade; ER / PR status; number of positive lymph nodes; risk of death and relapse.
Tsutsui *et al*., 2006	249 invasive ductal breast carcinomas.	IHC for BCL2, p27 and p53 and Ki-67 proteins.	Grade; ER status; p27; p53; Ki-67; HER2; DFS
Callagy *et al*., 2006	930 breast cancers.	Tissue microarray. IHC for 13 biomarkers.	Survival.
Lee, Im *et al*., 2007	151 curatively resected stage III breast cancer patients (3 males, 148 females, median age 46 years) with at least 4 positive lymph nodes and received doxorubicin/ cyclophosphamide / paclitaxel as adjuvant chemotherapy. Patients with positive ER and / or PR expression received 5 years of tamoxifen following doxorubicin / cyclophosphamide / paclitaxel.	IHC for BCL2, p53, ER, PR, HER2, Ki-67. Follow-up: median duration, 36 months; 37 patients (24.5%) experienced a recurrence.	Clinico-pathologic variables; DSF; OS.
Alireza *et al*., 2008	Breast tumour sections from 35 surgically removed patient samples and 35 normal or benign tissue samples; sera from both the patient and control groups.	IHC for BCL2 protein. Enzyme-linked immunosorbent assay technique for soluble BCL2 (BMs244/3 Kit).	Tumour type, grade, and size, patient's menopausal status and age.

Table 1.2. (end)

Callagy, Webber *et al*., 2008	PubMed reports (1994–2006). 18 series including 5892 cases with an average median follow-up of 92.1 months; 8 studies investigating disease free survival unadjusted for other variables in 2285 cases.	Meta-analysis of studies that investigated the role of BCL2 expression by IHC with a sample size greater than 100.	The prognostic significance of BCL2.
von Minckwitz *et al*., 2008	248 patients with a histologically confirmed diagnosis of previously untreated, operable primary breast cancer T2-3(≥3 cm) N0-2 M0, treated prospectively in randomised trial with 4 cycles of dose-dense doxorubicin and docetaxel, with or without tamoxifen, prior to surgery.	IHC for BCL2, ER, PR, Ki-67, HER2, p53 (196).	Menopausal status; clinical tumour size and nodal status; grade; clinical response after two cycles; pathological complete response.
Talley *et al*., 2008	100 IDC in pre-menopausal women, aged 45 years and younger. 100 IDC in post-menopausal women, aged 65 years and older. IDC were selected so that the proportions of high and low/moderate grade carcinomas were equal in the pre- and post-menopausal groups.	IHC for BCL2, EGFR, p185erbB-2 and p53. Multivariate analysis of survival.	Survival by age and biological factors.
Dawson *et al*., 2010	11 212 women with early-stage breast cancer, enrolled into any of 5 studies.	Meta-analysis of 5 studies. Cox model incorporating the time-dependent effects of each variable (tumour size, grade, lymph node status, treatment, mortality, BCL2, ER, PR and HER2 levels).	The prognostic significance of BCL2.
Zaha and Lazar, 2012	61 patients who had been followed up 5 years since diagnosis.	IHC for molecular subtyping and BCL2 assessment.	Age; tumour size; histological type and grade; clinical stage; lymph node status.

Abbreviations in the Table: IHC, immunohistochemistry; ER, oestrogen receptor; PR, progesterone receptor; HER, human epidermal growth factor receptor; DFS, disease free survival; OS, overall survival; IDC, infiltrating ductal carcinoma; EGFR, epidermal growth factor receptor.

Regarding the rate of BCL2 expression, controversial findings are reported. Positive BCL2 expression was detected in 46% of tumours [Rolland *et al*., 2007]. Higher frequencies have been described reaching 63-67.6% of breast cancer cases [Le *et al*., 1999; Alireza *et al*., 2008]. In contrast, Tsutsui *et al*. have analysed the breast cancer cases by loss of BCL2 expression. Decreased BCL2 protein expression was found in 42% of breast cancer cases [Tsutsui *et al*., 2006].

Clinical relevance of semiquantitative classification of the BCL2 immunostaining based on both the distribution and the intensity of the staining reaction was studied by Trere and colleagues. The proposed classification was validated in 69 breast cancer specimens by comparing the BCL2 immunostaining with the BCL2 messenger RNA levels evaluated by real-time reverse transcription – polymerase chain reaction. Highly significant association was found between protein and messenger RNA for BCL2 [Trere *et al*., 2007]. Similar experience was reported by Martinez-Arribas and colleagues. BCL2 expression in breast cancer by either IHC or real time PCR yields very similar results [Martinez-Arribas *et al*., 2007].

The hypothetic release of BCL2 protein into patient's serum has been evaluated. The BCL2 serum levels were 3.6 +/-1.1 ng/mL in the breast cancer patient group and 3.23 +/-0.06 ng/mL in the control group. A weak correlation was found between BCL2 serum levels and tissue expression of it (r=0.382, P=0.049). A positive and significant correlation was shown between BCL2 and menopause (r=0.523, P=0.005) and between age and serum BCL2 (r=0.488, P=0.011). Although a majority of the breast tumour tissue expressed BCL2, the mean BCL2 serum levels were not different between patient and control groups. Therefore, BCL2 expression should be analysed in tissue at the level of protein or messenger RNA expression, but the use of serum levels would be very limited for clinical or scientific purposes [Alireza *et al*., 2008].

There was a significant (P<0.001) inverse correlation between histological grade and immunoreactivity for BCL2 [Binder *et al*., 1995]. On univariate analysis BCL2 expression was correlated with the clinicopathological features of less aggressive disease [Rolland *et al*., 2007]. Also, decreased BCL2 protein expression, found in 105 (42%) cases, significantly correlated with a nuclear grade 3 [Tsutsui *et al*., 2006]. Any degree of BCL2 immunohistochemical staining significantly (P=0.0015) inversely correlated with high nuclear grade. The correlation was confirmed (P=0.006) by messenger RNA expression [Martinez-Arribas *et al*., 2007]. The BCL2 expression did not correlate with histological type of the tumour [Zaha *et al*., 2012]. The BCL2 protein

expression was significantly (P=0.048) associated with smaller tumour size [Martinez-Arribas *et al*., 2007]. Messenger RNA expression of the *BCL2* gene showed a significant (P=0.04) direct correlation with nodal invasion [Martinez-Arribas *et al*., 2007].

The correlation between BCL2 expression and hormone receptor status has been evaluated by several authors. A significant positive correlation (P<0.001) was observed between BCL2 expression and positivity for ER and PR by Binder *et al*., 1995. In addition, BCL2 expression was found to be significantly correlated with the expressions of ER and PR by Lee, Im *et al*., 2007. Loss of BCL2 was associated with ER negativity [Tsutsui *et al*., 2006]. BCL2 protein overexpression was significantly associated (P=0.0001) with hormone receptor positive tumours [Le *et al*., 1999]. The significant correlation between BCL2 and hormone receptor expression (ER, P=0.0003; PR, P=0.0002) was confirmed by messenger RNA expression (ER, P=0.0004; PR, P=0.001) in the study conducted by Martinez-Arribas *et al*., 2007.

BCL2 immunostaining was not related to positivity for HER1. It was negatively associated with overexpression of HER2 (P=0.04), whereas a strong positive correlation was found with expression of HER3 characterised by P=0.01 [Binder *et al*., 1995]. BCL2 expression showed significant inverse correlation with the expression of HER2 [Lee, Im *et al*., 2007]. The expression of BCL2 protein and messenger RNA significantly (P=0.0008 and P=0.016, respectively) inversely correlated with HER2 expression [Martinez-Arribas *et al*., 2007].

Significant differences were found analysing BCL2 expression regarding hormone receptor status and molecular subtype [Zaha *et al*., 2012].

Taking into account the anti-apoptotic action of BCL2, even low proliferative activity would be sufficient to establish tumour growth. Indeed, high proliferative activity as assessed by Ki-67 staining significantly (P<0.001) inversely correlated with BCL2 expression [Binder *et al*., 1995]. The expression of BCL2 protein significantly (P=0.02) inversely correlated with a Ki-67 labelling index >10% [Martinez-Arribas *et al*., 2007]. In the study of Lee *et al*., significantly inverse correlation was found between BCL2 expression and proliferative activity by Ki-67 [Lee, Im *et al*., 2007]. The incidence of a high nuclear grade and Ki-67 counts increased as the number of abnormal findings of BCL2, p27 and p53 protein expressions increased [Tsutsui *et al*., 2006].

More complex approach to the analysis of cell cycle regulation in BCL2 positive breast cancers has been undertaken as well. Experimental research work of Tsutsui *et al*. has shown that BCL2, which has been established as a key player in the control of apoptosis, participates in regulating the cell cycle and proliferation. Decreased BCL2 protein expression significantly correlated with decreased p27 protein expression, positive p53 protein expression, positive Ki-67 counts and a positive HER2 protein expression [Tsutsui *et al*., 2006]. The significant inverse correlation between BCL2 and p53 expression was confirmed by Lee, Im *et al*., 2007. Messenger RNA expression of the BCL2 gene showed a significant (P=0.014) inverse correlation with p53 expression [Martinez-Arribas *et al*., 2007].

The association between BCL2 and survival has been analysed as well. By univariate analysis, decreased BCL2 protein expression was significantly (P=0.0089) associated with a worse disease-free survival (DFS), while multivariate analysis indicated the lymph node status and Ki-67 counts to be independently significant prognostic factors. The prognostic value of BCL2 as well as p27 and p53 protein expression was dependent on the proliferation activity in breast cancer [Tsutsui *et al*., 2006]. Loss of BCL2 expression significantly (P<0.001 on univariate analysis) correlated with a reduction in survival [Rolland *et al*., 2007]. Better survival rates were observed for BCL2 positive tumours [Zaha *et al*., 2012].

BCL2 expression was one of the few factors significantly (P=0.002) associated with prolonged DFS. The other factors included ER positivity (P=0.013), and low p53 expression (P=0.032). Univariate analyses indicated that the tumour size (P=0.038) and the number of involved lymph nodes (P<0.001) significantly affected the recurrences. However, the type of surgery, the histology, histological grade, the presence of endolymphatic emboli, and a close resection margin did not. Knowing this, further multivariate analysis identified 10 or more involved lymph nodes (HR=7.366; P<0.001), negative BCL2 expression (HR=2.895; P=0.030), and HER2 overexpression (HR=3.535; P=0.001) as independent indicators of worse DFS. Patients with BCL2 expression had a significantly longer DFS even in the ER positive subgroup [Lee, Im *et al*., 2007].

As the prognostic evaluation of breast cancer by clinicopathologic variables is useful but imperfect, Callagy *et al*. evaluated the prognostic potential of Nottingham Prognostic Index (NPI) along with the expression of 13 biomarkers in 930 breast cancers on a tissue microarray. BCL2 was among those 8 markers, including also

hormone receptors, cyclin E, p53, proliferative activity, CK 5/6, and HER2 that showed a significant association with survival at 10 years on univariate analysis. On multivariate analysis BCL2 again was among the 3 markers that retained significance: BCL2 (HR=0.68; 95% CI=0.46-0.99; *P*=0.055); NPI [HR=1.35; 95% CI=1.16-1.56; *P*=0.0005] and ER status (HR=0.59; 95% CI=0.39-0.88; *P*=0.011). BCL2, used as a single marker, was more powerful than the use of a panel of markers. Based on these results, an independent series was used to validate the prognostic significance of BCL2. Here, BCL2 (HR=0.83; 95% CI=0.71-0.96; *P*=0.018) retained prognostic significance independent of the Nottingham Index (HR=2.04; 95% CI=1.67-2.51; *P*<0.001) with an effect that was maximal in the first 5 years [Callagy *et al*., 2006].

Initially, the prognostic effect of BCL2 was considered to be related to ER status. Dawson *et al.* tested the clinical validity of BCL2 as an independent prognostic marker. In univariate analysis, ER, PR and BCL2 positivity was associated with improved survival and HER2 positivity with inferior survival. For ER and PR this effect was time-dependent, whereas for BCL2 and HER2 the effect persisted over time. In multivariate analysis, BCL2 positivity retained independent prognostic significance (HR=0.76, 95% CI=0.66-0.88, *P*<0.001). BCL2 was a powerful prognostic marker independently of ER and HER2 status: in ER- (HR=0.63, 95% CI=0.54-0.74, *P*<0.001) and ER+ disease (HR=0.56, 95% CI=0.48-0.65, *P*<0.001), and in HER2- (HR=0.55, 95% CI=0.49-0.61, *P*<0.001) and HER2+ disease (HR=0.70, 95% CI=0.57-0.85, *P*<0.001), irrespective of the received adjuvant therapy [Dawson *et al*., 2010].

Studying 1000 high-risk breast cancer patients receiving radiotherapy, negative BCL2 expression was significantly associated with increased overall mortality, distant metastases and locoregional recurrence in multivariate Cox regression analyses. Kaplan-Meier probability plots showed a significantly improved overall survival for the BCL2 positive subgroup [Kyndi *et al*., 2008].

Multivariate analysis by Cox model showed that only 2 factors were independently linked to the risk of death: BCL2 protein overexpression, which decreased the risk (*P*=0.008) and number of metastasis in lymph nodes, which increased the risk (*P*=0.0001). When BCL2 overexpression was studied in relation to nodal status, hormone receptor status and treatment, no significant differences was observed. The BCL2 expression was also associated with a significantly lower risk of distant metastasis (*P*=0.04). Thus, it was suggested that BCL2 expression reveals

prognostically favourable phenotype of breast cancer, and it may therefore be used as a marker of long-term survival [Le *et al*., 1999].

Interestingly, BCL2 has been found to have prognostic role in particularly serious group of patients – young women affected by breast cancer. Young pre-menopausal women with breast carcinomas have an overall worse prognosis than older, post-menopausal women. Talley *et al*. assessed the BCL2 along with EGFR, HER2 and p53 in breast carcinomas in pre- and post-menopausal women with equivalent histologic grades. There were statistically significant differences in the BCL2, EGFR and p53 expression in carcinomas of high vs. low histologic grade. Despite the observation that there were no age-related differences in the expression frequency and/ or pattern, BCL2 and p53 had prognostic significance in overall study population and pre-menopausal, but not post-menopausal women [Talley *et al*., 2008].

Callagy, Webber *at al*. have also performed meta-analysis of the association between BCL2 expression and both disease-free survival and overall survival in female breast cancer. The re-analysis of 18 studies identified wide range of relative hazard estimates (0.85-3.03). However, pooled analysis of all groups (separating groups of studies unadjusted/ adjusted for other factors) invariably disclosed beneficial effect. The meta-analysis strongly supported the prognostic role of IHC-assessed BCL2 in breast cancer and showed that this effect is independent of lymph node status, tumour size and grade as well as a range of other biological variables on multivariate analysis [Callagy *et al*., 2008].

BCL2 was an independent predictor of clinical outcome, and its prognostic independence was maintained when lymph node-negative and -positive patients were considered separately. The subgroup of node-negative breast cancer patients with a negative BCL2 immunostaining had a very high probability of relapse or death (respectively about 5 and 7 times greater than patients with a positive BCL2 immunostaining). Moreover, BCL2 retained prognostic significance in subgroups of patients treated with adjuvant endocrine therapy or chemotherapy [Trere *et al*., 2007].

Von Minckwitz with colleagues investigated the predictive value of clinical and biological markers for a pathological complete remission after a preoperative dose-dense regimen of doxorubicin and docetaxel, with or without tamoxifen, in primary operable breast cancer. Pathological complete remission was observed in 9.7% of patients. Clinically negative axillary lymph nodes, poor tumour differentiation, negative ER and PR status, and loss of BCL2 were significantly predictive for a pathological

complete remission in a univariate logistic regression model, whereas in a multivariate analysis only the clinical nodal status and hormonal receptor status provided significantly independent information [von Minckwitz *et al*., 2008].

Thus, several investigators have confirmed that BCL2 is an independent predictor of breast cancer outcome [Callagy *et al*., 2006; Rolland *et al*., 2007; Tsutsui *et al*., 2006; Lee, Im *et al*., 2007; Kyndi *et al*., 2008; Dawson *et al*., 2010; Zaha *et al*., 2012]. Some investigators have limited the conclusion for early-stage breast cancer but extended it for all molecular types [Dawson *et al*., 2010], or focused on curatively resected stage III breast cancer patients [Lee, Im *et al*., 2007]. BCL2 expression in high-risk breast cancer has been evaluated as well [Kyndi *et al*., 2008]. Despite opposing tumour cell death, BCL2 is associated with biological features of the tumours which define a better intrinsic prognosis, such as hormone receptor expression, low proliferation and absence of HER2 and mutant *TP53* expression. This may in great part explain why BCL2 expression has been invariably found to correlate with a better prognosis of breast cancer [Martinez-Arribas *et al*., 2007].

The prognostic value of BCL2 expression could extend over the first 5 years after diagnosis [Callagy *et al*., 2006]. As addition of BCL2 to the Adjuvant! Online prognostic model, for a subset of cases with a 10-year follow-up, improved the survival prediction (P=0.0039), the effect can also have longer-lasting value [Dawson *et al*., 2010].

1.4.3. Cyclooxygenase-2 evaluation

The cyclooxygenase (COX) enzymes, COX-1 and COX-2, are critical in the biosynthesis of prostaglandins from arachidonic acid. Two isoforms of COX have been identified: COX-1, the constitutive isoform; and COX-2, the inducible form of the enzyme [Davies *et al*., 2002]. The human COX-1 protein is constitutively expressed in most tissues and maintains tissue homeostasis. Non-steroidal anti-inflammatory drugs decrease the inflammatory response by blocking both COX enzymes. COX-2 is a 70 kDa inducible enzyme that is responsible for prostaglandin synthesis at the site of inflammation. COX-2 can undergo rapid induction in response to many factors such as bacterial lipopolysaccharides, growth factors, cytokines and phorbol esters. COX-2 expression has also been linked to carcinogenesis, and specific COX-2 inhibitors have

been shown to have antitumour effects. Expression of COX-2 by IHC has been reported in a variety of normal tissues. The cellular staining pattern for anti-COX-2 is cytoplasmic [*Dako Denmark A/S, M3617*].

Many human cancers exhibit elevated prostaglandin levels due to upregulation of COX-2, a key enzyme in eicosanoid biosynthesis. Extensive pharmacologic and genetic evidence implicates COX enzymes in neoplasia [Harris *et al*., 2009]. COX-2 is also overexpressed in carcinoma of the breast [Davies *et al*., 2002]. COX-2 overexpression has been observed in about 40% of cases of invasive breast carcinoma and at a higher frequency in preinvasive DCIS tumours [Harris *et al*., 2009].

Interest in chemoprevention in oncology using suppressants of prostaglandin (PG) synthesis has been stimulated by epidemiological observations that the use of aspirin and other non-steroidal inflammatory drugs is associated with reduced incidence of some cancers, including cancer of the breast [Davies *et al*., 2002; Harris *et al*., 2009]. The breast cancer risk reduction can be as significant as 46% [Ashok *et al*., 2011]. However, the findings are unequivocal. It has been reported that prolonged use of COX-2 inhibitors is associated with an increased risk of breast and haematological carcinomas along with decreased risk of colorectal cancer [Vinogradova *et al*., 2011]. Lack or correlation has been reported as well [Cronin-Fenton *et al*., 2010].

COX-2-dependent activity is a necessary component for cellular and molecular mechanisms of breast cancer cell motility and invasion. COX-2 activity also modulates the expression of matrix metaloproteinases, which may be a part of the molecular mechanism by which COX-2 promotes cell invasion and migration. The studies suggest that COX-2 assists in determining and defining the metastatic signalling pathways that promote the breast cancer progression to metastasis [Larkins *et al*., 2006].

Complementary experimental studies have established that both conventional nonsteroidal anti-inflammatory drugs and selective COX-2 inhibitors suppress mammary tumour formation in rodent breast cancer models. Furthermore, knocking out COX-2 reduces mammary tumourigenesis and angiogenesis, and, conversely, transgenic COX-2 overexpression induces tumour formation. However, lack of correlation between COX-2 expression and microvessel density has been reported as well [Thorat *et al*., 2009]. In breast cancer cell model, COX-2 has been implicated in the epithelial-mesenchymal transformation and thus in the development of metastasis [Bisaro *et al*., 2012]. The COX-2 expression in mouse breast cancer controls development of bone metastasis [Karavitis *et al*., 2012]. The utility of COX/PG signalling as a target for

chemoprevention has been established by randomized controlled clinical trials. However, these studies also identified increased cardiovascular risk associated with use of selective COX-2 inhibitors. Thus, current efforts are directed toward identifying safer approaches to antagonizing COX/PG signalling for cancer prevention and treatment, with a particular focus on PGE2 regulation and signalling, because PGE2 is a key pro-tumourigenic prostanoid [Howe, 2007]. In breast cancer cell cultures, COX-2 has been implicated in the metastatic potential of chemotherapy-resistant breast cancer cells [Kang *et al*., 2011], in stem-like breast cancer cells and disseminated cancer cells [Singh *et al*., 2011].

Several authors have studied the role of COX-2 in breast cancer [Ristimäki *et al*., 2002; Leo *et al*., 2006; Nassar *et al*., 2007; Zerkowski *et al*., 2007; Lee *et al*., 2010; Ciris *et al*., 2011; Park *et al*., 2012]. The logistics and the applied methods of selected representative studies are highlighted in Table 1.3. but the main results are discussed subsequently.

Table 1.3.

Logistic and technological characteristics of selected studies devoted to cyclooxygenase-2 analysis in breast cancer tissues

Author	Patients and materials	Methods	The analysed correlations
Ristimäki *et al*., 2002	1576 invasive breast cancers.	Tissue array construction. IHC for COX-2, ER, PR, Ki-67, p53. FISH for *HER2* amplification.	Tumour size; grade; lymph node status; ER / PR status; proliferation rate.
Leo *et al*., 2006	39 patients: paired samples of invasive cancer and normal breast epithelium; 29 patients: also ductal carcinoma *in situ*.	IHC for COX-2 by monoclonal antibody.	COX-2 in normal tissue, *in situ* and invasive cancer.
Nassar *et al*., 2007	43 breast carcinomas and 5 normal breast tissues.	Tissue microarray construction. IHC for COX-2 by monoclonal antibody. Expression was assessed as intensity and scored as percentage of positive cells. Prognostic parameters and follow-up information: obtained from the hospital records.	Disease-free and overall survival.
Zerkowski *et al*., 2007	669 primary breast cancers, stage I-III.	Tissue microarray. IHC for COX-2. AQUA and X-tile: algorithms for quantitative analysis of protein expression and determination of optimal cut-points.	The total tumour and subcellular expression of COX-2; clinicopathologic factors and survival.
Lee *et al*., 2010	80 breast cancer patients (January 2005 – February 2007).	IHC for COX-2. COX-2 cutoff: 10%. Outcome: locoregional recurrence, 2; systemic metastasis, 8; died, 1 patient.	Age; tumour size and grade; nodal status; ER / PR status; HER2 positivity.
Park *et al*., 2012	861 breast cancers.	IHC for COX-2 and Ki-67. Univariate and multivariate analyses.	Clinicopathological parameters and survival.

Abbreviations in the Table: IHC, immunohistochemistry; COX, cyclooxygenase; ER, oestrogen receptor; PR, progesterone receptor; FISH, fluorescent *in situ* hybridization; HER, human epidermal growth factor receptor.

Controversial data are reported regarding the frequency of COX-2 overexpression in breast cancer. At least partially the discrepancies can be attributed to different evaluation systems. In the study of van Nes *at al*., the COX-2 expression was scored and the cases classified into low expression or high expression group in relation to the average value [van Nes *et al*., 2011]. Alternatively, the rate of COX-2 expression was 15% using the cut-off value of 10% neoplastic cells [Lee *et al*., 2010]. In another study, 95% of the breast carcinomas showed cytoplasmic COX-2 expression [Nassar *et al*., 2007]. In contrast, COX-2 overexpression was observed in 46.8% of surgical specimens [Guo *et al*., 2008]. Moderate to strong elevated expression of COX-2 protein was observed in 37.4% of breast cancers by Ristimäki *et al*., 2002. In a large cancer group, COX-2 was positive in 57.3% of invasive tumours [Park *et al*., 2012]. Most of authors have reported expression of COX-2 in tumor cells. However, stromal areas showed higher expression in the study of Richardsen *et al*., 2012. The observations of the role of macrophages inducing COX-2 expression in breast cancer cells [Hou *et al*., 2011] are in agreement with these findings as so far as the importance of stromal compartment is emphasized [Troester *et al*., 2009]. However, significant discrepancies exist in the primary data. COX-2 can be implicated in the epithelial-stromal interactions facilitating the transformation of *in situ* cancer into invasive tumour [Hu *et al*., 2009].

No statistically significant correlation was found between COX-2 expression and patient's age [Lee *et al*., 2010].

It has been reported that COX-2 expression was not associated with tumor size and grade [Lee *et al*., 2010]. In contrast, COX-2 intensity and percentage of positive cells correlated significantly with size of carcinoma (P=0.0271; P=0.0539, respectively), and COX-2 intensity correlated significantly with histologic grade (P=0.0182) as described by Nassar *et al*., 2007. In the study performed by Zerkowski *et al*., 2007, COX-2 expression also was directly associated with nuclear grade. Significant association between COX-2 overexpression and high histological grade (P=0.026) was also described by Ciris *et al*., 2011. The correlation between elevated COX-2 expression, large tumour size and a high histological grade was identified also by Ristimäki *et al*., 2002. Elevated COX-2 was associated a high proliferation rate and high p53 expression, (P<0.0001 for both comparisons), along with axillary node metastases and a ductal type of histology (P<0.0001 and P<0.0017, respectively) as described by Ristimäki *et al*., 2002. In contrast, association with lobular cancer type was reported by Holmes *et al*., 2011.

In normal breast, ductal carcinoma *in situ* and invasive ductal cancer, the COX-2 overexpression rate was 0%, 84%, and 58.8%, respectively. The differences in COX-2 expression between cancer and benign tissues were significant ($P<0.0001$) as well as the differences between in situ and invasive cancer ($P=0.042$) as described by Ciris *et al*., 2011. Recently it was demonstrated that COX-2 protein is mainly located in plasma membrane of lobular intraepithelial neoplasia cells suggesting localization in caveolae-like structures [Perrone *et al*., 2007]. Leo *et al*. has also hypothesised that COX-2 overexpression is an early event in breast carcinogenesis. Comparing COX-2 protein expression in normal breast tissue, carcinoma *in situ* and invasive breast cancer in samples from the same patients, the findings were concordant. COX-2 expression in normal breast tissue was an indicator for COX-2 expression in the paired breast tumours. There was no significant correlation between COX-2 expression and pathologic tumour stage, nodal status, hormone receptor status, tumour size, grading, and lymphovascular space involvement in this study [Leo *et al*., 2006]. Similar COX-2 expression in invasive and *in situ* cancer has been reported by meta-analysis; this also would lead to the conclusion that COX-2 expression is an early event in breast carcinogenesis [Glover *et al*., 2011].

Although COX-2 expression has been associated with lymph node metastasis [Nassar *et al*., 2007], this was not confirmed by Lee *et al*., 2010. In contrast, COX-2 expression was strongly correlated with vascular endothelial growth factor-C expression ($P<0.01$), lymphangiogenesis characterised by number of lymphatic capillaries ($P=0.032$) and metastatic lymph nodes ($P=0.035$) in the study performed by Guo *et al*., 2008. The correlation between COX-2 expresion and higher stage was reported also by Holmes *et al*., 2011.

There was no statistically significant correlation between COX-2 expression and hormone receptor status. However, among ER/ PR positive tumours, COX-2 expression was related to larger size ($P=0.001$ and $P=0.009$, respectively) and nodal status ($P=0.048$ and $P=0.009$, respectively) as reported by Lee *et al*., 2010. In contrast, tumours characterised by COX-2 and progesterone receptor co-expression have been characterised by smaller size and lower number of axillary lymph node metastasis [Almeida *et al*., 2011].

Lack of correlation between COX-2 and ER has been confirmed also by other research groups [Nassar *et al*., 2007; Holmes *et al*., 2011] but rejected by still other investigators. Interestingly, Holmes *et al*. observed positive association between COX-2

and hormone receptor status but worse prognosis [Holmes *et al*., 2011]. COX-2 expression has been inversely associated with ER and PR [Zerkowski *et al*., 2007]. Elevated COX-2 was associated with negative hormone receptor status [Ristimäki *et al*., 2002]. In contrast, significant positive association between COX-2 and hormone receptor expression (P<0.0010) has been reported recently [Dhakal *et al*., 2012].

There is evidence that COX-2 expression is associated with HER2 over-expression [Nassar *et al*., 2007; Rozenowicz *et al*., 2010]. Statistically significant correlation (P<0.0001) between COX-2 expression and *HER2* oncogene amplification was showed also by Ristimäki *et al*., 2002. In contrast, no statistically significant correlation was found between COX-2 expression and HER2 positivity in other, more recent studies [Lee *et al*., 2010; Miglietta *et al*., 2010]. COX-2 overexpression had a significant relationship with HER2 overexpression (P=0.026) as described by Ciris *et al*., 2011.

COX-2 expression has been studied in the context of fragile histidine triad – a putative tumour suppressor gene that is could be involved in the carcinogenesis of breast cancer. Loss of fragile histidine triad expression has been observed in up to 72% of breast cancers and has been associated with increased p53, a high proliferation index, and increased tumour size and grade. However, no association between COX-2 and expression of fragile histidine triad has been observed [Arun *et al*., 2005].

In breast cancer, the prognostic impact of COX-2 expression varies widely between studies. van Nes *et al*. examined the prognostic value of COX-2 expression in a large cohort of breast cancer patients treated with surgery (1985-1994) and followed-up for the median period of 19 years. COX-2 was scored using a weighted histoscore. The COX-2 expression was prognostically important regarding disease-free survival and overall survival. However, COX-2 did not remain independent in multivariate analysis. In patients with hormone receptor positive tumours, COX-2 expression had a negative influence on outcome (low versus high: disease free survival, HR=1.37, 95% CI=1.07-1.76, P=0.013). This effect disappeared when endocrine therapy was administered (low versus high: disease free survival, HR=0.93, 95% CI=0.51-1.70, P=0.811) while it remained statistically significant when endocrine therapy was omitted (low versus high: disease free survival, HR=1.48, 95% CI=1.12-1.94, P=0.005) as described by van Nes *et al*., 2011. Thus, these findings suggest that COX-2 plays role in hormonal signalling. In contrast, high COX-2 has been associated with local recurrence, cancer-related death

and reduced disease-free and disease-related overall survival in ER-negative but not ER-positive disease [Nassar *et al*., 2007].

COX-2 did not correlate with DFS and OS in the study of Nassar *et al*., 2007. In contrast, Guo *et al*. showed that patients with COX-2 positive tumours had a significant shorter survival time than those with negative tumours, including DFS and OS. The respective P values were 0.010 and 0.040 [Guo *et al*., 2008]. These findings are in agreement with the research performed by Zerkowski *et al*., 2007. Here, patients with high non-nuclear expression of COX-2 had significantly worse survival. Multivariate analysis showed that COX-2 remained a significant prognostic factor for survival independent of tumour size, nodal status, ER, *HER2/neu*, and grade [Zerkowski *et al*., 2007]. Stromal expression of COX-2 is shown to have negative prognostic significance [Richardsen *et al*., 2012]. Elevated expression of COX-2 protein was associated with unfavourable distant disease-free survival ($P<0.0001$) in the study of Denkert *et al.*, 2003. Association with unfavourable outcome was especially apparent in the subgroups defined by oestrogen receptor positivity, low p53 expression, and no *HER2* amplification ($P<0.0001$ for all comparisons). These results indicated that elevated COX-2 expression is more common in breast cancers with poor prognostic characteristics and is associated with an unfavourable outcome [Ristimäki *et al*., 2002].

Worse prognosis in COX-2 positive cancers was reported by Holmes *et al*., 2011 despite the association between COX-2 and hormone receptor positivity in their study group [Holmes *et al*., 2011]. Another challenging observation is the reported survival advantage in patients taking non-steroidal anti-inflammatory drugs [Holmes *et al*., 2011].

Interesting observations are brought by COX-2 expression studies by cell proliferation. COX-2 was associated with favourable markers, but was not related to survival outcome by itself. However, COX-2 in proliferative tumours [COX-2(+)/Ki-67(+)] were significantly associated with unfavourable factors and the worst survival, but COX-2 in non-proliferative tumours [COX-2(+)/Ki-67(-)] showed significantly favourable parameters and better outcomes. COX-2(-)/Ki-67(any) showed intermediate prognosis. The statistical significance was maintained in stage-matched and multivariate analyses. The results suggested that COX-2 expression may act in a different ways by the proliferation status of the tumour cells [Park *et al*., 2012].

Implications of COX-2 in early breast cancer treatment have been suggested [Filipovic *et al*., 2011]. Criteria for COX-2 inhibitor combinations in the treatment of

invasive breast cancer have been discussed [Kim *et al*., 2012] as COX-2 inhibitor celecoxib has antiproliferative effects on human breast cancer cell lines [Bocca *et al*., 2011]. A challenging study was triggered by the following observations. In a clinical research study combination of aromatase inactivator exemestane and COX-2 inhibitor celecoxib for the treatment of breast cancer was as effective as exemestane alone. The effect of celecoxib in clinical study was disappointing indicating that COX-2 may not be as crucial for the progression of human breast cancer as previously described. Authors searched for technological short-comings that could explain the previous data. They suspected lack of quantitative measurements in the immunohistochemistry and conventional real time PCR as the possible source of mistake. To quantitatively measure the expression of COX-2 in breast cancer tissues, tumour-adjacent tissues, and healthy breast tissues, Boneberg and colleagues performed real-time PCR analysis and normalized the expression of COX-2 to the mean expression of four different housekeeping genes. The expression of COX-2 messenger RNA was decreased in the breast cancer samples (mean COX-2 expression: 0.3, 95% CI=0.2-0.4) in comparison with tumour-adjacent tissues (mean: 2.0, 95% CI=1.3-2.7) and healthy tissues (mean: 1.0, 95% CI=0.5-1.4). The median expression of COX-2 in tumour tissues was only 20% of the median expression in healthy tissues (P<0.001) and only 10% of that of tumour-adjacent tissues (P<0.001). Some tumour samples showed only about 1% of the messenger RNA levels measured in tumour-adjacent tissues. Final results indicate that COX-2 is expressed at reduced levels in human breast cancer, not over-expressed as previously reported. Furthermore, the authors did not find any correlation of COX-2 messenger RNA expression in the tumour tissues with the messenger RNA expression of *HER2/neu*, ER or PR. The loss of COX-2 expression in established breast cancers that were observed could explain the failure of celecoxib to inhibit tumour growth in the cited clinical trial [Boneberg *et al*., 2008].

Reduced risk for bone metastasis in breast cancer patients who use COX-2 inhibitors were reported [Valsecchi *et al*., 2009]. COX-2 is linked to cancer cell infiltration in brain and lungs [Bos *et al*., 2009].

1.4.4. Cyclin D1 in breast cancer

Cyclin D1 (synonyms: PRAD-1, CCND1, Bcl-1) is a 36 kDa protein encoded by the *CCND1* (bcl-1) gene located on chromosome 11q13. Cyclin D1 associates with and activates cyclin dependent kinases (CDK): CDK4 and CDK6. The protein functions as a CDK-dependent regulator of the cell cycle by phosphorylating and inactivating the retinoblastoma protein, thereby allowing for progression through the G1-S phase of the cell cycle. Cyclin D1 is also involved in CDK-independent functions including associating with and regulation of a variety of transcription factors and transcriptional co-regulators, as well as the regulation of cellular metabolism, fat cell differentiation and cell migration. Cyclin D1 overexpression is associated with tumourigenesis, and cyclin D1 amplification and/or overexpression have been demonstrated in a variety of human tumours, including mantle cell lymphomas, breast carcinomas, head and neck squamous cell carcinomas and oesophageal cancers. Among lymphoid neoplasms, a subset of chronic lymphocytic leukaemia, small lymphocytic lymphoma and multiple myeloma have been reported to express cyclin D1 [*Dako Denmark A/S, M3642*].

The cellular staining pattern of cyclin D1 is predominantly nuclear. In breast, the positively staining tissue elements are ductal epithelial cells (<10%).

The materials and methods used in several representative studies of cyclin D1 in breast cancer [Umekita *et al*., 2002; Stendahl *et al*., 2004; Bilalović *et al*., 2005; Reis-Filho, Savage *et al.*, 2006; Guo *et al*., 2007; Lee, Park *et al*., 2007; Cho *et al*., 2008; Elsheikh *et al*., 2008; Rudas *et al*., 2008; Aaltonen *et al*., 2008; Lundgren *et al*., 2012] are presented in Table 1.4. The main findings are jointly discussed later.

Table 1.4.

Logistic and technological characteristics of selected studies devoted to cyclin D1 analysis in breast cancer tissues

Author	Patients and materials	Methods	The analysed correlations
Umekita *et al*., 2002	173 females with invasive ductal carcinoma that had had mastectomy (161) or breast-conserving surgery (12).	IHC for cyclin D1 by monoclonal antibody (clone DCS-6) and labelled streptavidin – biotin method in paraffin-embedded tissues. Follow-up: postoperative follow-up for 6-119 months (median 86 months).	Tumour size; histologic grade; lymph node status; ER status; OS; RFS.
Stendahl *et al*., 2004	167 postmenopausal breast cancer patients randomised to 2 years of tamoxifen treatment or no treatment.	Tissue array construction. IHC for cyclin D1. Follow-up: median, 18 years.	ER status; survival regarding tamoxifen treatment.
Bilalović *et al*., 2005	Medical records and formalin-fixed, paraffin-embedded breast cancer tissue of 48 patients (1998).	IHC for ER, PR, BCL2, and cyclin D1 by monoclonal antibodies. Follow-up: mean, 61 months (range: 4-103 months).	Tumour grade; size; lymph node status; ER/ PR status; BCL2; OS; RFS.
Reis-Filho, Savage *et al.*, 2006	245 breast cancer patients.	IHC for cyclin D1 by SP4 monoclonal antibody. CISH by Zymed CCND1 SpotLight probe. CISH signals were counted in 60 neoplastic cells. Amplification was defined as 45 signals per nucleus in more than 50% of cancer cells, or large gene copy clusters.	ER/ PR status; basal-like markers; overexpression; amplification; overall survival; disease-free survival.
Guo *et al*., 2007	Early breast carcinomas (18) and invasive ductal carcinomas (80).	IHC for cyclin D1, Ki-67, pRb, and p53. Southern blot and RT-PCR for alteration of *cyclin D1* gene and over-expression of cyclin D1 mRNA.	Ki-67; pRb; p53 expression; protein and mRNA overexpression; gene amplification; five-year survival.

Table 1.4. (end)

Lee, Park *et al.*, 2007	333 invasive breast cancer specimens.	IHC for cyclin D1 and ER. FISH and IHC to detect HER2 status.	ER; HER2; mortality.
Cho *et al.*, 2008	Tissue from the primary tumour and matching lymph node metastases of 73 breast cancer patients.	Cyclin D1 status evaluation along with HER2 and EGFR expression in primary tumours and metastases by IHC and CISH.	Overexpression in primary and metastatic tumour.
Elsheikh *et al.*, 2008	880 unselected invasive breast cancer cases.	Tissue microarray construction. IHC for cyclin D1 protein by rabbit monoclonal antibody SP4. CISH for *CCND1* amplification by Spotlight *CCND1* probe.	ER status; molecular subtype; amplification and overexpression.
Rudas *et al.*, 2008	Surgical specimens of 253 breast cancers, ABCSG Trial 05 and 948 cancers, ABCSG Trial 06.	IHC for cyclin D1. Cox models.	Overall survival; RFS.
Aaltonen *et al.*, 2008	53 *BRCA1*, 58 *BRCA2*, 798 familial non-*BRCA1/2*, and 439 sporadic breast tumours.	Tissue microarray construction. IHC for cyclins E and D1.	Grade; Ki-67; ER status.
Lundgren *et al.*, 2012	1155 postmenopausal, oestrogen receptor positive breast cancer patients.	IHC for cyclin D1 protein expression. CISH for CCND1 amplification status.	Recurrence; tamoxifen treatment effect.

Abbreviations in the Table: IHC, immunohistochemistry; ER, oestrogen receptor; OS, overall survival; RFS, relapse – free survival; PR, progesterone receptor; CISH, chromogenic *in situ* hybridisation; RT-PCR, real time - polymerase chain reaction; mRNA, messenger ribonucleic acid; FISH, fluorescent *in situ* hybridization; EGFR, epidermal growth factor receptor. ABCSG-Austrian Breast and Colorectal Cancer Study Group.

The frequency of cyclin D1 overexpression in breast cancer ranges from 42% to 60% [Umekita *et al*., 2002; Guo *et al*., 2007; Rudas *et al*., 2008]. The findings depend on the applied methods significantly. Thus, strong cyclin D1 expression and *CCND1* amplification were found in 67.4 and 14.5% of the cases, respectively. However, a strong correlation (P<0.0001) between cyclin D1 overexpression and *CCND1* amplification was demonstrated as well [Reis-Filho, Savage *et al*., 2006]. Similarly, positive rate of cyclin D1 protein in invasive ductal carcinoma (52.5%) was slightly higher than overexpression rate (40.8%) of cyclin D1 messenger RNA but significantly higher than amplification rate (18.4%) of cyclin D1 gene [Guo *et al*., 2007]. In the study of Elsheikh *et al*., 9.6% tumours showed *CCND1* amplification and 43.6% showed strong cyclin D1 expression. A strong positive correlation (P<0.001) between *CCND1* amplification and higher levels of cyclin D1 expression was found [Elsheikh *et al*., 2008]. However, cyclin D amplification was not related to protein overexpression in the study performed by Cho *et al*., 2008. Close correlation between Western blot and IHC findings are reported [Kamel *et al*., 2006]. The cyclin D1 expression is significantly affected by neoadjuvant chemotherapy [Penault-Llorca *et al*., 2008].

The concordance between the primary lesion and the metastatic regional lymph nodes is 63% for cyclin D1 protein overexpression and 85% for gene amplification status by chromogenic in situ hybridisation (CISH) as reported by Cho *et al*., 2008.

Regarding the spread and histological characteristics of breast cancer, cyclin D1 expression inversely correlated with tumour grade (P=0.010) and size (P=0.023) as described by Bilalović *et al*., 2005. In the ER-negative subgroup, overexpression of cyclin D1 significantly correlated with the lymph node status and tumour size [Umekita *et al*., 2002]. No expression of cyclin D1 was found in usual ductal hyperplasia and atypical ductal hyperplasia [Guo *et al*., 2007]. The expression of cyclin D1 significantly increased (P<0.05) in ductal carcinoma *in situ* [Zhou *et al*., 2009]. As this transition is accompanied by increasing number of ER-positive proliferating (Ki-67 positive) intraductal cells [Zhou *et al*., 2009], the expression of cyclin D1 can be not only early but also pathogenetically important event in breast carcinogenesis, hypothetically more related to low-grade cancer development [Abdel-Fatah *et al*., 2008].

The relation between ER and cyclin D1 has been highlighted by several researchers. Significant positive association with ER (P<0.000001) was found by Umekita *et al*., 2002 and confirmed by Bilalović *et al*., 2005 (P=0.001) and Lee, Park *et al*., 2007 (P<0.01). The findings are confirmed by results regarding PR expression and

CCND1 gene amplification. Cyclin D1 expression showed a positive correlation (*P*<0.0001) with ER and PR expression [Reis-Filho, Savage *et al*., 2006]. Both *CCND1* amplification and cyclin D1 expression were associated with positive ER status. *CCND1* gene amplification was an independent prognostic factor for patients with ER positive breast cancer. The results demonstrated a strong correlation between *CCND1* amplification and its protein expression in breast cancer. However, protein expression was more pervasive than gene amplification and associated with ER expression [Elsheikh *et al*., 2008]. It has been recommended to include cyclin D1 in co-expression pattern panels to distinguish estrogen receptor alpha / estrogen receptor beta pathways [Yang, Pfeiffer *et al*., 2007].

Antioestrogen treatment by tamoxifen is a well-established adjuvant therapy for ER positive breast cancer. Despite ER expression some tumours do not respond to tamoxifen. Stendahl *et al*. observed that 55 patients having strongly ER positive tumours with moderate or low cyclin D1 levels, responded to tamoxifen treatment whereas the 46 patients with highly ER positive and cyclin D1 overexpressing tumours did not show any difference in survival between tamoxifen and no treatment. Survival in untreated patients with cyclin D1 high tumours was slightly better than for patients with cyclin D1 low/moderate tumours. These findings suggest that cyclin D1 overexpression predicts tamoxifen treatment resistance in breast cancer [Stendahl *et al*., 2004].

The objective of Rudas and Austrian Breast and Colorectal Study Group study was to determine the clinical relevance of cyclin D1 expression in hormone receptor positive breast cancer patients who were treated with tamoxifen based therapy. Expression of cyclin D1 was associated with poor outcome. OS was significantly shorter in patients with cyclin D1 positive tumours compared with patients with cyclin D1 negative tumours (adjusted HR for death 2.47; 95% CI=1.08-5.63; *P*=0.03; adjusted HR for death 1.78; 95% CI=1.36-2.34; *P*<0.0001). RFS was also shorter in patients with cyclin D1 positive tumours than in patients with cyclin D1 negative tumours (adjusted HR for relapse 2.73; 95% CI=1.50-4.96; *P*=0.001; adjusted HR for relapse 1.52; 95% CI=1.14-2.04; *P*=0.005). Authors concluded that cyclin D1 expression is an independent poor prognostic factor in women with early-stage, hormone receptor - positive breast cancer who received adjuvant tamoxifen-based therapy [Rudas *et al*., 2008].

In ER-negative breast cancer patients, overexpression of cyclin D1 is an independent prognostic indicator. According to Cox's multivariate analysis in the ER-negative subgroup, overexpression of cyclin D1 had the most significant effect on overall (P<0.02) and RFS (P<0.0058), followed by nodal status and histologic grade [Umekita *et al*., 2002].

The overexpression of HER2 was associated with the high expression of cyclin D1 and the negative expression of ER (P<0.01 for both). In the HER2 overexpressing group of patients, low expression of cyclin D1 was associated with a significantly higher mortality and thus worse prognosis than high expression (RR=3.2; 95% CI=1.6-6.6) of cyclin D1 [Lee, Park *et al*., 2007]. No correlation between HER2, EGFR and cyclin D1 was observed by Cho *et al*., 2008.

An inverse correlation between IHC panel of basal-like markers and both cyclin D1 overexpression (P<0.0001) and *CCND1* amplification (P<0.0001) was reported by Reis-Filho, Savage *et al*., 2006. These findings are in agreement with the publication of Elsheikh *et al*., 2008 who reported infrequent *CCND1* amplification and cyclin D1 overexpression (P<0.001) in basal-like cancers [Elsheikh *et al*., 2008]. Cyclin D1 was not predictive of pathological complete response to neoadjuvant chemotherapy in triple-negative breast cancer [Li *et al*., 2011].

The expression of cyclin D1 is significantly positively associated with BCL2 (P=0.001) protein expression [Bilalović *et al*., 2005]. In Chinese patients with breast carcinoma, expression of cyclin D1 correlated with Ki-67 expression, but not with pRb and p53 expression [Guo *et al*., 2007].

Comparatively large volume of evidence is devoted to relation between cyclin D1 overexpression or gene amplification and the survival of breast cancer patients. However, conflicting results on the prevalence of cyclin D1 overexpression and its correlation with *CCND1* amplification and outcome of breast cancer patients have been reported. Part of the problems have been attributed to IHC technology as the limited sensitivity and specificity of most antibodies against cyclin D1 can result in problematic evaluation of cyclin D1 expression [Reis-Filho, Savage *et al*., 2006]. The splice variants of *CCND1* gene polymorphisms, cyclin D1b and cyclin D1a can have different influence on IHC findings and prognosis [Abramson *et al*., 2010].

Patients with higher cyclin D1 expression had longer OS (P=0.014) and RFS (P=0.037). Cox regression analysis for OS showed that lymph node status, ER

expression, therapy, and cyclin D1 expression were independent prognostic factors with *P* ranging 0.003-0.04 [Bilalović *et al*., 2005].

Univariate analysis revealed no association between overexpression of cyclin D1 and OS or RFS. However, in the ER-negative subgroup, overexpression of cyclin D1 significantly correlated with shorter OS ($P<0.018$) and RFS ($P<0.014$). In contrast, there were no significant associations between overexpression of cyclin D1 and clinical outcome in the ER-positive subgroup [Umekita *et al*., 2002].

When the group of the high cyclin D1 expression was analysed, the patients with negative expression of ER showed a significantly higher mortality than those with the positive expression of ER (RR=2.1; 95% CI=1.1-3.8). Positive expression of ER was associated with better prognosis in patients with high cyclin D1 expression [Lee, Park *et al*., 2007].

On univariate analysis cyclin D1 expression showed a correlation with longer OS. Neither cyclin D1 nor *CCND1* were independent prognostic factors for DFS or OS [Reis-Filho, Savage *et al*., 2006].

In Chinese patients with breast carcinoma, 5-year survival of the patients with positive expression of cyclin D1 (52.7%) was significantly lower than the cases with negative expression of cyclin D1 (72.1%). Thus, positive expression of cyclin D1 could serve as a poor prognostic marker independent of nodal metastasis and clinical stage. Expression of cyclin D1 protein is affected more directly by overexpression of cyclin D1 messenger RNA rather than cyclin D1 gene amplification [Guo *et al*., 2007].

Amplification of *CCND1* has been associated with increased risk of breast cancer recurrence. In contrast, nuclear expression of cyclin D1 protein was associated with decreased recurrence rate (Lundgren *et al*., 2012). High *CCND1* gene amplification is associated with poor prognosis in ER-positive breast cancer (Roy *et al*., 2010). The co-expression of cyclin D1b and cyclin D1a is associated with higher risk of breast cancer recurrence (Abramson *et al*., 2010).

Aaltonen *et al.* analysed the expression of critical cell cycle regulators cyclin E and cyclin D1 in familial breast cancer, focusing on *BRCA* mutation - negative tumours. Cyclin E expression in tumours of *BRCA1* or *BRCA2* carriers is higher and cyclin D1 expression lower, than in sporadic tumours. In univariate analysis, *BRCA1* tumours had significantly more frequently high cyclin E (88%) and low cyclin D1 (84%) expression than sporadic (54% and 49%, respectively) or familial non-*BRCA1/2* (38% and 45%, respectively) tumours. *BRCA2* tumours had significantly more frequently low cyclin D1

expression (68%) than sporadic or familial non-*BRCA1/2* tumours and significantly more frequently high cyclin E expression than familial non-*BRCA1/2* tumours. In a logistic regression model, cyclin expression, early age of onset, and ER and HER2 status were the independent factors most clearly distinguishing tumours of *BRCA1*-mutation carriers from other familial breast cancers. High cyclin E and low cyclin D1 expression were also independent predictors of *BRCA2* mutation when compared with familial non-*BRCA1/2* tumours. Lower frequency of high cyclin E expression also distinguished familial non-*BRCA1/2* tumours also from sporadic ones [Aaltonen *et al*., 2008].

Besides the prognostic role of cyclin D1, the predictive value of it has been explored regarding bortezomib treatment [Ishii *et al*., 2006].

1.4.5. Basal differentiation by cytokeratin 5/6

Cytokeratin 5/6 has been employed as a marker of basal differentiation resulting in association with triple negative molecular subtype that, in turn, has been related to younger age, high tumour grade, mitoses, high nuclear grade and p53 expression [Pillai *et al*., 2012; Rattan *et al*., 2012; Alshareeda *et al*., 2013]. However, the relationships between different basal cytokeratins and the basal-like or triple negative differentiation are complex [Alshareeda *et al*., 2013].

2. MATERIALS AND METHODS

2.1. Patients

Patients with primary, invasive breast carcinoma, diagnosed and routinely operated between January 2008 and December 2010 at Pauls Stradins Clinical University Hospital, Riga, were enrolled in the study. Patients without invasive component in tumour and those who have been treated with neoadjuvant chemotherapy before operation were excluded from study. All patients were women. In total, 383 consecutive cases corresponding to the above mentioned criteria were included in the study. The demographic characteristics of the cases are provided in the Results section.

Records of the Clinic of Surgery were reviewed to identify the clinical and treatment data. The morphological data were acquired in Institute of Pathology. The gross and microscopic evaluation was performed routinely on breast cancer protocol basis, aiming at complete description of morphological prognostic factors. The study was approved by the Committee of Ethics, Riga Stradiņš University.

2.2. Gross examination

Breast cancer protocol comprised two subsections: clinical information and pathology data, including gross and microscopic assessment. Clinical information (patient identification data, age, gender and performed therapy before operation) was filled in the Clinic of Surgery, but all morphological part (gross and microscopic data) was assessed and filled in Institute of Pathology by a single pathologist. Gross examination included measurement of the breast operation material weight and size in three dimensions, assessment of tumour localization in operation specimen, colour, edges of tumour (rounded, pushing vs. infiltrative), measurement of tumour size in three dimensions and shortest distance between tumour and surgical resection margin. Lymph nodes were sought for in the axillary tissue. The following tissue specimens were submitted for microscopic investigation: tissue from surgical resection lines (including shave examination of representative soft tissue and skin as well as perpendicular sections from tissues between tumour and the closest point of resection surface),

tumour, nipple, skin overlying tumour and mammary gland tissue outside the grossly evident tumour. All the identified lymph nodes were submitted for microscopic investigation as well.

2.3. Microscopy

The primary tumour tissue samples after gross examination were fixed in neutral buffered 10% formalin (Sigma-Aldrich, United States of America), processed in vacuum infiltration processor Tissue-Tek® VIP™ 6 (Sakura Seiki Co., Ltd., Nagano, Japan) and embedded in paraplast (Diapath S.r.l., Belgamo, Italy) using tissue embedding system TES 99 (Medite GmbH, Burgdorf, Germany). After embedding tissue samples from paraffin blocks were cut in four-micron-thick sections by microtome (Accu-cut SRM 200CW, Sakura Finetek Europa B.V., the Netherlands), put on slides (Santaks, Yanheng Huida Medical Instruments Co., Ltd, China) and stained with haematoxylin and eosin by automated tissue stainer (TST 44, Medite Medizintechnik, Germany). Stained slides were covered by cover glass (Prestige, Vemi S.R.L., Milano, Italy) employing automated cover slipper (Dako Coverslipper, Dako Denmark A/S, Glostrup, Denmark). Standard haematoxylin and eosin stained slides were examined under microscope to establish the following data: the tumour type, the differentiation grade, presence of secondary changes like necrosis, sclerosis, inflammation, microcalcifications, presence of peritumoural lymphatic, vascular and perineural invasion. Carcinoma *in situ*, surgical resection margins and status of lymph nodes were evaluated too. The tumours were diagnosed corresponding to the World Health Organization (WHO) classification of breast tumours [Tavassoli and Devilee, 2003], the tumour grade was appreciated based upon the Scarff - Bloom - Richardson classification modified by Elston and Ellis (Grade (G) 1 – well differentiated tumour; G 2 – moderately differentiated tumour; G 3 – poorly differentiated tumour) as described by Elston *et al.*, 1991. *In situ* ductal carcinoma lesions were classified in ductal carcinoma *in situ*, non-*comedocarcinoma* type, lobular carcinoma i*n situ* (LCIS) and ductal carcinoma *in situ*, *comedocarcinoma* type. The tumour pathological T and N characteristics (pathological TNM stage) was specified accordingly to the 7th edition criteria of *AJCC Cancer staging manual* [Edge *et al.*, 2010].

2.4. Immunohistochemistry

The formalin-fixed, paraffin-embedded tissues, cut at 3 micron thick sections on electrostatic slides (Histobond, Marienfeld, Germany) were investigated by immunohistochemistry, using heat-induced epitope retrieval in TEG buffer at pH 9.0 in microwave oven 3x5 min. Panel of primary antibodies against oestrogen receptor alpha (clone 1D5, dilution 1:1), progesterone receptors (clone PgR636, 1:1), E-cadherin (clone NCH-38, 1:50), actin (clone HHF35, 1:400), p53 (DO-7, 1:400), Ki-67 (clone MIB-1, 1:100), BCL2 oncoprotein (clone 124, 1:800), cyclooxygenase - 2 (clone CX-294, 1:200), cyclin D1 (clone EP12, 1:80) and cytokeratin 5/6 (clone D5/16 B4, 1:100) was employed. The optimal dilution, incubation time and antigen retrieval for BCL2 oncoprotein, cyclin D1, cyclooxygenase-2 and p53 protein was detected by total test. Peroxidase-conjugated polymeric visualisation system EnVision was applied for the detection of bound primary antibodies, followed by colour development by 3, 3'-diaminobenzidine. Cell nuclei were counterstained with haematoxylin. The slides were rinsed with distilled water and covered by cover glass (Prestige) using automated cover slipper (Dako Coverslipper). All IHC reagents were produced by Dako, Glostrup, Denmark. HER2 protein overexpression was detected by HercepTest^{TM} according to manufacturer's (Dako) instructions. Appropriate positive and negative controls were performed.

The cytoplasmic expression of actin was evaluated in the myoepithelial cell layer. Loss of myoepithelial cell layer in an appropriate morphological setting was considered an evidence of invasive breast cancer [Walker *et al.*, 2012].

The expression of E-cadherin was evaluated in cancer cell membranes as positive or negative. Positive expression of E-cadherine in appropriate morphological background was considered an evidence of ductal differentiation while complete loss of E-cadherin was the diagnostic criterion of lobular breast cancer [Arps *et al.*, 2013].

The evaluation of ER alpha and PR status was carried out according to the American Society of Clinical Oncology/ College of American Pathologists (ASCO/ CAP) guideline recommendations for IHC testing of ER and PR. The breast cancer case was considered positive if at least 1% of tumour cells showed positive nuclear staining of any intensity [Hammond *et al.*, 2010].

Membranous staining was scored for HER2 according to the HercepTest^{TM} as follows: 0 - no staining is observed or membrane staining is observed in less than 10%

of the tumour cells; 1 - a faint / barely perceptible membrane staining is detected in more than 10% of the tumour cells. The cells are only stained in part of their membrane; 2 - a weak to moderate complete membrane staining is observed in more than 10% of the tumour cells; 3 - strong complete membrane staining is observed in more than 30% of the tumour cells. HercepTest™ is interpreted as negative for HER2 protein overexpression (0 and 1+ staining intensity), weakly positive (2+ staining intensity), and strongly positive (3+ staining intensity) in accordance with *Dako HercepTest™, 16th ed*. By ASCO/CAP guideline recommendations for HER2 testing in breast cancer HER2 staining was regarded positive if >30% of cells showed distinct and complete membrane staining of HER2 in IHC or *HER2/neu* gene copies were amplified in fluorescent *in situ* hybridization with ratio of *HER2* to *CEP17* of > 2.2 or average *HER2* gene copy number > six signals/nucleus for those test systems without an internal control probe. The FISH technology is further described in more detail [Wolff *et al.,* 2007].

To evaluate the expression of Ki-67, the positively stained nuclei of neoplastic cells were counted and expressed as the percentage designated the Ki-67 index. The Ki-67 index was considered low if the value was below 14%, but high if it was equal or exceeded 14% of tumour cells [Goldhirsch *et al.,* 2011].

The percentage of tumour cells with nuclear staining for p53 was graded semi-quantitatively: score 0 = 0%, score 1 = 1-10%, score 2 = 11-50% and score 3 = >50%. For statistical analysis, p53 expression score 0 and 1 was considered as negative, but score 2 and 3 as positive [Yamashita *et al.*, 2006].

The BCL2 oncoprotein expression was considered true positive if it was present in the cytoplasm and/or membrane of cancer cells. The following semi-quantitative model of evaluation was employed: negative by score 0 - 0% and score 1 between 0 - 10%; positive if 10 - 50% positive cells score 2+; more than 50% positive cells score 3+ [Zaha *et al.,* 2012]. Callagy *et al*. recommended 10% cut-off value for BCL2 expression [Callagy *et al.,* 2006].

The evaluation of COX-2 expression regarded cytoplasmic reactivity. The evaluation was made by intensity scoring as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong) and by percentage of positive tumour cells. COX-2 was considered overexpressed when the intensity was scored 2 and 3 in more than 10% of positive tumour cells [Lee *et al.,* 2010].

Cyclin D1 immunostaining was evaluated as the percentage of cyclin D1 - stained nuclei. The cut-off point 10% cyclin D1 - positive tumour cells were applied [Rudas *et al.*, 2008].

Any cytoplasmic staining with the cytokeratin 5/6 in cancer cells was scored as positive [Callagy *et al.*, 2006].

Five breast cancer molecular subtypes were defined based on ER, PR, HER2 and Ki-67 levels determined by IHC. Positive ER and/or PR, negative HER2, low Ki-67 (<14%) corresponded to the luminal A subtype. Luminal B subtype was divided in two groups – luminal B (HER2 negative) and luminal B (HER2 positive). Luminal B (HER2 negative) was recognised by positive ER and/ or PR, negative HER2 and high Ki-67 (≥14%), but luminal B (HER2 positive) was identified by positive ER and/ or PR, positive HER2 or amplified *HER2/neu* and any level of Ki-67. HER2 positive breast cancer subtype was recognised by positive (3+) HER2 or amplified *HER2/neu*, in the absence of ER and PR. Absent ER, PR and HER2 defined triple negative breast cancer subgroup [Goldhirsch *et al.*, 2011].

The routine microscopy was viewed in Clinical Microscope ECLIPSE 55i (Nikon Corp., Tokyo, Japan), but image collection, IHC assessment and cell counting was made by Axiolab microscope (Carl Zeiss AG, Oberkochen, Germany). Nuclear immunostaining of ER, PR, Ki-67, p53 and cyclin D1 was evaluated by computed morphometry using the Kappa image base program (KAPPA opto-electronics INC., United States of America).

2.5. Fluorescent *in situ* hybridization

FISH was performed using the HER2 FISH pharmDx Kit (Dako, Glostrup, Denmark) according to the manufacturer's instructions on 4-µm-thick paraffin sections. Briefly, slides with tumour were deparaffinized and rehydrated at room temperature. After pretreatment, pepsin digestion, dehydration, probe application and seal of coverslip, automated denaturation and hybridization in Dako hybridizer (Dako, Glostrup, Denmark) was performed overnight (16 hours) at 45^0C. In next day after washing slides were counterstained with 4',6-diamino-2-phenyl indole and covered by coverslips. After 30 minutes fluorescence was observed in an Olympus CH30LF200 (Olympus Optical Co., LTD, Japan) fluorescence microscope at x1000 magnification

with a computerized imaging system. At least 50 cells in each histologic lesion and 50 control cells were evaluated for nuclear *HER2/neu* amplification. Results were expressed as amplification ratio, the ratio of the number of *HER2/neu* to those of *CEP 17* signals in the same cell. A score of 2 or greater was considered to indicate amplification according to the manufacturer's instructions. Normal ductal epithelia and lymphocytes in the same specimen served as control cells [Xu *et al.*, 2003].

2.6. Statistical analysis

All calculations were performed with the IBM SPSS Statistics Version 20.0 statistical software package (International Business Machines Corp., Armonk, New York, USA). Data were analysed using mean ± standard deviation, descriptive statistic methods as descriptive and cross tabulation with Chi-square, bivariate correlation as Spearman's rank correlation coefficient, non-parametric methods as Mann-Whitney U-test and Kruskal-Wallis one-way analysis of variance by ranks and parametric method - the one-way analysis of variance (ANOVA). Survival was evaluated by Kaplan-Meier analysis. A value of $P<0.05$ was considered statistically significant.

3. RESULTS

3.1. Basic characteristics of the study group

The study included 383 female with primary, invasive breast carcinoma, who were consecutively diagnosed and routinely operated between January 2008 and December 2010 at a single University Hospital, Riga, Latvia. The age of patients ranged from 27 to 88 years (mean ± standard deviation (SD), 59.59±12.22). The age distribution is shown in the Figure 3.1.

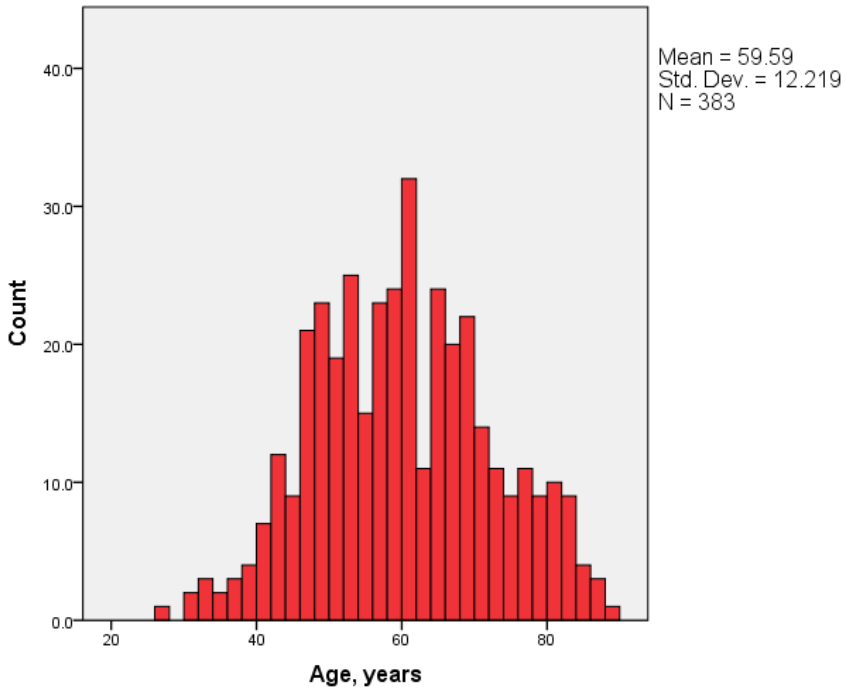


Figure 3.1. Age distribution of the recruited patients.

The following surgical approach was undertaken. Two hundred twenty nine patients underwent mastectomy (59.8%, 95% CI=54.8-64.5) as shown in Figure 3.2. and 154 ladies had segmental excisions (40.2%, 95% CI=35.5-45.2) of breast (Figure 3.3.). In 197 cases (51.4%, 95% CI=46.5-56.1) carcinoma affected the right breast, but in 186 cases (48.6%, 95% CI=43.9-53.5) – the left breast.

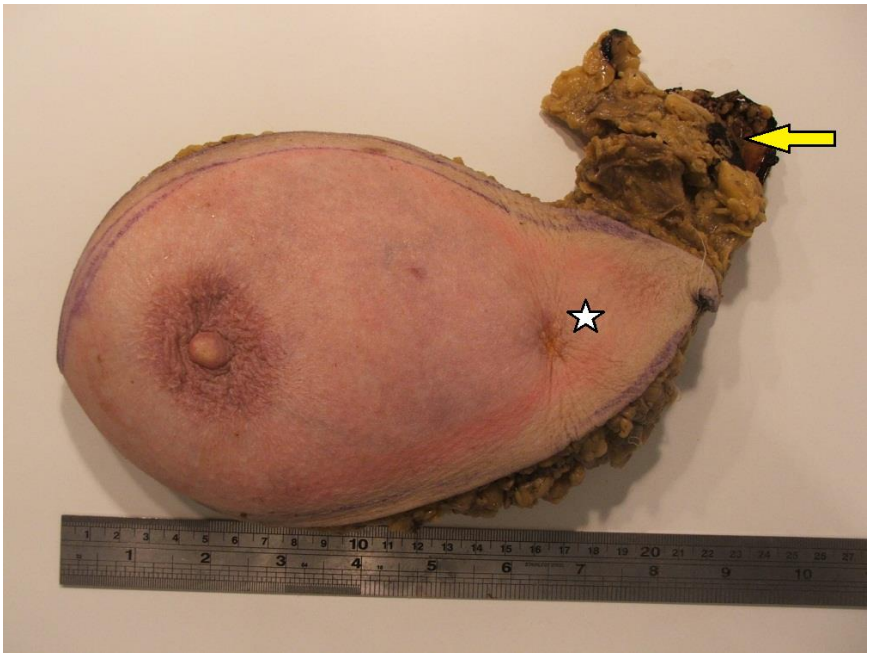


Figure 3.2. Mastectomy material by gross examination. Note the skin retraction due to breast cancer (star). *En bloc* axillary lymphadenectomy (arrow) has been performed as well. Photo image by A.Abolins.

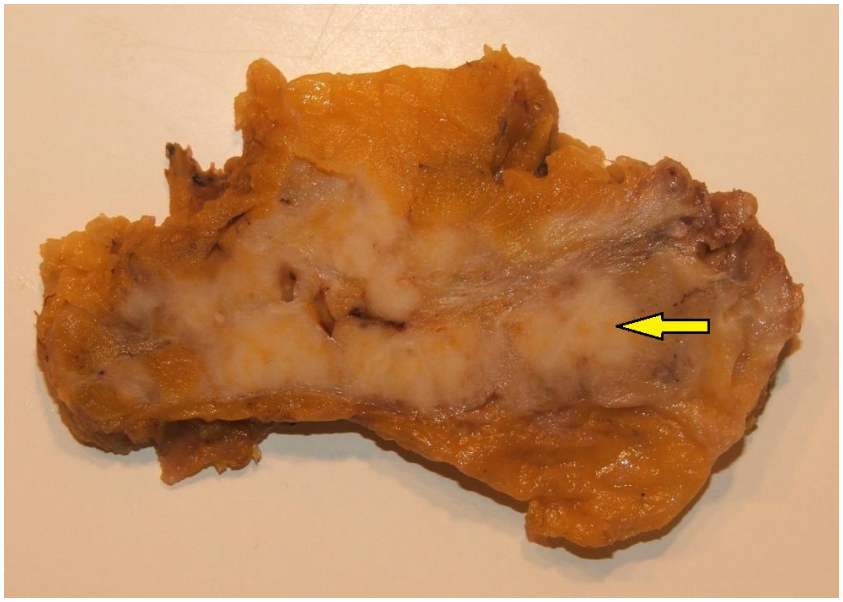


Figure 3.3. Segmental resection of breast tissue harbouring breast carcinoma (arrow). Photo image by A.Abolins.

Along with breast operation, 78 sentinel lymph node excisions (21.5%, 95% CI=17.7-26.0) and 284 axillary lymphadenectomies (78.5%, 95% CI=74.0- 82.3) were performed. Among all the 362 lymph node operations, the lymph nodes were successfully retrieved in 351 cases (97%, 95% CI=94.6-98.3). The mean count ± SD of retrieved axillary lymph nodes per case were 12.5±8.2. The number of breast carcinoma metastasis in the retrieved lymph nodes ranged 0 to 32 (mean amount ± SD, 2.8±5.0).

3.2. The morphological characteristics of the analysed tumours

Invasive ductal carcinomas constituted the largest part of cancers, respectively, 304 (79.4%) of 383 primary breast tumours. The other morphological types included 51 (13.3%) cases of invasive lobular carcinoma and 13 (3.4%) cases of mucinous breast cancer. A single case (0.3%) of tubular breast carcinoma also was diagnosed. The full spectrum of the revealed morphological types is shown in Table 3.1. and representative Figures 3.4.-3.7.

Table 3.1.

The distribution of breast carcinoma by histological type

Histological type of breast cancer	**Count**	**Frequency %**	**95% confidence interval, %**	
			Lower limit	Upper limit
Invasive ductal carcinoma (Figure 3.4.)	304	79.4	75.5	83.6
Invasive lobular carcinoma (Figure 3.5.)	51	13.3	9.9	16.7
Mucinous breast carcinoma (Figure 3.6.)	13	3.4	1.6	5.2
Apocrine carcinoma	4	1.0	0.3	2.1
Invasive cribriform carcinoma	3	0.8	0.0	1.8
Metaplastic breast carcinoma	2	0.5	0.0	1.3
Medullary breast carcinoma (Figure 3.7.)	2	0.5	0.0	1.3
Invasive papillary carcinoma	3	0.8	0.0	1.8
Tubular breast carcinoma	1	0.3	0.0	0.8
Total	383	100.0		

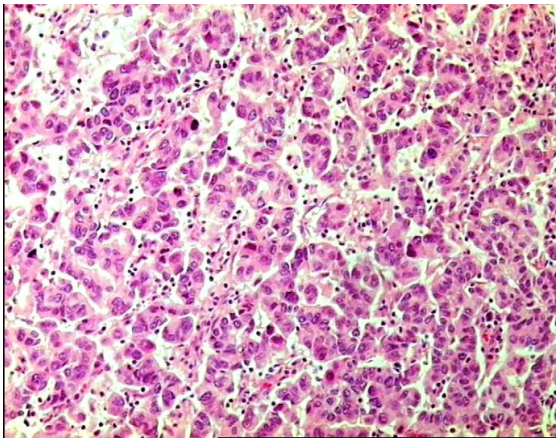


Figure 3.4. High grade invasive ductal breast carcinoma. Haematoxylin – eosin, original magnification 100 x.

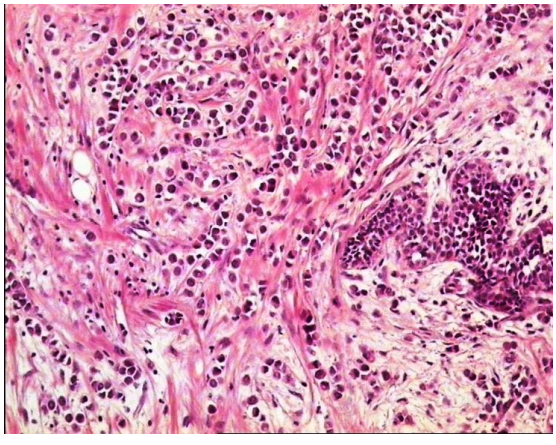


Figure 3.5. Invasive lobular carcinoma. Haematoxylin – eosin, original magnification 100 x.

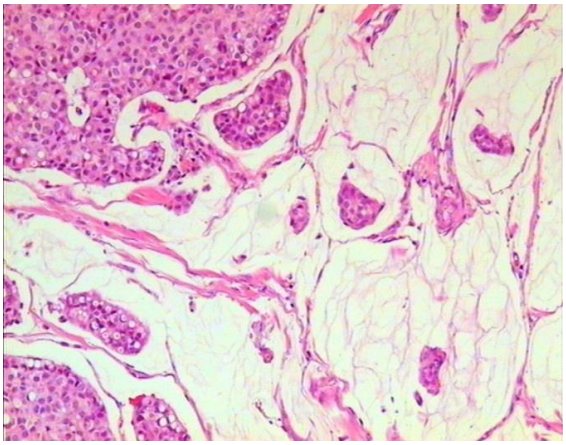


Figure 3.6. Mucinous breast carcinoma. Haematoxylin – eosin, original magnification 100 x.

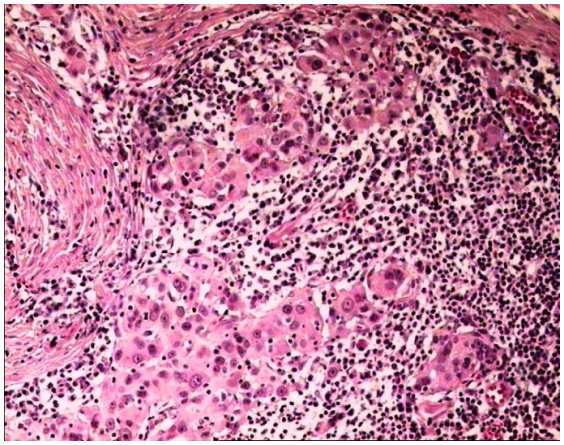


Figure 3.7. Medullary breast carcinoma. Haematoxylin – eosin, original magnification 100 x.

Microphotographs by A.Abolins.

According to pathological TNM classification, all 383 tumours were characterised as follows: pT1 – 161 tumours (42%); pT2 – 159 tumours (41.6%); pT3 – 35 tumours (9.1%) and pT4 – 28 tumours (7.3%). The relevant data are shown in Figures 3.8.-3.11.

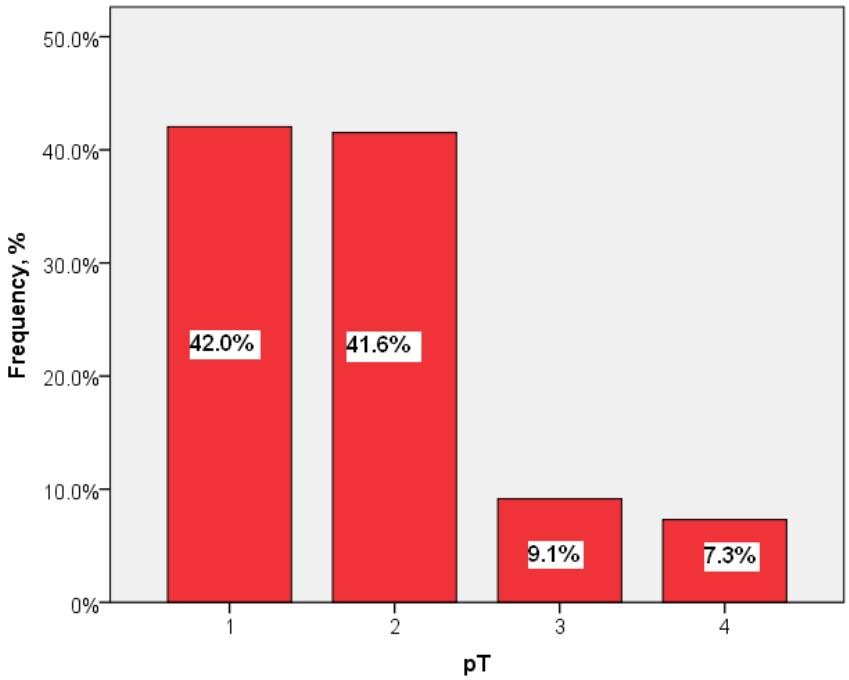


Figure 3.8. The distribution of breast carcinoma by local spread (pT).

Figure 3.9. pT1 by gross examination after segmental breast tissue excision. The represented tumour is characterised by marked *in situ* growth (arrow) that was associated with microinvasion.
Photo image by A.Abolins.

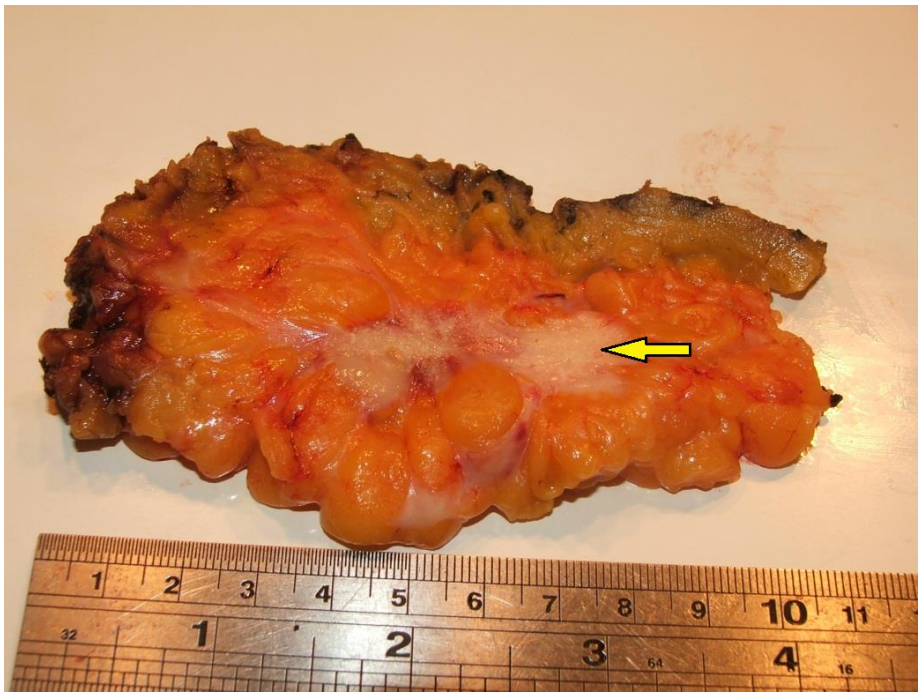


Figure 3.10. pT2 (arrow) by gross examination after segmental breast tissue excision.
Photo image by A.Abolins.

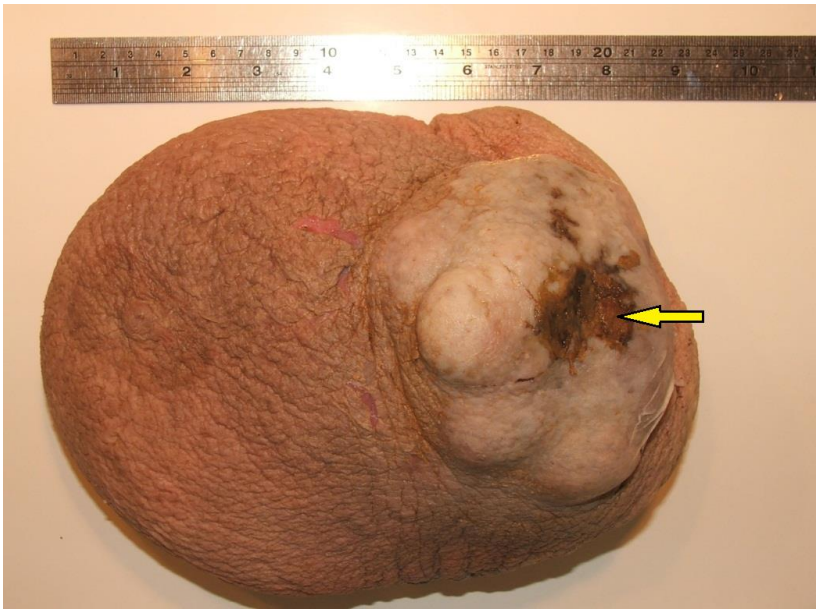


Figure 3.11. pT4 (arrow) by gross examination after mastectomy. Photo image by A.Abolins.

Regarding the pN category, there were 180 cases (47%) of pN0, 81 cases (21.1%) of pN1 (Figure 3.12.), 54 (14.1%) cases of pN2 (Figure 3.13.) and 36 cases (9.4%) of pN3. In 32 cases (8.4%), the lymph node status could not be established (pNx).

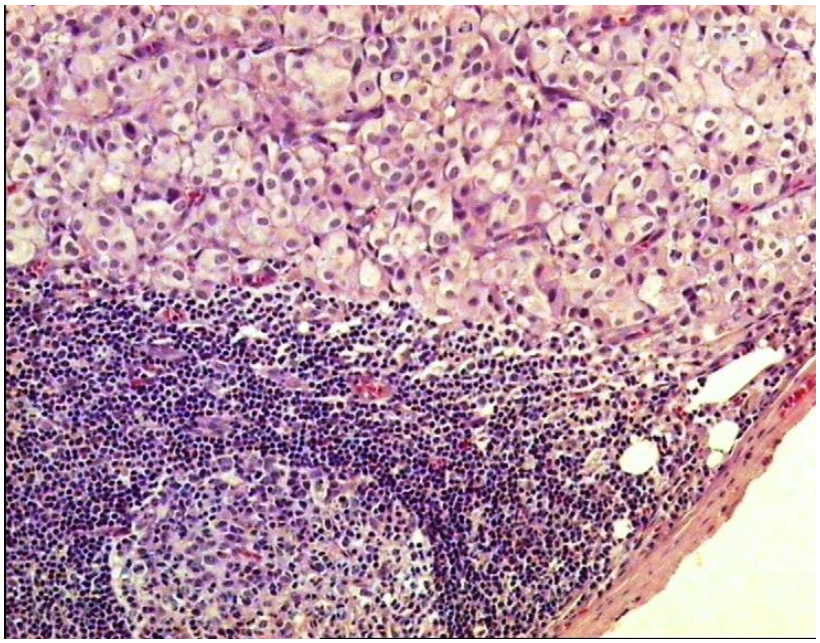


Figure 3.12. Metastasis of high grade ductal breast carcinoma in the lymph node. Haematoxylin – eosin, original magnification 100 x. Microphotograph by A.Abolins.

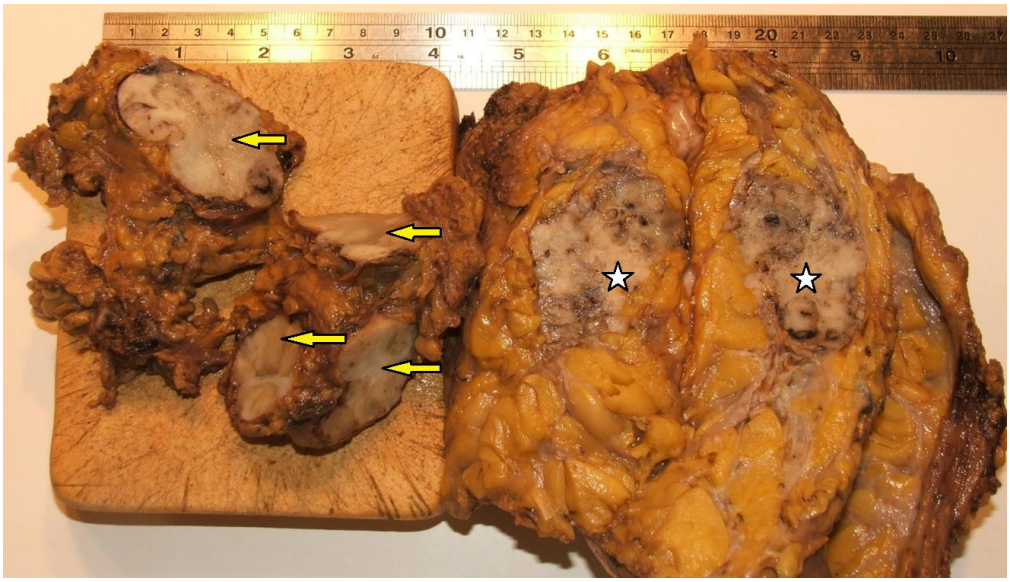


Figure 3.13. Breast carcinoma associated with metastases in axillary lymph nodes. The tumour is bisected (stars). Four bisected metastases (arrows) are evident in axillary tissues corresponding to pN2. Photo image by A.Abolins.

There were 12 patients (3.1%, 95% CI=1.8-5.4) affected by proved distant breast cancer metastases (M1) at the time of breast operation. Breast carcinoma metastases were found in bones – 33.3% (95% CI=13.8-60.9), central nervous system (brain) – 25% (95% CI=8.9-53.2), lungs – 25% (95% CI=8.9-53.2) and liver – 16.7% (95% CI=4.7-44.8).

In breast tissues (Figure 3.14.), all cases were classified by the histological grade as follows: G1 (Figure 3.15.) – 61 (16.0%, 95% CI=12.2-19.6), G2 (Figure 3.16.) – 138 (36.0%, 95% CI=31.9-41.1) and G3 (Figure 3.17.) – 184 cases (48.0%, 95% CI=43.8-52.8).

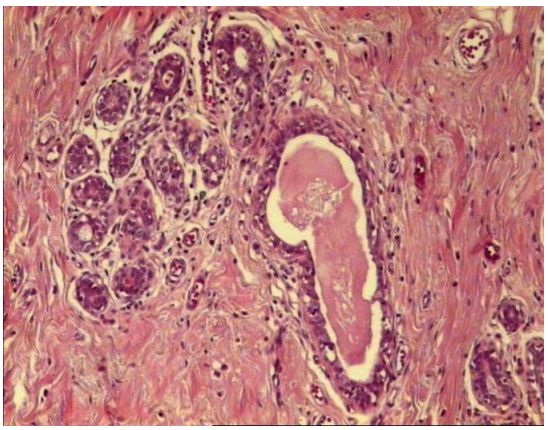


Figure 3.14. Breast tissues without malignant tumour. Haematoxylin – eosin, original magnification 100 x.

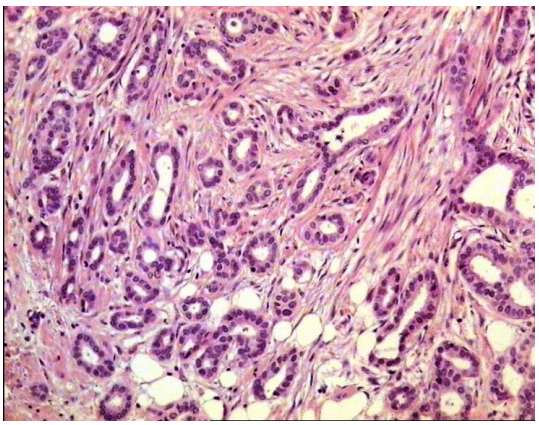


Figure 3.15. Low grade invasive ductal breast carcinoma. Haematoxylin – eosin, original magnification 100 x.

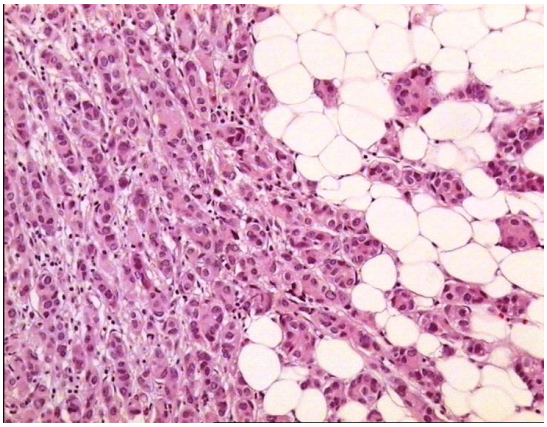


Figure 3.16. Intermediate grade invasive ductal breast carcinoma. Haematoxylin – eosin, original magnification 100 x.

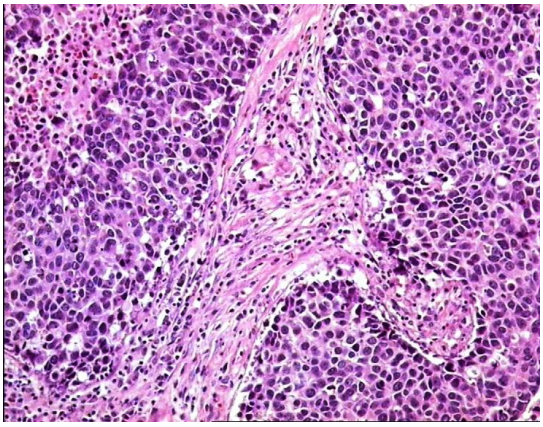


Figure 3.17. High grade invasive ductal breast carcinoma. Haematoxylin – eosin, original magnification 100 x.

Microphotographs by A.Abolins.

3.3. Immunohistochemical findings on molecular subtyping and prognostic variables

The immunohistochemical investigation was invariably applied to verify the histological diagnosis of invasive breast carcinoma as well as to detect the histological type. In all cases, loss of actin-positive myoepithelial cell layer in the appropriate morphological setting was used to confirm the invasive growth of breast carcinoma (Figures 3.18.-3.19.). The immunohistochemical data of E-cadherin expression in accordance with general morphology was used to classify the carcinoma as ductal or lobular cancer by presence or loss of E-cadherin in the malignant cells, respectively (Figures 3.20.-3.21.).

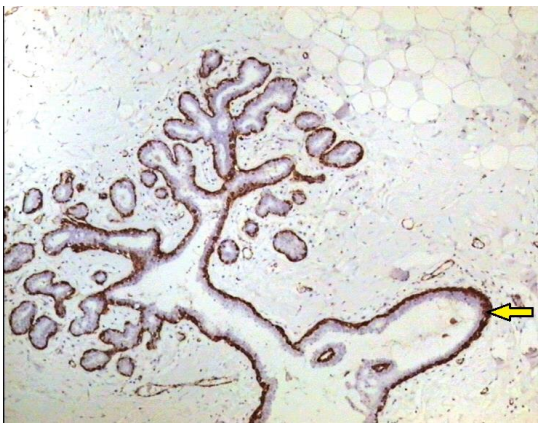


Figure 3.18. Actin expression in normal breast tissues. Note the positive reaction in myoepithelial cells (arrow). Anti-actin, immunoperoxidase, original magnification 50 x.

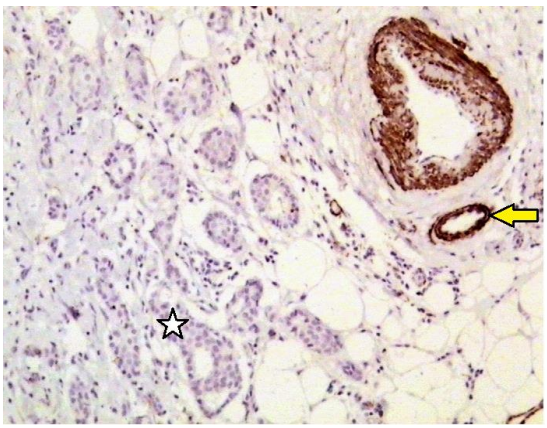


Figure 3.19. Lack of actin in invasive breast carcinoma (star). Note the positive reaction in muscular layer of small artery (arrow). Anti-actin, immunoperoxidase, original magnification 100 x.

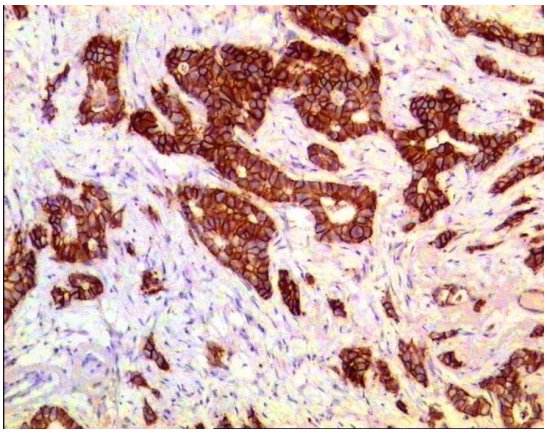


Figure 3.20. Intense membranous expression of E-cadherin in invasive ductal breast carcinoma. Anti-E-cadherin, immunoperoxidase, original magnification 100 x.

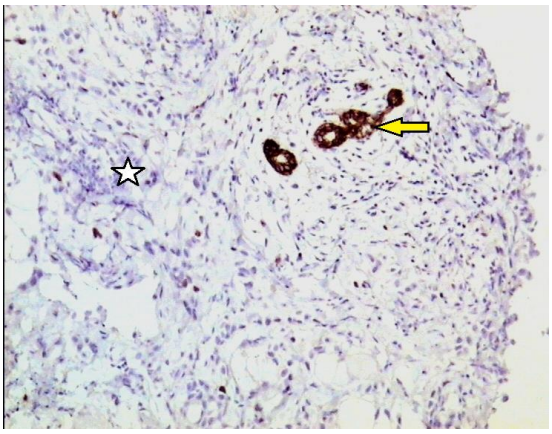


Figure 3.21. Lack of E-cadherin in invasive lobular breast carcinoma (star). Note the preserved positive reaction in benign lobule (arrow). Anti-E-cadherin, immunoperoxidase, original magnification 100 x.

Microphotographs by A.Abolins.

The further steps of immunohistochemical investigation included the evaluation of molecular subtype. The immunohistochemical analysis was than extended by evaluation of p53, cyclin D1, BCL2 and COX-2 protein expression (Table 3.2.).

By immunohistochemistry, expression of ER, PR, HER2 and Ki-67 was determined and incorporated in the molecular subtype of breast carcinoma along with appropriate FISH findings for HER2-positivity. Patient and tumour characteristics of the 383 cases based on molecular subtypes are summarized in Table 3.2. The majority of cases were luminal A (39.9%), followed by the luminal B (HER2 negative) subtype (32.6%). Triple negative breast cancer subtype constituted 12.8%, whereas only 8.4% and 6.3% of tumours were classified as HER2 positive and luminal B (HER2 positive), respectively.

Table 3.2.

Clinicopathological characteristics of molecular subtypes of breast cancer

Variable	All cases n=383	Luminal A n=153 (39.9%)	Luminal B (HER2 negative) n=125 (32.6%)	Luminal B (HER2 positive) n=24 (6.3%)	HER2 positive n=32 (8.4%)	Triple negative n=49 (12.8%)	*P* value
Age Mean ± SD (years)	59.59 ± 12.22	61.62 ± 11.61	59.66 ± 12.18	55.13 ± 11.80	57.56 ± 11.31	56.72 ± 13.88	0.025
Mean tumour volume ± SD (cm^3)	18.51 ± 110.53	6.46 ± 18.09	7.96 ± 13.78	36.53 ± 88.94	7.47 ± 10.02	79.98 ± 291.80	0.001
pT 1	161 (42%)	85 (55.6%)	37 (29.6%)	8 (33.3%)	11 (34.4%)	20 (40.8%)	0.002
pT 2	159 (41.5%)	50 (32.7%)	63 (50.4%)	9 (37.5%)	18 (56.3%)	19 (38.8%)	
pT 3	35 (9.2%)	6 (3.9%)	17 (13.6%)	4 (16.7%)	2 (6.3%)	6 (12.2%)	
pT 4	28 (7.3%)	12 (7.8%)	8 (6.4%)	3 (12.5%)	1 (3.1%)	4 (8.2%)	
pN 0	180 (51.3%)	90 (63.8%)	43 (37.7%)	9 (39.1%)	14 (48.3%)	24 (54.5%)	<0.0001
pN 1	81 (23.1%)	30 (21.3%)	33 (29.0%)	5 (21.7%)	8 (27.6%)	5 (11.4%)	
pN 2	55 (15.6%)	12 (8.5%)	26 (22.8%)	8 (34.8%)	3 (10.3%)	6 (13.6%)	
pN 3	35 (10.0%)	9 (6.4%)	12 (10.5%)	1 (4.4%)	4 (13.8%)	9 (20.5%)	
Nx	32	12	11	1	3	5	-
G 1	61 (16.0%)	53 (34.6%)	4 (3.2%)	2 (8.4%)	1 (3.1%)	1 (2.0%)	<0.0001
G 2	138 (36.0%)	72 (47.1%)	57 (45.6%)	5 (20.8%)	2 (6.3%)	2 (4.1%)	
G 3	184 (48.0%)	28 (18.3%)	64 (51.2%)	17 (70.8%)	29 (90.6%)	46 (93.9%)	

Table 3.2. (continued)

Variable	All cases n=383	Luminal A n=153 (39.9%)	Luminal B (HER2 negative) n=125 (32.6%)	Luminal B (HER2 positive) n=24 (6.3%)	HER2 positive n=32 (8.4%)	Triple negative n=49 (12.8%)	*P* value
Invasion in lymphatic vessels (Figure 3.22.)	88 (23.0%)	22 (14.4%)	33 (26.4%)	8 (33.3%)	12 (37.5%)	13 (26.5%)	0.012
Absence of invasion in lymphatic vessels	295 (77.0%)	131 (85.6%)	92 (73.6%)	16 (66.7%)	20 (62.5%)	36 (73.5%)	
Vascular invasion	22 (5.7%)	5 (3.3%)	9 (7.2%)	1 (4.2%)	2 (6.2%)	5 (10.0%)	0.386
Absence of vascular invasion	361 (94.3%)	148 (96.7%)	116 (92.8%)	23 (95.8%)	30 (93.8%)	44 (89.8%)	
Perineural invasion	57 (14.9%)	25 (16.3%)	23 (18.4%)	4 (16.7%)	1 (3.1%)	4 (8.2%)	0.148
Lack of perineural invasion	326 (85.1%)	128 (83.7%)	102 (81.6%)	20 (83.3%)	31 (96.9%)	45 (91.8%)	
Ductal carcinoma *in situ*, non-*comedo-carcinoma* type (Figure 3.23.)	108 (28.2%)	55 (35.9%)	34 (27.2%)	3 (12.5%)	6 (18.8%)	10 (20.4%)	<0.0001
Lobular carcinoma *in situ*	28 (7.3%)	18 (11.8%)	9 (7.2%)	1 (4.2%)	0 (0.0%)	0 (0.0%)	
Ductal carcinoma *in situ, comedo-carcinoma* type (Figure 3.24.)	124 (32.4%)	26 (17.0%)	53 (42.4%)	13 (54.1%)	20 (62.4%)	12 (24.5%)	
Carcinoma *in situ* not observed	123 (32.1%)	54 (35.3%)	29 (23.2%)	7 (29.2%)	6 (18.8%)	27 (55.1%)	

Table 3.2. (continued)

Variable	All cases n=383	Luminal A n=153 (39.9%)	Luminal B (HER2 negative) n=125 (32.6%)	Luminal B (HER2 positive) n=24 (6.3%)	HER2 positive n=32 (8.4%)	Triple negative n=49 (12.8%)	*P* value
Microcalcifications in the tumour (Figure 3.25.)	148 (38.6%)	68 (44.4%)	47 (37.6%)	8 (33.3%)	15 (46.9%)	10 (20.4%)	
Microcalcifications in the arteries	6 (1.6%)	2 (1.3%)	4 (3.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0.071
Microcalcifications elsewhere	3 (0.8%)	3 (2.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Microcalcifications not observed	226 (59.0%)	80 (52.3%)	74 (59.2%)	16 (66.7%)	17 (53.1%)	39 (79.6%)	
Invasive ductal carcinoma	304 (79.4%)	105 (68.5%)	108 (86.4%)	21 (87.5%)	30 (93.8%)	40 (81.7%)	
Invasive lobular carcinoma	51 (13.3%)	33 (21.6%)	12 (9.6%)	2 (8.3%)	1 (3.1%)	3 (6.1%)	
Mucinous breast carcinoma	13 (3.4%)	10 (6.5%)	3 (2.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Apocrine carcinoma	4 (1.0%)	0 (0.0%)	0 (0.0%)	1 (4.2%)	1 (3.1%)	2 (4.1%)	
Invasive cribriform carcinoma	3 (0.8%)	3 (2.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0.001
Metaplastic breast carcinoma	2 (0.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (4.1%)	
Medullary breast carcinoma	2 (0.5%)	0 (0.0%)	1 (0.8%)	0 (0.0%)	0 (0.0%)	1 (2.0%)	
Invasive papillary carcinoma	3 (0.8%)	1 (0.7%)	1 (0.8%)	0 (0.0%)	0 (0.0%)	1 (2.0%)	
Tubular breast carcinoma	1 (0.3%)	1 (0.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	

Table 3.2. (continued)

Variable	All cases n=383	Luminal A n=153 (39.9%)	Luminal B (HER2 negative) n=125 (32.6%)	Luminal B (HER2 positive) n=24 (6.3%)	HER2 positive n=32 (8.4%)	Triple negative n=49 (12.8%)	*P* value
Positive ER status (Figure 3.26.)	294 (76.8%)	151 (98.7%)	121 (96.8%)	22 (91.7%)	0 (0.0%)	0 (0.0%)	<0.0001
Negative ER status	89 (23.2%)	2 (1.3%)	4 (3.2%)	2 (8.3%)	32 (100.0%)	49 (100.0%)	
Positive PR status	270 (70.5%)	137 (89.5%)	113 (90.4%)	20 (83.3%)	0 (0.0%)	0 (0.0%)	<0.0001
Negative PR status	113 (29.5%)	16 (10.5%)	12 (9.6%)	4 (16.7%)	32 (100.0%)	49 (100.0%)	
Low Ki-67	170 (44.4%)	153 (100%)	0 (0.0%)	6 (25.0%)	5 (15.6%)	6 (12.2%)	<0.0001
High Ki-67 (Figure 3.27.)	213 (55.6%)	0 (0.0%)	125 (100%)	18 (75.0%)	27 (84.4%)	43 (87.8%)	
Positive HER2 status (Figure 3.28.)	56 (14.6%)	0 (0.0%)	0 (0.0%)	24 (100%)	32 (100.0%)	0 (0.0%)	<0.0001
Negative HER2 status	327 (85.4%)	153 (100%)	125 (100%)	0 (0.0%)	0 (0.0%)	49 (100.0%)	
Positive p53 status (Figure 3.29.)	92 (24.1%)	9 (5.9%)	34 (27.2%)	7 (29.2%)	17 (53.1%)	25 (51.0%)	<0.0001
Negative p53 status	291 (75.9%)	144 (94.1%)	91 (72.8%)	17 (70.8%)	15 (46.9%)	24 (49.0%)	
Positive BCL2 status (Figure 3.30.)	263 (68.7%)	133 (86.9%)	98 (78.4%)	17 (70.8%)	2 (6.2%)	13 (26.0%)	<0.0001
Negative BCL2 status	120 (31.3%)	20 (13.1%)	27 (21.6%)	7 (29.2%)	30 (93.8%)	36 (74.0%)	

Table 3.2. (end)

Variable	All cases n=383	Luminal A n=153 (39.9%)	Luminal B (HER2 negative) n=125 (32.6%)	Luminal B (HER2 positive) n=24 (6.3%)	HER2 positive n=32 (8.4%)	Triple negative n=49 (12.8%)	*P* value
Positive COX-2 status (Figure 3.31.)	5 (1.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (6.3%)	3 (6.1%)	0.021
Negative COX-2 status	378 (98.7%)	153 (100%)	125 (100%)	24 (100%)	30 (93.8%)	46 (93.9%)	
Positive cyclin D1 status (Figure 3.32.)	237 (61.9%)	100 (65.4%)	94 (75.2%)	14 (58.4%)	13 (40.6%)	16 (32.7%)	<0.0001
Negative cyclin D1 status	146 (38.1%)	53 (34.6%)	31 (24.8%)	10 (41.6%)	19 (59.4%)	33 (67.3%)	
Positive CK 5/6 (Figure 3.33.)	74 (19.3%)	21 (13.7%)	22 (17.6%)	3 (12.5%)	4 (12.5%)	24 (49.0%)	<0.0001
Negative CK 5/6	309 (80.7%)	132 (86.3%)	103 (82.4%)	21 (87.5%)	28 (87.5%)	25 (51.0%)	

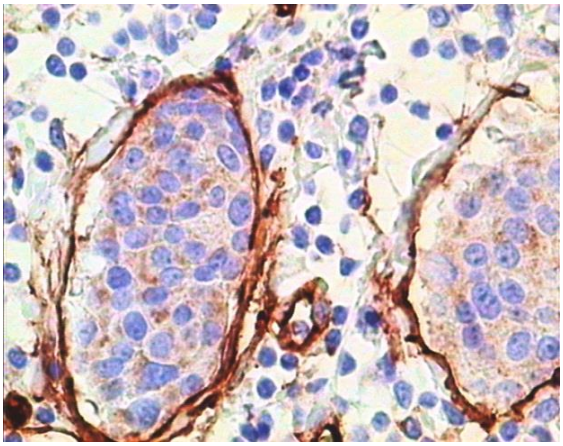


Figure 3.22. Cells of breast carcinoma in lymphatic vessels. Anti-CD34, immunoperoxidase, original magnification 400 x.

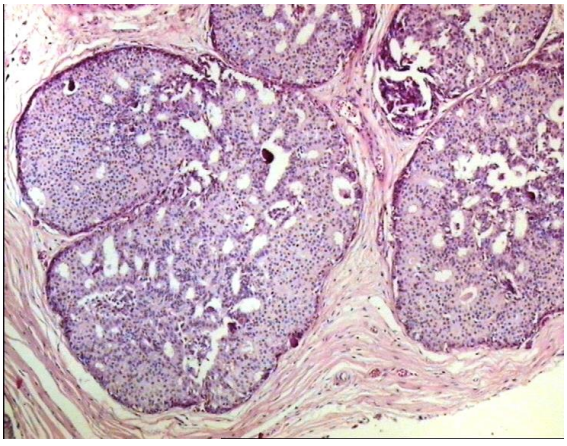


Figure 3.23. Ductal carcinoma *in situ*. Haematoxylin – eosin, original magnification 50 x.

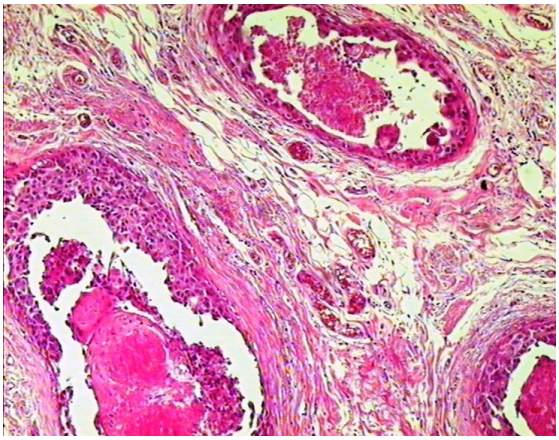


Figure 3.24. Ductal carcinoma *in situ*, *comedocarcinoma* type. Haematoxylin – eosin, original magnification 50 x.

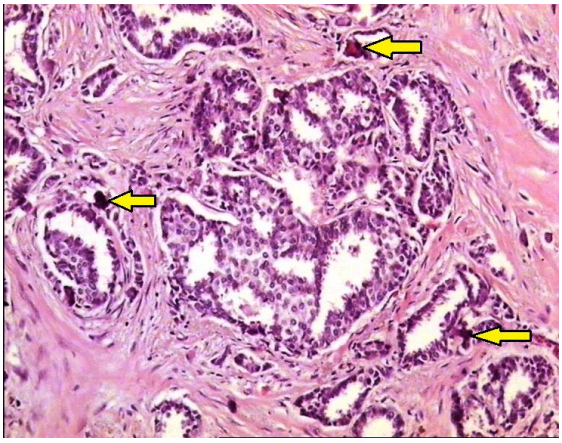


Figure 3.25. Microcalcifications (arrows) in invasive ductal breast carcinoma. Haematoxylin – eosin, original magnification 400 x.

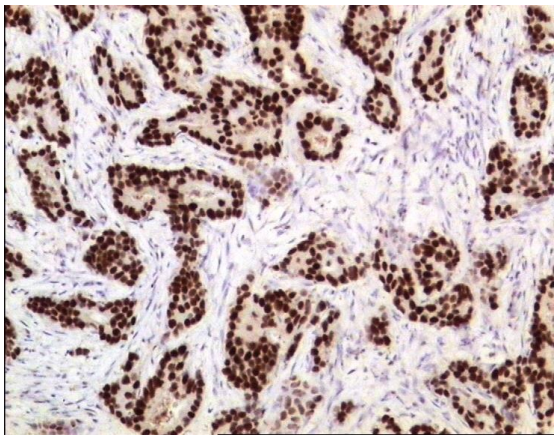


Figure 3.26. Nuclear oestrogen receptor expression in breast carcinoma. Anti-ER, immunoperoxidase, original magnification 100 x.

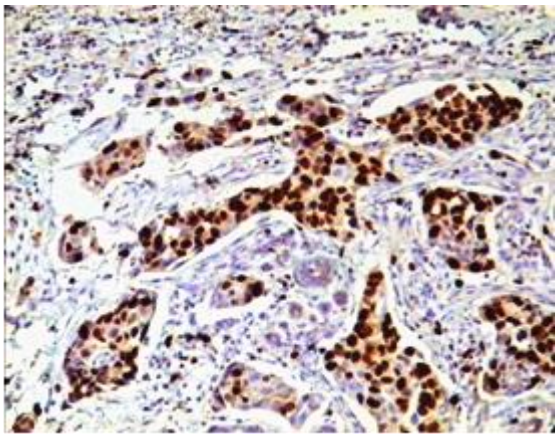


Figure 3.27. High proliferation activity by Ki-67 in high grade breast carcinoma. Anti-Ki-67, immunoperoxidase, original magnification 100 x.

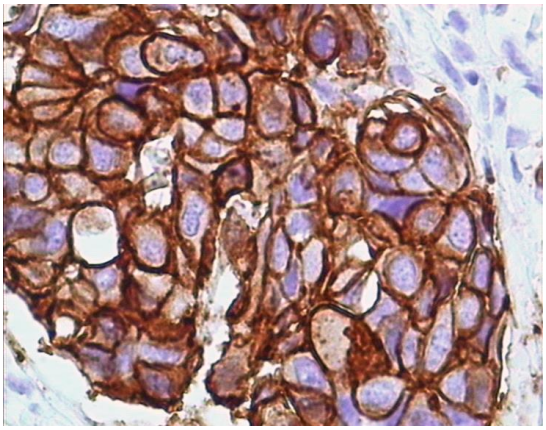


Figure 3.28. Membranous overexpression of HER2 protein (3+). HercepTest, original magnification 400 x.

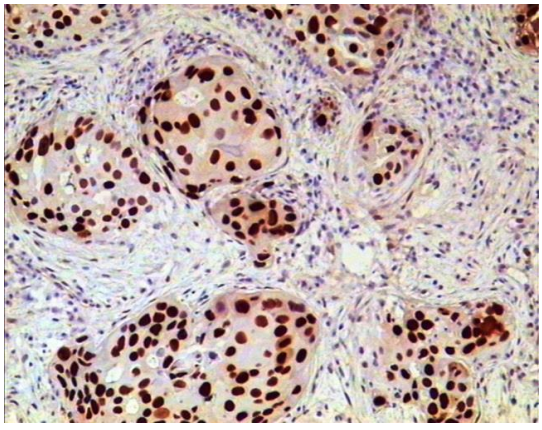


Figure 3.29. Nuclear expression of p53 in invasive breast carcinoma. Anti-p53, immunoperoxidase, original magnification 100 x.

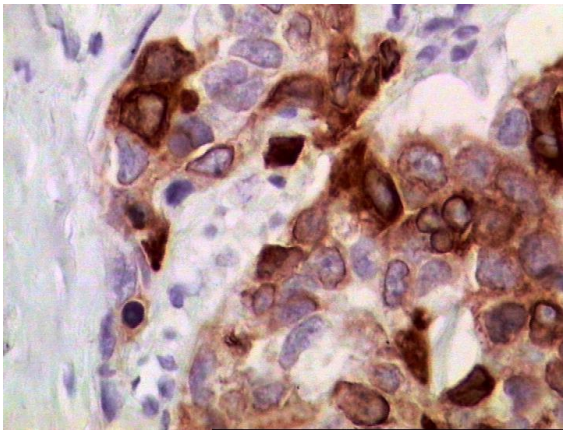


Figure 3.30. Cytoplasmic expression of BCL2 in breast carcinoma. Anti-BCL2, immunoperoxidase, original magnification 400 x.

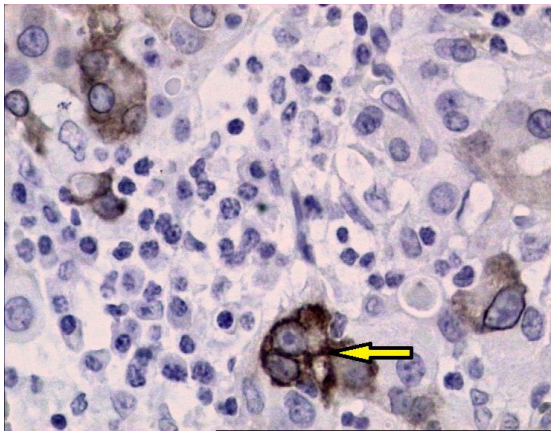


Figure 3.31. Cytoplasmic expression of COX-2 in breast carcinoma (arrow). Anti-COX-2, immunoperoxidase, original magnification 400 x.

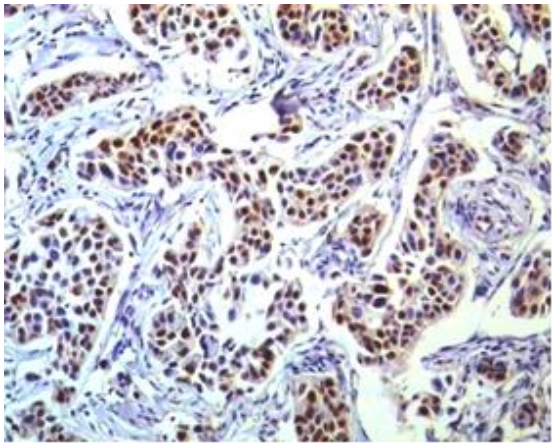


Figure 3.32. Nuclear expression of cyclin D1 in invasive ductal breast carcinoma. Anti-cyclin D1, immunoperoxidase, original magnification 100 x.

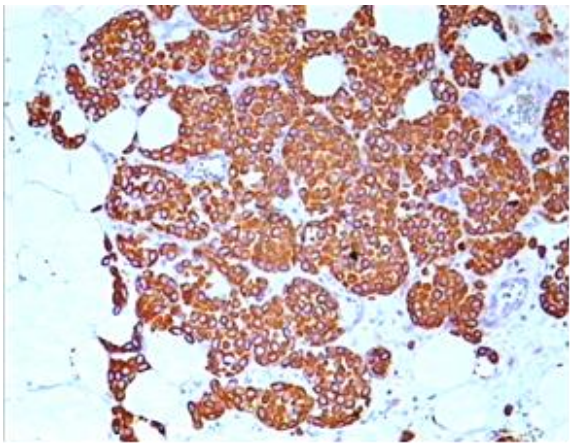


Figure 3.33. Diffuse cytoplasmic expression of CK 5/6 in breast carcinoma. Anti-CK 5/6, immunoperoxidase, original magnification 50 x.

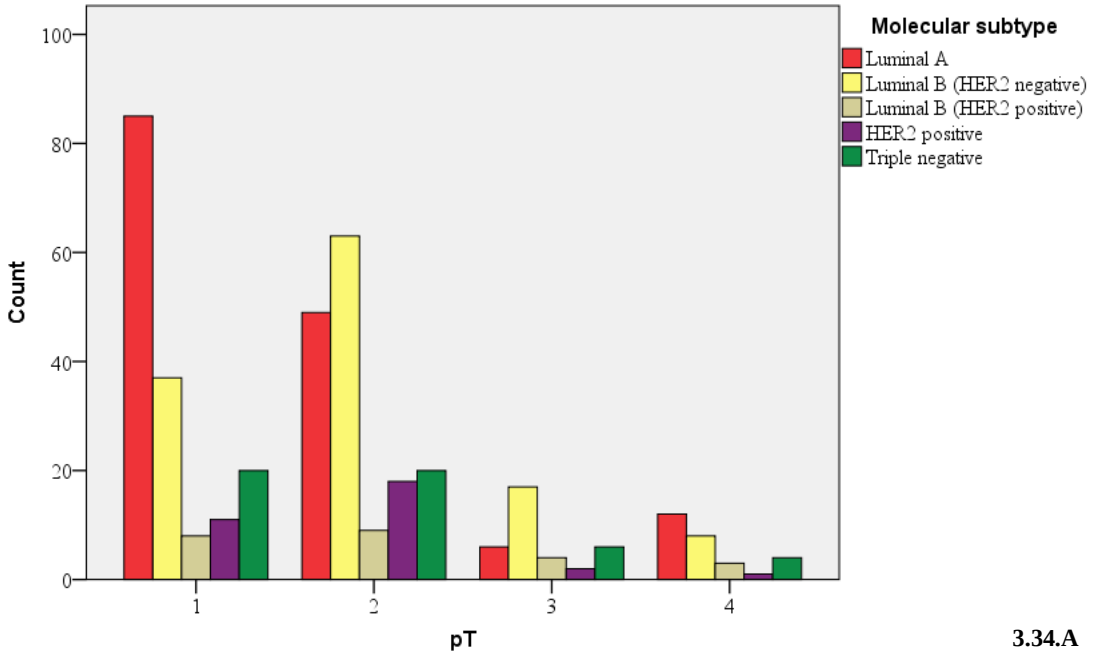
Microphotographs by A.Abolins.

3.4. The association between the molecular subtypes of breast carcinoma and known prognostic variables

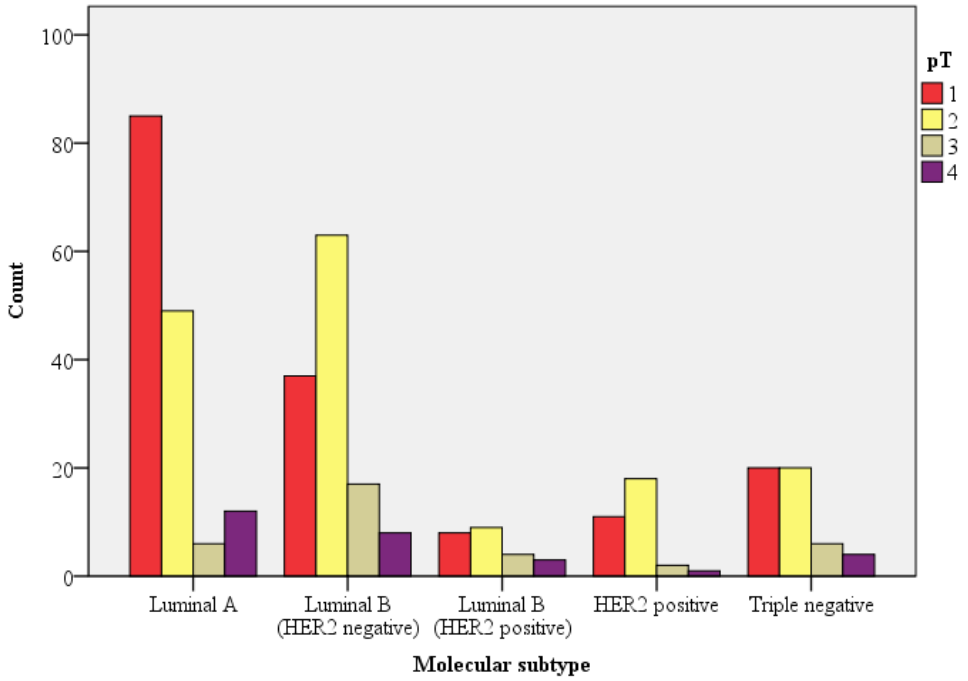
Although statistically significant differences were observed between the molecular subtypes regarding the mean age at diagnosis (ANOVA F test = 2.81, *P*=0.025) the mean values range within postmenopausal period, from 55.13 years in case of luminal B (HER2 positive) to 61.62 years regarding luminal A molecular subtype.

A significant difference was found when molecular subtypes were compared by mean tumour volume at the time of diagnosis (ANOVA F test = 5.04, $P<0.001$), with luminal B (HER2 positive) and triple negative breast cancers having larger volume (36.53 and 81.44 cm^3, respectively) than other molecular subtypes (luminal A - 6.47, luminal B (HER2 negative) - 7.96 and HER2 positive - 7.47 cm^3). Significant pair-wise differences were observed when the triple negative group was compared with luminal A ($P<0.0001$), luminal B (HER2 negative) ($P<0.0001$) and HER2 positive ($P=0.003$) subtypes.

There were statistically significant ($P=0.002$) differences, analysing the local spread (pT) by molecular subtype. The highest proportion of pT1 tumours were classified as luminal A. In contrast, pT2 and pT3 tumours showed predominance of luminal B (HER2 negative) subtype (Figure 3.34.A). The rate of pT1 tumours among luminal A breast cancers was as high as 55.9%. The proportion of pT2 was remarkably high in luminal B (HER2 negative) and triple negative subtypes, reaching 50.4% and 40.0%, respectively. Regarding statistical significance, higher proportion ($P=0.002$) of luminal A molecular subtype is revealed in pT1, but luminal B (HER2 negative) subtype – in pT2 stage (Figure 3.34.B).



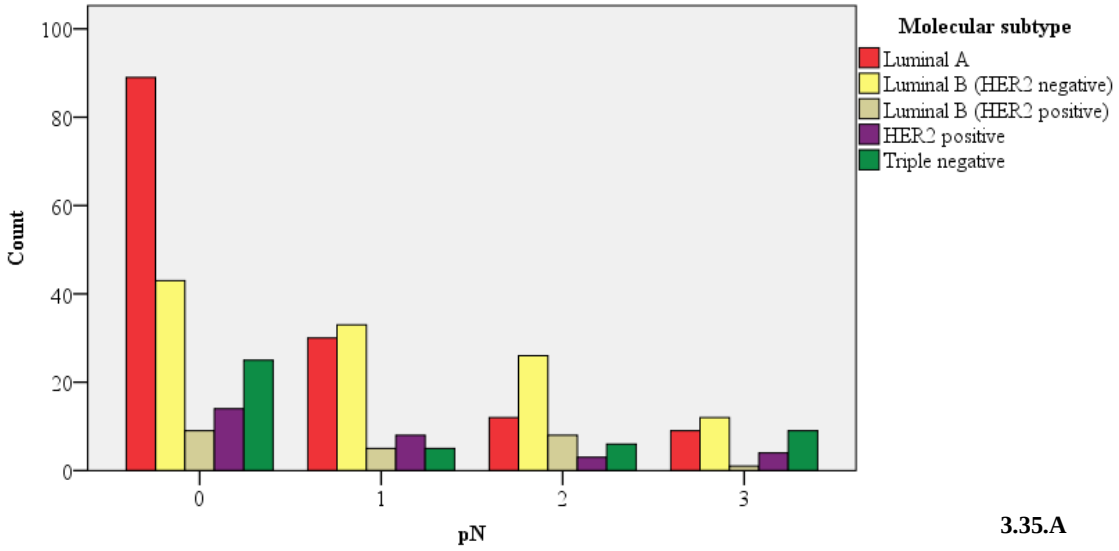
3.34.A



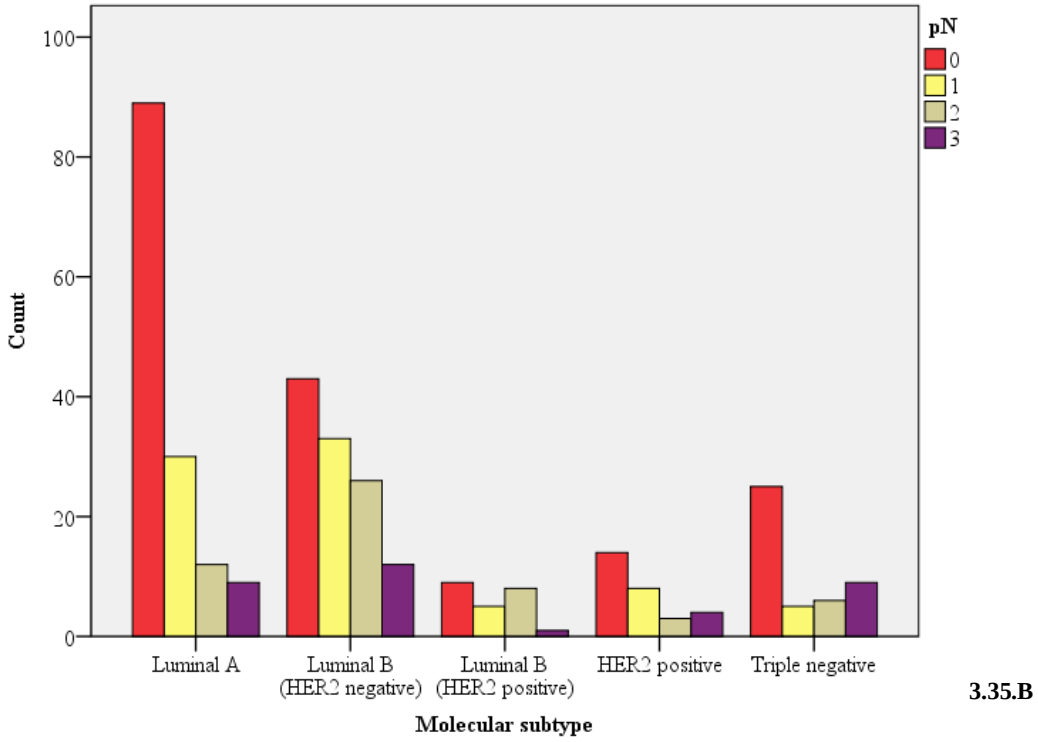
3.34.B

Figure 3.34. Relevance between pT and molecular subtypes of breast cancer. 3.34.A, by pT; 3.34.B, by molecular subtype.

Statistically significant (P<0.0001) differences were observed analysing molecular subtypes by pN stages. Tumours without metastases in regional lymph nodes belonged mainly to luminal A and luminal B (HER2 negative) molecular subtypes (Figure 3.35.A), but luminal A (63.9%) and triple negative (55.6%) molecular subtypes showed the highest proportion of pN0 tumours (Figure 3.35.B).



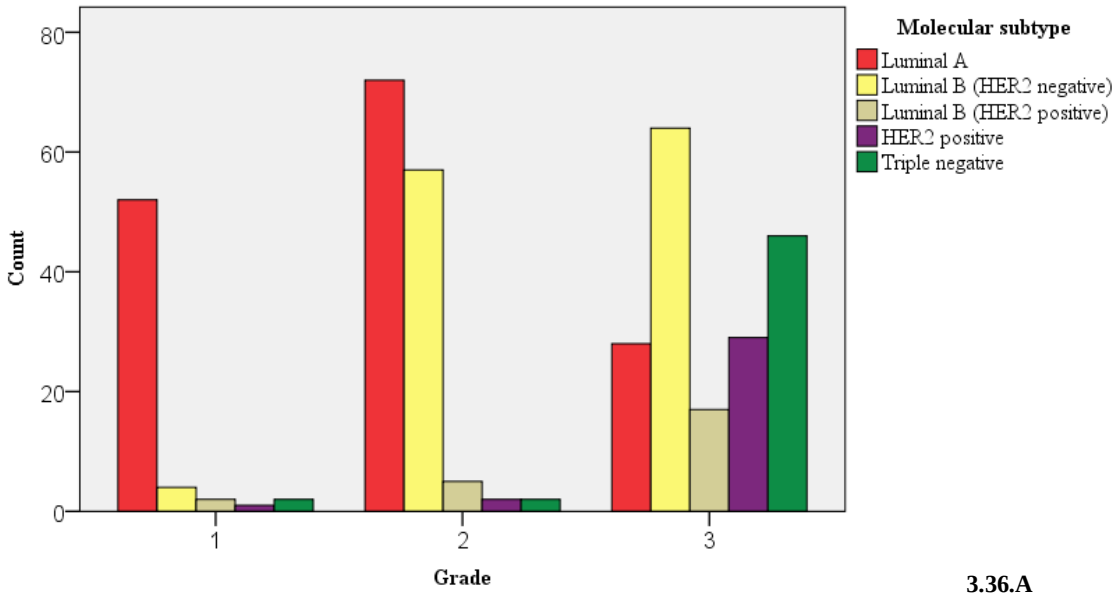
3.35.A



3.35.B

Figure 3.35. Relevance between pN and molecular subtypes of breast cancer. 3.35.A, by pN; 3.35.B, by molecular subtype.

Statistically significant differences (P<0.0001) were observed regarding breast cancer grade distribution between molecular subtypes. Significantly higher amount of G3 cases belong to luminal B (HER2 negative) and triple negative subtypes, while G1 breast cancers are mainly luminal A molecular subtype cancers (Figure 3.36.A). The highest proportions of high grade tumours were detected in luminal B (HER2 positive) (70.8%), HER2 positive (90.6%) and triple negative subtypes (Figure 3.36.B).



3.36.A

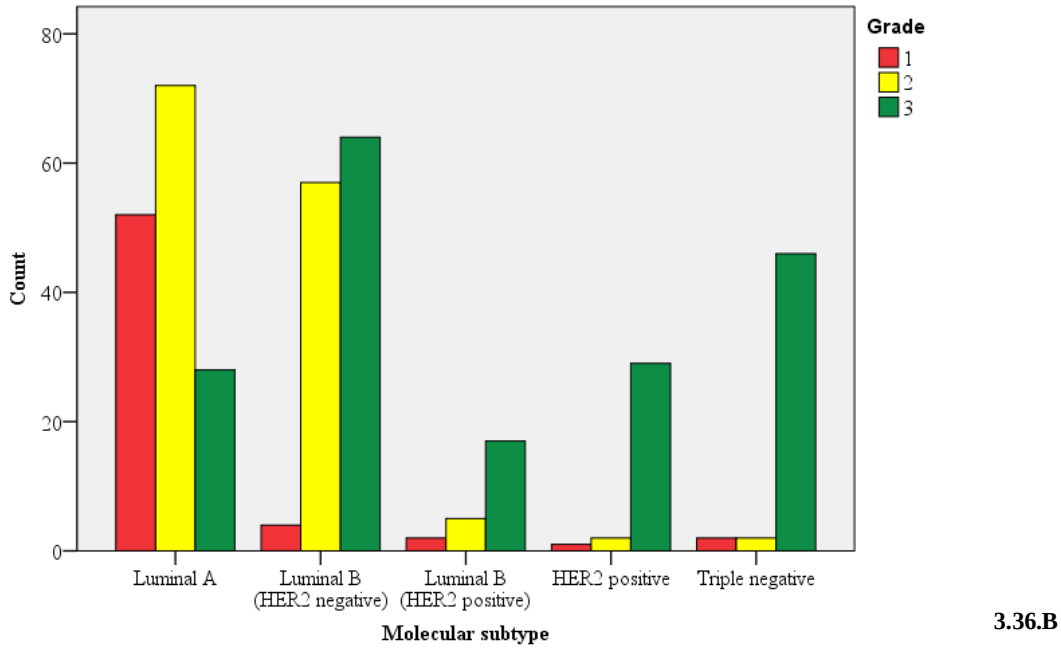
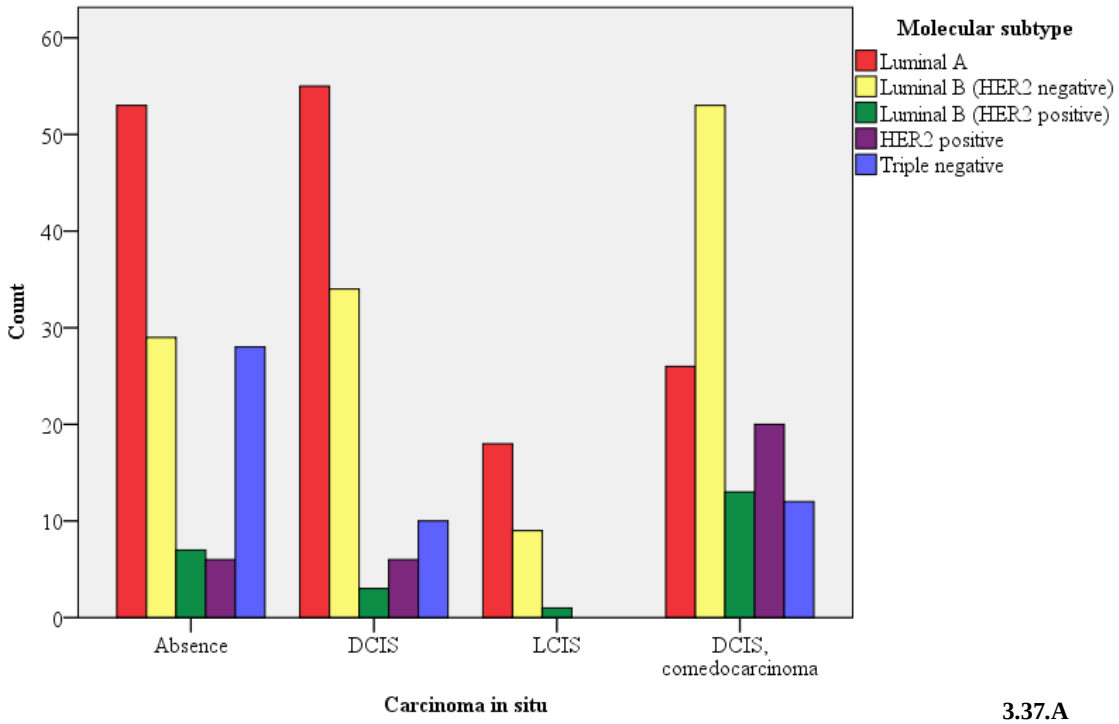
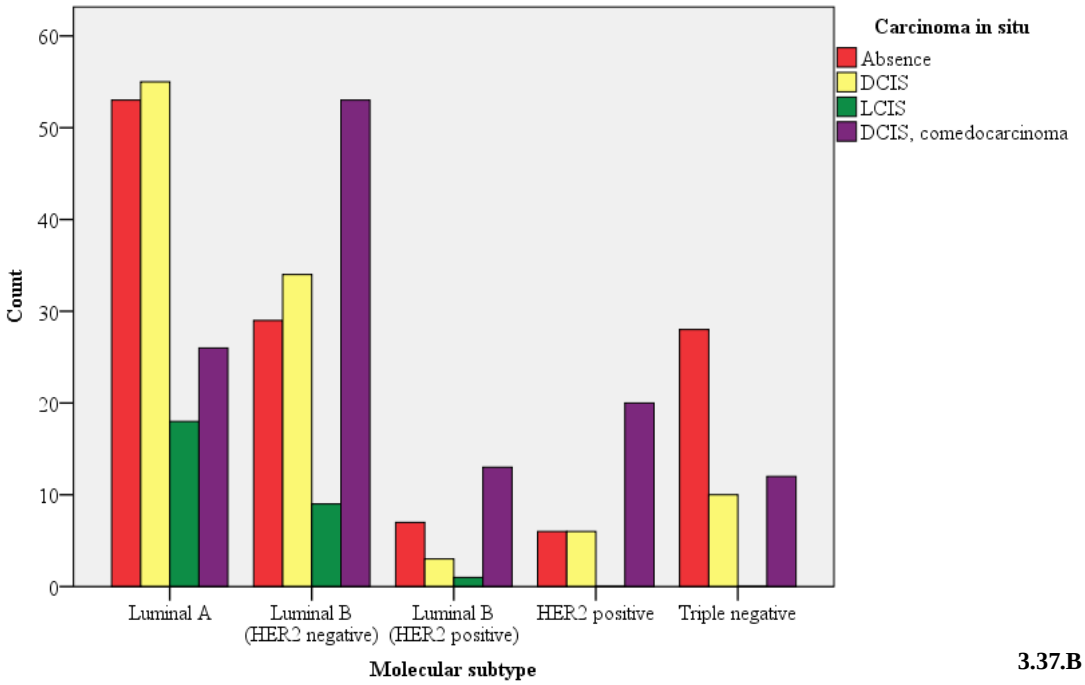


Figure 3.36. Relevance between grade and breast cancer molecular subtypes. 3.36.A, by grade; 3.36.B, by molecular subtype.

The molecular subtypes differed significantly considering the carcinoma *in situ* component in tumours ($P<0.0001$). Breast cancers lacking carcinoma *in situ* belonged mainly to luminal A, luminal B (HER2 negative) and triple negative groups. However, the highest proportions of DCIS-harboring breast cancers were of luminal A subtype as well. Presence of DCIS was also identified in luminal B (HER2 negative) molecular subtype. Ductal carcinoma *in situ, comedocarcinoma* type showed remarkably high frequency of luminal B (HER2 negative) molecular subtype as well as significant number of HER2 positive and luminal A molecular subtype cases (Figure 3.37.A). Most of luminal B (HER2 negative), luminal B (HER2 positive) and HER2 positive cases presented synchronous carcinoma *in situ* component, especially ductal carcinoma *in situ, comedocarcinoma* type (42.4%, 54.1% and 62.4%, respectively). Triple negative breast cancer molecular subtype showed the highest percentage (56.0%) of tumours lacking carcinoma *in situ* component (Figure 3.37.B).



3.37.A

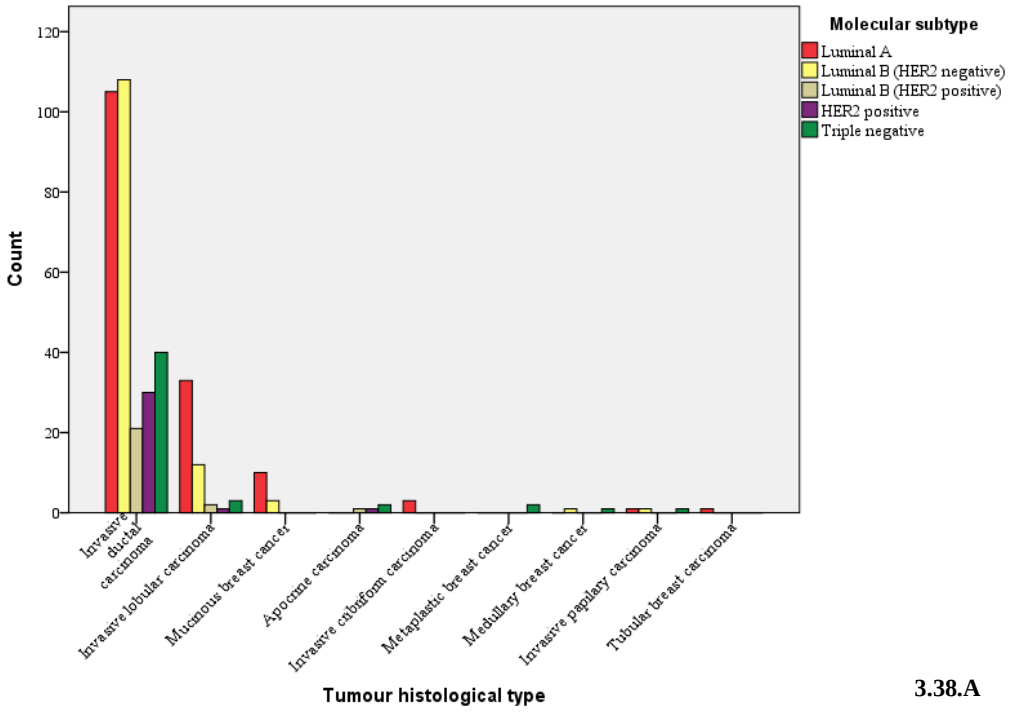


3.37.B

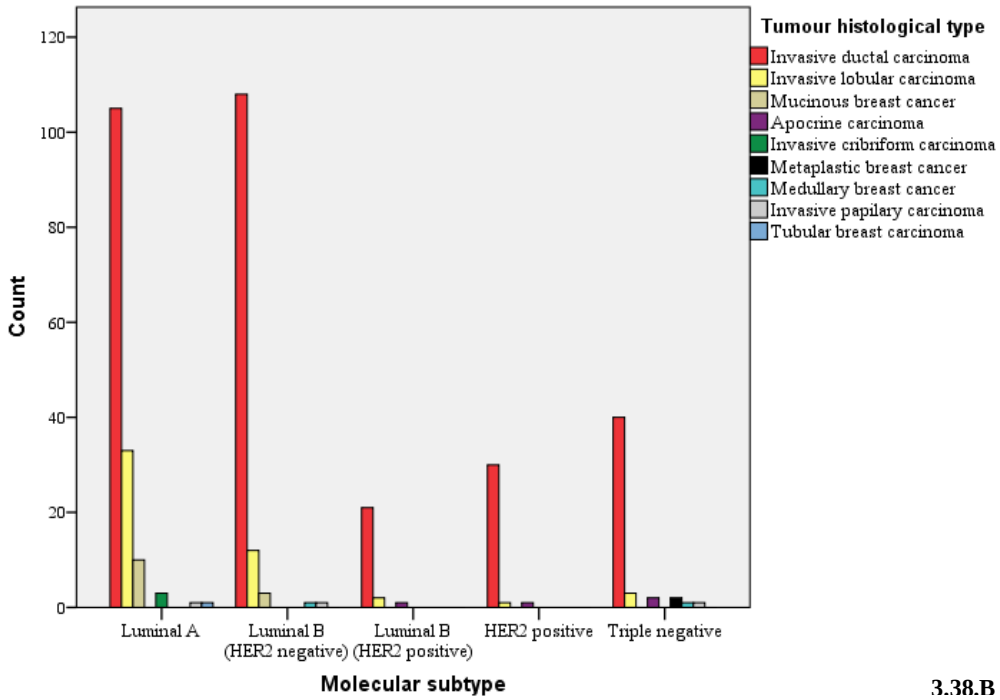
Figure 3.37. Frequency of carcinoma *in situ* components in different breast cancer molecular subtypes. 3.37.A, by carcinoma *in situ*; 3.37.B, by molecular subtype.

Categorizing the breast cancer cases by histological forms, significant difference between molecular subtype distribution (P<0.0001) was observed. Ductal breast cancer cases represent full spectrum of molecular subtypes with predominance of luminal B (HER2 negative) and luminal A, followed by triple negative molecular subtype. The lobular breast cancer is heterogeneous by the molecular subtype, but luminal A is the most frequent subtype followed by less frequent occurrence of luminal B (HER2

negative) molecular subtype in contrast with ductal breast carcinoma (Figure 3.38.A). Invasive ductal carcinoma was most frequent histological form of breast cancer in all molecular subtypes. Invasive lobular carcinoma was next frequent breast cancer form, especially among luminal A (21.7%) and luminal B (HER2 negative) molecular subtypes (16.9%), while all reported metaplastic or anaplastic carcinomas (9.6%) were of triple negative molecular subtype (Figure 3.38.B).



3.38.A



3.38.B

Figure 3.38. Distribution of the morphological type of breast carcinoma by molecular subtypes. 3.38.A, by tumour histological type; 3.38.B, by molecular subtype.

3.5. The mutual associations between morphological variables

The lymph nodes were detected and examined in 351 cases (91.6%). Among tumours measuring less than 2 cm in largest diameter (pT1), metastases in lymph nodes were mostly absent – not found in 107 cases (75.4%). In pT2 cancers, the pN distribution was different: metastases were not found in 39.3% of cases, 1-3 positive lymph nodes were indentified in 30% of cases, but 10 or more metastases in axillary lymph nodes were found in 10.7%. Larger tumours were associated with more aggressive spread as in pT4 cases there were 4-9 positive lymph nodes in 40%, but ≥10 in 36% of cases (Figure 3.39.A). In general, statistically significant differences were observed (P<0.0001), including the following findings.

Evaluating the lymph node – negative and positive cases, pT1 tumours (59.4%) predominated in pN0 group. In contrast, pT4 was rare finding in pN0 constituting only 1.1%. In the pN1 group showing 1-3 positive lymph nodes, pT2 was the most common finding constituting 55.6%. Similarly, pN2 (4-9 positive lymph nodes) and pN3 (≥10 positive lymph nodes) were dominated by pT2 composing 54.5% and 45.7%, respectively (Figure 3.39.B). Statistically significant differences were observed (P<0.0001).

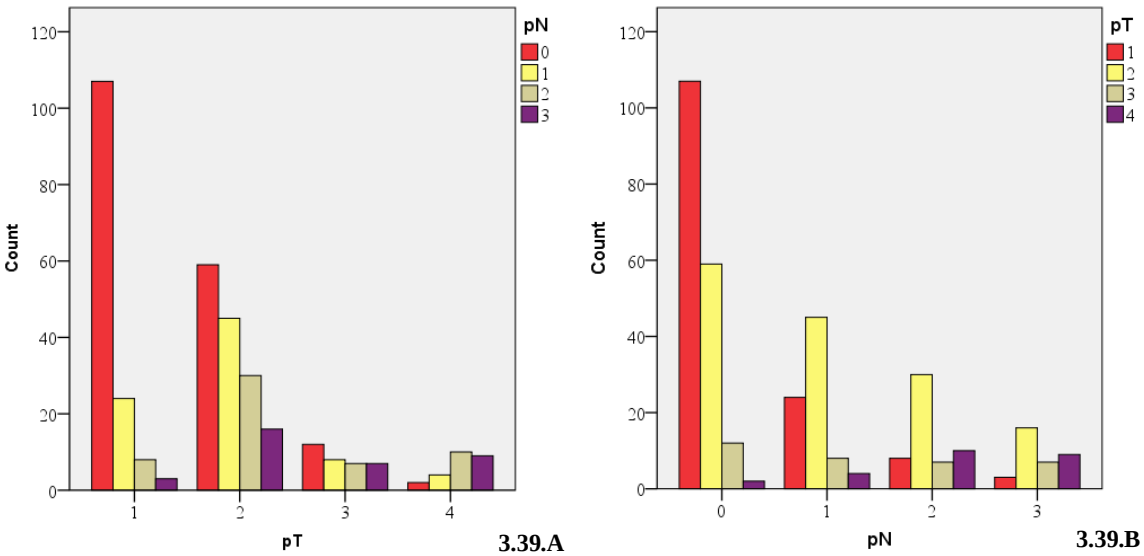


Figure 3.39. Relevance between pT and pN findings. 3.39.A, by pT; 3.39.B, by pN.

The association between pT and pN parameters was statistically insignificant (P=0.76) in cases undergoing only sentinel node examination. In contrast, the differences were statistically significant (P<0.0001) in cases where axillary lymph nodes dissection was performed.

Axillary lymph node dissection was attempted by the surgeon in 284 cases. In 275 (96.8%) of these cases, the nodes were detected. Among pT1 tumours, in 61 cases (67%) metastases were not found but 20.9% cases had 1-3 positive lymph nodes corresponding to pN1. Among pT2 breast cancers, metastases were absent in 31.5%, but pN1 was slightly more common finding (33.1%). Among pT3 tumours, there was slight predominance of pN1 finding (27.6%) in comparison with pN2, pN3 or pN0. Among pT4 tumours there was predominance of pN2 and pN3 showing the frequency of 40%, respectively. Just 8% of pT4 cases were free of lymph node metastases (Figure 3.40.A). Statistically significant differences were present (P<0.0001).

Evaluating lymph node negative and positive cases, the pN0 group mostly comprised pT1 cancers (55%). Presence of pT4 was rare finding in this group (8%). pN1, pN2 and pN3 cancers showed predominance of pT2 tumours with frequencies 58.1%, 54.5% and 45.7%, respectively (Figure 3.40.B). Statistically significant differences were identified (P<0.0001).

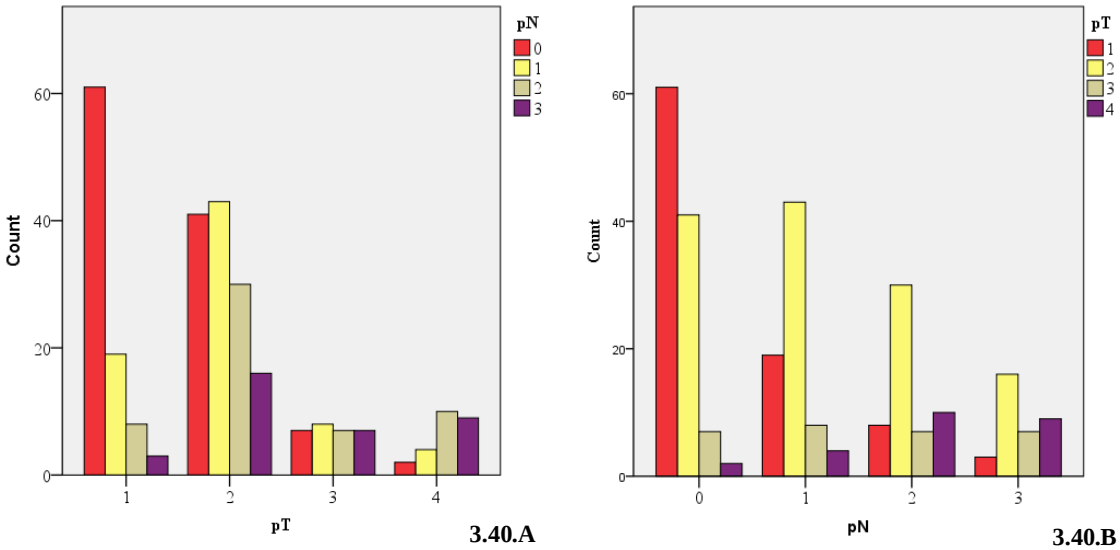


Figure 3.36. Relevance between pT and pN findings in axillary lymph node dissection group. 3.40.A, by pT; 3.40.B, by pN.

Statistical analysis has disclosed correlation between pT and tumour grade (P=0.003). In pT1 group, the frequency of G2 or G3 carcinoma was equal reaching 38.5%. In contrast, G3 was the most frequent finding in pT2 group (50.9%), pT3 (60%) and pT4 cases (71.4%) as shown in Figure 3.41.A. pT3 and pT4 cancers were characterised by rare occurrence of G1 (3.3% and 4.9% correspondingly).

Among G2 group, there was slight predominance of pT1 (44.9%), while G3 tumours were more frequently of pT2 (44%) as shown in Figure 3.41.B. Statistically significant differences were identified (*P*=0.003).

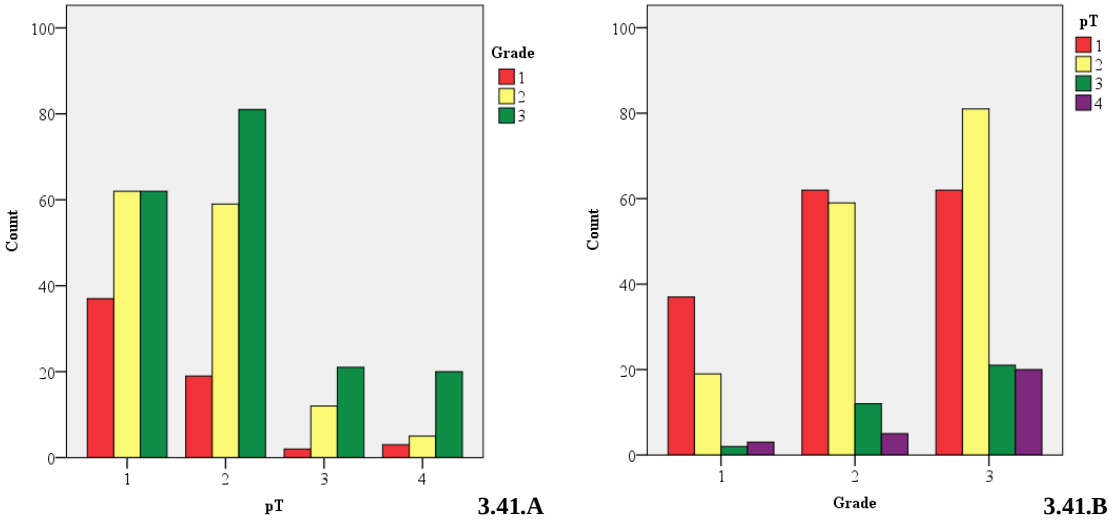


Figure 3.41. Relevance between the local tumour spread (pT) and grade. 3.41.A, by pT; 3.41.B, by grade.

Analysing the association between tumour grade and lymph node metastases, the following findings were obtained. Intermediate grade and high grade tumours constituted similar frequency (G2 - 40.6%, G3 – 39.4%) in tumour group without lymph node metastases. Among pN1 tumours, the frequency of G2 and G3 also was equal: 44.4% in each group. High number of metastases in lymph nodes (between 4-9, pN2 and ≥10, pN3, correspondingly) were associated with high grade tumours (Figure 3.42.A).

Among pN0 tumours, there were low grade, intermediate and high grade tumours, comprising 66.7%, 56.2% and 42.5% of the respective G group. Intermediate and high grade tumours have presented with low number of metastases in substantial number of patients: G2/pN1 - 27.7%, G3/pN1 - 21.6%. However, high grade tumours have been associated with higher number of metastases: G3/pN3 - 14.4%, G2/pN3 - 5.4% and G1/pN3 - 7.4%. The data are revealed in more detail in Figure 3.42.B. Statistically significant differences were identified (*P*=0.002).

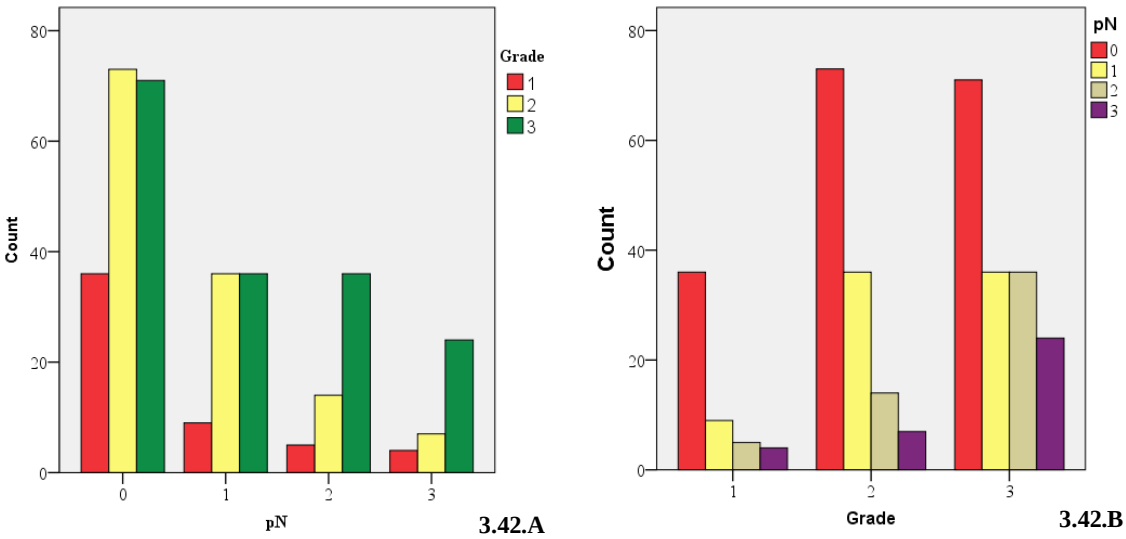


Figure 3.38. Relevance between tumour grade and extent of lymph node metastases (pN). 3.42.A, by pN; 3.42.B, by grade.

The invasion in lymphatic vessels was mostly absent (Figure 3.43.A). Among cases showing peritumorous invasion in lymphatic vessels, pT2 cases were predominant as shown in Figure 3.43.B (*P*=0.03).

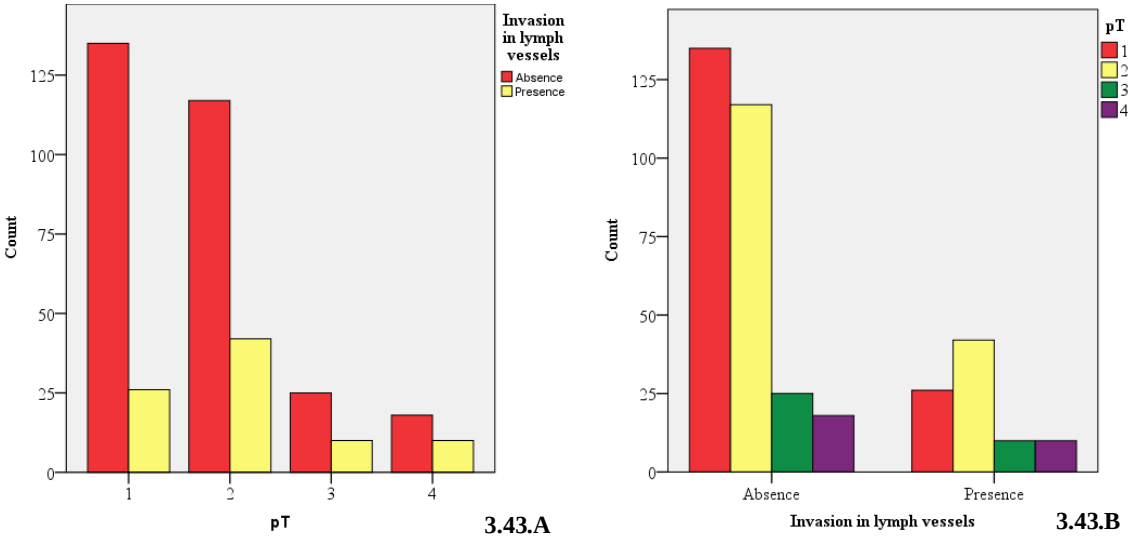


Figure 3.43. Relevance between local tumour spread (pT) and invasion in lymphatic vessels. 3.43.A, by pT; 3.43.B, by invasion in lymphatic vessels.

The invasion in veins was also mostly not observed (Figure 3.44.A). Among cases showing invasion in veins, pT2 cases were predominant as shown in Figure 3.44.B (*P*=0.04).

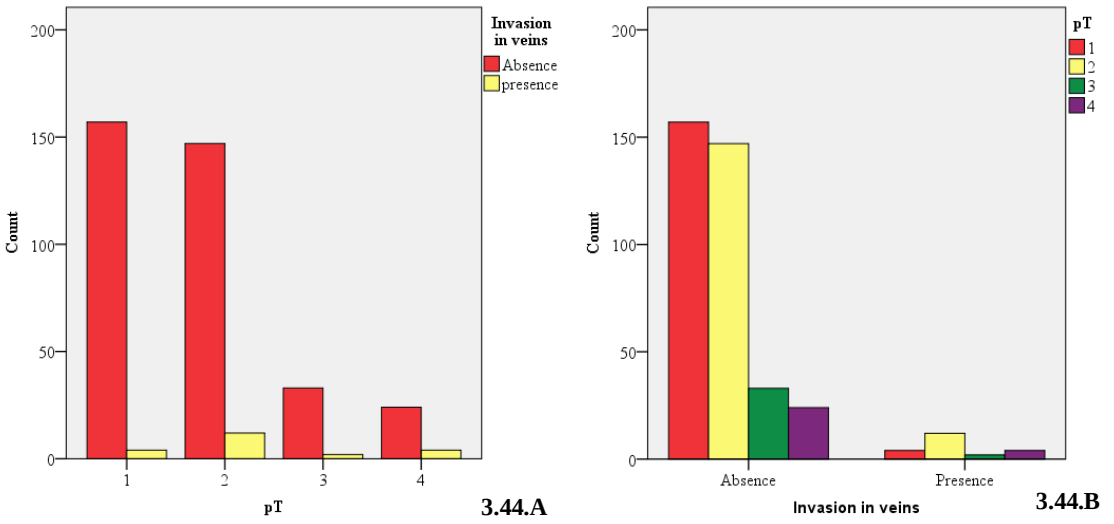


Figure 3.44. Relevance between pT and tumour invasion in veins based on these factors. 3.44.A, by pT; 3.44.B, by invasion in veins.

Most of the studied breast cancer cases did not show perineural growth (*P*=0.005). The data are shown in Figure 3.45.A. As it was seen in tumour invasion in lymphatic vessels and veins, perineural invasion was observed more in pT2 stage than other pT stages (Figure 3.45.B). The difference is statistically significant (*P*=0.005).

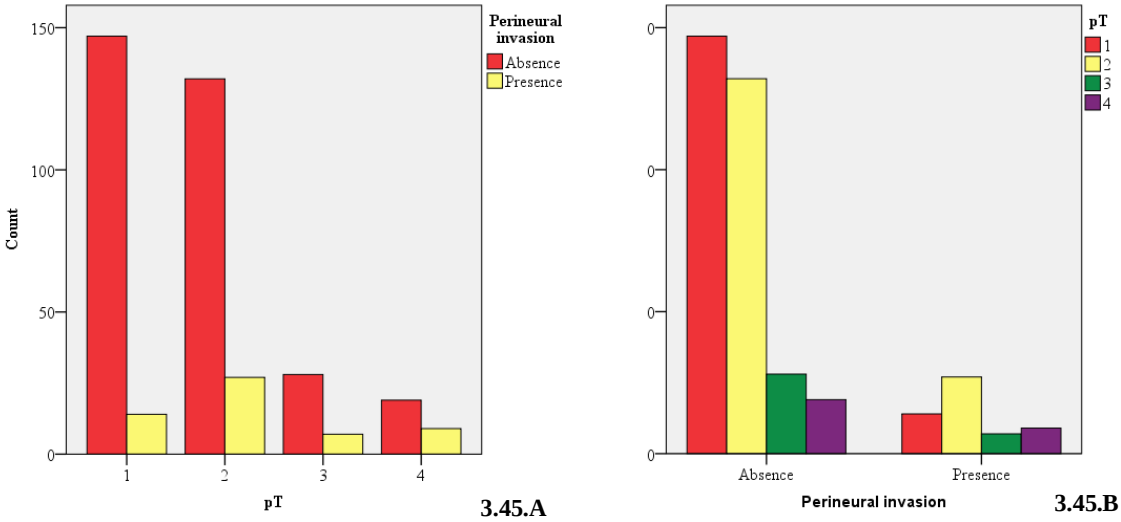
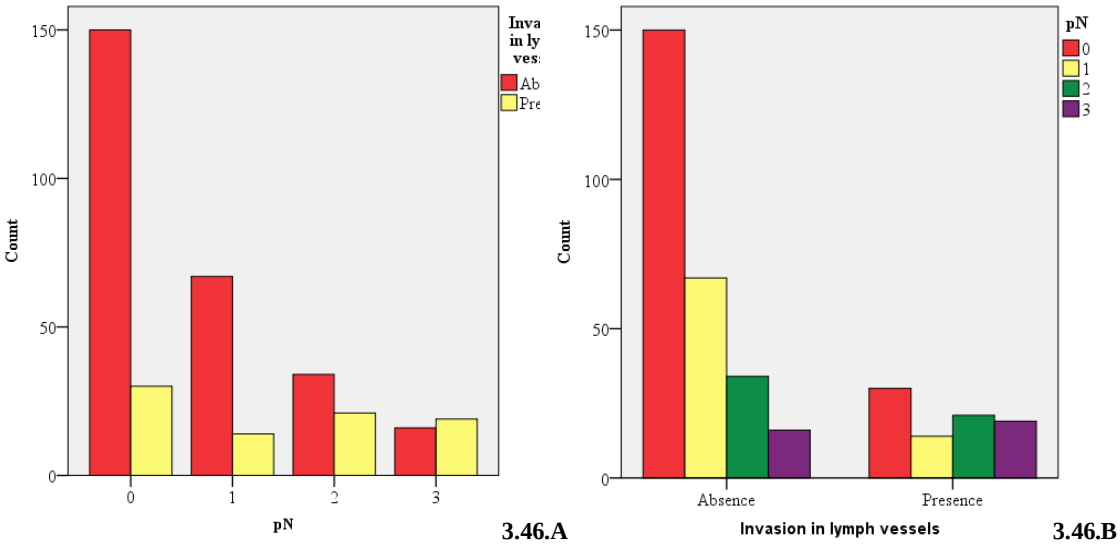


Figure 3.45. Relevance between pT and perineural tumour invasion. 3.45.A, by pT; 3.45.B, by perineural invasion.

Among pN0 cases, in 16.7% cases breast cancer cells can be identified in the peritumorous lymphatic vessels despite absence of breast cancer metastases in axillary lymph nodes (*P*<0.0001). Among pN3 cancers, the cases with identifiable lymphatic vessel invasion are more frequent than cases not displaying such clear-cut evidence of metastatic spread (Figure 3.46.A).

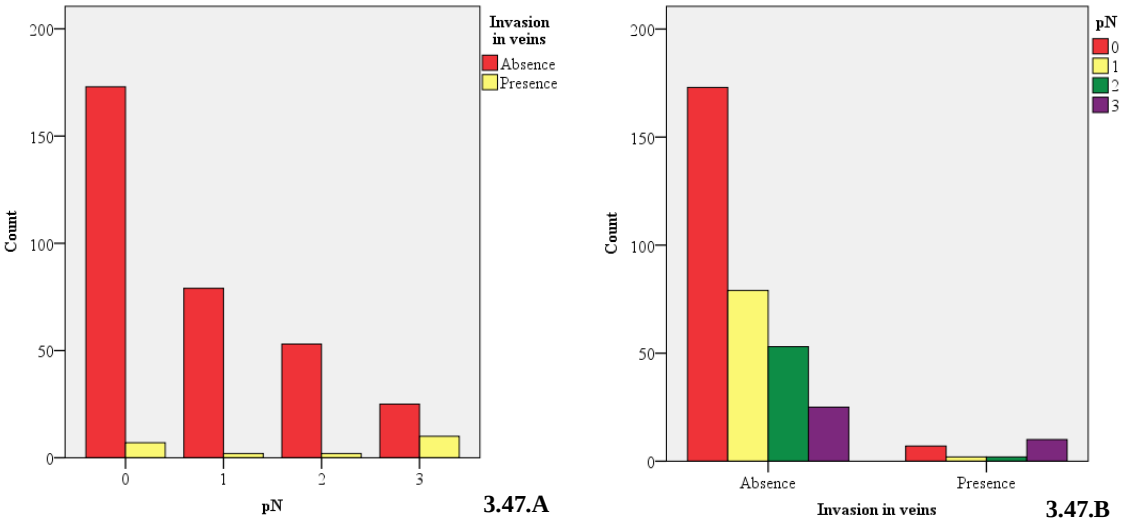
Considering breast carcinoma cases not showing evidence of lymphatic vessel invasion, the status of lymph nodes reflect the findings in the general group with pN0 predominance and progressively lower rate of more extensive metastatic process. Among the cases showing the presence of cancer cells in lymphatic capillaries, the predominance of pN0 is lost as shown in Figure 3.46.B (P<0.0001).



3.46.A 3.46.B

Figure 3.46. Relevance between pN and tumour invasion in lymphatic vessels. 3.46.A, by pN; 3.46.B, by invasion in lymphatic vessels.

Most of the studied breast cancer cases did not show invasion in veins; this observation was true for pN0, pN1, pN2 and pN3. The data are shown in Figure 3.47.A. As shown in Figure 3.47.B, invasion in veins is associated with higher occurrence of pN3 (P<0.0001).



3.47.A 3.47.B

Figure 3.47. Relevance between pN and tumour invasion in veins. 3.47.A, by pN; 3.47.B, by invasion in veins.

Most of the studied breast cancer cases did not show perineural growth (*P*=0.02) regardless of pN group. The data are shown in Figure 3.48.A. Among cases not showing perineural growth, pN0 show distinct predominance in contrast to perineurally invading cases (*P*=0.02). The data are presented in Figure 3.48.B.

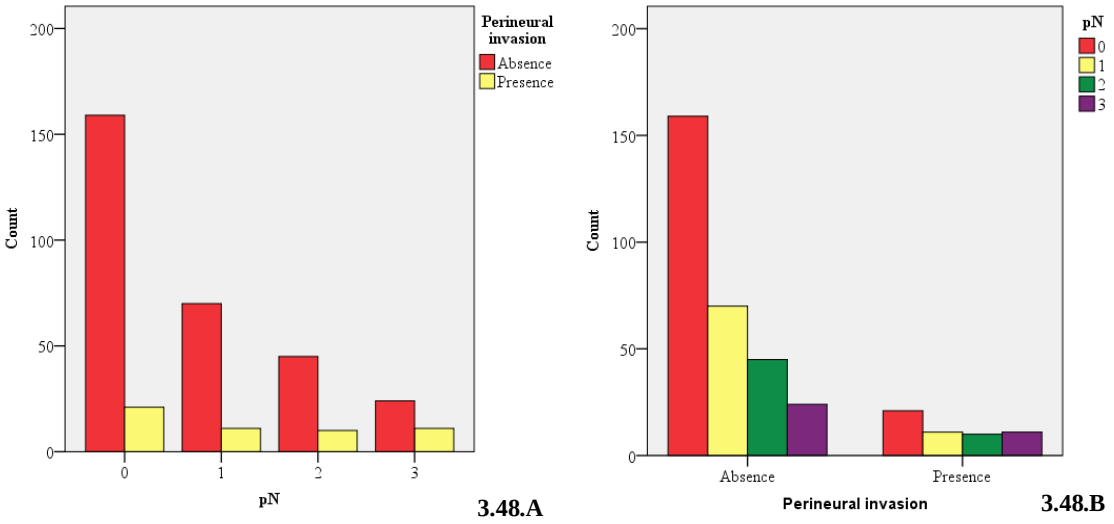


Figure 3.48. Relevance between pN and perineural tumour invasion. 3.48.A, by pN; 3.48.B, by perineural invasion.

The relevance between breast cancer grade and observed cancer invasion in lymphatic vessels was assessed as well. In all G groups, cancer invasion in the lymphatic vessels was mostly absent. However, G3 group harbour relatively more cases showing such invasion in contrast to G1 (Figure 3.49.A). Similarly, comparing the cancer groups presenting with lymphatic vessels invasion or lacking such evidence (Figure 3.49.B), high grade breast cancers are predominant but the frequency of G1 cancer is relatively higher among cases lacking lymphatic invasion (*P*=0.002).

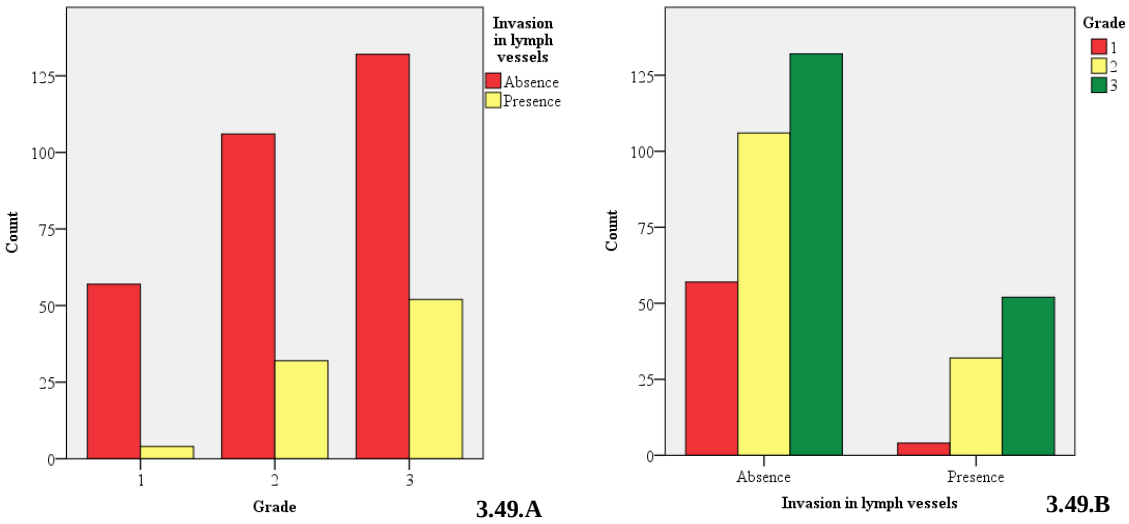
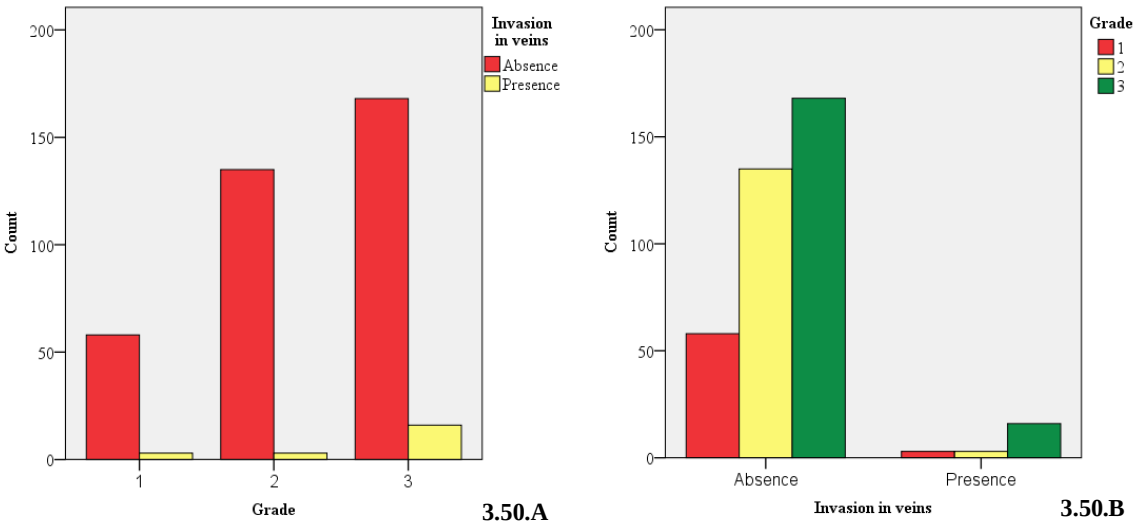


Figure 3.49. Relevance between breast cancer grade and invasion in lymphatic vessels. 3.49.A, by grade, 3.49.B, by invasion in lymphatic vessels.

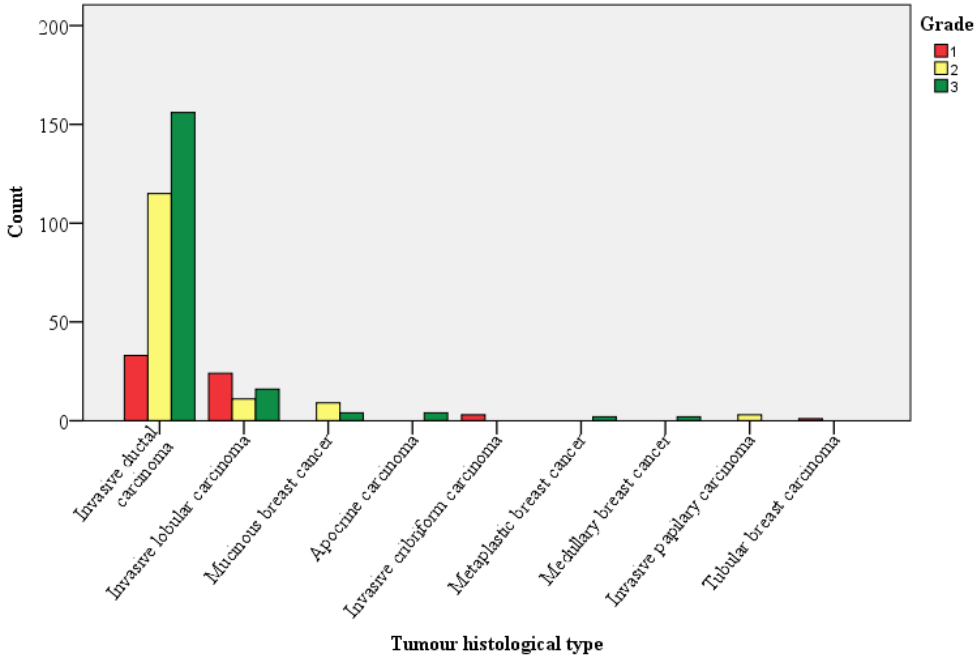
Breast cancer invasion in veins was rare observation in all grades (Figure 3.50.A), so the dominant finding was absence of such invasion. The invasion was identified with higher frequency in high grade breast cancer cases in comparison to intermediate or low grade breast cancers. High grade cancer cases compose the greatest part of tumours regardless of venous invasion (P=0.043); however, G1 cancers almost exclusively belong to the group lacking venous invasion (Figure 3.50.B).



3.50.A 3.50.B

Figure 3.46. Relevance between cancer grade and tumour invasion in veins. 3.50.A, by grade; 3.50.B, by invasion in veins

Ductal breast cancer frequently had high grade in contrast with lobular and tubular breast cancer characterised by low grade (Figure 3.51.A). Mucinous and invasive papillary breast cancers typically were G2 (P<0.0001). Medullary, apocrine and metaplastic breast cancers (Figure 3.51.B) are invariably G3 tumours (P<0.0001).



3.51.A

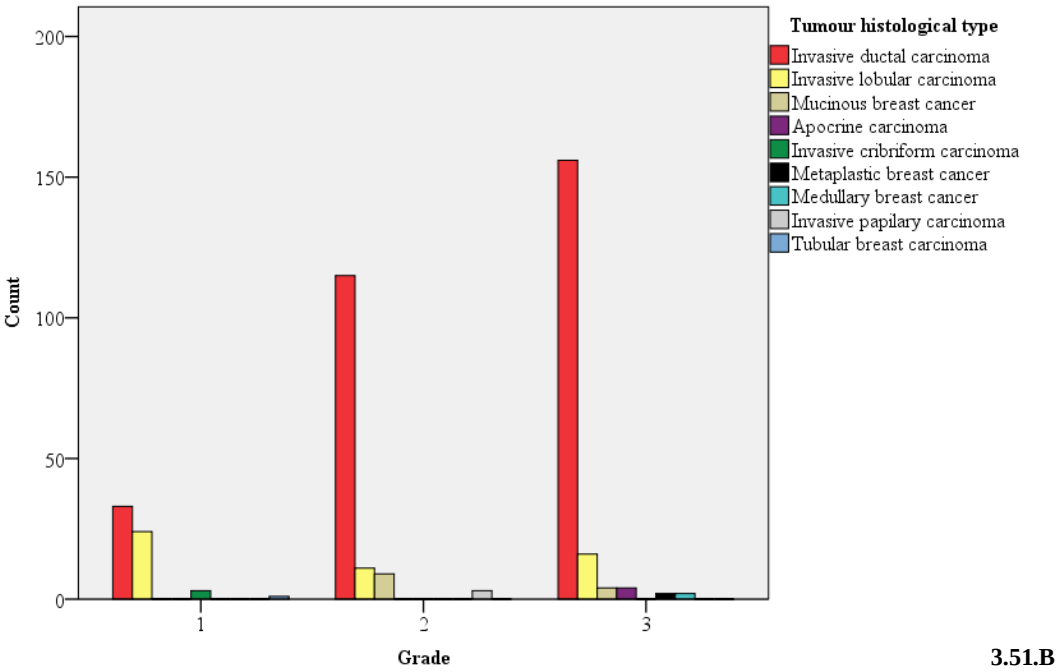


Figure 3.51. Relevance between breast cancer grade (G) and tumour morphological type. 3.51.A, by tumour histological type, 3.51.B, by G.

Among the breast cancers with high proliferation activity, pT2 tumours (measuring 2-5cm in largest diameter) were the most frequent finding, followed by pT1. In low Ki-67 group, pT1 cancers are dominating and pT3 – distinctly rare (Figure 3.52.A). Examining pT by Ki-67 (Figure 3.52.B), it can be concluded that higher size is associated with higher proliferation activity by Ki-67 (*P*=0.001).

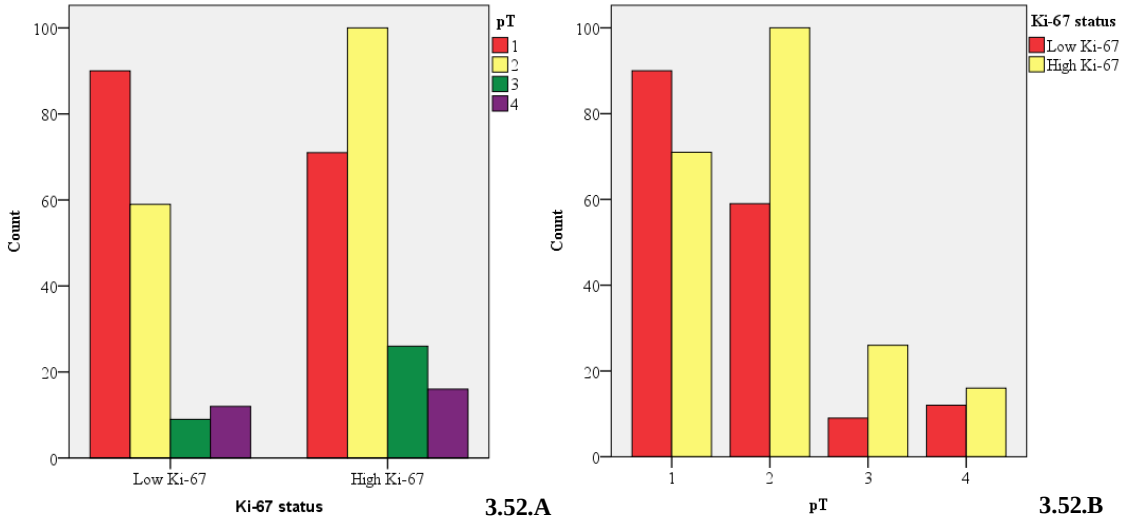


Figure 3.52. Relevance between breast cancer proliferation activity (Ki-67) and cancer size (pT). 3.52.A, by Ki-67 status; 3.52.B, by pT.

Statistical methods approved statistically significant association between presence and number of metastatic lymph nodes (pN) and PR status in breast cancer cells (*P*=0.03). Positive PR are more frequently found in breast cancers without

metastases in lymph nodes, but by increasing positive metastatic lymph node amount, positivity of PR decreases (Figure 3.53.A). However, in all pN groups PR positive breast cancers are more common than negative. By increasing involved lymph node amount (pN3), negativity of PR increases (P=0.03) as shown in Figure 3.53.B.

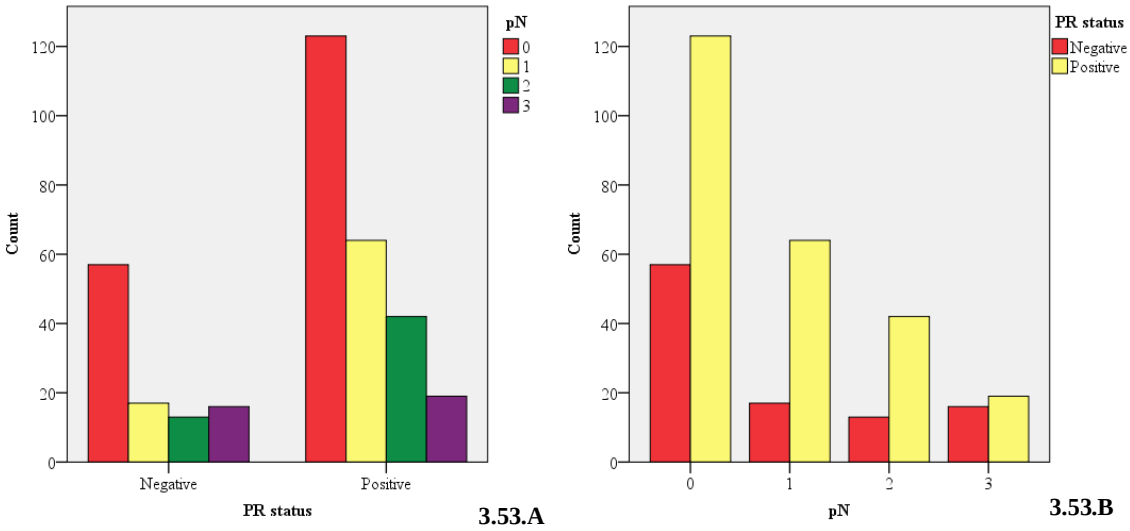


Figure 3.53. Relevance between breast carcinoma PR and lymph node status. 3.53.A, by PR status; 3.53.B, by pN.

Very strong, statistically significant association is found between breast cancer proliferation activity and involved lymph node status (P<0.0001). Despite the similarities between low and high Ki-67 groups where pN0 stage is more frequent than other pN stages (Figure 3.54.A), only pN0 group shows marked predominance of cases with low proliferation activity. As shown in Figure 3.54.B, by increasing number of metastases in axillary lymph nodes, Ki-67 activity (reflected by higher proportion of intensively proliferating cases) increases rapidly (P<0.0001).

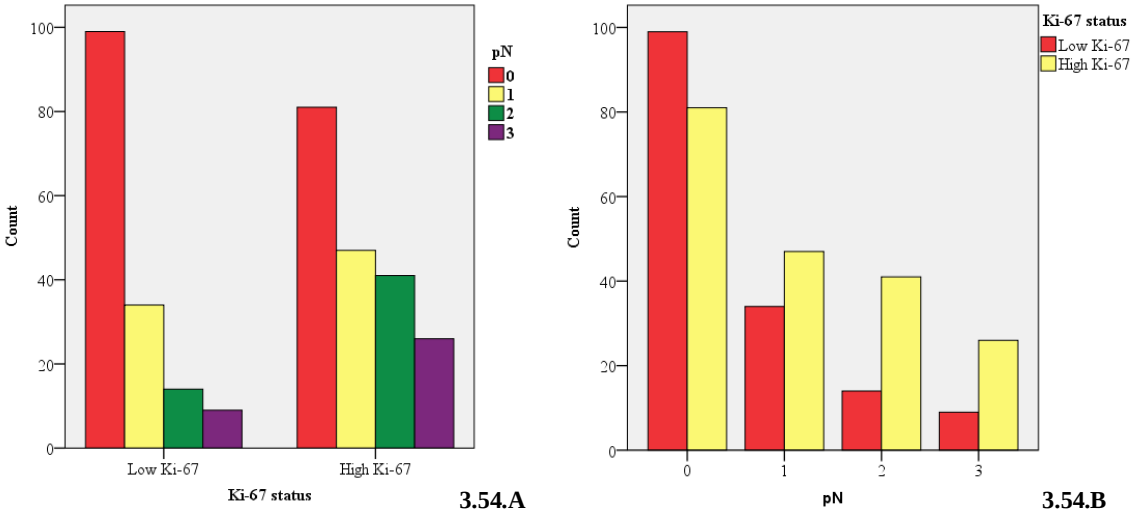


Figure 3.54. Relevance between breast cancer proliferation activity and lymph nodes status. 3.54.A, by Ki-67 status; 3.54.B, by pN.

Breast cancer grade and ER status (negativity or positivity) in breast cancer cells show statistically significant association (P<0.0001). Negativity of ER can be found more frequently in G3 breast cancers (P<0.0001), but it is distinctly rare finding in low or intermediate grade cancers (Figures 3.55.A and 3.55.B). The predominance of intermediate grade cancers among the ER positive group reflects lower number of G1 cases in the general group as well as tendency to ER negativity in high-grade cancers.

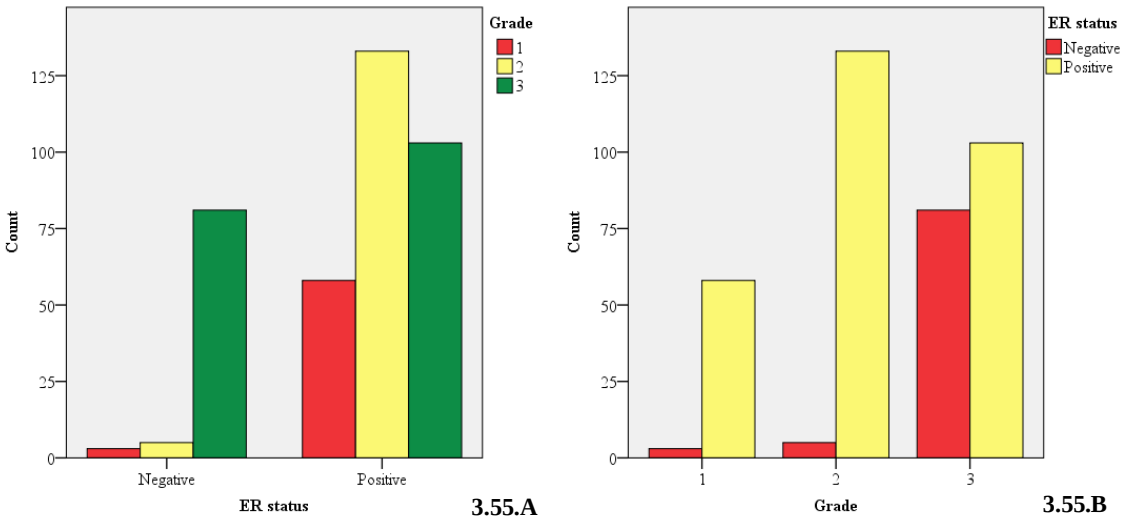


Figure 3.55. Relevance between cancer grade and ER.
3.55.A, by ER status; 3.55.B, by grade.

Breast cancer grade and negativity or positivity of PR in breast cancer cells shows statistically significant association (P<0.0001) similarly as between ER and G (Figures 3.56.A and Figure 3.56.B). There is association between G3 and negative PR status (P<0.0001).

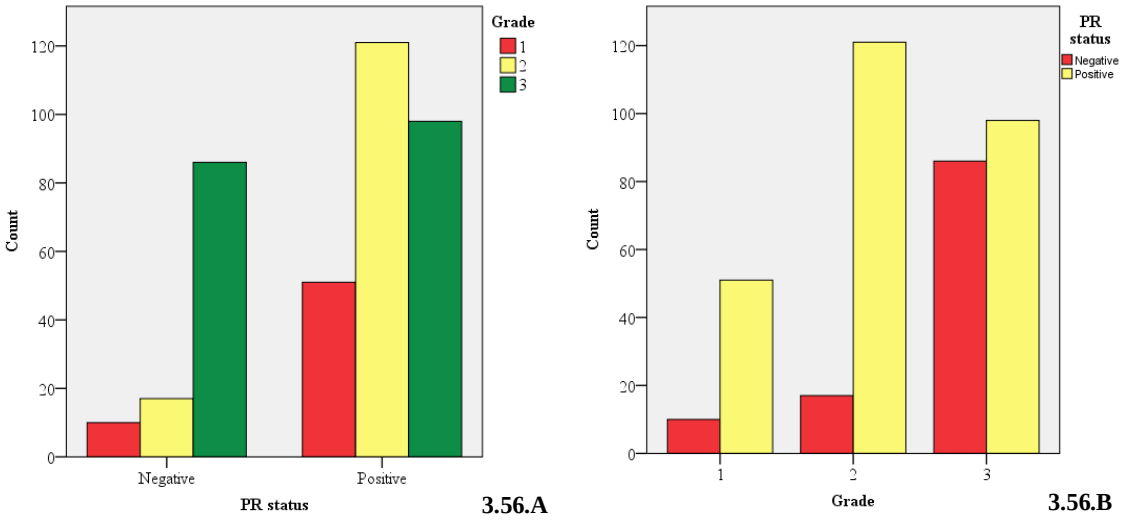


Figure 3.56. Relevance between cancer grade and PR.
3.56.A, by PR status; 3.56.B, by grade.

Analysing by Ki-67 status, tumours of all grades are almost equally represented in the low proliferation group, but tumours characterised by high proliferation rate show distinct predominance of high grade tumours (Figure 3.57.A). The observed differences are statistically significant (P<0.0001). In high grade cancer group, the proliferation activity was also high in most cases (Figure 3.57.B). The proportion of intensively proliferating cancers increases by growing tumour grade (P<0.0001).

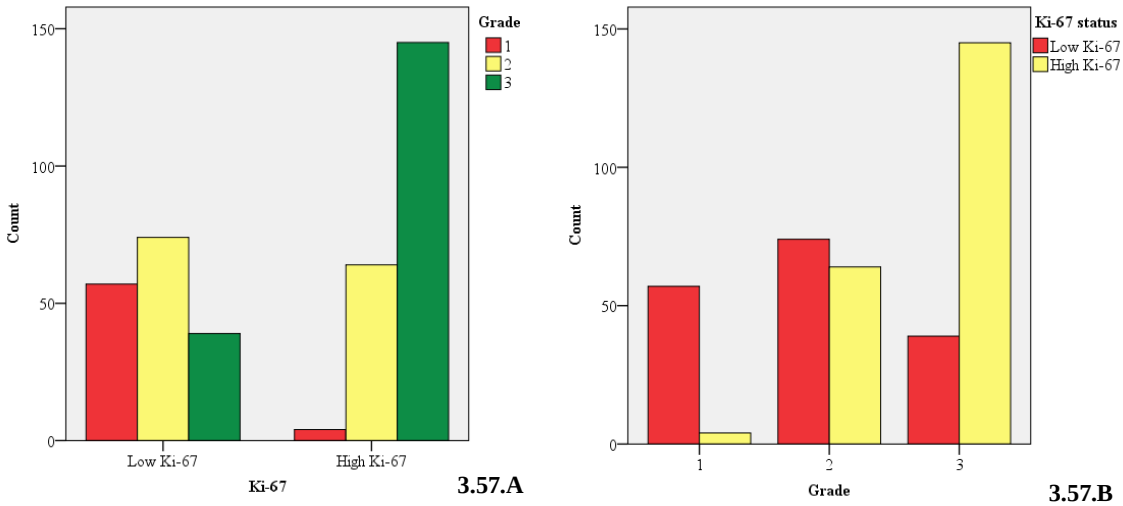


Figure 3.57. Relevance between breast cancer proliferation activity (Ki-67) and grade. 3.57.A, by Ki-67; 3.57.B, by grade.

HER2 receptor overexpression or absence in breast cancer cells is another prognostic and predictive factor. Subdividing all breast cancers in negative and positive HER2 receptor groups (Figure 3.58.A) there is evidence that HER2 negative group is heterogeneous by grade. In contrast, the HER2 positive breast cancer group shows clear-cut evidence of predominance of high grade breast cancers (P<0.0001). Analysing the breast cancer by grade (Figure 3.58.B), G1 and G2 cancers are predominantly HER2 negative, while G3 cancers comprise significant number of HER2 positive cases thus representing another evidence of heterogeneous structure (P<0.0001).

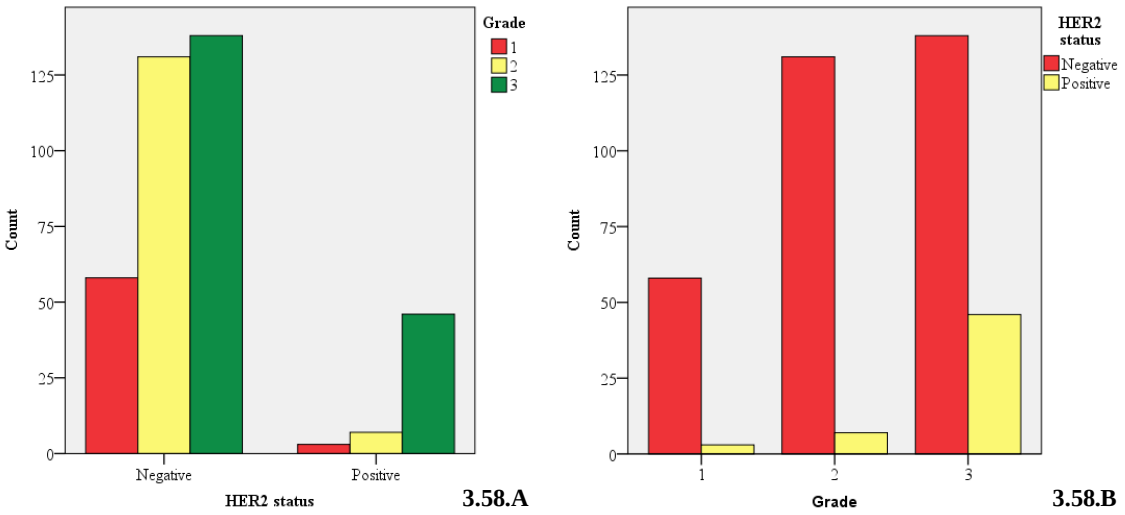


Figure 3.58. Relevance between breast cancer HER2 receptor status and grade. 3.58.A, by HER2 status; 3.58.B, by grade.

The p53-negative breast cancers represent heterogeneous group by cancer grade (Figure 3.59.A). If p53 is overexpressed it is seen in high grade breast cancer cells in high level (P<0.0001). Analysing the breast cancers by grade (Figure 3.59.B), higher number of p53 protein-positive cases is observed in G3 group (P<0.0001).

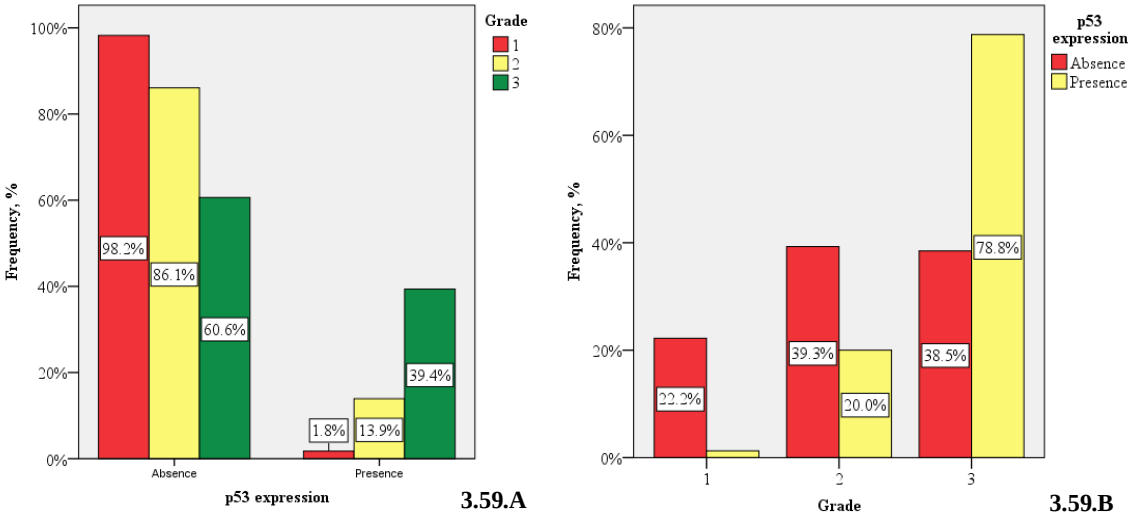


Figure 3.59. Relevance between breast cancer grade and expression of aberrant p53 protein. 3.59.A, by p53 expression; 3.59.B, by grade.

Among BCL2 negative cases, high grade cancers are the most frequent finding (Figure 3.60.A). BCL2 positive group is heterogeneous, showing high percentage of low and intermediate grade cancers (P<0.0001). The number of BCL2 negative cases increases by increasing grade (Figure 3.60.B). The difference is statistically significant (P<0.0001).

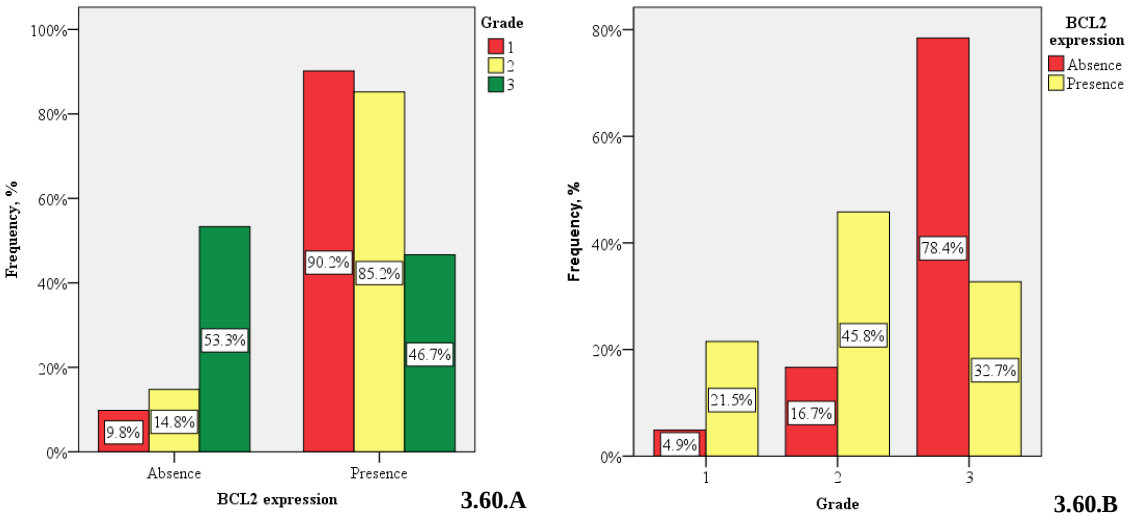


Figure 3.60. Relevance between breast cancer grade and BCL2 protein expression. 3.60.A, by BCL2 expression; 3.60.B, by grade.

The p53 negative breast cancer group shows distinct predominance of ER positive cases: 15.4% ER negative versus 84.6% ER positive breast cancer cases (Figure 3.61.A). The p53 positive group is heterogeneous regarding ER receptor status: 51.9% ER negative versus 48.1% ER positive cases (P<0.0001). The ER positive cases (Figure 3.61.B) are heterogeneous by p53 protein status but negative cases predominate in p53 positive group (P<0.0001).

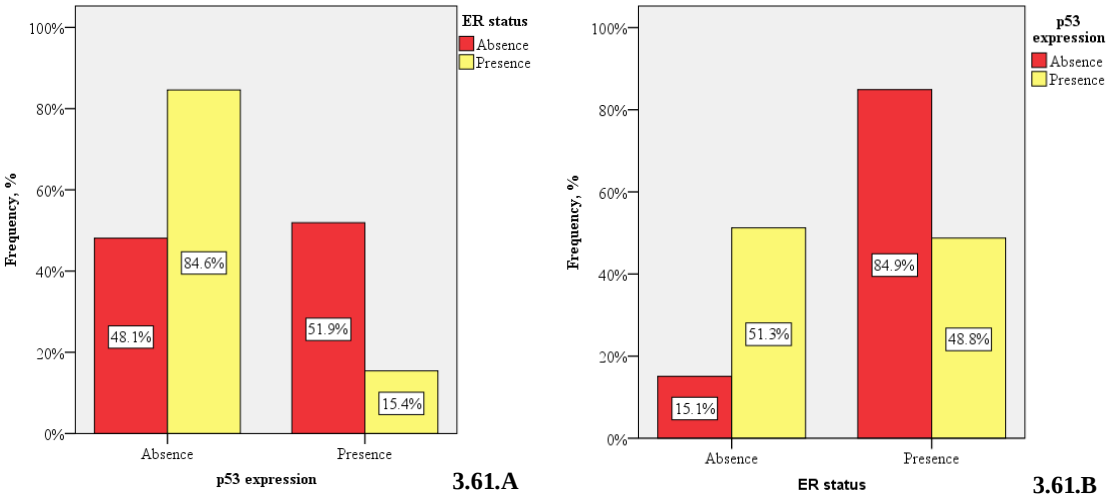


Figure 3.61. Relevance between breast cancer ER status and aberrant p53 protein expression. 3.61.A, by p53 expression; 3.61.B, by ER status.

Similar data were obtained considering the association between PR status and p53 protein expression (Figure 3.62.A). The PR expression is observed more frequently in p53 negative cases (*P*<0.0001). Comparing PR positive and negative breast cancer cases, the p53 expression is more frequent in negative PR group (Figure 3.62.B). The difference is statistically significant (*P*<0.0001).

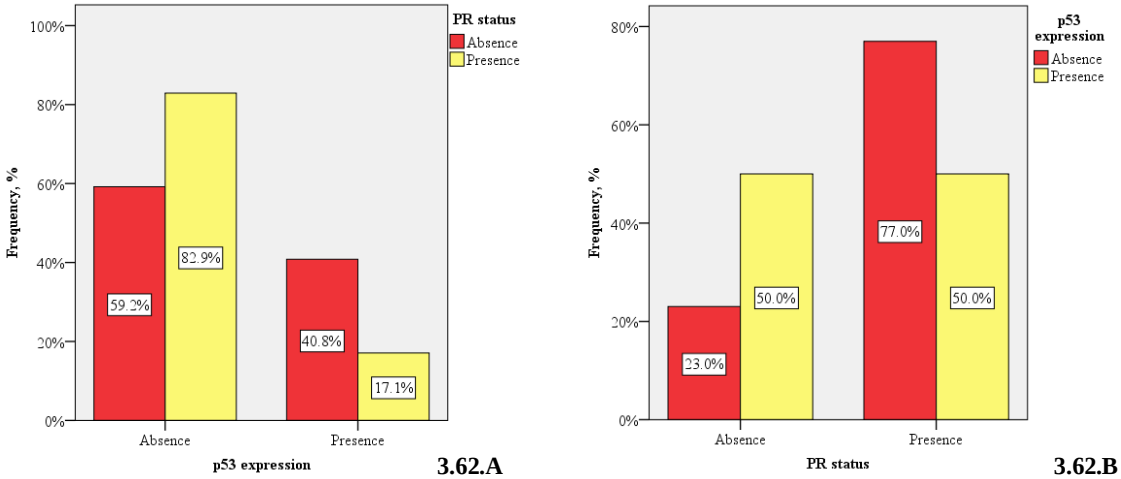


Figure 3.62. Relevance between breast cancer PR status and p53 protein expression. 3.62.A, by p53 expression; 3.62.B, by PR status.

Both p53 positive and negative groups share fraction of HER2 positive cases (Figure 3.63.A). However, p53 negative group includes higher number of HER2 negative cases (*P*=0.001). Dividing the study group into HER2 positive and negative cases (Figure 3.63.B), HER2 negative group contains more p53 negative cases as HER2 positive group (*P*=0.001).

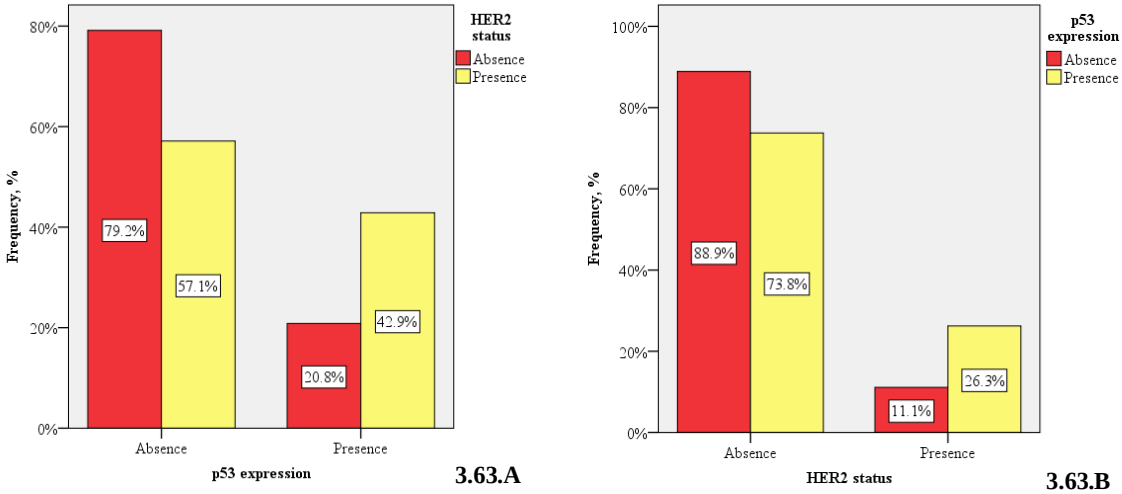


Figure 3.63. Relevance between breast cancer HER2 receptor status and aberrant p53 protein expression. 3.63.A, by p53 expression; 3.63.B, by HER2 receptor status.

The p53 negative group is heterogeneous regarding proliferation activity (Figure 3.64.A). However, p53 positive group contains more cases featuring high proliferative activity (P<0.0001). In low Ki-67 group (Figure 3.64.B) most of cancers are p53 negative. In highly proliferative group, there are more p53 positive cases (P<0.0001).

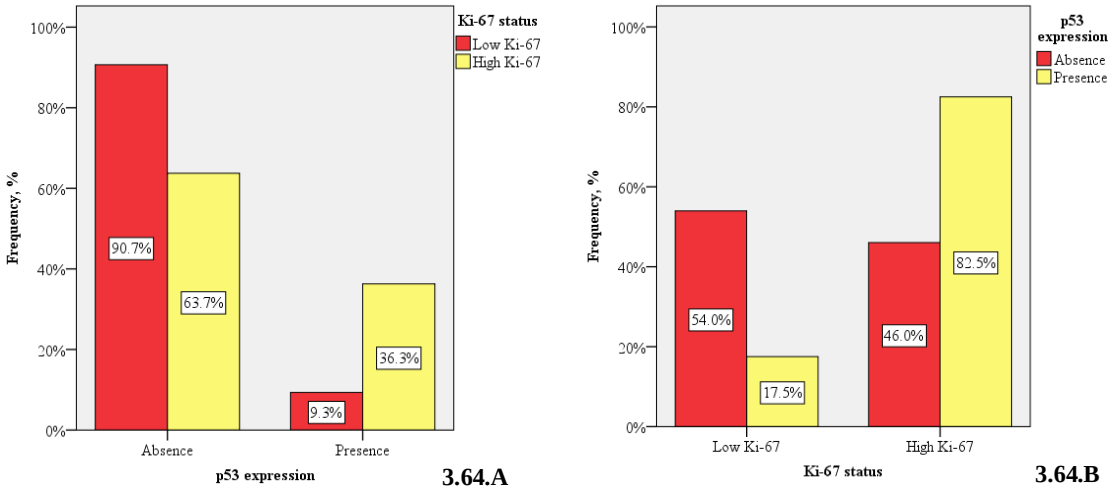


Figure 3.64. Relevance between breast cancer proliferation activity and expression of aberrant p53 protein. 3.64.A, by p53 expression; 3.64.B, by Ki-67 status.

Expression of aberrant p53 protein and BCL2 protein has tendency to mutual exclusion. The p53 negative group shows predominance of BCL2 positivity as shown in Figure 3.65.A where p53 negative group contains more BCL2 positive cases, but p53 positive group - BCL2 negative cases (P<0.0001). Analysing the cases by BCL2 expression (Figure 3.65.B) the findings are analogous (P<0.0001).

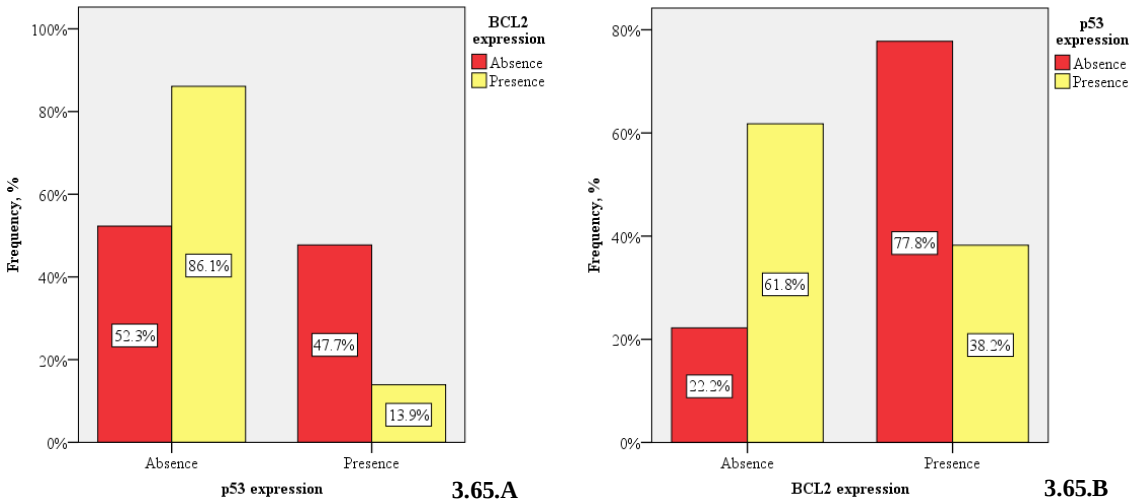


Figure 3.65. Relevance between BCL2 and aberrant p53 protein expression in the breast cancer. 3.65.A, by p53 expression; 3.65.B, by BCL2 expression.

There is statistically significant association (P=0.002) between CK 5/6 negativity and p53 negativity with predominance of CK 5/6 negativity in p53 negative

group (Figure 3.66.A). Similarly, subdividing the breast cancers by CK 5/6 expression in positive and negative groups (Figure 3.66.B) p53 negativity is more frequent in CK 5/6 negative breast cancer group (*P*<0.0001).

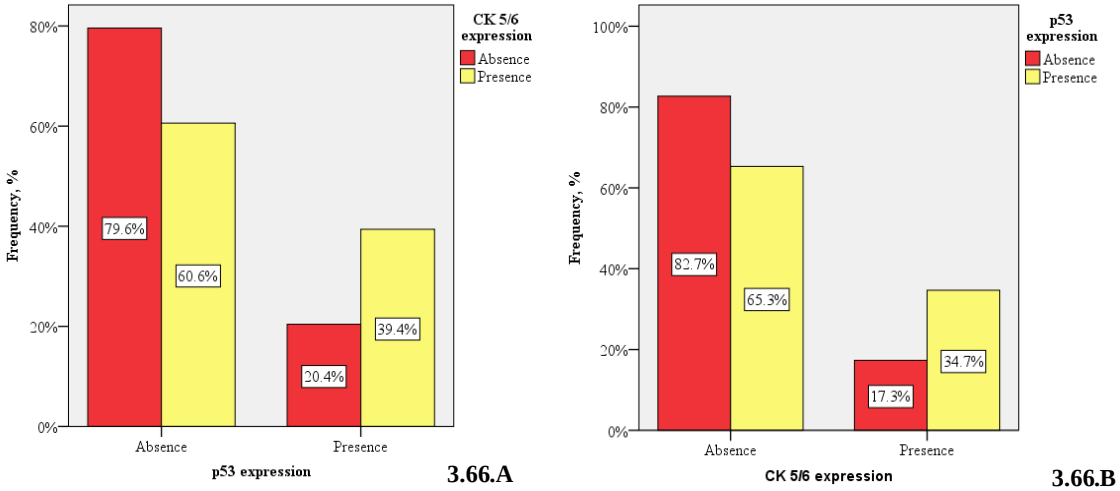


Figure 3.66. Relevance between CK 5/6 and aberrant p53 protein expression in breast cancer. 3.66.A, by p53 expression; 3.66.B, by CK 5/6 expression.

Determining BCL2 positivity or negativity in the breast cancer cells and comparing the findings with ER expression (Figure 3.67.A), the BCL2 positive group includes higher number of ER receptor positive cases (*P*<0.0001). Breast cancers with positive ER receptors are more frequently BCL2 positive (Figure 3.67.B). The difference is statistically significant (*P*<0.0001).

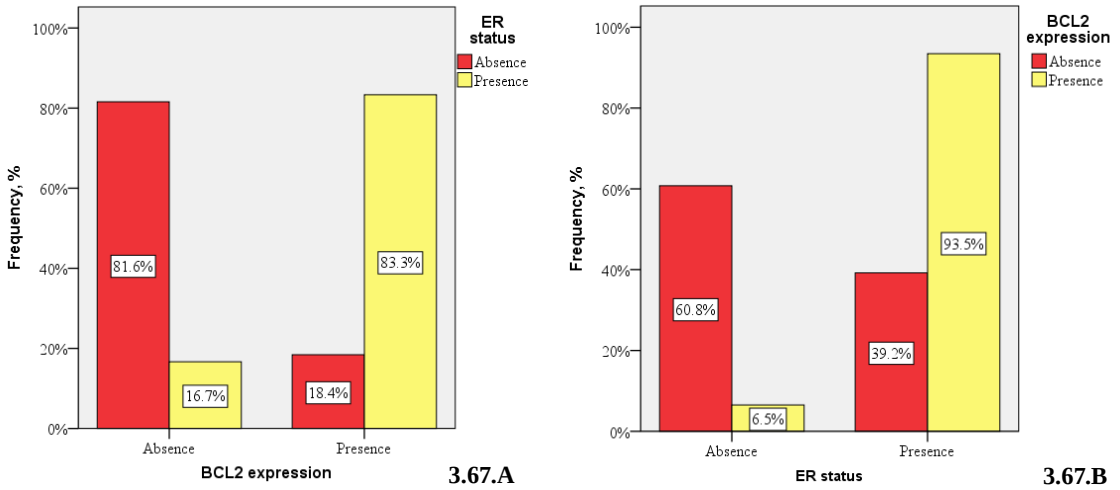


Figure 3.67. Relevance between breast cancer ER status and BCL2 protein expression. 3.67.A, by BCL2 expression; 3.67.B, by ER status.

Similar results are obtained regarding PR and BCL2 expression. In BCL2 negative group, there are more PR negative cases but BCL2 positive breast cancer cells mostly express PR (Figure 3.68.A). The difference is statistically significant

(P<0.0001). PR positive breast cancer cases show statistically significantly more frequent BCL2 positivity as well (Figure 3.68.B).

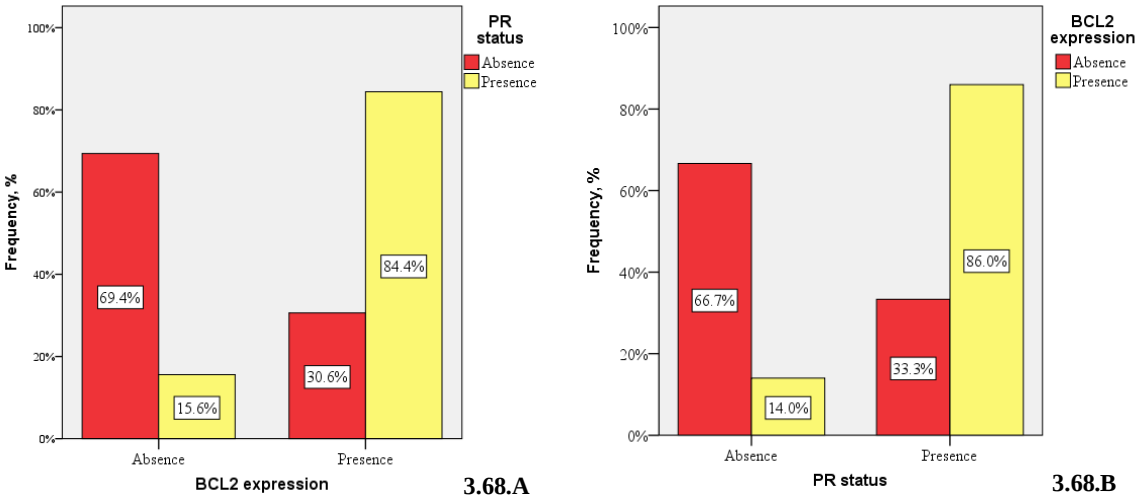


Figure 3.68. Relevance between breast cancer PR status and BCL2 protein expression. 3.68.A, by BCL2 expression; 3.68.B, by PR status

The BCL2 negative group is heterogeneous regarding HER2 overexpression (Figure 3.69.A). BCL2 positive group is predominantly HER2 negative (P<0.0001). Similarly, HER2 negative group contains more BCL2 positive cases as HER2 positive group (Figure 3.69.B) where more frequent occurrence of BCL2 negative cases is observed (P<0.0001).

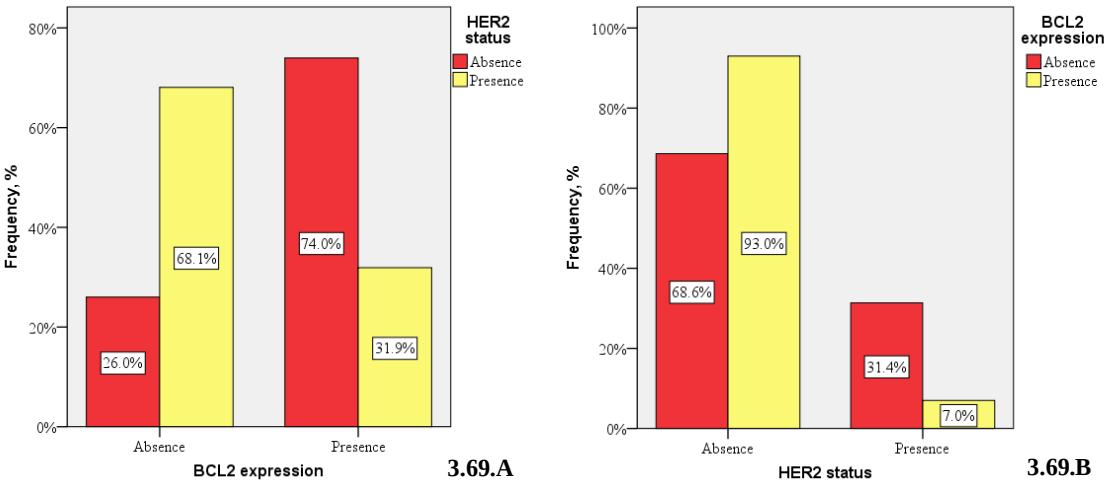


Figure 3.69. Relevance between breast cancer HER2 receptor status and BCL2 protein expression. 3.69.A, by BCL2 expression; 3.69.B, by HER2 receptor status.

Regarding proliferation activity in breast cancer cells, high proliferative activity is more frequent in BCL2 negative group while low proliferation is more typical finding within BCL2 positive group (Figure 3.70.A). The difference is statistically significant (P<0.0001). The association is even more clearly evident, if the groups are separated by

Ki-67 expression (Figure 3.70.B). The cases showing low proliferation activity also mostly are BCL2 positive (*P*<0.0001). Again, the heterogeneity of tumours is evident by Ki-67 and BCL2 analysis.

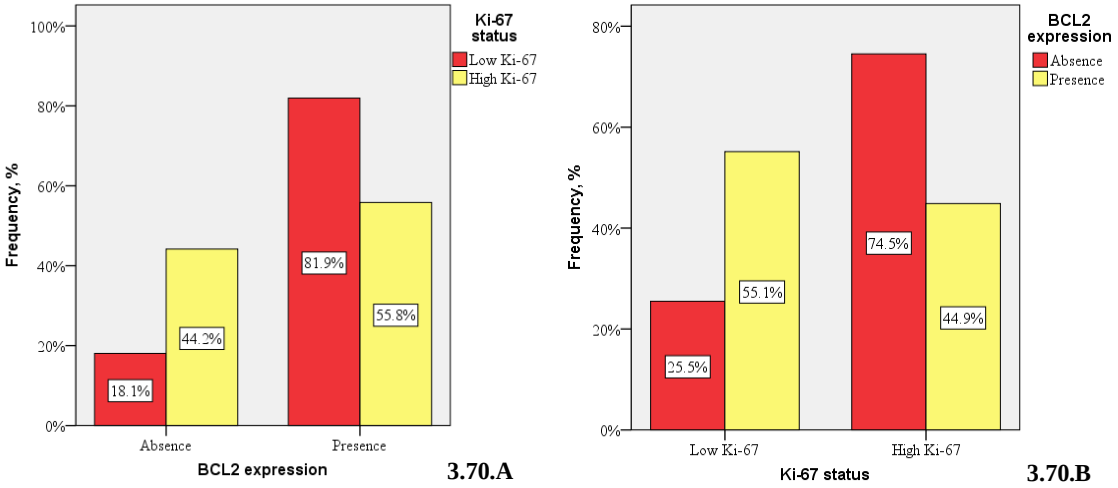


Figure 3.70. Relevance between breast cancer proliferation activity and BCL2 protein expression. 3.70.A, by BCL2 expression; 3.70.B, by Ki-67 status.

Statistically significant data are shown regarding the association between two new potentially prognostic and predictive factors as BCL2 and cyclin D1 (*P*<0.0001). The BCL2 positive group includes more cyclin D1 positive cases (Figure 3.71.A). Similarly, cyclin D1 negative group is heterogeneous by BCL2 expression (Figure 3.71.B). In contrast, cyclin D1 positive group show clear-cut predominance of BCL2 positive cases (*P*<0.0001).

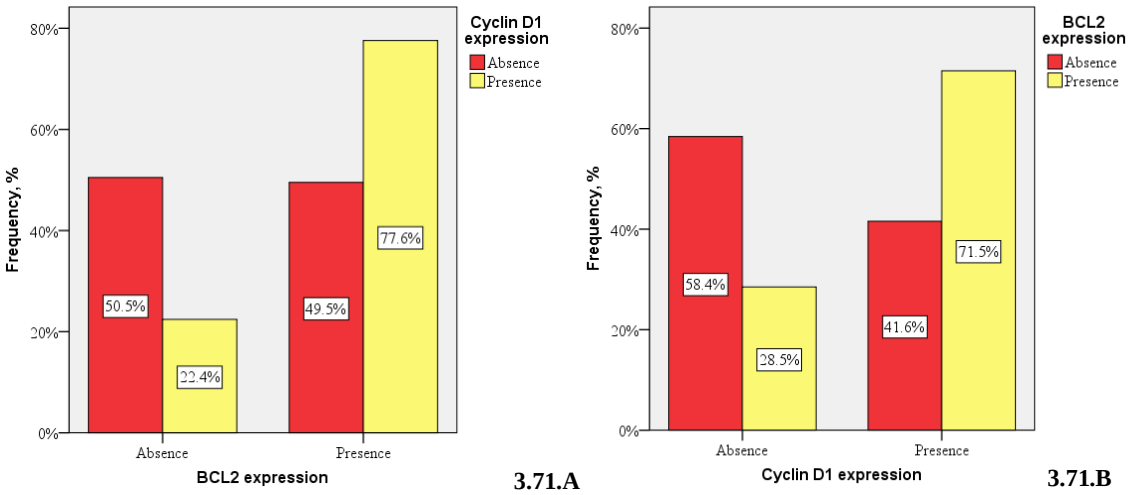


Figure 3.71. Relevance between cyclin D1 and BCL2 protein expression in breast cancer. 3.71.A, by BCL2 expression; 3.71.B, by cyclin D1 expression.

There are statistically significant data (*P*=0.02) regarding the association of other potentially prognostic and predictive factors as BCL2 and CK 5/6 (Figures 3.72.A and 3.72.B). The BCL2 positive group shows marked predominance of CK 5/6 negative cases (*P*=0.02).

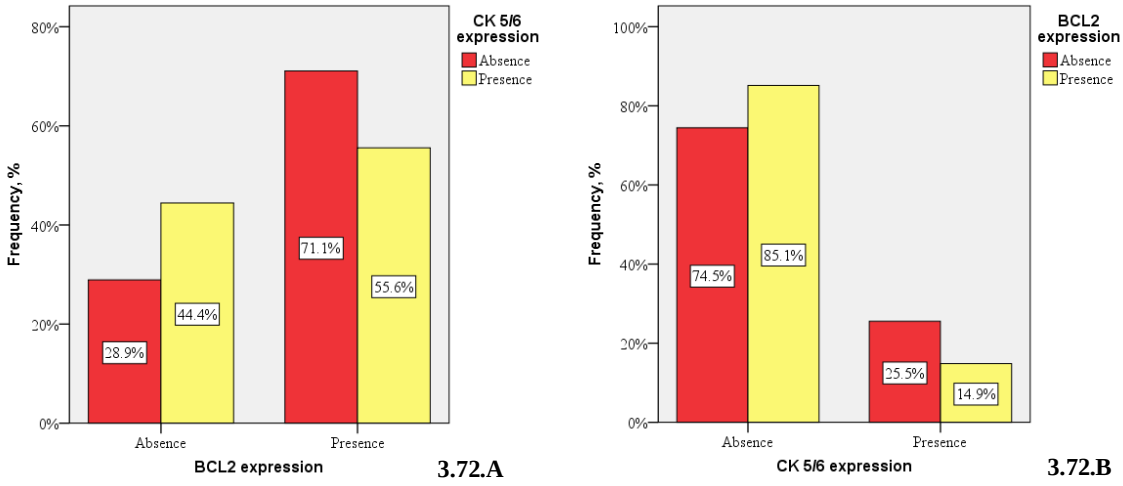


Figure 3.72. Relevance between CK 5/6 and BCL2 protein expression in breast cancer. 3.72.A, by BCL2 expression; 3.72.B, by CK 5/6 expression.

COX-2 negativity is statistically significantly associated with ER expression in the breast cancer (*P*=0.002) as shown in Figure 3.73.A and Figure 3.73.B.

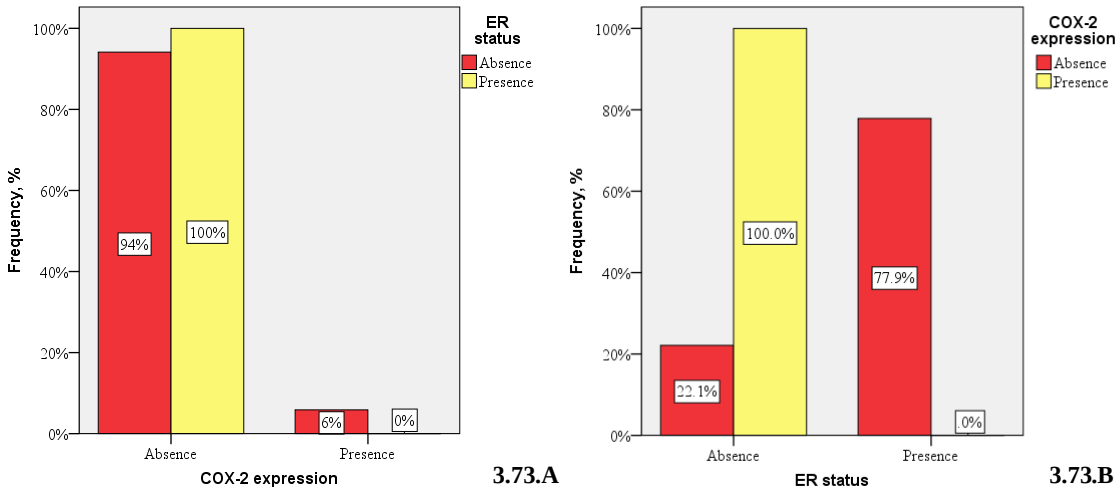


Figure 3.73. Relevance between breast cancer ER status and COX-2 expression. 3.73.A, by COX-2 expression; 3.73.B, by ER status.

Similar association was observed between COX-2 negativity and PR expression (*P*=0.008) and was shown in Figure 3.74.A and Figure 3.74.B.

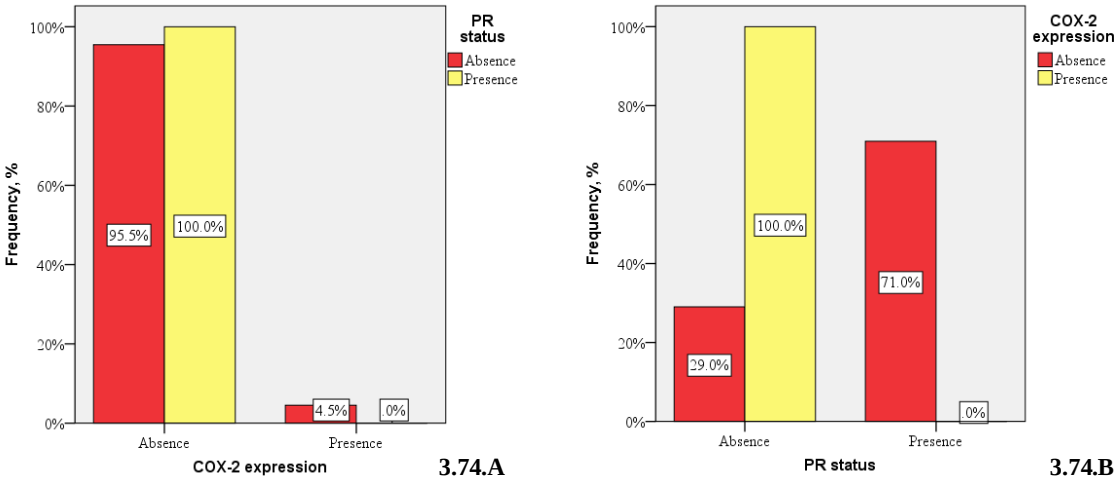


Figure 3.74. Relevance between breast cancer PR status and COX-2 expression. 3.74.A, by COX-2 expression; 3.74.B, by PR status.

Statistically significant association was found between the two potential prognostic and predictive factors as COX-2 and CK 5/6 (*P*=0.001). The COX-2 positivity is observed only in a subgroup of CK 5/6 positive cases (Figure 3.75.A) but is virtually absent in CK 5/6 negative cases (Figure 3.75.B) characterised by *P*=0.001. The COX-2 negative cases are observed both in CK 5/6 positive and CK 5/6 negative groups.

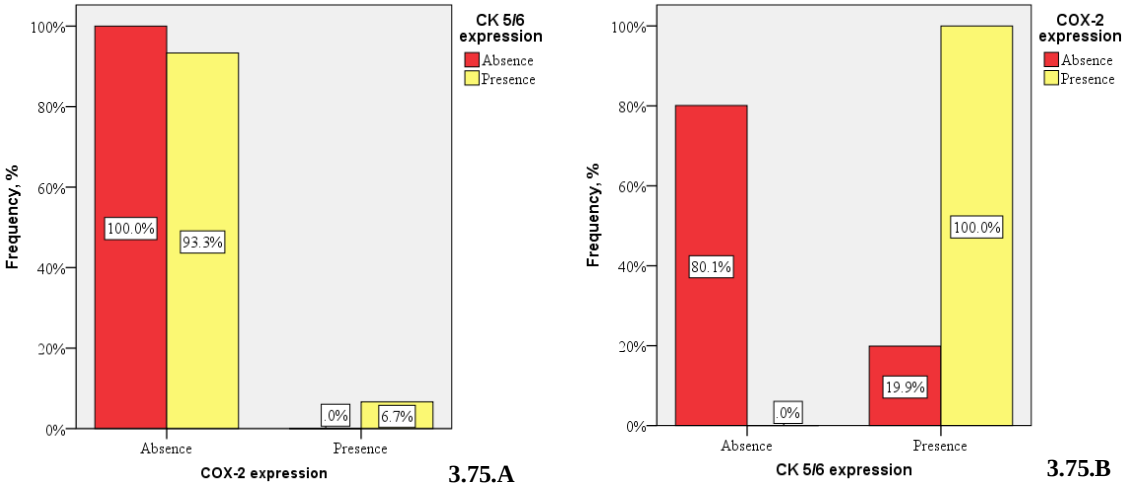


Figure 3.75. Relevance between CK 5/6 and COX-2 expression in breast cancer. 3.75.A, by COX-2 expression; 3.75.B, by CK 5/6 expression.

Cyclin D1 expression shows strong association with presence of ER (Figure 3.76.A). The finding is statistically significant (P<0.0001). The analysis is confirmed (P<0.0001) if cyclin D1 expression is evaluated by ER status (Figure 3.76.B).

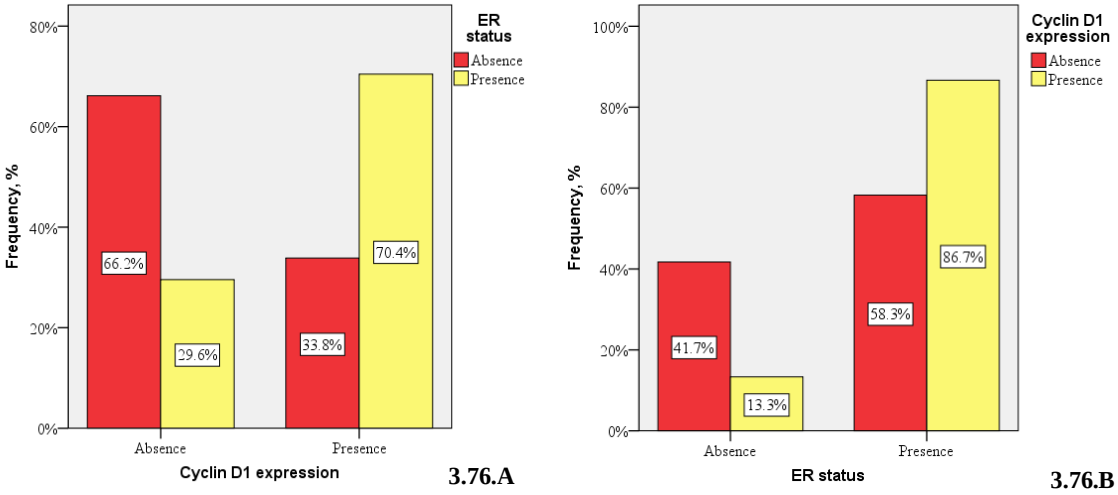


Figure 3.76. Relevance between breast cancer ER status and cyclin D1 expression. 3.76.A, by cyclin D1 expression; 3.76.B, by ER status.

Similar data are obtained regarding cyclin D1 and PR status (Figures 3.77.A and Figure 3.77.B), where majority of PR positive cases are in the cyclin D1 positive group (P<0.0001)

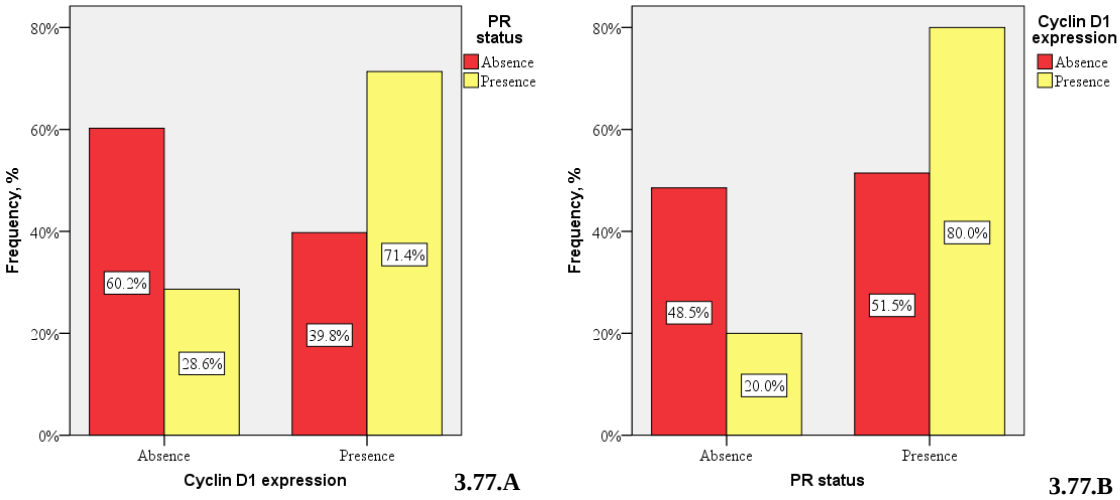


Figure 3.77. Relevance between breast cancer PR status and cyclin D1 expression. 3.77.A, by cyclin D1 expression; 3.77.B, by PR status.

CK 5/6 negativity is observed in both cyclin D1 negative and positive groups with predominance of CK 5/6 negativity in cyclin D1 positive group (Figures 3.78.A and Figure 3.78.B). The difference is statistically significant (*P*=0.004).

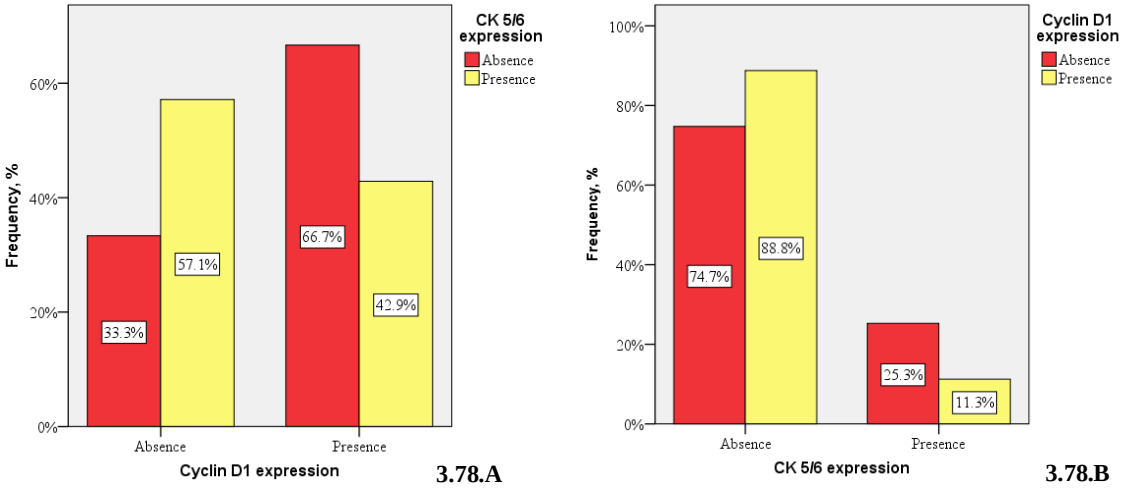


Figure 3.78. Relevance between cyclin D1 and CK5/6 expression in breast cancer. 3.78.A, by cyclin D1; 3.78.B, by CK 5/6 expression.

In the CK 5/6 negative group, ER positivity is observed more frequently than lack of ER expression (Figure 3.79.A). The CK 5/6 positive breast cancers show contrary reciprocal relationship between ER positive and negative cases. The difference is statistically significant (*P*=0.003). CK 5/6 negativity is associated with ER positive group, but ER negative group shows more CK 5/6 positive cases (Figure 3.79.B); the finding is significant as well (*P*=0.003).

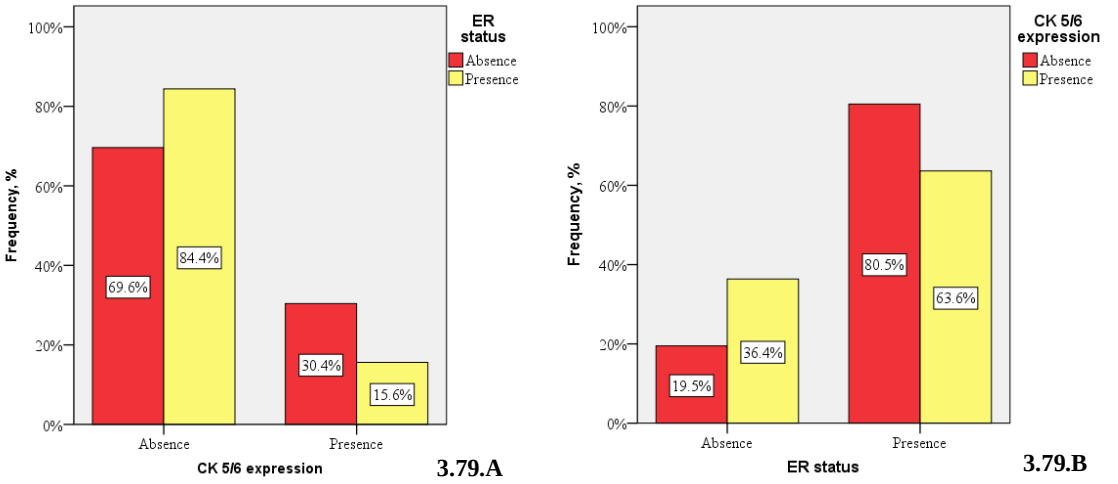


Figure 3.79. Relevance between breast cancer ER status and CK 5/6 expression. 3.79.A, by CK 5/6 expression; 3.79.B, by ER status.

Similar data are obtained regarding PR and CK 5/6 expression. The data are represented in Figures 3.80.A and 3.80.B. The association is statistically significant (*P*=0.004).

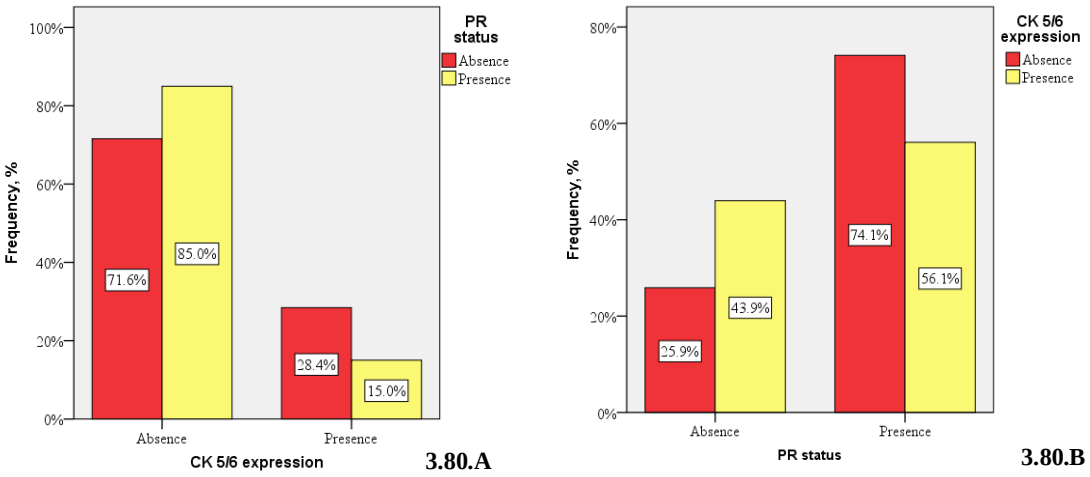


Figure 3.80. Relevance between breast cancer PR status and CK 5/6 expression. 3.80.A, by CK 5/6 expression; 3.80.B, by PR status.

The analysis of proliferation activity by cytokeratin 5/6 status is embarrassed by the fact that majority of breast cancer cases are CK 5/6 negative (Figure 3.81.A). However, the CK 5/6 negative group is characterised by predominance of cases exhibiting low proliferation activity in contrast to CK 5/6 positive group showing more frequent occurrence of breast carcinoma with high proliferation fraction (*P*=0.047). Among cases showing high proliferation activity (Figure 3.81.B) there are more CK 5/6 positive cases (*P*=0.047).

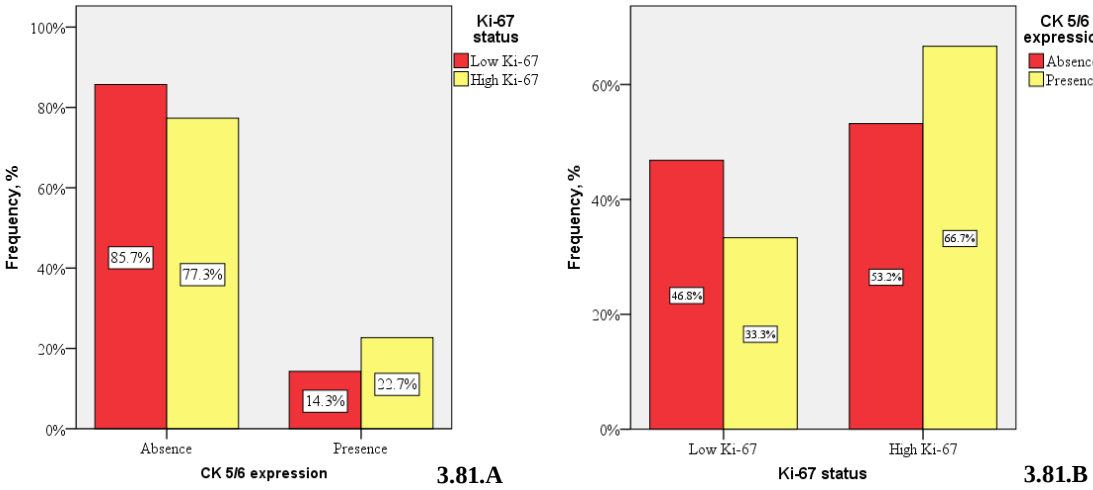


Figure 3.81. Relevance between breast cancer proliferation activity and CK 5/6 expression. 3.81.A, by CK 5/6 expression; 3.81.B, by Ki-67 status.

3.6. Survival

In the observed period, 39 patients have died from breast cancer. In the general group of 383 patients such number of undesirable events is low embarrassing statistic evaluation. However, Kaplan-Meier survival analysis by pT shows statistically significant association between pT stage and survival. pT4 stage is associated with higher death rate within the first year in comparison with pT2 or pT3 (P<0.0001) as shown in Figure 3.82.

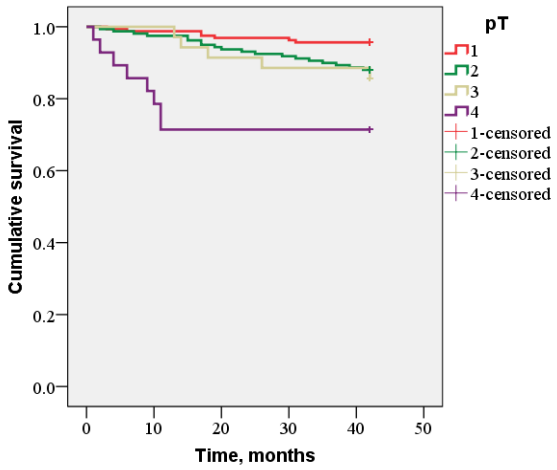


Figure 3.82. Kaplan-Meier breast carcinoma specific survival in relation to size (pT) of breast carcinoma.

More than 10 metastatic lymph nodes is associated with poor outcome 11 months earlier comparing to cases without metastases (P<0.0001) or 7 months earlier than pN2 cases (P<0.0001). Survival in patients having 1-3 positive lymph nodes corresponding to N1 is the same as cases without lymph node metastases (P<0.0001) as shown in Figure 3.83.

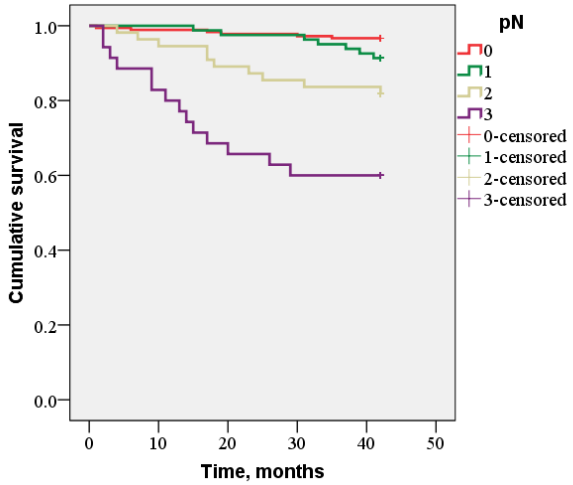


Figure 3.83. Kaplan-Meier breast carcinoma specific survival in relation to metastases in lymph nodes (pN).

Kaplan-Meier survival analysis by breast cancer grade shows statistically significant association between grade and survival (*P*=0.001), but analysed data are affected by small account of G1 cases (Figure 3.84.).

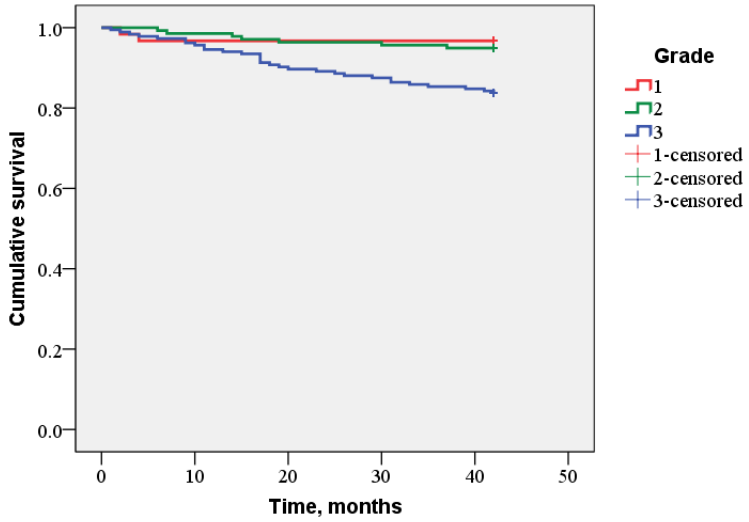


Figure 3.84. Kaplan-Meier breast carcinoma specific survival in relation to grade of breast carcinoma.

Patients with luminal A, luminal B (HER2 negative) and HER2 positive breast carcinoma molecular subtypes statistically significantly survive 6 months longer than patients with triple negative breast carcinoma molecular subtype (*P*<0.0001, *P*<0.0001, *P*=0.001, respectively). Patients with luminal B (HER2 positive) breast carcinoma molecular subtype live 5 months longer than triple negative molecular subtype patients (*P*=0.02) as shown in Figure 3.85.

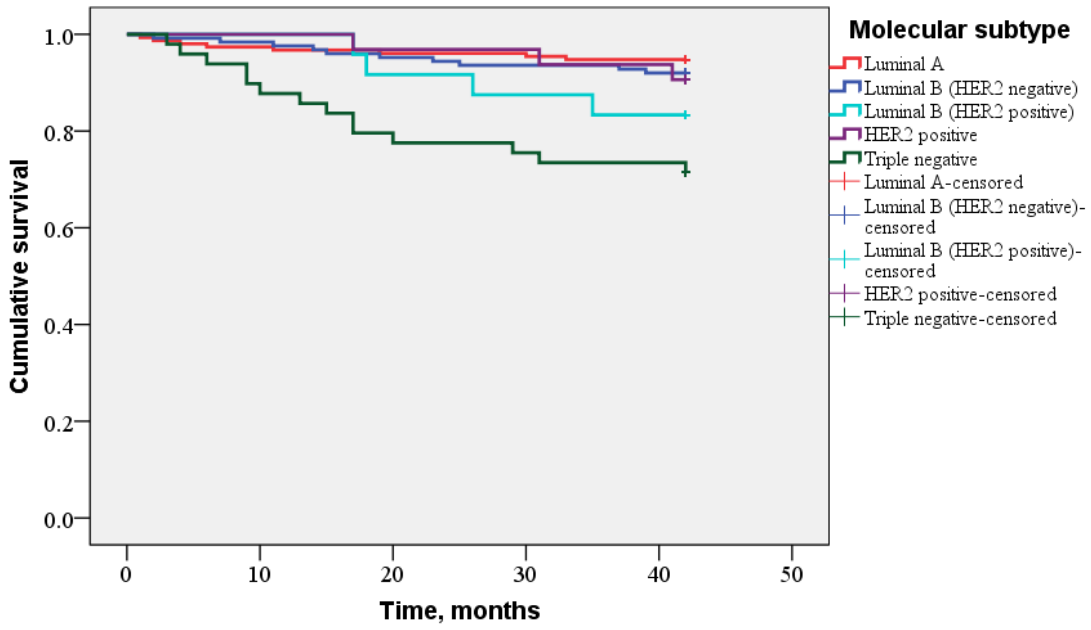


Figure 3.85. Kaplan-Meier breast carcinoma specific survival in relation to molecular subtypes of breast carcinoma.

Among the new potential immunohistochemical markers as p53, BCL2, COX-2, cyclin D1 and CK 5/6, Kaplan-Meier survival analysis shows statistically significant association between survival and expression or absence of p53 (P=0.03) and expression or absence of BCL2 (P=0.002). Patients without p53 expression in breast carcinoma survive 2 months longer than patients with p53 expression (Figure 3.86.). Patients with positive expression of BCL2 in breast carcinoma survive 2 months longer than patients lacking BCL2 expression (Figure 3.87).

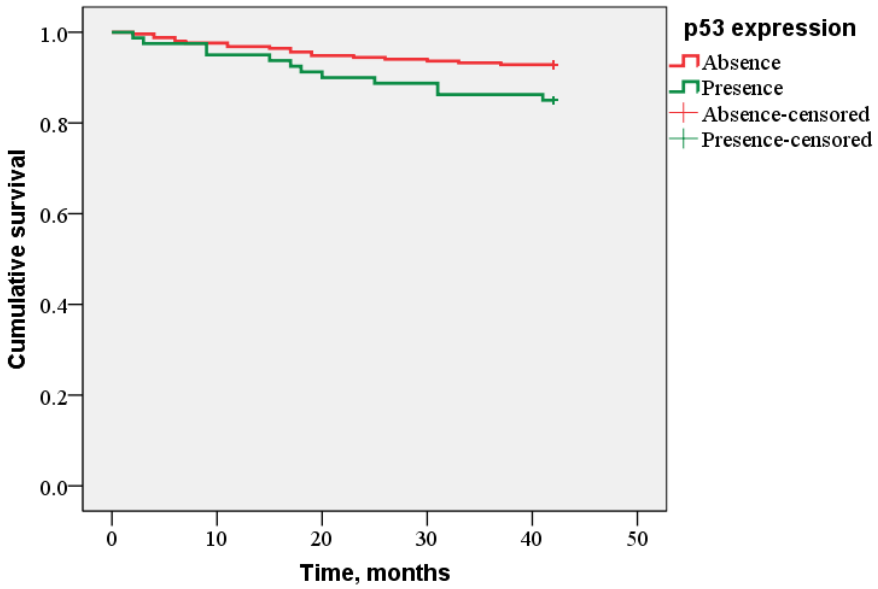


Figure 3.86. Kaplan-Meier breast carcinoma specific survival in relation to presence of p53 in breast carcinoma cells.

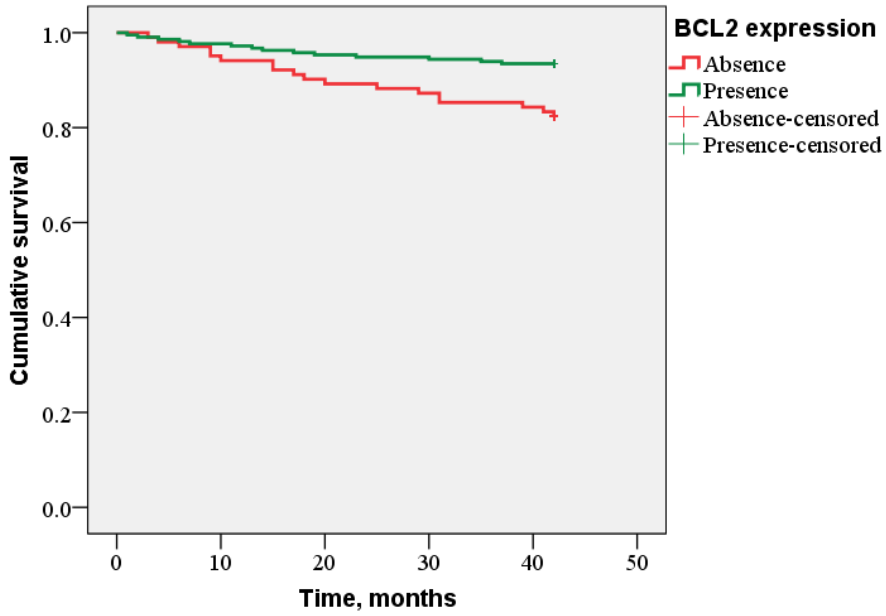


Figure 3.87. Kaplan-Meier breast carcinoma specific survival in relation to presence and absence of BCL2 in breast carcinoma cells.

3.7. Statistically non-significant results

No statistically significant associations were detected between breast cancer molecular subtypes and tumour invasion in lymphatic vessels (Figure 3.88.) or veins, perineural tumour growth and presence of microcalcifications (Figure 3.89.). Cancer invasion in arteries was not observed in any of investigated case.

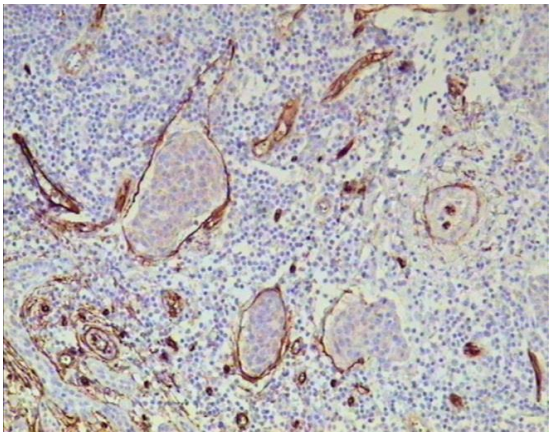


Figure 3.88. Breast carcinoma invading lymphatic vessels. Anti-CD34, immunoperoxidase, original magnification 50 x.

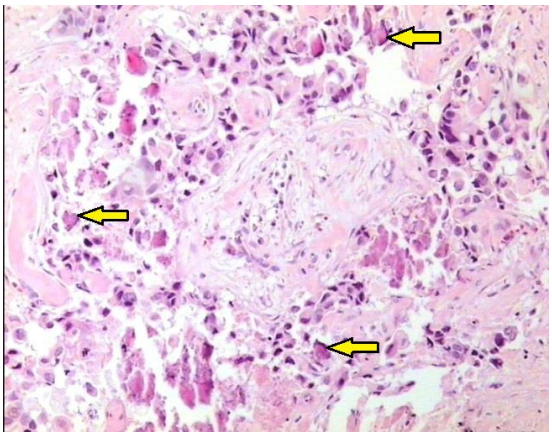


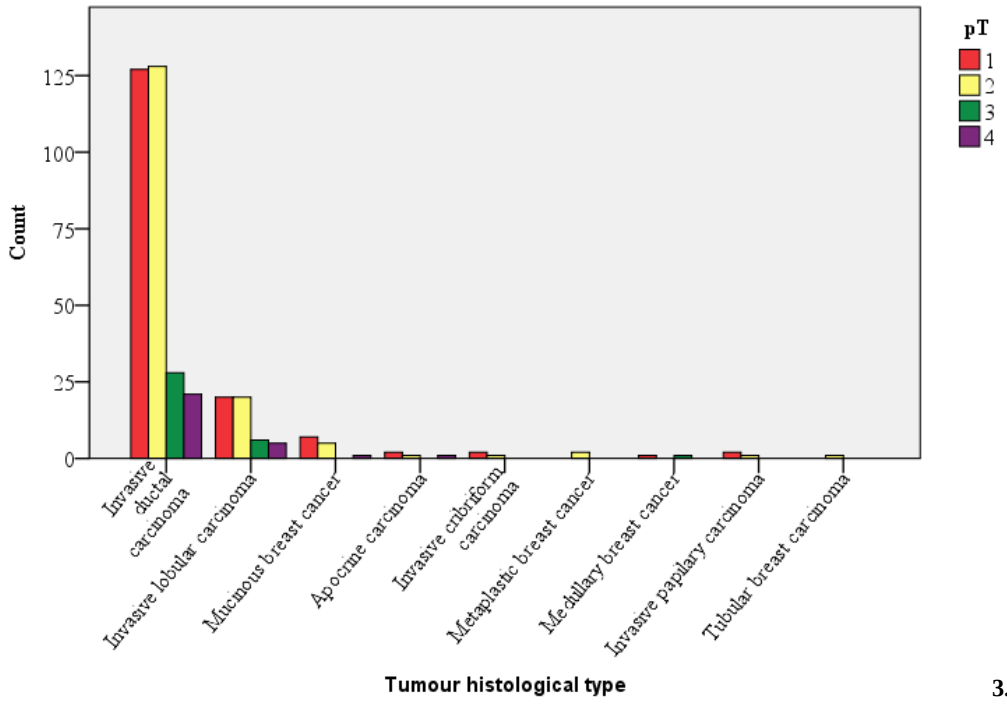
Figure 3.89. Microcalcifications (arrows) in high grade invasive ductal breast cancer. Haematoxylin – eosin. Original magnification 50 x.

Microphotographs by A.Abolins.

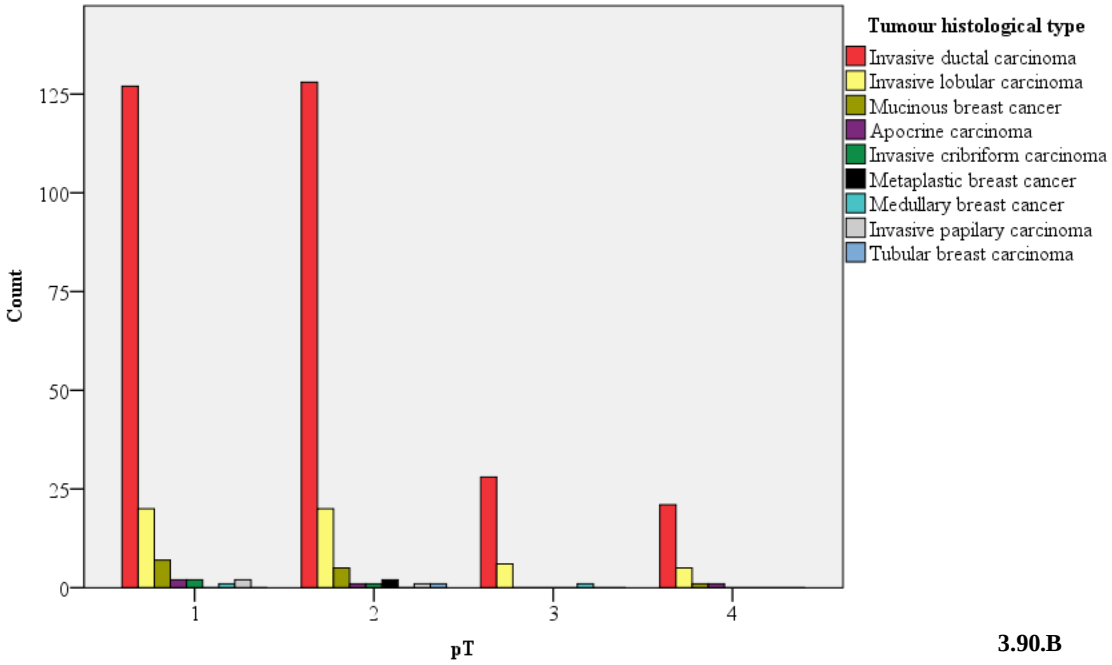
There was no statistically significant association between age and pT (P=0.06), pN (P=0.7) and breast cancer grade (P=0.1).

No statistically significant association was observed between breast cancer grade and perineural growth (P=0.2).

Ductal breast carcinoma is the most frequent morphological type of breast cancer. It was mostly diagnosed when the tumour measured less than 5 cm in largest diameter (pT1 and pT2). Metaplastic and tubular breast cancers were larger than 2 cm but not more than 5 cm (pT2). However, the number of such cases was limited possibly contributing to lack of statistically significant association between morphological type and local spread (pT) of the breast cancer (P=0.9). The relevant analysis is shown in Figures 3.90.A and 3.90.B.



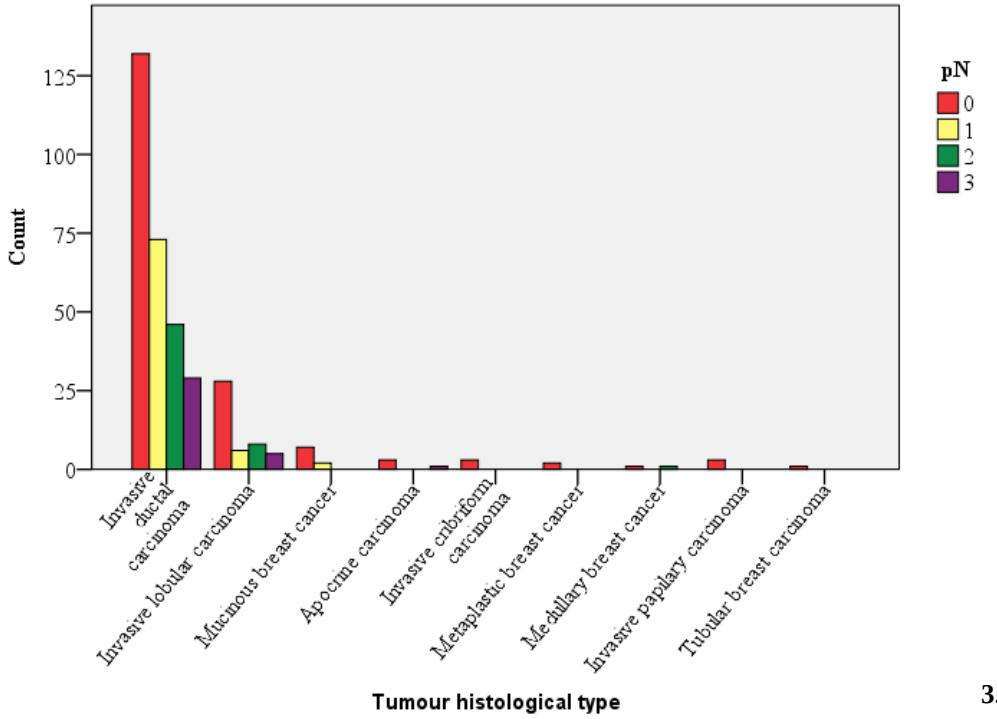
3.90.A



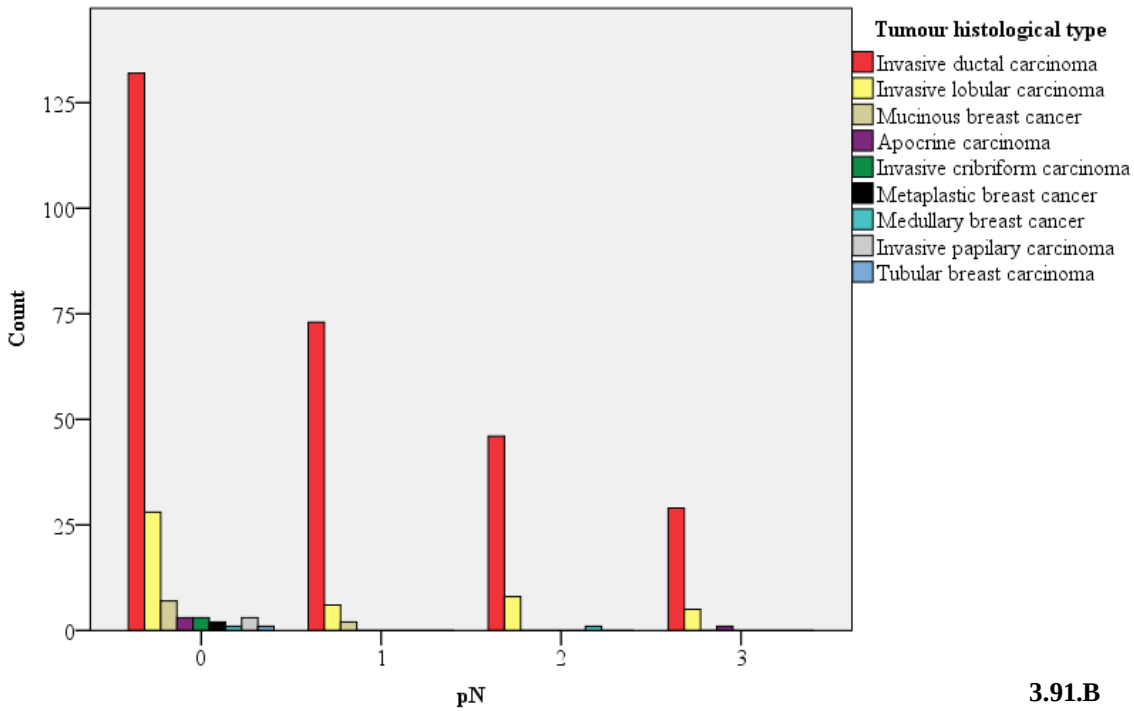
3.90.B

Figure 3.90. Relevance between morphological type and local spread (pT) of breast carcinoma. 3.90.A, by tumour histological type; 3.90.B, by pT.

There was no association between the presence of metastases in axillary lymph nodes and the morphological type of breast carcinoma (P=0.6) as shown in Figure 3.91.A. Cancers of all morphological types were associated with metastatic spread to lymph nodes except invasive cribriform, metaplastic, invasive papillary and tubular breast carcinoma (Figure 3.91.B).



3.91.A



3.91.B

Figure 3.91. Relevance between the morphological type and pN of breast cancer. 3.91.A, by tumour histological type; 3.91.B, by pN.

There were no statistically significant association between ER expression and local tumour spread (*P*=0.5) as shown in Figure 3.92.A and Figure 3.92.B.

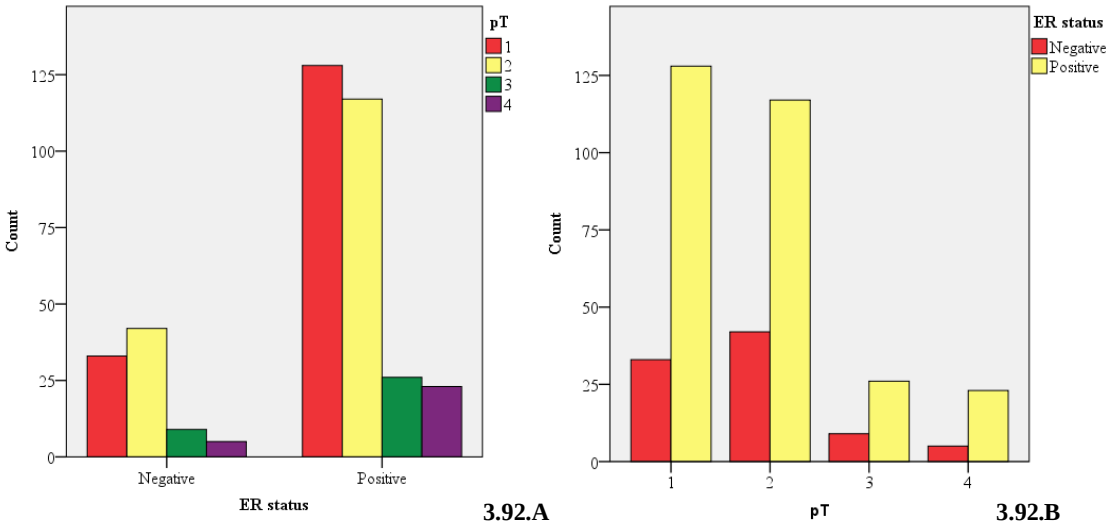


Figure 3.92. Relevance between ER status and local tumour spread (pT) of breast cancer. 3.92.A, by ER status; 3.92.B, by pT.

Similarly regarding the analysis of ER status vs. local tumour spread, no statistically significant association was found between PR status and local tumour spread (*P*=0.8). The data are presented in Figure 3.93.A and Figure 3.93.B.

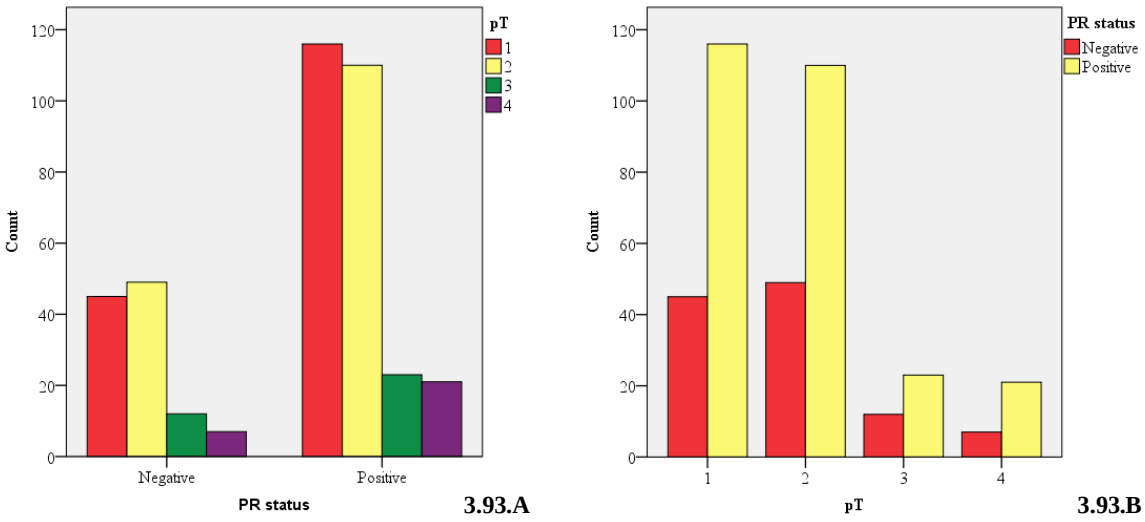


Figure 3.93. Relevance between breast cancer PR status and local tumour spread (pT). 3.93.A, by PR status; 3.93.B, by pT.

No association was identified between HER2 protein expression and local tumour spread (*P*=0.8). The analysis is highlighted in detail in the Figure 3.94.A and Figure 3.94.B.

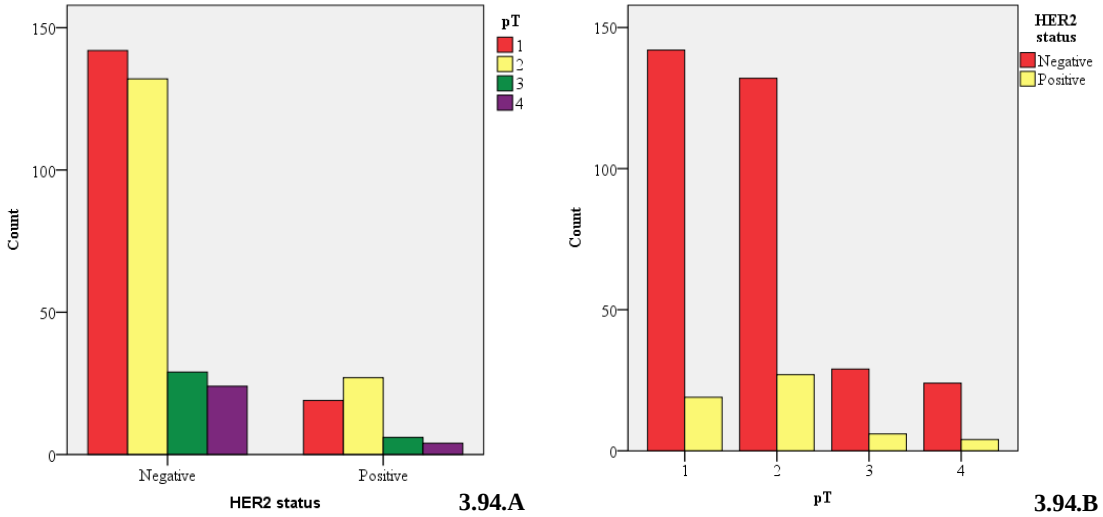


Figure 3.94. Relevance between breast cancer HER2 receptors and local tumour spread (pT). 3.94.A, by HER2 status; 3.94.B, by pT.

There is no statistically significant association between breast cancer size (pT) and new potential prognostic factor expression as p53 (*P*=0.1), BCL2 (*P*=0.9), COX-2 (*P*=0.1), cyclin D1 (*P*=0.1) and CK 5/6 (*P*=0.6).

Statistical methods did not reveal statistically significance between presence and count of metastatic lymph nodes reflected by pN parameter and such prognostic factors as ER (*P*=0.08) or HER2 (*P*=0.6). There is no statistically significant association between lymph node status and immunohistochemical expression of p53 (*P*=0.6), BCL2 (*P*=0.1), COX-2 (*P*=0.2), cyclin D1 (*P*=0.1) and CK 5/6 (*P*=0.1).

Statistical methods did not disclose statistical significance between breast cancer grade and expression of new potential prognostic factors as COX-2 (*P*=0.1), cyclin D1 (*P*=0.1) and CK 5/6 (*P*=0.07).

Ductal breast cancer is the most common histological type both in BCL2 positive and BCL2 negative groups. The BCL2 positive and BCL2 negative breast cancers thus show no significant differences by histological type (*P*=0.1).

There is no statistically significant association between p53 expression and breast cancer positivity for COX-2 (*P*=0.7) and cyclin D1 (*P*=0.4).

No statistically significant association was found between BCL2 and COX-2 expression (*P*=0.2).

COX-2 positivity did not show statistically significant association with HER2 receptors (P=0.3), proliferation activity (P=0.7) and expression of cyclin D1 (P=0.7) in breast cancer.

Expression of cyclin D1 is not statistically significantly associated with HER2 protein expression (P=0.09) and with degree of breast cancer proliferation activity (P=0.3).

CK 5/6 positivity or negativity does not show statistically significant association with breast cancer HER2 receptors (P=0.1).

No statistically significant association was shown between patients' survival and expression of COX-2 (P=0.1), cyclin D1 (P=0.2) and CK 5/6 (P=0.3).

4. DISCUSSION

As breast cancer represents a heterogenous group of tumours with variable biological and clinical characteristics, the identification of prognostic and predictive markers is clinically important. ER and PR, determined by IHC, are widely used both as predictive markers for hormonal therapy and as prognostic factors. HER2 status, as determined by IHC or FISH, indicates poorer survival. Possible benefits may be derived by therapeutically targeting these molecules. Recently, gene expression microarray studies have shown a strong prognostic power [Hamilton and Piccart, 2000], but immunohistochemistry remains a convenient and powerful means of prognostic evaluation in a clinical setting as it is less expensive and easier to perform [Lee, Im *et al*., 2007; Lee, Park *et al*., 2007].

The prognostic or predictive factors that currently are in use do not provide sufficient information to allow accurate individual risk assessment and treatment planning, emphasizing the need for additional prognostic and therapeutic factors [Lee, Im *et al*., 2007; Lee, Park *et al*., 2007].

Approximately half of all new breast cancers are diagnosed in the developing world, where the analysis of prognostic factors needs to be inexpensive and easy to replicate. Even in the developed world, microarray analysis has yet to fully replace classical IHC. Thus, in the absence of routine gene-expression profiling, surrogate IHC markers for molecular breast cancer subtypes have emerged as a more practical means of characterising breast cancer types according to prognosis and/or differential response to specific agents [Hudis and Gianni, 2011]. For example, a five-marker method, which examines ER, PR, HER2, CK 5/6, and EGFR have been proposed as a surrogate system for identifying basal-like breast cancer [Nielsen *et al*., 2004; Cheang *et al*., 2008]. Such an approach could have practical benefits. Moreover, it is becoming increasingly apparent that the success of new anticancer therapies is likely to be dependent upon the use of new biomarkers to detect patients who will benefit from a particular treatment [Penault-Llorca and Viale, 2012].

4.1. Surgical intervention

Surgical intervention has a central role in the treatment of breast cancer. In the presented study, 229 mastectomies (59.8%, 95% CI=54.8-64.5) and 154 segmental resections (40.2%, 95% CI=35.5-45.2) of breast were performed. In general, the extent of surgical operation corresponds to the data reported in other studies, Thus, Wiechmann *et al*. reported rate of mastectomy as 59% of all cases, offering breast conserving surgery to 41% of patients [Wiechmann *et al*., 2009]. Reverse ratio between mastectomy and breast conserving surgery is reported by Irigoyen *et al*., who have performed breast conserving operation in 59.2% of patients, but radical surgery - in 40.8% of patients. In this study, segmental resections were performed for most of luminal A and luminal B subtype breast cancers (65% and 63%, respectively). Mastectomies were performed for breast cancers which belonged to basal, HER2 positive and normal molecular subtype; the corresponding rate was 72.7%, 55.5% and 75%, respectively [Irigoyen *et al*., 2011]. The proportion of breast conserving operations is only moderately dependant on the year of study. Some older studies report higher frequency of mastectomy, e.g., in the study of Lee *et al*., radical modified mastectomy without removal of the pectoral muscles was performed in 79.5% patients but breast conserving surgery including quadrantectomy was performed in 20.5% [Lee, Im *et al*., 2007]. However, Le *et al*. [Le *et al*., 1999] described 175 patients with breast cancer who were locally treated by surgery, either lumpectomy (43%) or modified radical mastectomy (57%).

In our study, in 197 cases (51.4%, 95% CI=46.5-56.1) breast carcinoma was in right breast, but in 186 cases (48.6%, 95% CI=43.9-53.5) the tumour affected the left breast. The breast cancer laterality has been analysed by several authors. Spitale *et al*. showed data characterising breast cancer laterality in the general breast cancer group and by molecular subtypes. Interestingly, cancer development was slightly more frequent in the left breast (50.4% versus 49.6%), and this predominance was true also in luminal B (53.3% vs. 46.7%) and basal cell-like (58.9% vs. 41.1%) cases. Luminal A and HER2 positive cases were slightly more common in the right breast: 50.9% vs. 49.1% in luminal A and 51.5% vs. 48.5% in HER2 positive cases [Spitale *et al*., 2009]. In the study performed by Irigoyen *et al*., no statistically significant differences were identified between the molecular subtypes regarding tumour location in right or left

breast, or the affected quadrant of breast: *P*=0.43 and *P*=0.38, respectively [Irigoyen *et al*., 2011].

In general, our data regarding the surgical approach and cancer location are not different from the published evidence and thus the group could be considered representative for breast cancer evaluation.

4.2. Surgical approach to axillary lymph nodes

The surgical treatment of breast cancer involves also the evaluation of axillary lymph node status and treatment of metastatic disease. Thus, sentinel lymph node excision and / or axillary lymph node dissection can be performed. In the study of Lee *et al.*, sentinel node excisions were performed in 21.5%, 95% CI=17.7-26.0 and axillary lymphadenectomies in 78.5%, 95% CI=74.0-82.3 of cases [Lee, Im *et al*., 2007].

Bertolo *et al. s*tudied 60 women with invasive ductal cancer. Complete axillary dissection was done in 54 patients. In 43% of these cases the lymph nodes were positive. Lymphadenectomy was not considered in 6 patients because of age and/or health status [Bertolo *et al.*, 2008].

In older studies, more active surgical approach towards lymph nodes was attempted. Thus, axillary dissection was invariably performed in all patients undergoing mastectomy or breast conserving surgery [Le *et al*., 1999].

The number of recovered lymph nodes is an important quality indicator in the surgical and pathological diagnostic work. In our study group, 362 lymph node operations were performed and in 351 cases (97%, 95% CI=94.6-98.3) the lymph nodes were found at gross and microscopic pathology evaluation. The mean number ± SD of lymph node count per case was 12.5±8.2, and the number of breast cancer metastasis ranged from 0 to 32 (mean amount ± SD, 2.8±5.0). In other studies, comparable mean number of removed lymph nodes was found reaching 22.9 (range 7-54) or mean number of 14 (range 4-34) lymph nodes per patient in case of axillary lymphadenectomy [Le *et al*., 1999; Lee, Im *et al*., 2007].

4.3. The histological type of breast cancer

The general composition of breast cancer by histological types has been previously highlighted. In the present study, 304 (79.4%) of 383 primary breast tumours were invasive ductal carcinomas, 51 (13.3%) invasive lobular carcinomas and 13 (3.4%) represented mucinous breast cancer. The predominance of ductal breast cancer is recognised by other researchers as well, e.g., this histological type comprised 91.4% of breast cancers in the series of Lee, Im *et al*., 2007. Bennis *et al*. described the rate of invasive ductal carcinoma as 87.4% while invasive lobular carcinomas comprised only 4% of breast cancers, followed by metaplastic carcinoma (3%), medullary carcinoma (2%) and few cancers of rare histology (3%), summarized as other types [Bennis *et al*., 2012].

In the present study, ductal breast cancer is the most frequent histological type both overall and in all individual molecular subtypes. It is followed by lobular breast cancer, mucinous and other histological types as medullary or apocrine breast cancer constituting 3.9%. The data regarding the presence and type of breast cancer can be characterised as highly reliable. E-cadherin expression has been used systematically to distinguish ductal and lobular breast cancer [Arps *et al*., 2013; Rakha *et al*., 2013]. The loss of actin-positive myoepithelial cell layer has been used to confirm the invasive growth of breast cancer in all the evaluated cases [Lee, 2013].

Within the present research work, the relationship between proportions of ductal and lobular breast cancer is retained in all molecular subtypes. The following uncommon histological types of breast cancer are observed with different frequency in different molecular subtypes. Mucinous breast cancer is not observed in luminal B (HER2 positive), HER2 positive and triple negative molecular subtype groups. This shows that mucinous breast cancer never overexpresses HER2 receptors but is characterised by preserved hormone receptor expression and different, but frequently low proliferation fraction. Medullary breast cancer is characterised by lack of HER2 overexpression and/ or amplification and by high proliferation fraction leading to classification into triple negative or luminal B (HER2 negative) group. Metaplastic carcinoma is typically triple negative. Invasive cribriform carcinoma is invariably classified into luminal A type. The obtained data are in aggrement with other published studies but have high practical value.

The association between the histological types and molecular subtypes of breast cancer has been analysed by Yang *et al*. [Yang, Sherman *et al*., 2007]. Luminal A molecular subtype comprised ductal breast cancer (56%), followed by lobular breast cancer and mixed and other tumours (23% and 21%, respectively). Luminal B molecular subtype included more ductal breast cancer cases (75%), but fewer lobular breast cancer cases (10%). In this subtype, mixed or other types of breast cancers are more frequent than lobular histological type (14%). HER2 positive molecular subtype shows similar relationships between histological types as luminal B subtype, but with even more marked predominance of ductal carcinoma – ductal breast cancer constituted 89%, lobular breast cancer 5% and other / mixed types of breast cancer – 7%. Basal-like breast cancer also was characterised by high percentage of ductal breast cancer (80%).

Presence of *in situ* cancer component has also been analysed by molecular subtype. In the research of Spitale *et al*., most cases did not show synchronous *in situ* component. Only HER2 positive molecular subtype was characterised by frequent occurrence of *in situ* cancer (55.9% positive vs. 44.1% negative) while other molecular subtypes showed *in situ* component only occasionally [Spitale *et al*., 2009].

In the present study, more detailed classification was applied, describing separately ductal carcinoma *in situ*, non-*comedocarcinoma* type as well as ductal *comedocarcinoma* and lobular carcinoma *in situ*. In the whole group, synchronous *in situ* component was absent in one third of all cases. In more than half of cases of triple negative breast cancer, carcinoma *in situ* was not found. Ductal *in situ* carcinoma, non-comedocarcinoma type, was more frequent in luminal A subtype while rare in HER2 positive subtypes. In contrast, comedocarcinoma was less frequent in luminal A subtype but distinctly frequent in HER2 positive and luminal B (HER2 positive) subtypes.

In general, the association between the molecular subtypes and frequency and type of *in situ* cancer points towards early segregation of breast cancerogenesis towards different pathogenetic ways. The association between morphological type and molecular subtype can be clinically useful as hint towards the exact diagnosis.

4.4. The local tumour spread (pT)

According to pathological TNM classification, all 383 tumours were classified subsequently: pT1 – 161 tumours (42%); pT2 – 159 tumours (41.6%); pT3 – 35 tumours (9.1%) and pT4 – 28 tumours (7.3%).

The size of diagnosed breast cancers influencing pT differs significantly in many studies, reflecting the approaches to the diagnostic process and particularly breast cancer screening as well as the biological properties of the tumours. Thus, in the study group described by Callagy *et al*., most of tumours (48%) were of pT2, followed by pT1 (38%) and pT3 (18%). In the Nottingham study, pT1 cancers were dominating by 58% of all cases, followed by pT2 (48%) and pT3 comprising only 2% of tumours [Callagy *et al*., 2006]. The data obtained by Lee *et al*. show similar findings: 48.7% of tumour cases are <2 cm; 43.8% measure ≥2 but <5 cm and 7.5% are ≥5 cm [Lee *et al*., 2010].

All WHO TNM-defined T stages were represented in the study of Rouzier *et al*. T1 cancers were the smallest group (9%) but T2 with 56% of all cases composed the largest part of all breast cancer cases in study. The T3 tumours formed 18% and T4 - 17% of cases [Rouzier *et al.*, 2005]. pT4 cases were found also by Ambrogi *et al*., who observed pT1 in 61.6% of all breast cancer cases, pT2 in 29.4%, pT3 – 1.3% and pT4 in 7.7% of all study cases [Ambrogi *et al*., 2006]. Wiechmann *et al*. described group which included mostly T1 – 69%. T2 composed 24%, T3 – 3%, but Tx – 4% of all cases [Wiechmann *et al*., 2009]. In the publication of Le *et al*., 70% of cases were of T2 stage, 20% – T1 but 10% of cases were of T3 stage [Le *et al*., 1999]. In another study, characteristics of breast cancer by size were consequent: ≤2 cm, 7.3%; 2-5 cm – 72.8% and >5 cm – 19.9% of all investigated breast cancers [Lee, Im *et al*., 2007].

Yamashita *et al*. divided all tumours not by WHO TMN classification stages, but just in 2 groups: up to 2 cm and more than 2 cm. Consequently, only pT1 frequency can be compared with our data. In this study, pT1 tumours represented 41% of studied cases [Yamashita *at al*., 2004].

Spitale *et al*. classified breast cancers by TNM and resulted in the following distribution: T1, 62.1%; T2, 35.2% and T3, 2.7% of all cases. By molecular subtypes, luminal A group consisted of T1, 65.9%; T2, 31.4% and T3, 2.7%. Luminal B group comprised T1, 58.3% and T2, 41.7% cancers. HER2 positive molecular subtype group showed opposite data with dominance of relatively larger tumours measuring 2-5 cm: T2, 66.0% and T1, 34.0%. Basal-like breast cancer (7% from all cases) comprised

slightly higher number of T1 (48.1%) than T2 cancers (42.0%), and some cases (9.9%) were more than 5 cm large [Spitale *et al*., 2009].

In another large study, comprising 1134 breast cancer cases, 71.4% of tumours were T1. T2 cancers, measuring 2.1-5 cm formed 23.1% of the study group. The frequency of T3 was 4.7%, and of Tx – 0.8%. In such group, all molecular subtypes (luminal A, luminal B, HER2 positive and triple negative/basal-like cancer) showed highest percentages of tumours measuring ≤ 2 cm (78.9%, 62.1%, 47.1% and 54.0%, respectively), followed by T2 [Onitilo *et al*., 2009].

Irigoyen *et al*. describe more frequent occurrence of pT1 in luminal A and luminal B molecular subtype than in basal, HER2 positive or normal molecular subtypes that showed predominance of pT2. In this study, pT3 and pT4 composed only small fraction [Irigoyen *et al*., 2011].

The results obtained in the present study are comparable with the published evidence. In the general group, T1 and T2 are the predominant findings. However, 9.1% of breast cancers are diagnosed in stage T3 and 7.3% – in stage T4. The luminal A subtype showed the highest frequency of pT1 tumours. On other extreme, pT4 also have been observed in this group and are even more frequent than pT3; occasionally these pT4 cases were related to very long anamnesis (A. Abolins, unpublished case observations). Both luminal B (HER2 negative) and HER2 positive molecular subtypes showed larger amount of pT2 in comparison with the general group. Triple negative molecular subtype had the same pT distribution as the general group.

4.5. The evaluation of axillary lymph node status (pN)

In the present study, pN0 was observed in 180 cases (47%), pN1 – 81 cases (21.1%), pN2 – 54 (14.1%) and pN3 – 36 cases (9.4%). There were 32 cases (8.4%) of pNx as well. The general distribution of pN is within the published range although the available data show some diversity.

In the University of British Colombia study of 800 cases, lymph nodes were free of metastases in 30%, N1 was observed in 41% and more than 3 nodes were positive in 29% cases. The Nottingham case series used for the validation study consisted of 1,961 consecutive cases of primary operable breast carcinoma patients who presented from 1986 to 1998 and entered into the Nottingham Tenovus Primary Breast Carcinoma

Series. Nodal status of 1,938 cases was negative in 64%, positive in 1 to 3 nodes – 28%, and positive in more than 3 nodes in 8% [Callagy *et al*., 2006].

In the research article published by Lee *et al*., the following lymph node status was described: N0, 51.3% of the evaluated 80 patients; pN1, 22.5%, pN2, 11.2% and pN3, 15% of cases [Lee *et al*., 2010].

Yamashita *et al.* analysed 503 cases. In this group, metastases have not been found in 57% of cases, from 1 to 3 positive lymph nodes were identified in 24% cases, but more than 3 positive lymph nodes were found in 19% [Yamashita *at al*., 2004].

Spitale *et al*. divided breast cancer by metastases in lymph nodes as positive or negative cases. Positive lymph node status was in 39.6% of cases, but negative in 60.4% of cases [Spitale *et al*., 2009]. In this study, no statistically significant differences in lymph node status in different molecular subtypes were found in contrast to the present study as discussed further.

Similarly, Onitilo *et al*. classified the lymph node status into negative (61.2%) or positive (31%). In addition, lymph node investigation had not been done in 7.8% of cases [Onitilo *et al*., 2009].

Carey *et al*. reported absence of lymph node metastases in approximately 2/3 of investigated lymph nodes (61%) whereas 39% of cases presented with breast cancer metastases. Negative lymph node status was predominant in luminal A (66%), luminal B (53%), basal-like (61%) and unclassified (71%) breast cancer molecular subtypes. Positive lymph node status was more frequent among HER2 positive cases [Carey *et al*., 2006].

Le *et al*. performed complete lymph node investigation by TNM. N0 group comprised largest part of all investigated cases – 40.5%. There were 33.7% of cases corresponding to N1, 15.4% – N2 and 10.4% – N3 [Le *et al*., 1999].

In the present study, N0 cases are predominating in the general group as well as in all molecular subtypes. In luminal A and luminal B (HER2 negative) subtypes, number of cases decreases by increasing pN. Opposite data are observed in triple negative molecular subtype characterised by bimodal distribution: relatively more frequent occurrence of high number of metastases among N+ cases.

4.6. Distant metastases (M)

At the time of operation, proved distant breast cancer metastases (M1) were present in 12 cases (3.1%, 95% CI=1.8-5.4). Breast cancer metastases affected bones 33.3% (95% CI=13.8-60.9), brain – 25% (95% CI=8.9-53.2), lungs – 25% (95% CI=8.9-53.2) and liver – 16.7% (95% CI=4.7-44.8).

In the study performed by Spitale *et al.*, similar rate of distant metastases (4.8%) was described [Spitale *et al.*, 2009].

In Onitilo *et al*. study, recurrence was observed in 8.7%. Among these cases, local recurrence was found in 45.5% and the remaining developed metastases in bone (39.4%), liver (22.2%), lung (15.1%), mediastinal lymph nodes (10.1%), brain (7.1%) and other sites (11.1%). All molecular subtypes except luminal B molecular subtype more frequently recurred locally than progressed to distant metastases into particular site. Luminal B subtype breast cancers frequently metastasized to bones (61.5%), liver (38.5%) or recurred locally (38.5%) as reported by Onitilo *et al.* 2009.

In the study of Lee *et al.*, 151 patients were followed up for median duration of 36 months (range 8-78). During this time, 24.5% patients experienced breast cancer recurrence, and 34 of these had distant metastases. Frequent sites of distant metastases were bone (46%), lung (29.7%), liver (18.9%), and brain (5.4%) [Lee *et al.* 2007].

Although the cancer recurrence after treatment and presence of distant metastases at the time of primary diagnostics differs by time of disease progression, our data are in general agreement with the cited studies. The higher frequency of brain metastases reaching statistical significance can be attributed to relatively low number of events in the study group although cancer recurrence after treatment and presence of distant metastases at the time of primary diagnostics can also involve different mechanisms.

4.7. Histological grade

By histological grade all cases in the presented research work were classified as follows: G1 – 16.0%, 95% CI=12.2-19.6; G2 – 36.0%, 95% CI=31.9-41.1 and G3 – 48.0%, 95% CI=43.8-52.8. Very similar data are reported by Bertolo *et al.*: G1, 18%;

G2, 35%; and G3, 47% of cases [Bertolo *et al*., 2008]. Lee *et al*. classified 19.2% of cases as G1, 35.9% as G2 and 44.9% as G3 [Lee *et al*., 2010].

Callagy *et al*. reported slightly different composition by grade showing statistically significantly lower rate of low grade cancers and higher – of high grade cancers: G1, 9%; G2, 32% and G3, 59%. In contrast, Nottingham series showed similar results: G1 – 19%, G2 – 33%, G3 – 48% of cases, respectively, without statistically significant differences from the presented study [Callagy *et al*., 2006].

Histological grade in Yamashita *et al*. study was following: G1 in 17%, G2 in 59%, but G3 in 24% of cases. Le *et al.* describe breast cancer grade subsequently: G1 – 7.9%, G2 – 53.7% and G3 – 39%. In both these studies statistically significant excess of G2 cancers was found [Le *et al*., 1999; Yamashita *et al*., 2004].

Spitale *et al*. classified the analysed cases into 2 grade levels including well/ moderately differentiated cancer (72.9% of cases) and poorly differentiated cancer, constituting 27.1% of cases [Spitale *et al*., 2009]. Well/ moderately differentiated breast cancers constituted the largest part of luminal A and luminal B molecular subtypes (84.6% in luminal A and 52.5% in luminal B group). HER2 positive and basal-like cancers were predominantly poorly differentiated with notable difference, e.g., 75.9% of poorly differentiated breast cancers versus 24.1% of well/moderately differentiated breast cancers in basal-like molecular subtype group. Carey *et al*. also divided breast cancer by the histological grade in two groups – poorly and well/moderately differentiated breast cancer. In this study, poorly differentiated cancer predominated in the general group (65% of all cases) as well as in all molecular subtypes constituting 58% in luminal A, 56% in luminal B, 70% in HER2 positive, and 82% and 81% of basal-like and unclassified breast cancer molecular subtype groups respectively [Carey *et al*., 2006].

Onitilo *et al*. analysed breast cancer histological grades by three-tiered system. Their study group comprised G3 tumours (35.9%), G2 tumours (38.4%) as well as relatively small proportion of G1 tumours (21.2%). Data were missing in few cases (4.6%). Luminal A molecular subtype group contained more G2 breast cancers (44.9%) followed by well differentiated (28.9%) and poorly differentiated (21.5%) breast cancers. Luminal B subtype breast cancers were less differentiated containing more poorly differentiated tumours (49.1%), the moderately differentiated – 41.4%, followed with few cases of well differentiated breast cancers – 6%. In HER2 positive and triple negative molecular subtypes, poorly differentiated breast cancers were frequently

observed (77.7% and 76.3%, respectively), followed by moderately differentiated (20.0% and 12.5%, respectively) and well differentiated (1.2% and 4%, respectively) cancers [Onitilo *et al*., 2009].

IHC subtypes were significantly different by histological grade (P=0.0053) in Bennis *et al*. study. The unclassified, basal-like and HER2 positive subtypes showed higher percentage of cases with histological grade 3 (53%; 47.6% and 42.2% respectively), and a very low percentage of tumours with histological grade 1: 0%, 4.8% and 13.3%, respectively [Bennis *et al*., 2012].

Similar tendency is shown in the present study data where triple negative molecular subtype group of breast cancer practically contain poorly differentiated breast cancers (92% of G3 vs. 8% of G1/G2 group). The same situation is in HER2 positive molecular subtype group and Luminal B (HER2 positive) group. If G1 and G2 group cases are counted together then amount is greater than single G3 group in luminal A breast cancer subtype like in Spitale *et al.* study [Spitale *et al.,* 2009]. The differences that were identified within the frames of the present research are statistically significant.

4.8. Expression of oestrogen and progesterone receptors

Classifying all investigated breast cancers by ER positivity or negativity, up to 80% of all breast cancers show ER positivity. By definition, HER2 positive and triple negative molecular subtypes do not posses ER positivity. The present study is in agreement with Spitale *et al.* [Spitale *et al.,* 2009].

The proportion of ER positive cases was lower in the study performed by Carey *et al*. Among 496 cases, ER receptors were found in 60% of all cases [Carey *et al*., 2006], being absent in basal-like, HER2 positive and unclassified breast cancer molecular subtypes. In luminal A subtype, 86% cases showed ER positivity, but in luminal B almost all cases were positive – 97% [Carey *et al*., 2006].

The rate of ER positivity was 55% in the study performed by Lee *et al*. Among the investigated 151 cases, PR receptor positivity was observed in 39.1 % of cases. A cut-off value of 10% or more positively stained nuclei in 10 high-power fields was used to define ER and PR positivity [Lee, Im *et al*., 2007]. In the present study, the evaluation of ER alpha and PR status was carried out according to the ASCO/CAP guideline recommendations for IHC testing of ER and PR. The breast cancer case was

considered positive if at least 1% of tumour cells showed positive nuclear staining of any intensity [Hammond *et al.*, 2010].

The data about PR positivity parallels the ER expression. Up to 70% or more all breast cancers show PR positivity [Spitale *et al.*, 2009] in agreement with the present study. The expression of PR is absent in basal-like, HER2 positive and unclassified breast cancer molecular subtype as well as in 16% and 14% of cases in luminal A and luminal B molecular subtypes, respectively [Carey *et al.*, 2006].

4.9. Proliferation activity by Ki-67

High tumour proliferation activity recognised by high levels of Ki-67 expression is associated with worse outcomes [de Azambuja *et al.*, 2007]. The proliferation marker Ki-67 should be included in routine clinical investigation because the labelling index is crucially important in the distinction between luminal A and luminal B (HER2 negative) subtypes. If reliable Ki-67 labelling index assessment is not available, some alternative measure of proliferation such as histological grade may be used in making this distinction. Ki-67 labelling index presents substantial challenges, as important guidelines for this test are still under development. The cut-off point <14% for Ki-67 labelling index was established by comparison with PAM50 intrinsic subtypes meaning that a higher score defines luminal B tumours with a worse prognosis [Cheang *et al.*, 2009; Goldhirsch *et al.*, 2011].

Regarding the cut-off point, different researchers have used different decision-changing values of Ki-67 labelling index. Spitale *et al.* divided breast cancer cases by Ki-67 labelling index in 3 groups: ≤5%, 5-20% and >20%. Consequently, most of breast cancer cases belonged to the group with 5-20% of Ki-67 positive cells. Analysing Ki-67 labelling index by molecular subtypes, luminal A and luminal B subtypes were associated with index up to 20%. Luminal B molecular subtype showed also high rate of cases belonging to actively proliferating group recognised by Ki-67 index over 20%. Basal cell-like and HER2 molecular subtypes were associated with high Ki-67 labelling index.

In the article by Lee *et al.*, more than 5% of positive cells were presumed as cut-off point. Consequently, the proliferative activity was lower than 5% in 44.4 % of all investigated patients and exceeded this cut-off in 55.6% of all cases [Lee *et al.*, 2007].

In accordance with the published evidence, the present study showed association between luminal A subtype and low Ki-67 labelling index. All other subtypes were associated with high Ki-67 labelling index. However, the cut-point was 14% in accordance with the recent St. Gallen recommendations [Goldhirsch *et al.*, 2011].

4.10. The overexpression of HER2 protein and amplification of *HER2/neu* gene

HER2 overexpressing breast cancer patients are more likely to suffer from relapse and tend to have a shorter overall survival. Amplification of the *HER2/neu* gene and RNA/protein overexpression correlates strongly [Pegram *et al*., 2000; Lee, Park *et al*., 2007]. After development of trastuzumab, which is the monoclonal antibody and targets HER2, the amplification status of HER2 became a highly predictive biomarker [Slamon *et al*., 1987; Mass *et al*., 2005]. Overexpression and amplification of *HER2/neu* can be detected in about 15% of all primary breast cancers, and this group of patients benefit significantly from anti-HER2 therapies. HER2 status should be assessed in every diagnosed case of breast cancer [Romond *et al*., 2005; Smith *et al*., 2007]. HER2 status is currently assessed in most cases initially by immunohistochemistry, and in cases of equivocal protein expression levels, *HER2/neu* gene copy number is measured via FISH or CISH techniques [Wolff *et al*., 2007]. In addition, detection of HER2 status along with expression of ER and PR is useful for defining the molecular subtypes.

Carey *et al*. examined HER2 overexpression in all consecutive breast cancer cases [Carey *et al*., 2006]. According to molecular subtype classifications, HER2 expression was observed in HER2 positive and luminal B molecular subtype. Luminal A, basal-like and unclassified breast cancers did not possess HER2 positivity by definition. In that study membranous staining for HER2 was scored as: 0, faint incomplete staining in 10% or less of cells; 1, faint incomplete staining in more than 10% of cells; 2, weak to moderate complete staining in more than 10% of cells; 3, strong complete staining in more than 10% of cells. Thus, no expression of HER2 was present in 43% of cases but 1+ was observed in 23.8% of investigated cases. Borderline HER2 expression as 2+ was observed in 13.2% of cases. HER2 overexpression scoring 3+ was present in 19.9% of cases [Lee *et al*., 2007]. Again, it must be emphasized that the cut-off levels for HER2 positivity have changed over years.

HER2 positivity in the present study is shown in 14% of cases which is similar to other studies [Spitale *et al*., 2009]. In the present study, there was no association between HER2 expression and the local tumour spread characterised by pT (P=0.8). In contrast, Lee *et al.* found correlation between the overexpression of HER2 and larger tumour size (P=0.03) and more extensive axillary lymph node involvement as characterised by P=0.02 [Lee, Im *et al*., 2007; Lee, Park *et al*., 2007]. In present study no correlation was found between pN parameter and HER2 overexpression.

4.11. Immunohistochemistry and breast cancer molecular subtype

Breast cancer is the second most common neoplasm among women in Latvia [*spkc.gov.lv*], as well as in the world, accounting for one third of newly diagnosed malignancies [Jemal *et al*., 2011]. Breast cancers with similar histopathological appearances can exhibit different clinical presentation, disease aggressiveness and response to treatment. These differences cannot be explained completely by morphology therefore the application of the current WHO classification of breast cancers is limited. Systematic investigations of gene expression patterns and their correlation with specific features of phenotypic diversity are changing the way of classifying, at the molecular level, the phenotypes of breast cancers, as well as of other tumours [Spitale *et al*., 2009]. In addition, profiles of gene expression analysis and immunophenotype suggest that breast cancer is not a single entity but a heterogeneous disease, which is composed of a growing number of recognized biological subtypes. Nowadays classification of human breast tumours was developed through a hierarchical clustering of genes on the basis of similarity in the expression pattern [Spitale *et al*., 2009]. Breast cancers were categorized into at least five main groups which differ markedly in terms of incidence in distinct races/ ethnicities, risk factor distribution, prognosis, response to treatment, clinical outcomes and both OS and RFS: luminal cell-like tumours, subdivided into luminal A and B, basal cell-like and/ or triple negative phenotype [ER and PR negative tumours with amplification of genes usually expressed by basal/myoepithelial cells], HER2 tumours (amplification of the *HER2/neu* gene) and normal breast-like group [Sotiriou *et al*., 2003; Carey *et al*., 2007; Spitale *et al*., 2009].

Recent publications have shown that molecular classification of breast cancer also has important prognostic value [Pusztai *et al*., 2006]. Luminal A tumours were

shown to be associated with good prognosis and a less aggressive behaviour if compared with the basal-cell like or *HER2/neu* groups [Sotiriou *et al*., 2003]. Basal-cell like subtype has been associated with aggressive behaviour, poor clinical outcomes and lack of response to the usual endocrine therapies, shorter survival and presence of *BRCA1* mutations [Spitale *et al*., 2009]. Several studies have shown that breast carcinomas may be stratified in subtypes similar to those defined by expression profiling using a panel of IHC markers [Tang *et al*., 2008; Spitale *et al*., 2009].

Subtyping breast cancer using microarrays for gene expression analysis is the best way to perform molecular classification, but it is not always feasible to obtain gene expression array information according to high costs or inaccessibility of fresh tissues, therefore simplified classification has been adopted as useful shorthand [Cheang *et al*., 2009].

Molecular subtypes defined by clinicopathological criteria are similar to but not identical to intrinsic subtypes and represent a convenient approximation. This approach uses immunohistochemical definition of ER and PR, the detection of overexpression and/or amplification of *HER2/neu* gene, and Ki-67 labelling index, a marker of cell proliferation, as the means of identifying tumour subtypes. Clearly, this clinicopathological classification requires the availability of reliable measurements of its individual components. Guidelines have been published for ER and PR determination [Hammond *et al*., 2010] and for the detection of HER2 positivity [Wolff *et al*., 2007]. Ki-67 labelling index presents more substantial challenges, but important guidelines for this test are still under development [Cheang *et al*., 2009].

Routine IHC evaluation of breast cancers may provide not only crucial information to guide clinical management but also represents a valid alternative to costly genotyping assays. The relationship between IHC markers and responsiveness to therapeutic treatments has been extensively studied [Goldhirsch *et al*., 2011]. IHC-based classification systems are still useful in clinical practice, and have been shown to correlate well with intrinsic classification using gene expression microarrays: ER/PR+,HER2+ with luminal B; ER/PR+, HER2- with luminal A; ER/PR-, HER2+ with HER2 positive and ER/PR-,HER2- with triple negative/ basal-like tumours [Carey *et al*., 2006, Carey *et al*., 2007].

On 2011, in 12th St. Gallen International Breast Cancer Conference expert panel adopted a new approach to the classification of patients for therapeutic purposes based on the recognition of intrinsic biological subtypes within the breast cancer spectrum.

Intrinsic subtypes of breast cancer are luminal A, luminal B, HER2 overexpressed and basal-like, but corresponding clinico-pathological surrogate classification with definitions to intrinsic subtypes includes luminal A, luminal B (HER2 negative), luminal B (HER2 positive), HER2 positive (non-luminal) and triple negative breast cancer [Goldhirsch *et al*., 2011].

In the present work, the molecular subtypes of breast cancer were detected according to this new classification and IHC data. The majority of cases belonged to luminal A (39.7%), followed by the luminal B (HER2 negative) subtype (32.6%). Triple negative breast cancer subtype constituted 13.1%, whereas only 8.4% and 6.3% of tumours were classified as HER2 positive and luminal B (HER2 positive), respectively. As the St. Gallen classification (2011) is new, few scientists have published data according to it.

4.12. Age and molecular subtype

In a study of breast cancer molecular subtypes and response to different preoperative chemotherapy, Rouzier *et al*. [Rouzier *et al*., 2005] included 82 females whose mean age was 52 years (range 29-79 years). In another study evaluating 151 breast cancer cases, the mean age was 46 years ranging 28-70 years. Among them, 73.5% of patients were younger than 50 years of age and only 26.5% were older than 50 years of age [Lee, Im *et al.*, 2007].

In Spitale *et al*. study, evaluating 1214 breast cancer cases, the mean age of patients was 62.7±14.0 years. After classification of breast cancer by molecular subtypes, the mean age in the basal cell-like or triple negative phenotype group was 58.5±14.6 years, in HER2 positive breast cancer group – 62.3±12.5 years. In other breast cancer molecular subtypes like luminal A and luminal B, the mean age ± standard deviation was 63.4±13.7 and 61.4±15.0 years, respectively [Spitale *et al*., 2009].

Onitilo *et al*. included in study 1134 patients, whose mean age was 62.7±13.8 years. By molecular subtype, luminal A or ER/PR+, HER2- group contained patients with highest age 64.4±13.2 years. HER2 molecular group included patients with the age 59.9±12.7 years, but ER/PR+, HER2 and ER/PR-, HER2- included patients with the mean age 58.9±14.6 and 58.1±14.7years, respectively [Onitilo *et al*., 2009].

The mean patient age was 56 (range, 22-95) years in Wiechmann *et al*. study [Wiechmann *et al*., 2009]. Subdividing this group into four molecular subtypes, the mean age was following – luminal A, 58 years; luminal B, 52 years; HER2 positive, 53 years and basal subtype, 54 years.

Carey *et al*. in her study of 496 cases described the mean age of 50 years with SD 12 years. By molecular subtype, the mean age in luminal A group was 52 years, luminal B – 50 years, HER2 positive – 47 years, basal- like – 46 years, but the lowest mean age was in unclassified breast cancer molecular subtype group [Carey *et al*., 2006].

Phipps, Malone *et al*. evaluated 2616 breast cancer cases subdividing the study group in luminal, HER2 overexpressing and triple negative cases. The mean age in luminal group was 68.6 years, but in HER2 overexpressing group – 67.6 years. Triple negative cases included patients with mean age 66.0 years [Phipps, Malone *et al*., 2008].

In the present study, 383 consecutive female patients with primary, invasive breast carcinoma were included. The mean age ± SD was 59.59±12.22 years. Patient's age ranged from 27 to 88 years old. In the published studies, the mean age ranges from 46 to 62.7 years. Our data are within this interval. In accordance with other studies, the highest mean age is in luminal A molecular subtype group, followed by luminal B (HER2 negative) molecular subtype and HER2 positive group. The lowest mean age is observed in luminal B (HER2 positive) group and triple negative group. Several researchers have applied classification of breast cancer by molecular subtypes including just 4 categories. The information about recently defined luminal B (HER2 positive) can be lacking than. However, the observation that triple negative breast cancer is diagnosed in younger patients is in agreement with other studies.

4.13. Expression of aberrant p53 protein

The tumour suppressor protein p53 has central role in cell protection against malignant change. It is activated in response to cellular stresses and initiates a variety of cellular responses including cell cycle arrest and apoptosis [Liu *et al*., 2013]. p53 protein regulates also gene transcription and DNS synthesis and repair [Ma *et al*., 2013].

Apoptosis or programmed cell death plays a critical role in the development of cancer. Inhibition of apoptosis leads to loss of the tumour-suppressor phenotype and to

accumulation of mutations. Apoptosis is a complex process controlled by multiple genes, and investigations on cell models in culture have demonstrated that the proto-oncogene BCL2 and the tumour-suppressor gene p53 play a fundamental role in its regulation. The BCL2 protein is a potent inhibitor of cell death, whereas wild-type p53 protein activates the apoptotic pathway. Mutated p53 protein loses this function [Reed *et al*., 1997]. Inappropriate expression of BCL2 gene also modulates the function of p53 and triggers cell proliferation and transformation [Ryan *et al*., 1994].

The recent trends to target mutant p53 for cancer treatment [Liang *et al*., 2011; Liu *et al*., 2013] underline the necessity to develop technologies for p53 detection and to detect the damage in *TP53* gene and the respective protein levels. The regulatory pathways can be deranged as well. As the frequency of p53 mutation can be associated with socioeconomic deprivation, race and other factors [Ma *et al*., 2013; Starks *et al*., 2013], the frequency of p53 pathway dysfuction must be determined separately for the particular population. The present study is the first research devoted to p53 analysis in breast cancer tissues in Latvia.

In the present study, we used immunohistochemistry to detect p53 protein as the method has been widely accepted for p53 analysis in proteome level [Laurinavicius *et al*., 2012; Shapocka *et al*., 2012; Liu *et al*., 2013]. By this approach, expression of p53 protein was observed in 24% of consecutive breast cancer cases.

Lee *et al*. found nuclear staining of p53 protein in 23% of tumours from 175 breast cancer cases using 10% cut-off value of tumour cells displaying strong nuclear staining [Le *et al*., 1999]. We have used analogous evaluation scale as described by Yamashita *et al*., 2006, and the obtained data are in aggrement with the published findings.

Other autors have used different evaluation scales. In occasional publications, the number of cells stained for p53 have been scored semi-quantitatively as follows; 0%, 1–25%, 26–50%, 51–75%, or > 75%. According to this classification p53 was expressed consequently: absent – 31.1%; present in 1-25% of tumour cells – 38.4%; present in 26-50% of neoplastic cells – 7.3%; present in 51-75% of neoplastic cells – 5.3% and present in 76-100% of neoplastic cells – 17.9% of cases. Expression of aberrant p53 protein in more than 25% of the tumour cells was observed in 30.5% of all cases [Lee *et al*., 2007]. Although the general trends are similar, guidelines for p53 detection and evaluation should be evaluated in near future. Immunohistochemistry seems to be one of the mainstays in these guidelines as the findings in protein level are

different from genetic data but protein conformation and functionality changes could be targeted by future treatments [Liu *et al*., 2013].

Le *et al*. showed statistically insignificant association (P=0.6) between breast cancer size and p53 expression. However, p53 nuclear overexpression in breast cancer cells was found to be associated by lymph node metastases [Le *et al*., 1999]. In the presented study, the association between p53 status, pT and pN was statistically insignificant.

Statistically significant relations (P=0.0001) between breast cancer histological grade and p53 were described by Le *et al*. G1 breast cancers did not show p53 nuclear overexpression (0%). The amount of p53 overexpressing cases increased in paralled with increasing cancer grade: G2, 13% and G3, 41% [Le *et al*., 1999]. In triple negative breast cancer, expression of p53 has been associated with high nuclear grade [Nakagawa *et al*., 2011]. In women with *TP53* germline mutations, most of breast cancers (81%) also are of high grade [Masciari *et al*., 2012]. In the present study, expression of p53 protein is more frequent in high grade breast cancers than in other groups (P<0.0001). Thus, our findings are in accordance with the world experience.

The rate of ER expression is statistically significantly associated with p53 negativity. In the present study, the ER+ cases predominate in p53 negative group in contrast to p53 positive group showing marked excess of ER negative breast cancer cases. The difference is statistically significant at P<0.0001. The same association is observed between p53 and PR expression (P<0.0001). p53 overexpression is more frequent in ER and/ or PR negative breast cancer [Rossner *et al*., 2009; Ma *et al*., 2013] and have caused great interest in triple negative breast cancer [Nakagawa *et al*., 2011; Kumar *et al*., 2012]. Statistically significant association between hormone receptor and p53 status was shown by Le *et al*. Among hormone receptor positive cases, the rate of synchronous nuclear expression of p53 was 16% in contrast to 41% in cases without hormone receptor expression [Le *et al*., 1999]. However, breast cancer in women with *TP53* germline mutations is characterised by frequent expression (84%) of ER and/or PR [Masciari *et al*., 2012]. p53 expression is also shown to be adverse prognostic factor in luminal breast cancer [Millar *et al*., 2011].

The p53 expression is heterogeneous regarding HER2 protein expression. However, most of HER2 negative cases are also negative for p53 (P=0.001). In the present study, there is statistically significant association between p53 expression and

higher proliferative activity (P<0.0001). Trend towards higher proliferation in p53-positive breast cancer has been described [Jung *et al*., 2011].

Regarding the 5 molecular subtypes, HER2 positive molecular subtype included more p53-positive than negative cases. In triple negative molecular subtype, the ratio between positive and negative cases was 1:1. Luminal A and luminal B (HER2 negative) molecular subtypes are these groups where the present data suggest no necessity to do the p53 investigations due to usually negative results. Ratio between expression and non-expression of p53 is lower in luminal B (HER 2 positive) group emphasizing HER2 aggressive nature and higher possibility of p53 expression. More studies can be recommended regarding this group as well as triple negative molecular subtype although the obtained data are statistically significant. The data about p53 expression by molecular type are still highly controversial [Jung *et al*., 2011].

p53 and BCL2 are two opposite findings (P<0.0001). Inverse correlation of expression of BCL2 and p53 was described by Le *et al*. Among the 64 BCL2 negative tumours, 36% were p53 positive, whereas among the 111 BCL2-positive tumours only 16% were also p53 positive corresponding to P=0.003 [Le *et al*., 1999]. The findings were confirmed by Lee *et al*., 2007. In their study, p53 ≤ 25% correlated with high BCL2 expression (68.6%). One third of cases did not show p53 and BCL2 expression. Breast cancer cases which expressed more than 25% of p53 lacked BCL2 expression in 56.5% of cases. 43.5% of cases were p53 and BCL2 positive [Lee, Im *et al*., 2007]. No significant association between p53 and BCL2 expression was found in Korean triple negative breast cancer despite sufficient heterogeneity of data [Ryu and Lee, 2012].

In the present study, CK 5/6 negativity was associated with lack of p53 expression (P=0.002). The CK 5/6 positive group is heterogeneous regarding expression of p53 protein; however, the association is statistically significant (P<0.0001).

The survival analysis by Kaplan-Meier has identified p53 expression as significant negative prognostic factor in agreement with the previously published findings that p53 status, as determined by immunohistochemistry, has prognostic impact and provides additional prognostic information for intrinsic subtypes and St. Gallen consensus classification [Guarneri *et al*., 2010; Jung *et al*., 2010]. However, controversial results have been reported that are partially associated with different cut-off levels, evaluation of gene and protein dysfunction and variable findings in specific subgroups of patient [Rossner *et al*., 2009; Ryu and Lee, 2012].

4.14. Expression of BCL2 oncoprotein

BCL2 belongs to a group of related proteins that are key regulators of apoptosis or programmed cell death. The tumorigenic potential of inappropriate BCL2 protein expression was first described as a result of the chromosomal translocation t(14;18) in non-Hodgkin's lymphoma. Since this discovery, overexpression of BCL2 protein has been identified in a variety of solid tumours, including breast cancer. BCL2 protein expression in breast cancer has been associated with low grade, slowly proliferating, ER positive breast tumours [Dawson *et al*., 2010]. However, BCL2 still has been suggested but not accepted as a prognostic factor in breast cancer due to conflicting results and insufficient level of evidence [Hwang *et al*., 2012]. Thus, more studies are necessary in this field.

This paradoxical favourable prognostic effect of BCL2 in breast cancer could be related to its non-apoptotic functions. The exact mechanism of differential BCL2 protein expression in breast cancer is complex. BCL2 is expressed in normal breast glandular epithelium and is known to be unregulated by oestrogen, possibly as a direct result of transcriptional induction [Wang and Phang, 1995; Leung and Wang, 1999]. In breast cancer, BCL2 positivity is not simply a surrogate for ER positivity as 14% of BCL2 positive tumours were ER negative and 31% of BCL2 negative tumours were ER positive [Dawson *et al*., 2010]. The prognostic impact of BCL2 positivity was observed regardless of other tumour characteristics, including tumour size, grade and lymph node status. BCL2 was a prognostic factor in women with both ER negative and ER positive disease. It is important that women with ER positivity/ BCL2 negativity were found to have a worse prognosis than those with ER negativity/ BCL2 positivity of the tumour. BCL2 was also a strong prognostic marker in women with both HER2 negative and HER2 positive disease and women with triple negative disease. The interaction between treatment and the prognostic role of BCL2 has also been addressed, showing that the prognostic impact of BCL2 is independent of adjuvant therapy received [Dawson *et al*., 2010].

In the present study, positive expression of BCL2 was found in 67.6% of cases. If only cytoplasmic staining was scored as positive for BCL2, regardless of the intensity of the stained cells, BCL2 positivity has been observed in 92 (60.9%) of all cases by immunohistochemical evaluation of 151 patients [Lee, Im *et al*., 2007]. Choosing the cut-off value for BCL2 protein positivity in breast cancer as 30% of tumour cells

showing moderate to strong cytoplasmic staining, positive reaction was observed in 63% of tumours [Le *et al*., 1999].

In the present study, there was no statistically significant association between BCL2 expression and the local spread of breast cancer as characterised by pT (P=0.9) similarly to Won *et al*., 2010. In contrast, Le *et al*. showed statistically significant association between tumour size and cytoplasmic expression of BCL2 protein (P=0.03). The distribution of BCL2 positive cases by T stage was consequent: T1 – 20%, T2 – 25% and T3 – 17%. The frequency of BCL2 expression reached 83% among pT1 cases, 58% – pT2 and 61% – pT3 [Le *et al*., 1999]. Recently, Zaha *et al*. have shown association between BCL2 and pT characterised by P=0.04 [Zaha *et al*., 2012]. Hwang *et al*. have compared the expression of BCL2 in breast cancers exceeding the size of 2 cm with smaller tumours, and the difference was found statistically significant [Hwang *et al*., 2012].

BCL2 expression also did not correlate with axillary lymph node status. Previously, contrasting data are published. By increasing amount of positive lymph nodes with breast cancer metastases, the BCL2 overexpression rate statistically significantly decreased (P=0.01). The frequency of BCL2 overexpression was 70% in N0, 64% in N1, 59% in N2 and 39% in N3 [Le *et al*., 1999]. Recently, statistically significant association between BCL2 expression and N0 has been reconfirmed [Hwang *et al*., 2012]. However, no statistically significant association between BCL2 expression and presence or absence of lymph node metastasis was found by Won *et al*., 2010.

Comparing the presence of BCL2 protein in different histological types of breast cancer, the expression was observed mostly in ductal breast cancer followed by lobular breast cancer. However, as the ductal breast cancer is the most frequent histological type the difference is not statistically significant (P=0.1). Similar data were described by Lee *et al*., 2007. In this study, 60.1% of ductal breast cancer cases and 69.2% cancers of other histological types expressed BCL2 and the difference was not statistically significant, characterised by P=0.521 [Lee, Im *et al*., 2007]. In the group described by Zaha *et al*., the frequency of BCL2 expression was 54.8% in invasive ductal carcinomas and 66.6% in invasive lobular and mixed lobular carcinomas. Medullary carcinomas were negative. These differences were statistically insignificant (P=0.1) in accordance with the present study [Zaha *et al*., 2012]. Lee *et al*. analysed BCL2 expression in the endolymphatic tumour emboli. The emboli were present in 95%

of cases; 62.9% were positive for BCL2 [Lee, Im *et al*., 2007]. In the present study, lymphatic invasion was rarely observed therefore no definite conlusions can be drawn.

Absence of BCL2 in breast cancer cells is more frequent in high grade breast cancers (P<0.0001). Zaha *et al*. found that BCL2 expression decreased with increasing tumour grade. G3 tumours did not present positive BCL2 at 3+. All well differentiated infiltrating ductal carcinomas (G1) were BCL2 positive and score 3+ was found in 75% of the cases. Moderately differentiated tumours had a rate of 54.5% BCL2 positive cases. G3 carcinomas presented positive reaction for BCL2 in only 38.4% of cases, all scoring 2+. Although these results are statistically significant, the difference had a borderline statistical significance with P=0.048 [Zaha *et al*., 2012].

Statistically significant association (P=0.003) between BCL2 overexpression and tumour histological grade was revealed by Le *et al*. Well differentiated breast cancers (G1) showed absolute (100%) BCL2 cytoplasmic overexpression while among moderately differentiated breast cancers (G2), only 61% cases showed BCL2 positivity. Slightly more than a half (51%) of high grade (G3) breast cancers was positive for BCL2 protein [Le *et al*., 1999].

In the joint group of G1 and G2 tumours, the rate of BCL2 expression was 78.8%. In G3 tumours more than a half of all investigated cases (54.5%) did not show BCL2 cytoplasmic expression [Lee *et al*., 2007]. In more recent study, similar data were obtained. Among BCL2 positive tumours, there were 53.4% G1-G2 and 34.1% G3 cancers. The BCL-2 negative group comprised 31.3% G1-G2 and 59.3% G3 tumours. The differences were statistically significant [Hwang *et al*., 2012]. Similarly, statistically significant correlations between BCL2 expression and low nuclear grade were found [Hwang *et al*., 2012].

In the present study, BCL2 protein expression was significantly associated with ER expression (P<0.0001). Analysing the published evidence, similar observations can be found. High level of BCL2 expression is strongly associated with positive oestrogen and progesterone receptors. BCL2+ ER+ and PR+ cases represent half of the cases exhibiting the best response to hormone therapy and the lowest rate of recurrence [Zaha *et al*., 2012]. The relationships between BCL2 and the hormone receptor markers were evaluated also by Lee *et al*. Ninety two percent of ER positive tumours showed co-expression with BCL2. Among the cases with negative ER expression, 76.5% of cancers were negative for BCL2 as well [Lee, Im *et al*., 2007]. Statistically significant association between hormone receptor expression and BCL2 positivity was shown also

by Le *et al*. Eighty three percent of cases with positive hormone receptor expression showed co-expression of BCL2 [Le *et al*., 1999]. The results regarding PR and BCL2 expression are similar (P<0.0001) and in accordance with the published evidence [Lee, Im *et al*., 2007].

In contrast to positive association between BCL2 and hormone receptor expression, HER2 overexpression was more frequently observed in the BCL2 negative group (P<0.0001). The data are in accordance with the published evidence [Lee, Im *et al*., 2007; Hwang *et al*., 2012]. Negativity of HER2 (from 0 to 2+) was observed along with frequent marked BCL2 expression (68.6%). Overexpression of HER2 (3+) was observed in 56.7% of BCL2 negative cases, but 43.3% of BCL2 positive tumours [Lee, Im *et al*., 2007]. Hwang *et al*. have recognised HER2 positivity as 2+ or 3+ expression by immunohistochemistry, or by positive FISH findings. Despite different cut-off levels, the same conclusion was reached. The HER2 positivity was found in 34.6% of BCL-2 negative and 21.8% of BCL-2 positive cases, and the difference was statistically significant with P<0.001 [Hwang *et al*., 2012].

The BCL2 positive cases more frequently show low proliferation activity. Opposite situation is observed in the negative BCL2 group where there are more high Ki-67 level of breast cancer cells (P<0.0001). Controversial relationships between BCL2 and proliferative activity have been described. Some authors have shown that the expression of BCL2 is significantly more frequent in breast cancers with low Ki-67 index. Others authors insist that there is no association between BCL2 and Ki-67 status.

In highly proliferative tumours, lack of BCL2 expression is common while cancers having lower proliferation show BCL2 positivity in a subgroup of such cancers (P=0.00049). Regarding BCL2 positive tumours, 66.6% of cases had a low Ki-67, 18.8% – intermediate type and only 15.1% – high. In contrast, BCL2 negative tumours are highly proliferative [Zaha *et al*., 2012].

In contrast, Ki-67 expression was not found to be inversely correlated with BCL2 as it was with other parameters in Lee *et al*. study. The breast cancers were classified into 2 groups based on the Ki-67 labelling index less than 5% or exceeding 5%. In the group of low proliferative activity, 73.1% of cases expressed BCL2. In contrast, the proliferatively active cancers exhibited BCL2 positivity in 51.2% of cases. The difference did not reach statistical significance [Lee, Im *et al*., 2007].

Molecular subtype of breast cancer and BCL2 expression has been evaluated by Zaha *et al*. BCL2 expression was observed in 54.1% of cases. Assessing the BCL2

expression according to molecular classification, luminal A tumours expressed BCL2 at a rate of 92.3%, luminal B subtype cancers in 60% of cases, while the remaining molecular subtypes showed no expression. Among non-luminal tumours, the basal-like tumours has a negative score 0 in 77.8% cases, the rest being 1+. HER2-type tumours were classified the score 0 [Zaha *et al*., 2012]. In the present study, positive expression is observed in luminal A and both luminal B subtype cases, but HER2 positive and triple negative molecular subtypes are frequently negative. However, the heterogeneity exists and should not be neglected.

In the present study, statistically significant association is shown between two new potentially prognostic and predictive factors as BCL2 and cyclin D1 ($P<0.0001$). The BCL2 positive group also include most of cyclin D1 positive cases ($P<0.0001$). This finding corresponds to the the known molecular mechanisms, including up-regulation of BCL-2 and cyclin D1 by common pathway including oestradiol-repressed microRNAs. This pathway is associated both by presence of oestrogen receptors ensuring the binding of oestradiol, and growth suppression in response to oestradiol [Yu *et al*., 2012]. In addition, BCL2 positivity in the present study is associated with lack of CK 5/6 expression ($P=0.02$).

The survival analysis by Kaplan-Meyer has identified BCL2 expression as important prognostic factor in agreement with Hwang *et al*., 2012. However, conflicting findings are reported [Ryu and Lee, 2012].

4.15. Expression of cyclooxygenase-2 protein

Many factors are known to have prognostic value in case of breast cancer, including tumour size, nodal status, hormone receptors, and *HER2/neu* expression. For targeted treatment, hormone therapy and *HER2/neu* application have been successful and they have become the mainstay of treatment of breast cancer patients along with chemotherapy and radiation therapy. Adding to these treatment strategies, different factors are being studied, one of which is COX-2 and the associated pathways. COX-2 is an inducible enzyme involved in the conversion of arachidonic acid to eicosanoids. In cancerogenesis, COX-2 has been associated with angiogenesis, tumour cell proliferation, invasion, metastatic spread and escape from apoptosis [Khan *et al*., 2011].

COX-2 expression can be evaluated by immunohistochemistry. However, the technological variability can exceed the biological differences [Strumfa, 2005], and the applied scoring systems also are diverse. Cho *et al.* determined COX-2 by multiplying the staining intensity score and staining quantity score, while Ristimäki *et al*. and Haffty *et al*. considered COX-2 moderately to strongly positive when **>** 10% was stained in cancer cells [Cho *et al.*, 2006; Ristimäki *et al*., 2002; Haffty *et al*., 2008]. Analogous approach was applied in the present study.

Consequently to the technological and scoring variability, contrasting data are reported. Lee *et al*. evaluated COX-2 cytoplasmic expression by intensity scoring as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong) and by percentage of positive tumour cells. COX-2 was considered overexpressed when the intensity was scored 2 or 3 in more than 10% of tumour cells. By these cut-off values, the positivity rate was 15% [Lee *et al*., 2010]. In the present study, positive COX-2 expression was found in 1.3% of investigated breast cancer cases. In general, the reported positivity of COX-2 in breast cancer varies from 4.5% to 85% [Brueggemeier *et al.,* 2005; Lee *et al*., 2010]. The difference can be attributed both to biological properties of the evaluated tumours or to the immunohistochemical staining protocol, especially the clonality and affinity of the primary antibody.

COX-2 immunohistochemical staining was scored using a weighted histoscore by van Nes *et al*. in their work. The proportion of cells with cytoplasmic staining was multiplied by the intensity of staining to provide a score of 0 to 300. The following calculations were applied: score = (0 x percentage of cytoplasm not stained) + (1 x percentage weakly stained) + (2 x percentage moderately stained) + (3 x percentage strongly stained). Median tumour COX-2 histoscore was 148.33 (range 3-278), meaning high COX-2 had a histoscore >148 and low COX-2 a histoscore ≤148. Increased COX-2 expression was observed with increasing grade, stage (P<0.0001), and age (P<0.004) as reported by van Nes *et al*., 2011. The correlation between COX-2 positivity and grade and size of breast cancer was identified also by Jana *et al*., 2012 and Kim *et al*., 2012. In the present study, there was no correlation between COX-2 expression and cancer grade or pT. Also, no association between pN and COX-2 expression was found in agreement with van Nes *et al*., 2011. As described by Lee, Im *et al*., 2007, no statistical significance was found between COX-2 overexpression and patient's age (P=0.76), tumour size by pT (P=0.143), nodal status by pN (P=0.236), distant

metastases (P=0.407) and death rate (P=0.674). Similarly, in multivariate analysis, no correlation was found between clinico-pathologic parameters and COX-2 expression although COX-2 overexpression was more common in larger tumours and higher nodal status (P<0.001 and P=0.048, respectively) by univariate evaluation [Lee *et al*., 2010].

In the present study, expression of COX-2 was limited to a subgroup of ER negative cases (P=0.002). Analogous association was found regarding COX-2 and PR expression (P=0.008). These data are in agreement with the observation that ER and PR negative tumours exhibit higher COX-2 expression levels than hormone receptor positive tumours [van Nes *et al*., 2011]. Also, significant correlation between COX-2 overexpression and ER negativity was found by Jana *et al*., 2012. In contrast, no statistical significance was found between COX-2 overexpression and hormone receptor (ER and PR) status (P=0.286 and P=0.272, respectively) by Lee, Im *et al*., 2007.

Brueggemeier *et al*. analysed the regulation of oestrogen in relation to COX-2, and concluded that elevated COX-2 results in increased aromatase activity via autocrine and paracrine mechanisms which underlie the pathogenesis of breast cancer. Although there are theories involving the relationship between COX-2 and breast cancer, clinical relevance or prognostic values are still controversial [Brueggemeier *et al.*, 2005; Lee *et al*., 2010].

There was no significant correlation between COX-2 expression and Ki-67 or HER2 expression in aggrement with van Nes *et al*., 2011. As described by Lee *et al*., 2007, no statistical significance was found between COX-2 overexpression and HER2 expression (P=0.277) or Ki-67 expression by cut-off value 20% characterised by P=0.23 [Lee, Im *et al*., 2007]. Recently, correlation between COX-2 and HER2 posivity in invasive ductal carcinoma was found [Jana *et al*., 2012].

In the present study, positive COX-2 expression was observed in HER2 positive and triple negative molecular subtypes. Luminal A and both luminal B subtype cases did not express COX-2. In general the data are in agreement with Nes *et al*., 2010 as in the cited study the presence of COX-2 is observed in ER and PR-negative tumours. The different cut-off values yield mathematically different results.

Statistically significant association was identified between two potential prognostic and predictive factors as COX-2 and CK 5/6 (P=0.001). Only subgroup of CK 5/6 positive cases exhibited COX-2 reactivity.

As described by Lee *et al*., 2007, no statistical significance was found between COX-2 overexpression and p53 positivity (*P*=0.126). Analogous data are achieved in the present study.

No correlation between COX-2 expression and survival was observed in agreement with Lee, Im *et al*., 2007. The small number of COX-2 positive cases limited the study.

4.16. Overexpression of cyclin D1

Cyclins ensure the positive regulation of cyclin dependant kinases that upon activation promotes the progression of cell cycle. The cyclin family comprises several proteins, including cyclins A, B1, D1 and E. Regarding breast cancer, more information is available on cyclins A and E that are associated with poor prognosis, and on cyclin B1 showing correlation with tumour grade, proliferation activity by Ki-67, mitotic count and adverse clinical outcome [Boström *et al*., 2009]. The cyclin D1 controls the G1 to S phase transition that represents critical checkpoint controlling cell entry into division [Aaltonen *et al*., 2009; Velasco-Velázquez *et al*., 2011].

Cyclin D1 overexpression is reported to be more prevalent than amplification, with the reported frequency ranging from 28% to 83%. The wide variation has been linked with different antibodies, techniques and thresholds (cut-off points). Cyclin D1 overexpression, with or without *CCND1* amplification, has received great attention in the literature in the last three years due to results of *in vitro* studies and data from clinical trials implicating cyclin D1 overexpression in resistance to tamoxifen treatment.

Immunohistochemical assessment of cyclin D1 expression has been performed by different technologies. Several authorities have reported that immunohistochemical assessment of cyclin D1 in pathology practice is difficult, erratic, "technically challenging" and "not routinely used because of the frequent demonstration of equivocal results" despite the development of a new rabbit monoclonal antibody against cyclin D1 [Reis-Filho, Savage *et al.*, 2006].

Reiss-Filho *et al*. assessed the intensity and distribution of immunohistochemical distribution of cyclin D1 in semiquantitative way using the Allred score method. With this method, the intensity of the immunohistochemical reaction as viewed under the light microscope was recorded as 0, negative (no staining of any nuclei even at high

magnification); 1, weak (only visible at high magnification); 2, moderate (readily visible at low magnification); or 3, strong (strikingly positive even at low power magnification). The proportion of tumour nuclei showing positive staining was also recorded as either: 0, none; 1, less than 1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3 and 5, more than 2/3. The proportion and intensity scores were added to obtain a total score, which ranged from 0 to 8.35. Tumours were then categorised into four groups: negative/weak expression (total scores 0-2), intermediate expression (total scores 3-5) and strong expression (total scores 6-8). Only nuclear staining was considered specific. Among 224 breast cancers, 13 were scored as 0; 1 as 2; 12 as 3; 16 as 4; 31 as 5; 57 as 6; 59 as 7 and 35 as 8 [Reis-Filho, Savage *et al*., 2006]. In present study positive cyclin D1 expression is observed in 61.6% of breast cancer cases. Boström *et al*., 2009 reported cyclin D expression in 3-90% of breast cancer cells. Evidently, the diversity of scoring systems embarrasses the comparison between different studies.

Research data from Lee *et al*. showed statistically significant association between breast cancer size or pT parameter and expression of cyclin D1 in breast cancer cells characterised by P=0.04 [Lee *et al*., 2007]. Statistically significant associations were found between cyclin D1 expression and tumour size (P=0.01) in postmenopausal women with early-stage, hormone receptor positive breast cancer [Rudas *et al*., 2008]. However, in the present study, the association was not statistically significant (P=0.1) in agreement with Reis-Filho, Savage *et al*., 2006 and Boström *et al*., 2009. No statistically significant association with pN was found in agreement with Reis-Filho, Savage *et al*., 2006; Lee *et al*., 2007; Rudas *et al*., 2008 and Boström *et al*., 2009.

In the present study, the expression of cyclin D1 is associated with ER expression (P<0.0001). Comparing ER positive and negative breast cancer groups it was evident that more cyclin D1 positive cases belong to ER positive group but the ER negative group includes more cyclin D1 negative cases (P<0.0001). Similar association was found between expression of cyclin D1 and PR (P<0.0001). The findings are in aggrement with the following literature data. Reis-Filho, Savage *et al*., 2006 reported strong direct correlation between cyclin D1 positivity and expression of ER and PR (both, P<0.0001). The positive correlation between cyclin D1 and hormone receptor status was confirmed also by Aaltonen *et al*., 2009 and Boström *et al*., 2009.

Expression of cyclin D1 in the present study was not associated with HER2 status (P=0.09) and proliferation activity (P=0.3). Both findings are in agreement with

Boström *et al*., 2009. Lack of significant correlation (*P*=0.08) between cyclin D1 and HER2 overexpression is reported also by Reis-Filho, Savage *et al*., 2006 and Rudas *et al*., 2008. The absence of correlation between cyclin D1 and proliferation activity is indirectly confirmed by the reported lack of association between cyclin D1 expression and breast cancer grade [Reis-Filho, Savage *et al*., 2006; Boström *et al*., 2009]. Controversial data regarding grade are presented by Rudas *et al*., 2008. However, tumours with high levels of cyclin D1 expression showed lower proliferation rates (*P*<0.0001) when compared to cyclin D1 low tumours [Reis-Filho, Savage *et al*., 2006].

In the present study, luminal A and luminal B (HER2 negative) molecular subtype groups expressed cyclin D1 more frequently than HER2 positive or triple negative breast cancer. Strong positive correlation between cyclin D1 expression and luminal molecular subtype and significant inverse correlation (*P*<0.0001) between basal-like immunophenotype and cyclin D1 overexpression has been described [Nielsen *et al.,* 2004; Reis-Filho, Savage *et al*., 2006].

Expression of cyclin D1 was significantly associated with CK 5/6 negativity (*P*=0.004). Reis-Filho, Savage *et al*., 2006 also reported inverse correlation of cyclin D1 with the expression of basal markers (*P*<0.0001), including CK 5/6 (*P*<0.0001) as well as EGFR (*P*<0.0001), CK 14 (*P*=0.0014), and CK 17 (*P*<0.0001).

There has been controversy in explaining the meaning of the cyclin D1 expression as a prognostic or predictive marker. Cyclin D1 overexpression has been associated with poor prognosis in breast cancer. However, some researchers have reported it to be of no prognostic significance while others have reported that cyclin D1 overexpression is associated with a better prognosis in breast cancer [Lee *et al*., 2007].

Stendahl *et al*. suggested that, when no hormone therapy was involved, patients with breast cancers expressing high cyclin D1 levels had a better survival outcome than those with cyclin D1 low/moderate breast cancers, but cyclin D1 overexpression is a negative predictive factor for the response to tamoxifen in postmenopausal breast cancer patients [Stendahl *et al*., 2004]. Aaltonen *et al*. suggested that cyclin D1 in ER positive tumours correlates with high grade and proliferation activity as well as high concentration of cyclins A and E, but in ER negative tumours it is associated with low grade and low proliferation activity suggesting different pathogenetic mechanisms and different regulation of cell proliferation [Aaltonen *et al*., 2009]. Ahnström *et al*. reported that combined cyclin D1 and HER2 overexpression among breast cancer patients is associated with a high rate of recurrence and suggested that cyclin D1 and

HER2 can cooperate to produce a more malignant tumour type with worse prognosis [Ahnström *et al*., 2005]. The present study showed no significant relationship between the expression of cyclin D1 and survival in patients with invasive breast cancer.

4.17. Basal differentiation by cytokeratin 5/6

The expression of CK 5/6 was found in 19.0% of consecutive invasive breast cancer cases. The frequency of CK 5/6 presence is within the published range [Rattan *et al*., 2012; Alshareeda *et al*., 2013]. CK 5/6 showed statistically significant association with triple negative molecular subtype in accordance with Pillai *et al*., 2012. However, positive cases were found in all molecular subtypes by reasonable rate. Statistically significant associations between the presence of CK 5/6 and lack of oestrogen and progesterone receptors as well as cyclin D1 expression also were identified. The CK 5/6 positive cases were significantly associated with higher proliferation. These findings are in agreement with the published evidence [Pillai *et al*., 2012; Rattan *et al*., 2012; Alshareeda *et al*., 2013]. However, the heterogeneity of CK 5/6 expression is an important finding.

5. CONCLUSIONS

1. The breast cancer can be categorised into mutually exclusive molecular types by immunohistochemistry for ER, PR, proliferation activity and HER2 protein. Immunohistochemistry is also technologically adequate method to detect aberrant p53 protein, BCL2 protein, COX-2, cyclin D1 and CK 5/6 in breast cancer tissues.
2. The molecular subtypes differ by tumour volume and local tumour spread pT. Statistically significant differences between molecular subtypes are found regarding axillary lymph nodes status by pN, invasion in lymph vessels, presence of carcinoma *in situ* and histological type by WHO classification.
3. The expression of p53, BCL2, COX-2, cyclin D1 and CK 5/6 differs between molecular types suggesting different pathways of molecular pathogenesis.
4. The expression of p53 protein, observed in 24.0% of breast cancer cases, is associated with negative ER ($P<0.0001$), PR ($P<0.0001$) and BCL2 ($P<0.0001$). It is heterogeneous regarding HER2 over-expression and proliferative activity.
5. The molecular portrait of BCL2 protein-expressing breast cancer includes positive ER ($P<0.0001$), PR ($P<0.0001$) and cyclin D1 ($P<0.0001$) expression, as well as lack of HER2 over-expression ($P<0.0001$) and CK 5/6 expression ($P<0.0001$). There is statistically significant association with lower proliferative activity ($P<0.0001$) although heterogeneity is observed. The rate of BCL2 protein expression (67.6%) is well suited for clinical analysis.
6. Expression of COX-2 in breast cancer is rare event (1.3%), limited to ER ($P=0.002$) and PR ($P=0.008$) negative, CK 5/6 ($P=0.001$) positive cases.
7. The cyclin D1 expression in breast cancer has reasonable frequency (61.6%). It shows strong association with positivity for hormone receptors ER ($P<0.0001$) and PR ($P<0.0001$) and also a strong inverse correlation with the expression of basal-like CK 5/6 ($P=0.004$).
8. The expression of CK 5/6, found in 19.0% of breast cancer, is associated with ER ($P=0.003$) and PR ($P=0.004$) negativity.
9. The survival is significantly influenced by pT, pN, cancer grade, molecular subtype and expression of p53 and BCL2.

6. PRACTICAL RECOMMENDATIONS

1. The practical morphological examination of breast cancer tissues and data reporting must be carried out by protocol approach. It is highly recommended to extend the protocol by conclusion about the molecular subtype in addition to primary data. Five molecular subtypes should be determined using immunohistochemistry as economically adequate surrogate method and the significant factor associated with survival.
2. Taking into account the high frequency of p53 expression in the context with published evidence and the significant association with survival, it is recommended to include immunohistochemistry for aberrant p53 protein in the morphological diagnostic protocol of breast cancer.
3. Taking into account the high frequency BCL2 expression in the context with published evidence and the significant association with survival, it is recommended to include immunohistochemical evaluation of BCL2 protein expression in the morphological diagnostic protocol of breast cancer.

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