doi:10.25143/prom-rsu_2013-17_dts

RĪGAS STRADIŅA UNIVERSITĀTE

Arnis Abolins

# MOLECULAR SUBTYPES AND IMMUNOHISTOCHEMICAL PROFILES IN BREAST CANCER

For obtaining the degree of a Doctor of Medicine
Speciality – Pathology

Riga, 2013

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The doctoral thesis was carried out in Rīga Stradiņš University

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The defence of doctoral thesis will be held on June 10th, 2013 at 15.00 in the open meeting of the Promotion Council in Surgery, Rīga Stradiņš University, in the Hippocrate auditorium, 16 Dzirciema Street, Riga.

The doctoral thesis is available at the library of RSU and on the RSU home page: www.rsu.lv

The doctoral thesis was supported by European Social Fund Project "A new multidisciplinary research group formation for the study of breast cancer, therapy effectiveness and patient quality of life improvement", agreement Nr. 2009/0230/1DP/1.1.1.2.0/09/APIA/VIAA/070



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# LIST OF ABBREVIATIONS

ASCO – American Society of Clinical Oncology

BCL2 – BCL2 oncoprotein

CAP – College of American Pathologists

CI – 95% Confidence interval

CK – Cytokeratin

COX-2 – Cyclooxygenase – 2

ER – Oestrogen receptor alpha

FISH – Fluorescent *in situ* hybridization

G - Grade

HER – Human epidermal growth factor receptor

IHC – Immunohistochemistry

PR – Progesterone receptor

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 classics of breast cancer characteristics are 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 molecular subtypes initially were discovered by gene expression profiling in high throughput microarray technologies as the genes in breast cancer became up-regulated or down-regulated in larger groups [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 [Sorlie, 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 visualization 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. MATERIALS AND METHODS

## 1.1. Patients

Three hundred eighty three consecutive women 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.

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.

## 1.2. 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 applied treatment before operation) was submitted in the Clinic of Surgery, but all morphological details (gross and microscopic data) were assessed 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, 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.

### 1.3. Microscopy

The primary tumour tissue samples after gross examination were fixed in neutral buffered 10% formalin, processed in vacuum infiltration processor Tissue-Tek® VIP™ 6 (Sakura Seiki Co., Ltd., Nagano, Japan) and embedded in paraplast using tissue embedding system TES 99 (Medite GmbH, Burgdorf, Germany). Four-micron-thick sections were cut on glass slides by microtome Accu-cut SRM 200CW (Sakura Finetek Europa B.V., the Netherlands) and stained with haematoxylin and eosin by automated tissue stainer TST 44 (Medite Medizintechnik, Germany). The stained slides were covered by cover glass 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 as well. The tumours were diagnosed corresponding to the World Health Organization (WHO) classification of breast tumours [Tavassoli and Deville, 2003], the tumour grade was appreciated based upon the Scarff-Bloom-Richardson classification modified by Elston and Ellis (Grade (G) 1 – well differentiated/ low grade tumour; G2 – moderately differentiated/ intermediate grade tumour; G3 – poorly differentiated/ high grade tumour) as described by Elston *et al.,* 1991. *In situ* ductal carcinoma lesions were classified as ductal carcinoma *in situ,* non-*comedocarcinoma* type,

lobular carcinoma i*n situ* and *comedocarcinoma* type of ductal carcinoma *in situ*. The tumour pathological T and N characteristics (pathological TNM stage) were specified accordingly to the 7th edition criteria of *AJCC Cancer staging manual* [Edge *et al.,* 2010].

### 1.4. Immunohistochemistry

The formalin-fixed, paraffin-embedded tissues, cut at 3 micron thick sections on electrostatic slides were investigated by immunohistochemistry. 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, cyclin D1, COX-2 and p53 protein was detected by total test. All IHC reagents were produced by Dako, Glostrup, Denmark. HER2 protein overexpression was detected by HercepTest™.

The cytoplasmic expression of actin was evaluated in the myoepithelial cell layer. Loss of myoepithelial cell layer in an appropriate morphological setting was considered the 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-cadherin 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 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™ 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 and 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™ was interpreted as negative for HER2 protein overexpression (0 and 1+ staining intensity), weakly positive (2+), or strongly positive (3+) 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 by IHC or *HER2/neu* gene copies were amplified in fluorescent *in situ* hybridization (FISH) with ratio of *HER2/neu* to *CEP17* of > 2.2 or average *HER2/neu* gene copy number > six signals/nucleus. 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 showing 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 (BCL2) 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% of neoplastic cells; positive if 10-50% tumour cells score 2+; or more than 50% neoplastic cells score 3+ [Zaha and Lazar, 2012]. Callagy *et al.* recommended the cut-off value of 10% for BCL2 expression [Callagy *et al.,* 2006].

The evaluation of cyclooxygenase-2 protein (COX-2) expression regarded cytoplasmic reactivity. The evaluation was performed 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 tumour cells [Lee *et al.*, 2010].

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

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

Five molecular subtypes of breast cancer 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) breast cancer. Luminal B (HER2 negative) molecular subtype was recognised by positive ER and/ or PR, negative HER2 and high Ki-67 (≥14%), but luminal B (HER2 positive) subtype was identified by positive ER and/ or PR, positive HER2 by IHC or FISH 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 [Goldhirsch *et al.,* 2011].

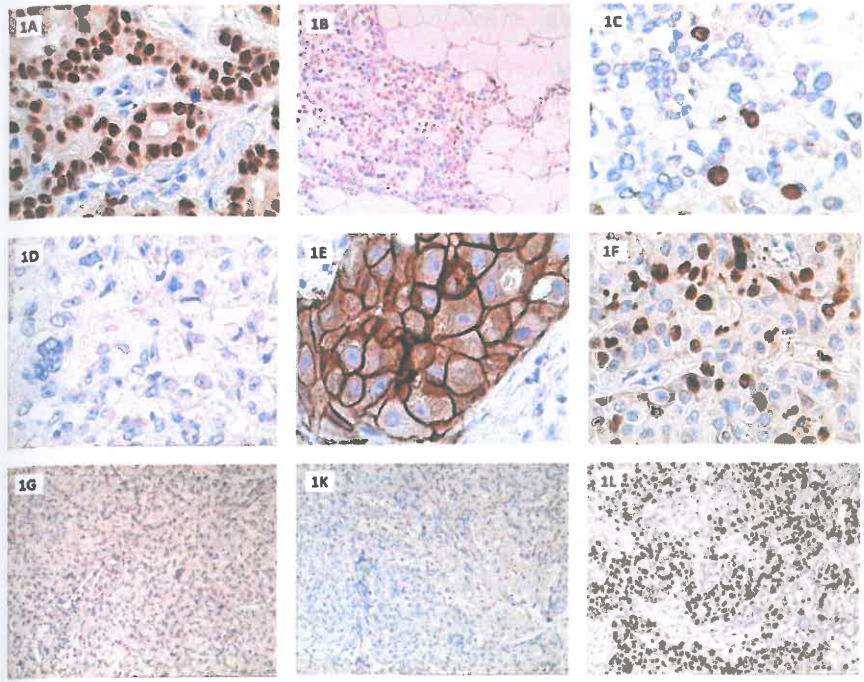



**Figure 1.1. Molecular subtypes of breast cancer. A-C, Luminal breast cancer: A, Oestrogen receptor expression; B, Lack of HER2 protein; C, Low proliferation fraction. D-F, HER2 overexpressing breast cancer: D, Lack of oestrogen receptors; B, HER2 protein overexpression; C, Moderate proliferative fraction. 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.**

### 1.5. Fluorescent *in situ* hybridization

FISH was performed using the *HER2/neu* FISH pharmDx Kit (Dako, Glostrup, Denmark) on 4-μm-thick paraffin sections according to the manufacturer's instructions. Briefly, slides 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$^0$C. In next day, slides were washed, counterstained with 4',6-diamino-2-phenyl indole and coverslipped. After 30 minutes, fluorescence was observed in an Olympus CH30LF200 (Olympus Optical Co., LTD, Japan) fluorescence microscope at 1000 magnification with digital 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].

### 1.6. 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.

## 2. RESULTS

### 2.1. Basic characteristics of the study group

The study included 383 females with primary, invasive breast carcinoma. The age of patients ranged from 27 to 88 years (mean age ± standard deviation (SD), 59.59±12.22).

Two hundred twenty nine mastectomies (59.8%, 95% confidence interval (CI) = 54.8-64.5) and 154 segmental excisions of breast were performed. In 197 cases (51.4%, 95% CI = 46.5-56.1) breast carcinoma affected right breast, but in 186 cases (48.6%, 95% CI = 43.9-53.5) it was located in left breast. 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 362 lymph node operations, in 351 cases (97%, 95% CI = 94.6-98.3) the lymph nodes were successfully retrieved. The mean count ± SD of retrieved axillary lymph nodes per case was 12.5±8.2. The number of breast cancer metastasis in the retrieved lymph nodes ranged 0 to 32 (mean amount ± SD, 2.8±5.0).

### 2.2. The morphological characteristics of the analysed tumours

According to pathological TNM classification all 383 tumours were designated as follows: pT1 – 161 tumours (42%, 95% CI = 37.2-47.0); pT2 – 159 tumours (41.6%, 95% CI = 36.7-46.5); pT3 – 35 tumours (9.1%, 95% CI = 6.6-12.4) and pT4 – 28 tumours (7.3%, 95% CI = 5.1-10.4).

Regarding the pN category, there were 180 cases (47%, 95% CI = 42.1-52.0) of pN0, 81 cases (21.1%, 95% CI = 17.4-25.5) of pN1, 54 (14.1%, 95% CI = 11.0-17.9) cases of pN2 and 36 cases (9.4%, 95% CI = 6.9-12.7) of

pN3. In 32 cases (8.4% 95% CI = 6.0-11.6), the lymph node status was not established (pNx).

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 cancer operation. Breast cancer metastasis were found in bones – 33.3% (95% CI = 13.8-60.9), in central nervous system (brain) – 25% (95% CI = 8.9-53.2), in lungs – 25% (95% CI = 8.9-53.2) and in the liver – 16.7% (95% CI = 4.7-44.8).

In breast tissues (Figure 2.1.), all cases were classified by the histological grade as follows: G1 (Figure 2.2.) – 61 (16.0%, 95% CI=12.2-19.6), G2 (Figure 2.3.) – 138 (36.0%, 95% CI=31.9-41.1) and G3 (Figure 2.4.) – 184 cases (48.0%, 95% CI=43.8-52.8).

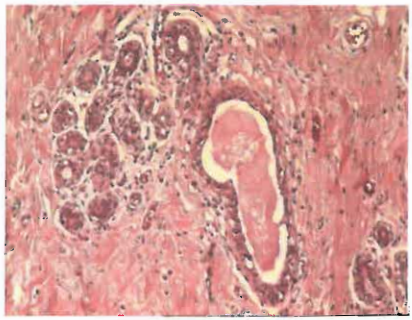


**Figure 2.1. Breast tissues without malignant tumour. Haematoxylin – eosin, original magnification 100 x..**

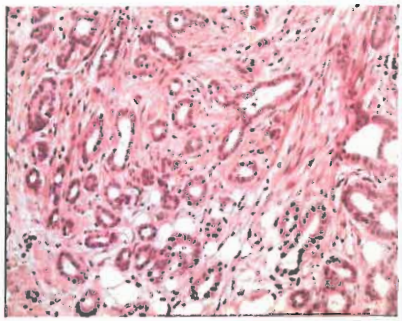


**Figure 2.2. Low grade invasive ductal breast carcinoma. Haematoxylin – eosin, original magnification 100 x.**

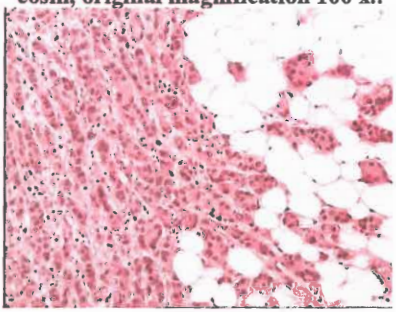


**Figure 2.3. Intermediate grade invasive ductal breast carcinoma. Haematoxylin – eosin, original magnification 100 x.**

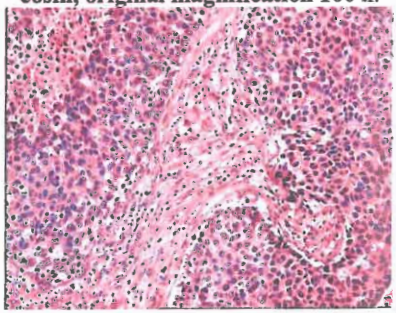


**Figure 2.4. High grade invasive ductal breast carcinoma. Haematoxylin – eosin, original magnification 100 x.**

**Microphotographs by A.Abolins.**

Invasive ductal carcinomas constituted 304 or 79.4% of 383 primary breast tumours. The full spectrum of the revealed morphological types is shown in Table 2.1.

**Table 2.1.**

**The frequency of histological types of breast carcinoma**

| **Histological type of breast carcinoma** | **Count** | **Frequency, %** | **95% confidence interval, %** | |
|---|---|---|---|---|
| | | | Lower | Upper |
| Invasive ductal carcinoma (Figure 2.5) | 304 | 79.4 | 75.5 | 83.6 |
| Invasive lobular carcinoma carcinoma (Figure 2.6) | 51 | 13.3 | 9.9 | 16.7 |
| Mucinous breast carcinoma carcinoma (Figure 2.7) | 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 carcinoma (Figure 2.8) | 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 | | |

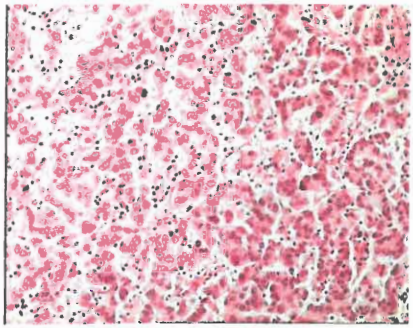


**Figure 2.5. High grade invasive ductal breast carcinoma. Haematoxylin – eosin, original magnification 100 x.**

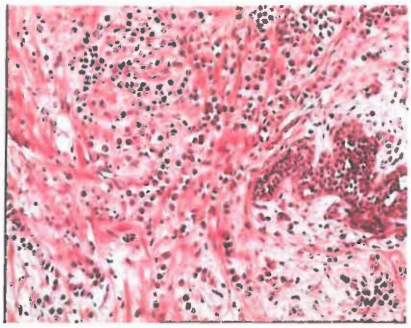


**Figure 2.6. Invasive lobular carcinoma. Haematoxylin – eosin, original magnification 100 x.**

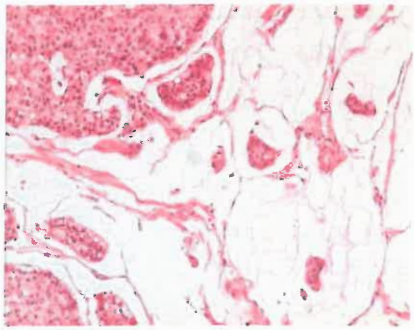


**Figure 2.7. Mucinous breast carcinoma. Haematoxylin – eosin, original magnification 100 x.**

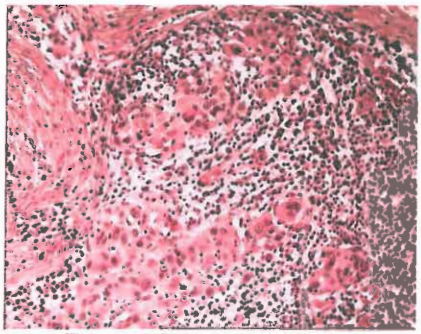


**Figure 2.8. Medullary breast carcinoma. Haematoxylin – eosin, original magnification 100 x.**

**Microphotographs by A.Abolins.**

By immunohistochemistry, expression of ER, PR, HER2 and Ki-67 was determined resulting in detection of molecular subtypes of breast cancer. Patient and tumour characteristics of the 383 cases based on IHC subtypes are summarized in Table 2.2.

**Table 2.2.**

## 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 | 56.7 ± 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%) | |
| Invasion in lymphatic vessels | 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%) | |

**Table 2.2. (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 |
|---|---|---|---|---|---|---|---|
| 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 | 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 | 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%) | |
| | | | | | | | |
| Microcalcifications in the tumour | 148 (38.6%) | 68 (44.4%) | 47 (37.6%) | 8 (33.3%) | 15 (46.9%) | 10 (20.4%) | 0.071 |
| Microcalcifications in the arteries | 6 (1.6%) | 2 (1.3%) | 4 (3.2%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | |
| 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%) | |

**Table 2.2. (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 | 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 | 213 (55.6%) | 0 (0.0%) | 125 (100%) | 18 (75.0%) | 27 (84.4%) | 43 (87.8%) | |
| | | | | | | | |
| 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 2.2. (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 HER2 status | 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 | 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 | 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%) | |
| Positive COX-2 status | 5 (1.3%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 2 (6.3%) | 3 (6.1%) | 0.024 |
| Negative COX-2 status | 378 (98.7%) | 153 (100%) | 125 (100%) | 24 (100%) | 30 (93.8%) | 46 (93.9%) | |
| Positive cyclin D1 status | 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 | 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%) | |

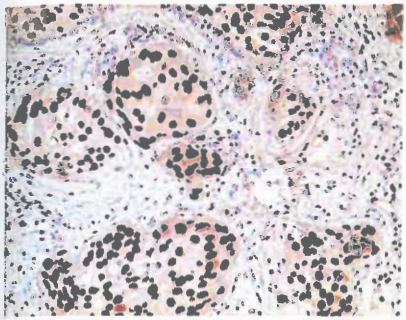


**Figure 2.9. Nuclear expression of p53 in invasive breast carcinoma. Anti-p53, immunoperoxidase, original magnification 100 x.**

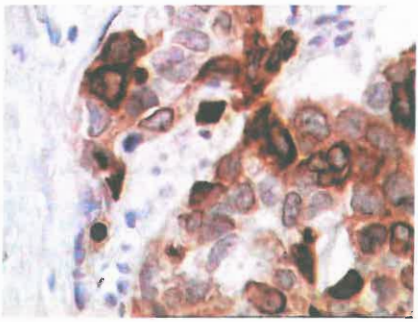


**Figure 2.10. Cytoplasmic expression of BCL2 in breast carcinoma. Anti-BCL2, immunoperoxidase, original magnification 400 x.**

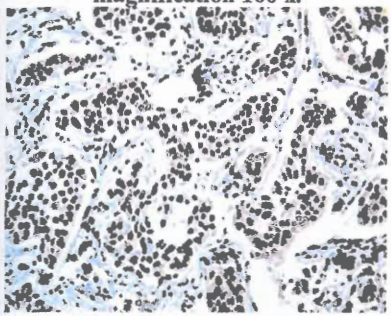


**Figure 2.11. Nuclear expression of cyclin D1 in invasive ductal breast carcinoma. Anti-cyclin D1, immunoperoxidase, original magnification 100 x.**

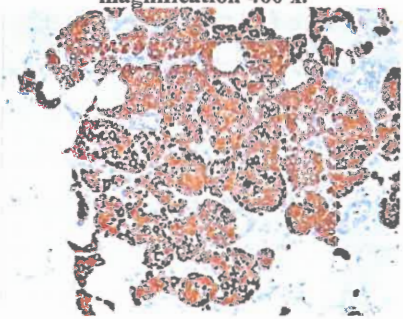


**Figure 2.12. Diffuse cytoplasmic expression of CK 5/6 in breast carcinoma. Anti-CK 5/6, immunoperoxidase, original magnification 50 x.**

**Microphotographs by A.Abolins.**

## 2.3. The association between the molecular subtypes of breast carcinoma and the morphological and prognostic variables

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 ranged within postmenopausal period, from 55.13 years in case of luminal B (HER2 positive) molecular subtype to 61.62 years regarding luminal A subtype.

The molecular subtypes differed significantly by mean tumour volume (ANOVA F test = 5.04, $P<0.001$). Luminal B (HER2 positive) and triple negative breast cancers had larger volume (36.53 and 79.98 $cm^3$, respectively) than other molecular subtypes (luminal A – 6.46, 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) as revealed by $P<0.0001$ and HER2 positive ($P=0.003$) subtypes.

There were statistically significant ($P=0.002$) differences, analysing the local tumour spread (pT) by molecular subtype. The highest proportion of pT1 tumours was classified as luminal A. In contrast, pT2 and pT3 tumours showed predominance of luminal B (HER2 negative) subtype. 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 was revealed in pT1, but luminal B (HER2 negative) subtype – in pT2 stage.

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

Breast cancer grade distribution between molecular subtypes showed statistically significant differences ($P<0.0001$). Significantly higher amount of G3 cases belonged to luminal B (HER2 negative) and triple negative subtypes, while G1 cases were mainly luminal A molecular subtype cancers. The highest proportions of G3 tumours were detected in luminal B (HER2 positive), HER2 positive and triple negative subtypes.

The molecular subtypes differed significantly considering the carcinoma *in situ* component ($P<0.0001$). Breast cancers lacking carcinoma *in situ* belonged mainly to luminal A, luminal B (HER2 negative) and triple negative subtypes. However, the highest proportion of DCIS-harboring breast cancers was of luminal A subtype as well. Ductal carcinoma *in situ, comedocarcinoma* type was remarkably frequent in luminal B (HER2 negative) molecular subtype as well as in HER2 positive and luminal A molecular subtype. 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.

Categorizing the breast cancer cases by histological types, significant differences between molecular subtype distribution ($P<0.0001$) were observed. Ductal breast cancer cases represented full spectrum of molecular subtypes with predominance of luminal B (HER2 negative) and luminal A, followed by triple negative molecular subtype. The lobular breast carcinoma was heterogeneous by the molecular subtype, but luminal A was the most frequent subtype followed by luminal B (HER2 negative) molecular subtype in contrast with ductal breast carcinoma. Invasive ductal carcinoma was most frequent histological type of breast cancer in all molecular subtypes. Invasive lobular carcinoma was the next frequent breast cancer type, especially among luminal A (21.7%) and luminal B (HER2 negative) molecular subtypes (16.9%), while metaplastic carcinomas were triple negative.

Among pT1 cases, metastases in lymph nodes were mostly absent – 75.4%. In pT2 cancers, the pN distribution was different: N0 in 39.3%, N2 – 30%, and N3 – 10.7% of cases. Larger tumours were more aggressive as among

pT4 cases there were 4-9 positive lymph nodes in 40%, but ≥10 in 36% of cases. The differences were statistically significant ($P$<0.0001).

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%. Among pN1 cancers, pT2 was the most common finding constituting 55.6%. Similarly, pN2 and pN3 were dominated by pT2 (54.5% and 45.7%, respectively). Statistically significant differences were observed ($P$<0.0001). 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 node dissection was performed.

The analysis disclosed association between pT and tumour grade ($P$=0.003). In pT1 group, 38.5% of cancers were G2 or G3 each. However, G3 was the most frequent finding in pT2 (50.9%), pT3 (60%) and pT4 cases (71.4%). pT3 and pT4 cancers were characterised by rare occurrence of G1 (3.3% and 4.9%, respectively). Among G2 cases, there was slight predominance of pT1 (44.9%), while G3 cancers were more frequently of pT2 (44%).

Intermediate and high grade cancers constituted almost similar fraction (G2 – 40.6%, G3 – 39.4%) in tumour group without lymph node metastases. Among pN1 tumours, the frequency of G2 and G3 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.

Among pN0 tumours, there were low, intermediate and high grade tumours, comprising 66.7%, 56.2% and 42.5% of the respective G group. Intermediate and high grade tumours presented with low number of metastases in substantial number of patients: G2/ pN1 – 27.7%, G3/ pN1 – 21.6%. However, high grade tumours were associated with higher number of

metastases: G3/ pN3 – 14.4%, G2/ pN3 – 5.4% and G1/ pN3 – 7.4%. Statistically significant differences were identified ($P$=0.002)

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

Among the breast cancers with high proliferation activity, pT2 tumours were the most frequent finding, followed by pT1. In low Ki-67 group, pT1 cancers are dominating and pT3 – distinctly rare. Examining pT by Ki-67, higher size was associated with higher proliferation activity ($P$=0.001).

Statistically significant association was found between breast cancer proliferation activity and involved lymph node status ($P$<0.0001). Despite the fact that pN0 stage was the most frequent pN measurement both in low and high Ki-67 groups, only pN0 group showed predominance of cases with low proliferation activity. By increasing number of metastases in axillary lymph nodes, Ki-67 activity increased rapidly ($P$<0.0001).

Breast cancer grade showed statistically significant association with ER and PR status in neoplastic cells ($P$<0.0001). Negativity of ER and PR was found more frequently in G3 cancers ($P$<0.0001), but it was distinctly rare finding in low or intermediate grade cancers. The predominance of intermediate grade cancers among the ER and PR positive group reflected lower number of G1 cases in the general group as well as tendency to ER and PR negativity in high grade cancers.

Analysing by Ki-67 status, tumours of all grades were almost equally represented in the low proliferation group, but tumours characterised by high proliferation rate showed distinct predominance of G3 tumours ($P$<0.0001).

HER2 receptor overexpression in breast cancer cells is another important prognostic and predictive factor. The HER2 negative breast cancer group was heterogeneous by grade. In contrast, the HER2 positive breast

cancers showed clear-cut evidence of predominance of high grade breast cancers ($P<0.0001$). Analysing the breast cancer by grade, G1 and G2 cancers were predominantly HER2 negative, while G3 cancers comprised significant number of HER2 positive cases thus representing another evidence of heterogeneous structure ($P<0.0001$).

The p53-negative breast cancers represented heterogeneous group by cancer grade. If p53 was overexpressed it was seen in G3 breast cancer ($P<0.0001$).

The p53 negative breast cancer group showed distinct predominance of ER and PR positive cases – 84.6% ($P<0.0001$). The ER and PR positive cases were heterogeneous by p53 protein status but negative cases predominated in p53 positive group ($P<0.0001$).

Both p53 positive and negative groups shared fraction of HER2 positive cases. However, p53 negative group included higher number of HER2 negative tumours ($P=0.001$). Dividing the study group into HER2 positive and negative cancers, HER2 negative group contained more p53 negative cases as HER2 positive group ($P=0.001$).

The p53 negative group was heterogeneous by proliferation activity. However, p53 positive group contained more cases featuring high proliferative activity ($P<0.0001$). In low Ki-67 group most of cancers were p53 negative ($P<0.0001$).

Expression of aberrant p53 protein and BCL2 protein had tendency to mutual exclusion. The p53 negative group showed predominance of BCL2 positivity but p53 positive group – BCL2 negative cases ($P<0.0001$).

There was 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. Similarly, subdividing the breast cancers by CK 5/6 expression into positive and negative groups p53 negativity was more frequent in CK5/6 negative breast cancer group ($P<0.0001$).

BCL2 positive group was heterogeneous by grade, showing high percentage of low and intermediate grade cancers. The number of BCL2 negative cases increased by increasing grade. The difference was statistically significant ($P<0.0001$).

BCL2 positive tumours included higher number of ER and PR receptor positive cases ($P<0.0001$). Breast cancers with positive ER and PR receptors were more frequently BCL2 positive ($P<0.0001$).

The BCL2 negative group was heterogeneous regarding HER2 over-expression. In contrast, BCL2 positive group was predominantly HER2 negative ($P<0.0001$). Similarly, HER2 negative group contained more BCL2 positive cases than HER2 positive group where slightly more frequent occurrence of BCL2 negative cases was observed ($P<0.0001$).

High proliferative activity was more frequent in BCL2 negative group. The association was even more clearly evident, if the groups were separated by Ki-67 expression: the cases showing low proliferation activity also mostly were BCL2 positive ($P<0.0001$).

Statistically significant data were obtained regarding the association between two new potentially prognostic and predictive factors as BCL2 and cyclin D1 ($P<0.0001$). The BCL2 positive group included more cyclin D1 positive cases. Cyclin D1 negative group was heterogeneous by BCL2 expression. In contrast, cyclin D1 positive group showed clear-cut predominance of BCL2 positive cases ($P<0.0001$).

There were statistically significant data ($P=0.02$) regarding the association between BCL2 and CK 5/6 expression. The BCL2 positive group showed predominance of CK 5/6 negative cases.

COX-2 negativity was statistically significantly associated with ER and PR expression in the breast cancer ($P=0.002$ and $P=0.008$, respectively).

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 was observed only in a subgroup of CK 5/6 positive cases but was virtually absent in CK 5/6 negative cases ($P$=0.001).

Cyclin D1 expression showed strong association with presence of ER and PR ($P$<0.0001). The analysis was confirmed ($P$<0.0001) if cyclin D1 expression was evaluated by ER and PR status.

CK5/6 negativity was observed in both cyclin D1 negative and positive groups with predominance of CK 5/6 negativity in cyclin D1 positive group ($P$=0.004).

ER and PR positivity was associated with lack of CK 5/6 ($P$=0.003 and $P$=0.004, respectively). ER and PR negative group showed more CK 5/6 positive cases ($P$=0.003 and $P$=0.004, respectively). The analysis of Ki-67 by CK 5/6 status was embarrassed by the fact that majority of breast cancer cases are CK 5/6 negative. In CK 5/6 negative group the difference between low and high proliferation activity was not as marked as in CK 5/6 positive group ($P$=0.047). Among cases showing high proliferation activity there are more CK 5/6 positive cases ($P$=0.047).

### 2.4. Survival

In the observed period, 39 patients 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 showed statistically significant association between pT stage and survival ($P$<0.0001). pT4 stage was associated with higher death rate within the first year in comparison with pT2 or pT3 (Figure 2.2.A).

Presence of more than 10 metastatic lymph nodes was 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 pN1 was the same as in cases without lymph node metastases (Figure 2.2.B).

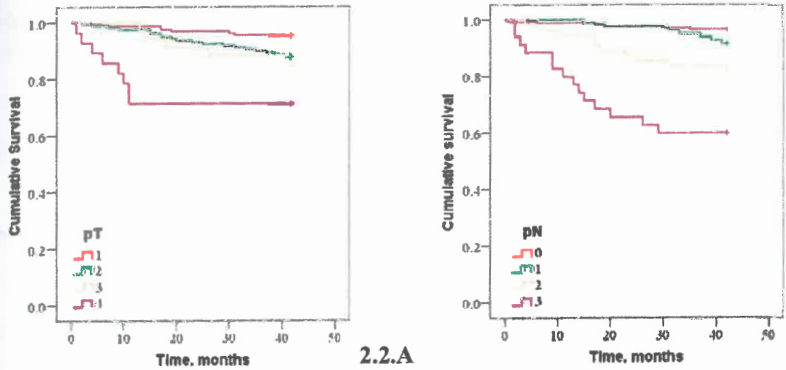



**Figure 2.2. Kaplan-Meier breast carcinoma specific survival in relation to size (pT) and metastases in lymph nodes (pN). 2.2.A, by pT; 2.2.B, by pN.**

Kaplan-Meier survival analysis by breast cancer grade showed statistically significant association between grade and survival ($P$=0.001), but the analysed data were affected by small account of G1 cases (Figure 2.3.).

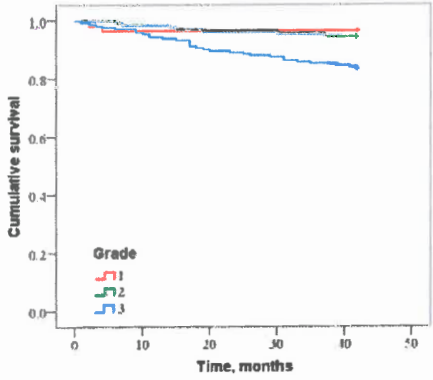



**Figure 2.3. Kaplan-Meier breast carcinoma specific survival in relation to grade of breast carcinoma.**

Patients with luminal A, luminal B (HER2 negative) and HER2 positive breast carcinoma molecular subtypes statistically significantly survived 6 months longer than patients with triple negative breast carcinoma molecular

subtypes ($P$<0.0001, $P$<0.0001, $P$=0.001, respectively). Patients with luminal B (HER2 positive) breast carcinoma molecular subtype survived 5 months longer than triple negative molecular subtype patients ($P$=0.02) as it shown in Figure 2.4.

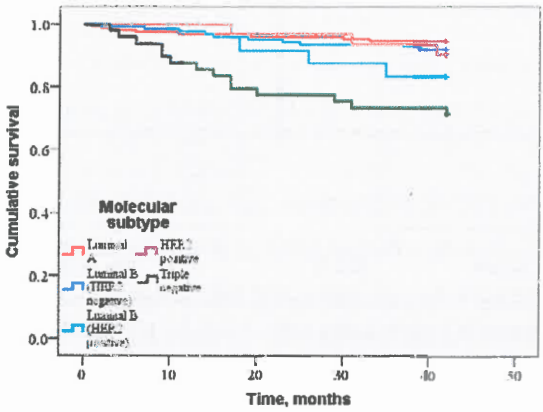
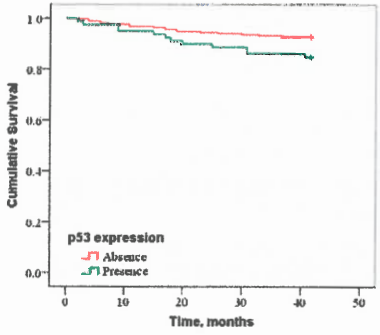
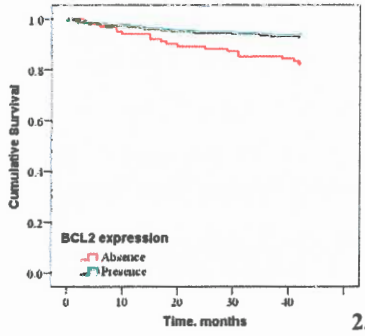



**Figure 2.4. Kaplan-Meier breast carcinoma specific survival in relation to molecular subtypes of breast carcinoma.**

Regarding the new potential immunohistochemical markers as p53, BCL2, COX-2, cyclin D1 and CK 5/6, Kaplan-Meier survival analysis showed statistically significant positive association between survival and absence of p53 ($P$=0.03) and expression of BCL2 ($P$=0.002) as it shown in Figure 2.5.A and Figure 2.5.B, respectively.




2.5.A




2.5.B

**Figure 2.5. Kaplan-Meier breast carcinoma specific survival in relation to presence of p53 and BCL2 in breast carcinoma cells. 2.5.A, by p53; 2.5.B, by BCL2.**

### 2.5. Statistically non-significant results

There was no statistically significant association between age and breast cancer pT ($P$=0.06), pN ($P$=0.7) and grade ($P$=0.1). No statistically significant association was observed between the cancer grade and perineural growth ($P$=0.2).

Ductal breast carcinoma was 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). There was no significant association between local tumour spread or between the presence of metastases in axillary lymph nodes and the morphological type of breast cancer ($P$=0.6). Cancers of all morphological types were associated with metastatic spread except invasive cribriform, metaplastic, invasive papillary and tubular breast cancer.

There was no statistically significant association between breast cancer size (pT) and expression of new potential prognostic factors 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 analysis did not reveal significant associations between metastatic lymph node damage and different prognostic factors as ER ($P$=0.08), HER2 ($P$=0.6), 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).

The BCL2 positive and BCL2 negative breast cancers showed no significant differences by histological type ($P$=0.1).

There was no statistically significant association between p53 expression and ability of breast cancer cells to express COX-2 ($P$=0.7) and cyclin D1 ($P$=0.4). COX-2 positivity did not show statistically significant association with breast cancer grade ($P$=0.1), HER2 receptors ($P$=0.3), proliferation activity ($P$=0.7), expression of cyclin D1 ($P$=0.7) and BCL2 ($P$=0.2) in breast cancer.

Expression of cyclin D1 was not statistically significantly associated with breast cancer grade ($P$=0.1), HER2 protein expression ($P$=0.09) and low or high proliferation activity ($P$=0.3). CK 5/6 positivity did not show statistically significant association with breast cancer grade ($P$=0.07) and 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).

# 3. 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, have been used both as predictive markers for hormonal therapy and as prognostic factors. HER2 status, as determined by IHC or FISH, indicates worse survival. Possible benefits may be derived by therapeutically targeting these molecules. Recently, gene expression microarray studies have demonstrated a strong prognostic power [Hamilton *et al.*, 2000], but immunohistochemistry remains a convenient and powerful means of prognostic evaluation in the clinical setting as it is less expensive and easier to perform [Lee, Im *et al.*, 2007].

The prognostic or predictive factors currently 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].

## 3.1. 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. Similar data were presented in Wiechmann *et al.* and Irigoyen *et al.* study where mastectomies were performed in 59% and 59.2%, but breast conserving surgery in 41% and 40.8% of cases [Wiechmann *et al.*, 2009; Irigoyen *et al.*, 2011]. In Irigoyen *et al.* 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].

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

### 3.2. 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.,* 78 sentinel node excisions (21.5%, 95% CI = 17.7-26.0) and 284 lymphadenectomies (78.5%, 95% CI = 74.0-82.3) were performed. The mean number of removed lymph nodes was 22.9 (range 7-54) [Lee, Im *et al.*, 2007]. Systematic axillary node dissection was performed for all 175 patients with breast cancer in Le *et al.* study with a mean number of 14 axillary nodes per patient (range 4-34) [Le *et al.*, 1999]. In our study, the rate of lymph node operations and the number of harvested lymph nodes corresponds to the published data.

### 3.3. The histological type of breast cancer

In the present study, 304 (79.4%) of 383 primary breast tumours were invasive ductal carcinomas, 51 (13.3%) invasive lobular carcinoma and 13 (3.4%) mucinous breast cancer. The predominance of ductal breast cancer is recognised by other researchers as well. The data by Lee *et al.* were consequent: ductal breast cancer 91.4%, other histological types of breast cancer tumours – 8.6% [Lee, Im *et al.*, 2007]. Bennis *et al.* diagnosed invasive

ductal carcinoma in 87.4% cases while invasive lobular carcinoma comprised 4%, metaplastic carcinoma – 3% and medullary carcinoma – 2% [Bennis *et al.*, 2012]. 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 agreement with other published studies but have high practical value.

The association between histological types and molecular subtypes of breast cancer were analysed by Yang *et al.* Luminal A subtype comprised mainly ductal breast cancer (56%) followed by lobular breast cancer (23%). In contrast, luminal B molecular subtype included more ductal cancer cases and less (10%) lobular breast cancer cases [Yang *et al.*, 2007].

### 3.4. The local tumour spread (pT)

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

Callagy *et al.* described the tumour size in mm, applying 3 categories - ≤20, >20 and >50 mm. Consequently, highest score was in T2 group – 48%,

followed by T1 (≤20 mm) comprising 38% [Callagy *et al.*, 2006]. The data obtained by Lee *et al.* were similar: 48.7% of tumours were <2 cm; 43.8% measured ≥2 but <5 cm and 7.5% are ≥5 cm [Lee *et al.*, 2010].

All T stages as defined by WHO TNM were represented in the study of Rouzier *et al.* T1 were the smallest group (9%) but T2 composed the largest part (56%) of all breast cancer cases in study. The T3 tumours comprised 18% and T4 – 17% of cases [Rouzier *et al.,* 2005].

Spitale *et al.* classified breast cancers by TNM and resulted in 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 predominance of relatively larger tumours measuring 2-5cm: T2, 66.0% and T1, 34.0%. Basal-like breast cancer (close to 7%) comprised slightly higher number of T1 (48.1%) than T2 cancers (42.0%); some cases (9.9%) were more than 5 cm large [Spitale *et al.*, 2009].

The results obtained in the present study are comparable with the published evidence. pT1 and pT2 are the predominant findings. However, 9.2% of breast cancers are diagnosed in stage pT3 and 7.3% – in stage pT4. The luminal A subtype comprised mainly pT1 tumours. On other extreme, pT4 also were observed in this group and were even more frequent than pT3; occasionally these pT4 cases were related to very long anamnesis (A. Abolins, unpublished case observations). Both luminal B subtype groups and HER2 positive molecular subtype showed largest amount of cases in pT2 followed by pT1. Triple negative molecular subtype showed similar amount of pT1 and pT2 cases.

### 3.5. 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 %). In the research article published by Lee *et al.*, the following lymph node status was described: N0, 51.3% of the enrolled patients; pN1, 22.5 %, pN2, 11.2% and pN3, 15% of cases [Lee *et al.*, 2010]. 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%) molecular subtypes. Positive lymph node status was more frequent among HER2 positive cases [Carey *et al.*, 2006].

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

### 3.6. Distant metastases (M)

At the time of operation, proved distant breast cancer metastases (M1) were present in 12 cases (3.1%), affecting bones (33.3% of M1), brain (25%), lungs (25%) and liver (16.7%). 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 occurred in 8.7% of cases, including local recurrence (45.5% of recurrent cases) as well as metastases in bone (39.4%), liver (22.2%), lung (15.1%), mediastinal lymph nodes (10.1%), brain (7.1%); other sites were affected in 11.1% [Onitilo *et al.* 2009]. 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 and to the fact that cancer recurrence after treatment and presence of distant metastases at the time of primary diagnostics can also involve different mechanisms.

### 3.7. Histological grade

By histological grade all cases were classified as follows: G1 – 61 (16.0%, 95% CI = 12.2-19.6), G2 – 138 (36.0%, 95% CI = 31.9-41.1) and G3 – 184 (48.0%, 95% CI = 43.8-52.8). Very similar data are reported: G1 in 11 (18%), G2 – 21 (35%), and G3 – 28 (47%) 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]. Onitilo *et al.* study group comprised G3 tumours (35.9%), G2 tumours (38.4%) as well as relatively small proportion of G1 tumours (21.2%). 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 G3 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, G3 breast cancers were frequently observed (77.7% and 76.3%, respectively), followed by G2 (20.0% and 12.5%, respectively) and G1 (1.2% and 4%, respectively) cancers [Onitilo *et al.*, 2009].

### 3.8. Expression of oestrogen and progesterone receptors

If all investigated breast cancers are classified within ER positive or negative group then up to 80% or more all breast cancers show ER positivity. The proportion of ER positive cases was lower in the study performed by Carey *et al.* where ER expression was found in 60% of all cases [Carey *et al.*, 2006]. The rate of ER positivity was 55% in the study performed by Lee *et al.* [Lee, Im *et al.*, 2007].

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 (by definition) as well as in 16% and 14% of cases in luminal A and luminal B molecular subtypes, respectively [Carey *et al.*, 2006].

### 3.9. Proliferation activity by Ki-67

Expressing of Ki-67 in high levels 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. The cut-off point <14% for Ki-67 labelling index was established by comparison with PAM50 intrinsic subtyping meaning that a higher score defines luminal B tumours with a worse prognosis [Cheang *et al.*, 2009; Goldhirsch *et al.*, 2011].

Different researchers have used different cut-off points of Ki-67 labelling index. Spitale *et al.* divided Ki-67 labelling index results in 3 groups - ≤5%, 5-20% and >20%. Consequently, most of breast cancers cases were in group possessing 5-20% of Ki-67 positive cells. Analysing Ki-67 labelling

index by molecular subtypes, luminal A and luminal B subtypes were more associated with index up to 20%. Luminal B molecular subtype showed high amount of cases in group showing Ki-67 in more 20% of neoplastic cells. Basal cell-like and HER2 molecular subtypes were associated with high Ki-67 labelling index [Spitale *et al.*, 2009].

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-off point was 14% in accordance with the recent St. Gallen recommendations [Goldhirsch *et al.*, 2011].

### 3.10. 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. HER2 status should be assessed in every diagnosed case of breast cancer [Romond *et al.*, 2005]. Currently, HER2 status is initially assessed by IHC in most cases and in tumours showing equivocal protein expression levels, *HER2/neu* gene copy number is measured via FISH or chromogenic *in situ* hybridization [Wolff *et al.*, 2007]. In addition, detection of HER2 status along with expression of ER and PR is useful for defining the molecular subtypes.

HER2 positivity in present study is shown in 14% of cases similarly to other studies [Spitale *et al.*, 2009]. There was no association between HER2 expression and the local tumour spread characterised by pT ($P$=0.8) or lymph node status. In contrast, Lee *et al.* found correlation between the overexpression of HER2 and larger tumour size ($P$=0.03) and axillary lymph node involvement characterised by $P$=0.02 [Lee, Im *et al.*, 2007].

### 3.11. Immunohistochemistry and breast cancer molecular subtype

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 include luminal A, luminal B (HER2 negative), luminal B (HER2 positive), HER2 positive (non-luminal) and triple negative subtypes [Goldhirsch *et al.*, 2011].

Recent publications have shown that the newer 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-like or *HER2/neu* groups [Sotiriou *et al.*, 2003]. Basal-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].

In the present work, the molecular subtypes of breast cancer were detected according to this new classification and IHC data. The majority of cases were luminal A (39.7%), followed by the luminal B (HER2 negative) subtype (32.6%). Triple negative breast cancer subtype was 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.

### 3.12.Age and molecular subtype

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-like or triple negative phenotype group was 58.5±14.6 years, in HER2 positive breast cancer group – 62.3±12.5 years. In luminal A and luminal B subtype, the mean age ± SD was 63.4±13.7 and 61.4± 15.0 years, respectively [Spitale *et al.*, 2009]. The mean patient age was 56 (range, 22-95) years in Wiechmann *et al.* study. By subdividing molecular subtypes, the mean age was following – luminal A, 58 years; luminal B, 52 years; HER2 positive, 53 years and basal-like subtype, 54 years [Wiechmann *et al.*, 2009]. Carey *et al.* described the mean age of 50 years with SD 12 years. By molecular subtype, the mean age in luminal A subtype 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 [Carey *et al.*, 2006].

In the present study, 383 consecutive female patients with primary, invasive breast carcinoma were included. The mean age ± standard deviation was 59.59±12.22 years (range, 27-88). In the published studies, the mean age range from 50 to 62.7 years. Our data are within this interval. In accordance with other studies, the highest mean age is observed in luminal A molecular subtype but triple negative breast cancer is diagnosed in younger patients.

### 3.13. Expression of aberrant p53 protein

Le *et al.* found nuclear staining of p53 protein in 23% of tumours among 175 breast cancer cases using 10% cut-off value of tumour cells displaying strong nuclear staining [Le *et al.*, 1999]. Expression of aberrant p53

protein was observed in 30.5% of all cases in Lee *et al.* study [Lee, Im *et al.*, 2007].

By immunohistochemistry, expression of p53 protein was observed in 24% of cases included in the present study. 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 perform the p53 investigations due to usually negative results. Relationship between expression and non-expression of p53 is lower in luminal B (HER 2 positive) group that marks aggressive nature of HER2 and higher possibility of p53 expression. More studies can be recommended regarding this group as well as triple negative molecular subtype despite the obtained data are statistically significant.

Statistically significant correlation ($P$=0.0001) between histological grade of breast cancer and p53 was evaluated by Le *et al.* study where G1 breast cancers did not show p53 nuclear overexpression (0%), but the amount of p53 overexpressing cases increased by higher cancer grade: G2, 13% and G3, 41% [Le *et al.*, 1999]. In the present study, expression of p53 protein is more frequent in high grade breast cancers ($P$<0.0001). Thus, our findings are in accordance with the world experience.

The rate of ER and PR expression was statistically significantly associated with p53 negativity. The ER positive cases in p53 positive and negative breast cancer represented 15.4% vs. 84.6%, respectively ($P$<0.0001). 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].

p53 and BCL2 are two opposite factors ($P$<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 characterised by $P$=0.003 [Le *et al.*, 1999]. The findings were confirmed by Lee *et al.*, 2007. In their study, expression of p53 ≤ 25% (of malignant cells) correlated with high BCL2 expression (68.6%). Breast cancer cases that expressed p53 in more than 25% of malignant cells lacked BCL2 expression in 56.5% of cases [Lee, Im *et al.*, 2007].

In the presented study, 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].

### 3.14. Expression of BCL2 protein

Molecular subtype of breast cancer and BCL2 expression (54.1% of cases) was evaluated by Zaha and Lazar. Luminal A subtype tumours expressed BCL2 at a rate of 92.3% and the luminal B – in 60% of cases, while the remaining molecular subtypes showed no expression [Zaha and Lazar, 2012]. BCL2 positivity by IHC was present in 60.9% of all cases in Lee *et al.* study [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 in Le *et al.* research [Le *et al.*, 1999].

In the present study, the rate of BCL2 expression in breast cancer was 67.5% of cases. Positive expression was observed in luminal A and both luminal B subtype cases, but HER2 positive and triple negative molecular subtypes were frequently negative.

Absence of BCL2 in breast cancer cells is more frequent in high grade breast cancers ($P<0.0001$). Zaha and Lazar found that BCL2 expression decreases with increasing tumour grade [Zaha and Lazar, 2012]. 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, Im *et al.*, 2007].

A high level of BCL2 expression is strongly associated with positive ER and PR and possible good response to hormonal therapy. Close association between BCL2 and ER and/ or PR expression has been described [Zaha and Lazar, 2012]. Among ER-positive cases, 92% [Lee, Im *et al.*, 2007] and 83% [Le *et al.*, 1999] of tumours showed BCL2 co-expression. The results regarding PR and BCL2 expression are similar ($P<0.0001$) and in accordance with the published evidence [Lee, Im *et al.*, 2007].

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]. 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 [Lee, Im *et al.*, 2007].

The BCL2 positive cases more frequently show low Ki-67 ($P<0.0001$). 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 [Lee, Im *et al.*, 2007; Zaha and Lazar, 2012].

In Le *et al.* research statistically significant association was identified between pT and BCL2 expression ($P$=0.03). The expression of BCL2 in pT1 cases was 83%, in pT2 – 58% and in pT3 – 61% of cases [Le *et al.*, 1999]. Zaha and Lazar have also shown association between BCL2 and pT characterised by $P$=0.04 [Zaha and Lazar, 2012].

In the group described by Zaha and Lazar, 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 and Lazar, 2012].

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].

### 3.15. Expression of cyclooxygenase-2 protein

In the present study positive COX-2 expression was observed in only 1.3% of investigated breast cancer cases. The previously described positivity of COX-2 in breast cancer varies from 4.5% to 85% [Brueggemeier *et al.*, 2005; Lee *et al.*, 2010]. The differences can be attributed to biological properties of the evaluated tumours, to technological diversity, including the immunohistochemical staining protocol, especially the clonality and affinity of the primary antibody, or to differences in scoring.

Positive 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 agreement with van Nes *et al.* as the presence of COX-2 is observed in ER and PR negative tumours [van Nes *et al.*, 2011]. The different cut-off values yield mathematically different results.

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).

Lee *et al.* compared the differences in clinico-pathologic factors between COX-2 overexpressing and COX-2 negatives cases. COX-2 overexpression was more common in larger tumours and higher nodal status ($P$<0.001 and $P$=0.048, respectively). However, in multivariate analysis, no correlation was found between clinico-pathologic parameters and COX-2 expression. There were no differences ($P$=0.424) in disease free survival according to COX-2 positivity [Lee *et al.*, 2010] similarly as in the present study.

As described by Lee *et al.* 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), hormone receptors (ER and PR status ($P$=0.286 and P=0.272, respectively)), HER2 expression ($P$=0.277), Ki-67 expression by cut-off value 20% ($P$=0.23), p53 positivity ($P$=0.126) and death rate ($P$=0.674) [Lee *et al.*, 2010].

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.

### 3.16. Overexpression of cyclin D1

Cyclin D1 overexpression is reported to be more prevalent than amplification, with the reported frequency ranging from 28 to 83% [Reis-Filho *et al.*, 2006]. In the present study, positive cyclin D1 expression was observed in 61.6% of breast cancer cases. Luminal A and luminal B (HER2 negative) molecular subtype groups expressed cyclin D1 more frequently than HER2 positive or triple negative breast cancer.

Research data from Lee *et al.* revealed statistically significant association between expression of cyclin D1 and pT parameter ($P$=0.04), but associations with lymph node metastasis, menstrual status or patient's age were not found showing $P$>0.05 of each [Lee, Park *et al.*, 2007].

There has been controversy in explaining the meaning of the cyclin D1 expression as a prognostic or predictive marker. Some studies have reported that cyclin D1 overexpression indicates a poor prognosis in breast cancer and some 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 cancers [Lee, Park *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 tamoxiphen in postmenopausal breast cancer patients [Stendahl *et al.*, 2004]. 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 did not show significant relationship between the expression of cyclin D1 and the survival outcome in patients with invasive breast cancer.

### 3.17. 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.

## 4. 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 overexpression 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 overexpression ($P<0.0001$) 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.

## 5. 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 considering the significant association 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 of 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.

## 6. REFERENCES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## 7. PUBLICATIONS

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