Original article

Differences in the Value of Proliferation Index (Ki67) and Immunophenotypes Between Invasive Breast Cancers With Respect to the Axillary Lymph Node Status

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Abstract

Introduction: Present study aimed to determine the frequency of invasive breast cancer (IBC) immunophenotypes in the General County Hospital Vinkovci, examine a difference between the ages of patients with respect to immunophenotypes and axillary lymph node (ALN) status, and determine differences in the frequency of positive ALNs with respect to immunophenotypes and the proliferation index (Ki67), regardless of the immunophenotype.

Materials and Methods: A monocentre cross-sectional study which included 289 patients diagnosed with invasive breast cancer was conducted in the period from 1 January 2011 to 31 December 2018. The expression of IBC biomarkers was determined by immunohistochemistry.

Results: The most common immunophenotype (41.54 %) was luminal B-like HER2-negative (LumB/HER2-). The mean age was 65.24 (± 12.04), with no age difference with respect to immunophenotypes ($F = 0.64$, $P = 0.43$) or ALN status ($t = 1.59$; $P = 0.11$). A total of 167 patients (58 %) had their ALNs removed, 66 % of which were positive. LumB/HER2- appeared to have significantly more positive ALNs compared to the luminal A-like immunophenotype ($P < 0.01$), while a difference in the size of primary tumours between metastatic breast cancers of these two immunophenotypes has not been detected ($P = 0.17$). ALNs were more likely to be positive in those tumours with Ki67 values higher than 20 % compared to the tumours in which Ki67 was lower than or equal to 20 % ($P < 0.01$).

Conclusions: LumB/HER2- is the most prevalent IBC immunophenotype in patients in our institution and has significantly more positive ALNs compared to the luminal A-like immunophenotype. Also, metastases to ALNs, regardless of the immunophenotype, are more common in patients with Ki67 higher than 20 % than in those with Ki67 lower than or equal to 20 %..

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Introduction

Breast cancer is the most common malignancy in women in the European Union (1). Breast cancers are divided into non-invasive (in situ), microinvasive and invasive carcinomas with recognisable subtypes. Non-invasive carcinomas, unlike invasive carcinomas, do not have the ability to invade blood and lymphatic vessels and their tumour cells are limited to ducts. Non-invasive carcinomas include ductal carcinoma in situ (DCIS), intraductal papillary carcinoma, and lobular carcinoma in situ (LCIS) (2). Histologically, invasive carcinomas can be divided into ductal (80%) and lobular (10%), while the rest are special forms of breast cancer (Paget's disease of the nipple, colloid mucinous carcinoma, tubular carcinoma, and solid papillary carcinoma with invasion) (3). According to literature, different types of breast cancers originate from the luminal and basal cells of the terminal duct lobular unit epithelium and they form a heterogeneous group of cancers that cannot be distinguished by histology (4, 8).

When it comes to breast cancer treatment, monitoring and survival rates, molecular subtypes determined by microarrays with more than 2.000 genes take precedence over the standard histological phenotype. This method is not easily attainable and it is quite costly, so it is generally accepted to use the immunophenotypic classification instead, as determined by the immunohistochemical expression of oestrogen receptors (ER), progesterone receptors (PR), proliferation index (Ki67) and amplification of the human epidermal growth factor receptor 2 (HER2) (2, 5). According to the new guidelines (Croatian Association for Cancer Research, 2018), ER and PR are considered weak prognostic and strong predictive factors because hormone receptor-positive cancers have a better survival rate, since they predictively indicate a potential response to hormonal therapy (5-6). ER indicates a response to cancer treatment via hormonal therapy such as tamoxifen, which blocks the growth of oestrogen-stimulated cancers, and a response to aromatase inhibitors that suppress oestrogen production (5). The next immunophenotypic marker, HER2, is associated with a lower survival rate and is also a predictive factor. One of the main reasons for determining the HER2 status is the identification of candidates for targeted anti-HER2 therapy (trastuzumab, lapatinib, pertuzumab, and trastuzumab emtansine) related to HER2-positive breast cancers. The most controversial marker used is Ki67, as it is determined in hot-spot areas, and the result often depends on the quality of tissue fixation, formulation, and the type of antibody used (5). As a rule, a more distinct expression is associated with a lower survival rate, both in general and after the administration of neoadjuvant therapy (2, 5, 7, 8). In addition to being connected with immunophenotypes, the survival rate and treatment also depend on the penetration of malignant cells into blood and lymphatic vessels, tumour necrosis, age of the patient (younger age is a negative prognostic factor), size of the tumour, status of surgical margins and lymph nodes (2, 5).

Studies from different countries show a different prevalence of respective immunophenotypes of breast cancer, which points not only to a number of factors that may play a role in carcinogenesis, but also to possible differences due to non-standardised protocols in pathohistological laboratories. In addition, the diagnosis of the correct immunophenotype is the cornerstone of proper treatment. Therefore, it is necessary to evaluate one's own work, among other things, comparing it with other laboratories in Croatia and other countries in order to detect possible problems in time and improve or at least maintain quality at an acceptable level. Therefore, the objective of this research was to determine the overall incidence of breast cancer and individual immunophenotypes in General County Hospital Vinkovci (CBC), to examine whether there is a difference between the age of women diagnosed with cancer related to immunophenotypes and lymph node status, and to determine the differences in the frequency of positive axillary lymph nodes with respect to immunophenotypes and especially with regard to high Ki67 values, regardless of the immunophenotype.

Materials and Methods

Study Structure and Materials

The paper is based on a monocentre cross-sectional study on historical data (9). The findings of patients diagnosed with breast cancer in the period from 1 January 2011 to 31 December 2018 were collected from the archives of the Department of Pathology and Cytology of the General County Hospital Vinkovci. From a total of 296 patients diagnosed with breast cancer, three were men, who were excluded from further processing. Also, because of the criteria of invasiveness, four female patients diagnosed with carcinoma in situ were excluded. Therefore, the final sample consisted of medical findings of 289 female patients with said diagnosis. The research was approved by the Ethics Committee of the General County Hospital Vinkovci.

Methods

Data of interest in the findings expressed ER, PR, HER2, and KI67 and were obtained using routinely prepared histological samples, formalin fixed paraffin embedded – FFPE, and finally immunohistochemically stained. The preparations were stained with Dako EnVision FLEX kit by treating the sample with Peroxidase Blocking Reagent (for 5 minutes), rinsed with EnVision FLEX wash buffer and treated with the primary antibody called FLEX Monoclonal Rabbit Anti-Human Estrogen receptor α, Clone EP1 (Dako), or other antibodies like Mouse Anti-Human Progesterone Receptor, Clone PgR 636 (Dako), FLEX Monoclonal Rabbit Anti-Human Ki67 Antigen, Clone MIB-1 (Dako) and Monoclonal Rabbit Anti-Human Her 2 Protein (Dako) (for 20 min), rinsed with EnVision FLEX wash buffer and stained with EnVision FLEX Hematoxylin. Finally, the preparations were covered with Sakura Tissue-Tek film in an automatic glass coating device Sakura Tissue-Tek Film (10). Analysis and interpretation of the immunohistochemical staining findings were carried out according to the WHO guidelines (8). The ER and PR findings are considered positive if 1 % or more tumour cells show immunohistochemical nuclear positivity, and negative in case of absence of nuclear positivity or strong nuclear positivity in less than 1 % of total tumour tissue. If tumour cells show absence of membrane positivity or very weak membrane positivity to the HER2 antibody, the HER2 status is indicated with 0 or 1+ and the finding is considered negative. In contrast, if more than 10 % of tumour cells have membrane positivity, the finding is considered positive and marked with 3+. When tumour cells show incomplete membrane positivity in 10 % of tumour tissue or if the positivity is at the borderline of 10 % of the total tumour tissue, the HER2 status is denoted by 2+. In this case, additional in situ hybridisation (FISH/CISH) is required to determine the existence of amplification of the HER2 gene and categorise the tumour as HER2-positive or HER2-negative (8).

According to immunohistochemically determined expression, we distinguish the following five immunophenotypes: luminal A-like, luminal B-like HER2-negative, luminal B-like HER2-positive, HER2-positive and triple-negative breast cancer (Table 1). Luminal A-like immunophenotype has the best survival rate, while a triple-negative immunophenotype has the worst survival rate (2). Luminal B-like HER2-negative breast cancer immunophenotype should exhibit higher Ki67 values than luminal A-like. Both immunophenotypes are HER2-negative and ER-positive, but the luminal B-like HER2-negative immunophenotype has a lower survival rate, so the separation of these two breast cancer immunophenotypes is essential for therapy prescription. According to the recommendations of the St. Gallen Oncology Conferences from 2013 and the 2017 Croatian Guidelines for Quality Assurance in Breast Cancer Screening and Diagnosis, the cut-off value for this factor is 20 % (11-12). The cut-off value is important in clinical practice because it indicates which patients should receive a more intensive therapy (13).

Table 1. Immunophenotypes of breast cancer

Immunophenotype	ER	PR	Her2	Ki67
Luminal A-like	+	+	-	< 20 %
Luminal B-like HER2-negative*	+	- or low*	-	> 20 %*
Luminal B-like HER2-positive	+	+/-	+	+/-
HER2-positive	-	-	+	+/-
Triple-negative	-	-	-	+/-

*At least one of the following criteria is required

After thorough processing, the pathohistological findings indicate tumour size, malignancy, Ki67, hormone receptor status, HER2 status, tumour-to-resection-margin ratio, number of examined and positive axillary lymph nodes, and tumour-to-blood and lymphatic vessel ratio. Finally, after processing these data according to the TNM staging system, the stage of breast cancer is determined indicating the probability of being cured (14).

Statistical methods

The variables were collected in MS Excel and processed in the program R (15). The sample was described using descriptive statistical methods. The Shapiro–Wilk W-test (or, in case of a large number of samples, the Kolmogorov–Smirnov test) was used to examine the distribution of numerical variables. A comparison of categorical variables was carried out using the Pearson's chi-squared test and the Fisher's exact test. ANOVA was used when comparing several groups of numerical variables, given the distribution of data was normal, and an adequate post-hoc test was used in cases of statistically significant results. The parametric Student's t-test was used to compare two groups of numerical variables with normal distribution. In this paper, the level of statistical significance for all tests used for comparisons was defined at $P < 0.05$.

Results

The research included 289 patients with breast cancer. The highest number of patients (45) was in 2018, and the lowest (26) in 2011 ($\chi 2 = 5.09$, $P = 0.65$). A difference was observed in the frequency of occurrence of certain immunophenotypes throughout the research period: the luminal B-like HER2-negative immunophenotype was more common in 2013 than in other years ($\chi 2 = 3.41$, $P = 0.03$), while the luminal B-like HER2-positive immunophenotype was prevalent in 2017 ($\chi 2 = 3.89$, $P < 0.01$) (Figure 1). According to the pathohistological diagnosis, the majority of patients (238) were diagnosed with invasive ductal carcinomas. Invasive lobular breast cancers were present in 30 patients and invasive mucinous carcinomas in 10. The rest of the patients had less common pathohistological types of breast cancer (Table 2).

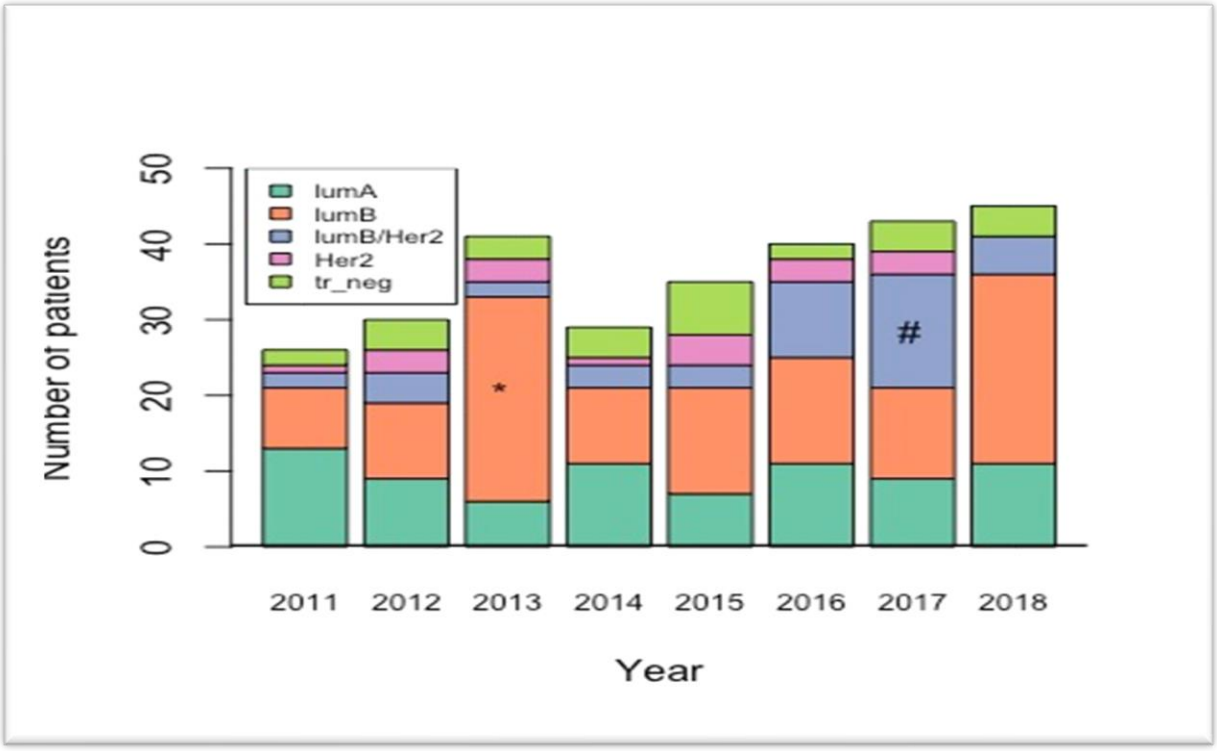

Figure 1. Distribution of patients with breast cancer with regard to immunophenotypes by years during the research period

Luminal B-like HER2-negative immunophenotype was more common in 2013 than in other years (* χ 2 = 3.41, P = 0.03), while the luminal B-like HER2-positive immunophenotype was most common in 2017 (# χ 2 = 3.89, P < 0.01). When it comes to other immunophenotypes, there was no difference in the frequency between the years within the research period (Figure 1). Index: lumA = luminal A-like; lumB = luminal B-like HER2-negative; lumB/Her2 = luminal B-like HER2-positive; Her2 = HER2-positive, tr_neg = triple-negative

Table 2. Pathohistological diagnosis of breast cancer (n (%))

Histological type of breast cancer	Number (%) of patients
Infiltrating duct carcinoma NOS	238 (82.3)
Lobular carcinoma NOS	30 (10.4)
Mucinous adenocarcinoma	10 (3.5)
Solid papillary carcinoma with invasion	5 (1.7)
Metaplastic carcinoma NOS	3 (1.0)
Tubular carcinoma	2 (0.7)
Apocrine adenocarcinoma	1 (0.4)
Total	289 (100)

The mean age of the patients was 65.24 (95 % CI 63.84 – 66.63). The sample follows the normal distribution (d = 0.05; P = 0.4). Regarding the immunophenotype of breast cancer, there was no difference in the age of the diseased patients (F = 0.64, P = 0.43) (Figure 2)..

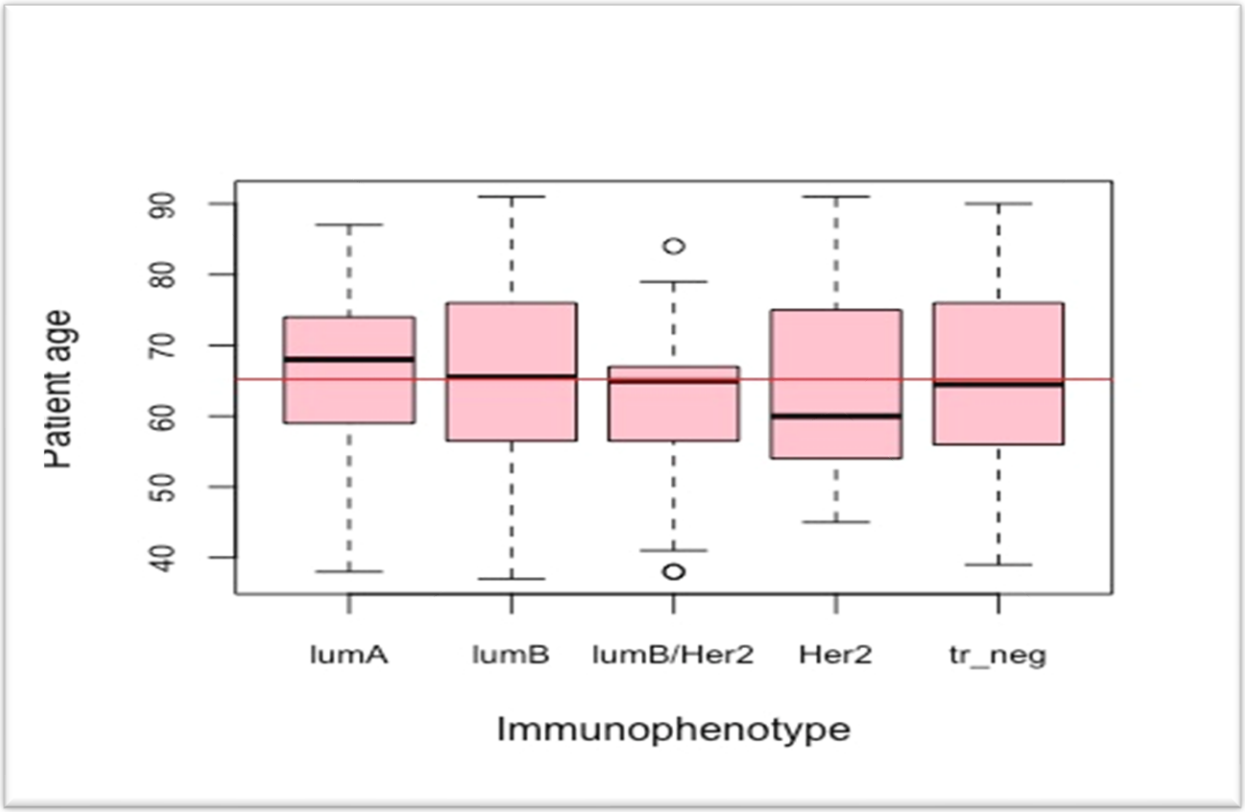

Figure 2. Age of patients with breast cancer with respect to immunophenotypes

The Shapiro-Wilk test helped determine that the age of all patients with respect to the immunophenotypes was distributed according to the normal distribution. There was no difference in the age of patients with regard to the breast cancer immunophenotype (F = 0.47, P = 0.49). The red line indicates the mean age of all patients, which is 65.24 (Figure 2). Index: lumA = luminal A-like; lumB = luminal B-like HER2-negative; lumB/Her2 = luminal B-like HER2-positive; Her2 = HER2-positive; tr_neg = triple-negative.

Axillary resections were performed in our institution on a total of 167 (58 %) patients. Of these 167 patients, 111 (66 %) had positive axillary lymph nodes (proven metastases of cancer tissue), and 56 (34 %) had negative results. The difference between respective immunophenotypes was determined with regard to the presence of positive axillary lymph nodes. The luminal B-like HER2-negative immunophenotype has positive axillary lymph nodes significantly more frequently than the luminal A-like (P < 0.01) (Figure 3). However, no difference in the primary tumour size (relative to pT) was observed between these immunophenotypes with positive axillary lymph nodes (P = 0.17) (Table 3). Furthermore, no age difference was observed between patients with positive and those with negative axillary lymph nodes (t = 1.59; P = 0.11). Patients with a Ki67 value higher than 20 % were more likely to have positive axillary lymph nodes than those with a Ki67 value lower than or equal to 20 %, regardless of the immunophenotype (χ 2 = 9.26, P < 0.01) (Figure 4)..

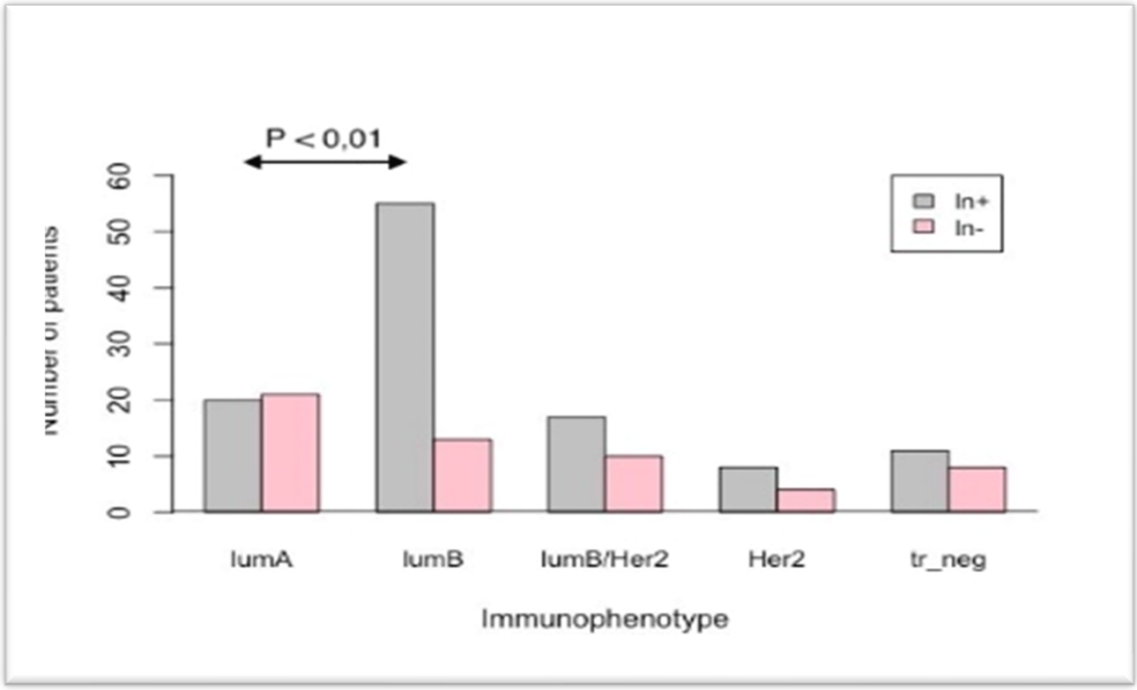

Figure 3. Incidence of positive axillary lymph nodes with respect to immunophenotypes of breast cancer

A difference was observed between the immunophenotypes of breast cancer in the frequency of detected positive axillary lymph nodes (χ 2 = 12.87, P < 0.05), and a post-hoc analysis found that the luminal B-like immunophenotype has positive axillary lymph nodes much more frequently compared to the luminal A-like immunophenotype (P < 0.01) (Figure 3). Index: lumA = luminal A-like; lumB = luminal B-like HER2-negative; lumB/Her2 = luminal B-like HER2-positive; Her2 = HER2 positive; tr_neg = triple-negative; ln+ = positive axillary lymph nodes; ln- = negative axillary lymph nodes.

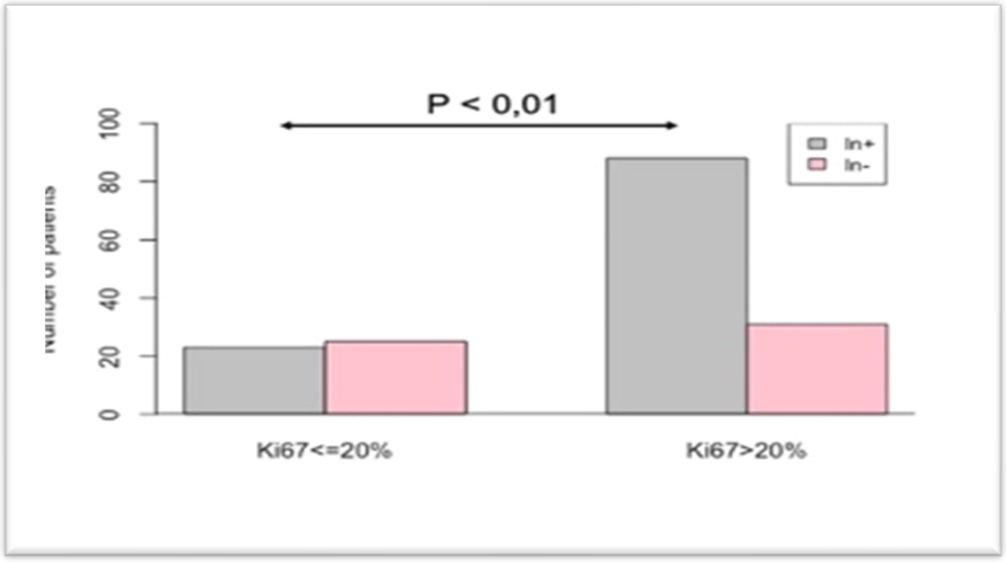

Figure 4. Number of patients with positive and negative axillary lymph nodes depending on the level of the proliferation index (Ki67)

Positive axillary lymph nodes are more common in breast cancer patients with Ki67 higher than 20 % (Ki67 > 20%) compared to those with Ki67 lower than or equal to 20 % (Ki67 ≤ 20 %), regardless of the immunophenotype in question (χ 2 = 9.26, P < 0.01) (Figure 4). Index: ln+ = positive axillary lymph nodes; ln- = negative axillary lymph nodes

Table 3. Primary tumour size in luminal A-like and luminal B-like HER2-negative carcinomas that have positive axillary lymph nodes.

	Primary tumour size				
Immunophenotype	pT1	pT2	pT3	pT4	Total
Lum A	10	7	1	2	20
Lum B/Her2-	17	26	10	2	55
Total	27	33	11	4	75

There was no difference in the size of the primary tumour between the luminal A-like and luminal B-like Her2-negative immunophenotypes of breast cancers with positive axillary lymph nodes (P = 0.17). Index: LumA = luminal A-like; lumB/Her2 = luminal B-like HER2-negative

Discussion

This research has shown that the luminal B-like HER2-negative immunophenotype is the most common immunophenotype of breast cancer in patients who have undergone surgery in our facility. In addition, positive axillary lymph nodes are more common in case of this immunophenotype than the luminal A-like immunophenotype, regardless of the size of the primary tumour. By way of explanation, due to the known correlation between tumour size and metastases of breast cancer to axillary lymph nodes (8), the observed difference in the incidence of metastatic breast cancers regarding these immunophenotypes was further analysed with respect to primary tumour size (pT). Although the difference in the size of the primary tumour subject to examination has not been confirmed, it should be emphasised that the possibility that this could be determined by research on a larger number of samples cannot be ruled out. Also, this paper indicates that metastases to axillary lymph nodes, regardless of the immunophenotype, are more common in patients with breast cancer with a Ki67 value higher than 20 % than in those with a Ki67 value lower than or equal to 20 %. The analysis of 289 patients with breast cancer found that the most common histological type was invasive duct carcinoma (82.3 %), followed by invasive lobular carcinoma (10 %) and invasive mucinous carcinoma (3.5 %). The same order of histological subtypes can be found in studies conducted in Germany (16), Saudi Arabia (17), Pakistan (18) and Nigeria (19), while the invasive ductal type was slightly less common (59 %) in one Italian study, and it was followed by invasive lobular type with 14 % (20). On the other hand, invasive ductal carcinoma is the most common in China (94 %), but it is followed by invasive mucinous carcinoma, with invasive lobular carcinoma in the last place (21).

The mean age of the patients included in this research was 65.24 years, which is similar to the results in clinical hospital centres, general hospitals, clinics and polyclinics all across the Republic of Croatia (22), as well as Serbia (65.59 ± 10.17) (23). A slightly lower mean age (55-57) was detected in studies carried out in Germany (16), Japan (24) and Brazil (25), while the lowest mean age (43-48) was in Sweden (26), Nigeria (19) and Pakistan (18) (Table 3). The significantly lower age of breast cancer patients in Africa is explained by Nigerian authors by the thesis that breast cancer occurs up to 15 years earlier in black people (19).

An almost equal representation of respective immunophenotypes as found in our sample was noted in another research conducted in Croatia (22), and in a country bordering Croatia – Serbia (27), but also in Sweden (26) (Table 4). The luminal A-like immunophenotype is most prevalent in certain European countries (Germany and Italy) (16, 20), Asia (China and Japan) (21, 24), as well as in Saudi Arabia and the United States (17, 28). It is interesting to single out countries such as Pakistan (18), Vietnam (26), and especially Nigeria (19), in which HER2-positive and triple-negative immunophenotypes that are more common in younger people, are more aggressive and have a lower survival rate,

prevail partially or predominantly (29). This is a possible explanation as to why these three countries also have slightly lower age statistics when it comes to patients with breast cancer (Table 4). In addition to the presence of said immunophenotypes, the age of patients is certainly affected by the implementation of prevention programs, which is evident in the example of Sweden, which, despite the distribution of immunophenotypes similar to that in our research, has a much lower median age. In Sweden, the age threshold of women included in the prevention program is 40, i.e. 10 years younger than in the Republic of Croatia, where the threshold is 50 (30, 12). The observed geographical grouping of certain immunophenotypes suggests that, in addition to genetic, a certain significant role in the carcinogenesis of breast cancer is also played by environmental factors, which should certainly be investigated in future research.

When it comes to the frequency of positive axillary lymph nodes, results from our sample made it clear that the luminal B-like immunophenotype metastasises significantly more frequently to axillary lymph nodes compared to the luminal A-like immunophenotype. A group of authors from Serbia obtained similar results (27). Contrary to our result, the authors of studies from Italy (20) and Brazil (25) did not come across significant differences. However, it should be pointed out that a general limitation of these comparisons with other studies lies in the fact that few of them have used a slightly different methodology in the determining of breast cancer immunophenotypes (indicated in Table 4). Minor differences in the methodology of the first group of studies is a consequence of a change in the cut-off value of the proliferation index from 14% to 20%, accepted after the conclusions of the St. Gallen Oncology Conferences in 2013 (11). In our opinion, although these important differences in determining the proliferation index have a huge impact on the type of treatment of each individual patient with breast cancer, they have a minor effect on the total immunophenotype ratio because a small minority of cases could be reclassified into a different immunophenotype if another classification system (the same as in our study) was used. On the other hand, major differences in the methodology presented in the second group of studies are the result of a lack of use of the proliferation index, which can be seen from the absence of the luminal B-like HER2-negative immunophenotype category (Table 4). Therefore, the previously discussed comparison of our results with the results from these studies could be biased.

Regardless of the immunophenotype, in our research, axillary lymph nodes were more frequently positive in those patients with a Ki67 value higher than 20 %. A group of authors from Serbia obtained a similar result, indicating the connection between positive axillary lymph nodes and elevated Ki67, but, in contrast to our study, it had to do with tumours in which Ki67 was higher than 14 % (27). In a research conducted in Turkey, it was found that patients with higher stages of positive lymph nodes (pN2 and pN3) have a higher average level of Ki67 expression than those with lower stages and negative lymph nodes (pN0 and pN1) (31). Contrary to these conclusions, a research in Ethiopia found that there was no difference in the level of the Ki67 proliferation index between breast cancers that have positive and negative axillary lymph nodes, regardless of whether all cancers were divided into three groups according to the Ki67 level (Ki67 < 15 %; 15 % < Ki67 < 30 %; Ki67 > 30 %) or the average level of the proliferation index (13). Since the level of the Ki67 proliferation index amounting to 20 % is the limit that groups certain breast cancers into luminal A-like and luminal B-like immunophenotypes, the result obtained indirectly confirms that the luminal B-like immunophenotype has a lower survival rate than the luminal A-like immunophenotype, which is consistent with the literature (11).

Table 4. Frequency (%) of immunophenotypes in different countries of the world

Country	Sample size	Age (in years)	lumA (%)	lumB (%)	lumB/Her2 (%)	Her2 (%)	tr_neg (%)	Reference
Germany	4102	57	44.7	31.8	6.2	5.0	12.3	(16)#
Japan	363	56.7	30.6	26.2	19.0	11.3	12.9	(24)#
Brazil	269	55.4	23.79	10	34.61	14.50	17.10	(25) #
Serbia	108	61	25.92	47.22	19.44	1.85	5.55	(27) #
Italy	1487	-*	34.09	25.21	11.49	10.15	19.03	(20) #
China	3198	51	65.3	-	19.0	6.5	9.2	(21) §
Saudi Arabia	359	49.8	58.5	-	14.5	12.3	14.8	(17) §
Nigeria	118	46.9	28.8	-	6.7	17.9	46.6	(19)§
Pakistan	285	43.3	21.05	-	48.77	18.4	11.22	(18) §
USA	50 571	-*	72.7	-	10.3	4.6	12.2	(28) §
Vietnam	237	47.7	10.6	33.5	23.0	19.3	13.6	(26)
Sweden	237	51.3	31.6	41.3	8.9	7.6	10.6	(26)
Croatia	1868	62.3	31.32	45.67	11.67	4.50	6.80	(22)
Croatia	289	65.24	26.64	41.54	15.22	6.22	10.38	This research

* The data on patients' age are not expressed as mean or median.

cut-off value of proliferation index is 14%

§ proliferation index was not used

lumA = luminal A-like; lumB = luminal B-like HER2-negative; lumB/Her2 = luminal B-like HER2-positive; Her2 = HER2-positive; tr_neg = triple negative

However, it is important to point out one of the limitations of these comparisons. In clinical pathology, the above-mentioned immunohistochemical marker Ki67 is observed by a microscope, using a semiquantitative method, the interpretation of which is subject to numerous factors, such as the experience of the pathologist, quality of equipment, and quality of sample processing. Therefore, that may be the cause of interlaboratory discrepancies in the interpretation of samples, and therefore lead to different results.

This paper has potentially useful clinical as well as public health implications. Primarily, this study describes the distribution and characteristics of breast cancer, which may be useful in planning, adjusting, and improving treatment options, but also in assessing the risk of disease recurrence and death depending on the immunophenotype of breast cancer. In addition, a slight but

continuous increase in the number of diagnosed cancers during the research period, as well as the data on the age of patients, can serve as criteria in evaluating the preventive program for early detection of breast cancer and in planning further steps in its improvement.

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Competing interests. None to declare.

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