International Journal of Science and Research (IJSR)
ISSN: 2319-7064
ResearchGate Impact Factor (2018): 0.28 | SJIF (2018): 7.426

Molecular Subtypes of Breast Carcinoma in South Indian Women and their Correlation with Histomorphological and Clinical Features

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Abstract: ***Breast carcinoma is the commonest malignant tumour and the leading cause of carcinoma death in women worldwide with an incidence rate greater than 30% of all cancers in urban Indian women. Though its detection is on the rise due to widespread screening programmes, there is no considerable fall in the mortality rate and survival. Breast cancers are heterogeneous in their morphology, clinical course and response to treatment. The conventional estrogen and progesterone receptors along with HER2/neu are notable for their differential expression among the subgroups. A crucial development in therapy has been the understanding that the presence of these markers correlates well with response to hormone therapy and chemotherapy. The determination of these receptors is regarded at present as the most powerful predictors in breast cancer management. The other important prognostic factors currently in use are the lymph node status, tumor size and grade. In spite of the numerous prognostic factors that have been identified, the clinical outcome continues to be hard to predict. Screening programmes and continuing advances in diagnostic and therapeutic techniques are allowing for the detection of smaller breast tumours magnifying the immediate need for newer prognostic markers. Normal breast ducts contain 3 types of epithelial cells. Luminal (glandular) cells, basal (myoepithelial) and stem cells. Basal cells typically express CK 5/6, CK 14 and CK 17 while luminal cells express CK 8 and 18.Cancers expressing basal cytokeratins 5 and 14 constitute a tumour subgroup that is typically hormone receptor negative, having a high grade and a high proliferative index. Basal like tumour markers are not routinely used in the standard histological diagnosis of breast cancers resulting in basal-like and non basal-like tumours being treated similarly. This could explain the poorer clinical outcome, higher recurrence rate, shorter disease free interval and different patterns of mortality over time. In patients without lymph node metastases, the prognostic significance of CK 5/6 is independent of tumour size, grade and hormone receptor status. CK 8/18 indicates the increasing degree of tumour.***

Keywords: Breast carcinoma ,Molecular subtypes, Immunohistochemical markers ,Luminal A, Luminal B , Triple negative, Basal like, Estrogen Receptor , Progesteron Receptor, HER2/neu overexpression , Ki-67 proliferative Index

Glossary of Abbreviations

IDC- Invasive ductal carcinoma
ILC- Invasive Lobular carcinoma
IMC- invasive medullary carcinoma
MC –mucinous carcinoma
IPC- Invasive papillary carcinoma
UIQ- upper inner quadrant
LIQ- lower inner quadrant
UOQ- upper outer quadrant
LUO – lower outer quadrant

1. Introduction

Breast carcinoma is one of the most common malignant tumours and the leading cause of carcinoma death in women worldwide with an incidence rate greater than 30% of all cancers in urban Indian women[1].Though its detection is on the rise due to widespread screening programmes, there is no considerable fall in the mortality rate and survival. [1]. They are heterogeneous in their morphology, clinical course and response to treatment. The conventional oestrogen and progesterone receptors along with HER2/neu are notable for their differential expression among the subgroups. A crucial development in therapy has been the understanding that the presence of these markers correlates well with response to hormone therapy and chemotherapy.[2]The classic characteristics of breast cancer is represented in the classification of breast tumours by the World Health Organization [3]. Even tumours belonging to the same histologic type can have different clinical course. Even the largest group ductal carinomas shows the highest heterogeneity. Additional information can be obtained from molecular subtyping of breast cancer. 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 [4]. At present, immunohistochemistry (IHC) is accepted as adequate surrogate marker [5] benefitting from higher economic effect and simpler technology despite less robust data in predictive sense [6].

The best-known molecular classification and nomenclature according to St Gallen 2013 consensusof breast cancer include luminal, human epidermal growth factor receptor (HER) 2 positive and triple negative(basal like) tumours [7]. 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 [8]. 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 [5, 8, 9, 10].HER2 positive breast cancer lacks expression of ER and PR, but is defined by HER2 9 protein over expression by IHC and/or *HER2/neu* gene amplification by *in situ* hybridisation [8]. 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. Basal cells typically express CK 5/6, CK 14 and CK 17, while luminal cells express CK 8 and 18 [11]. Cancers expressing basal cytokeratins constitute a tumour subgroup that is typically hormone receptor negative, having a high grade and a high proliferative index. Basal like tumour markers are not routinely used in the standard histological diagnosis of breast cancers resulting in basal-like and non basal-like tumours being treated similarly. This could explain the poorer clinical outcome, higher recurrence rate, shorter disease free interval and different patterns of mortality over time[`12]. In patients without lymph node metastases, the prognostic significance of CK 5/6 is independent of tumour size, grade and hormone receptor status. The determination of these receptors is regarded at present as the most powerful predictors in breast cancer management [2]. The other important prognostic factors currently in use are the lymph node status, tumor size and grade. In spite of the numerous prognostic factors that have been identified, the clinical outcome continues to be hard to predict. Screening programmes and continuing advances in diagnostic and therapeutic techniques are allowing for the detection of smaller breast tumours magnifying the immediate need for newer prognostic markers [13].

Aims and Objectives

1) To evaluate the expression of immunohistochemical markers on histologic sections and classify breast carcinomas into various phenotypes as basal, luminal-A, luminal-B or triple negative.
2) To correlate the immunohistochemical expression of biomarkers with clinical and pathological prognostic factors.
3) To compare the distribution of molecular subtypes of breast cancer in the study population with other groups.

Ethical concerns

The study was approved by the Committee of Ethics, Lourdes Hospital Kochi

2. Review of Literature

Breast cancer accounts for about one-fourth of all cancers in Indian women and about half of all cancer-related deaths. It is the most common malignant tumour and the leading cause of carcinoma death in women, with more than 10, 00, 000 cases occurring worldwide annually. [14]

The development of breast cancer involves a progression through series of intermediate processes, starting with ductal hyper proliferation, followed by subsequent evolution to carcinoma in situ, invasive carcinoma, and finally into metastatic disease [15]. Given the variability in clinical progression of disease, the identification of markers that could predict tumor behavior is particularly important in breast cancer [16]. Also, the determination of tumor markers is a useful tool for the clinical management of cancer patients, assisting in diagnostic procedures, staging, evaluation of therapeutic response, detection of recurrence and distant metastasis and prognosis, helping in the development of new treatment modalities [17].

It is known that breast cancer represents a complex and heterogeneous disease that comprises distinct pathologies, histological features, and clinical outcome. Also, it is well established that this neoplasia has well-defined molecular subgroups based on gene expression profiling closely related to the behavior of these molecular subtypes [18]. Sotiriou and Pusztai pointed out that result from studies of gene expression

Profiling have altered the view of breast cancer and provided a new tool for molecular diagnosis. The status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor type 2 (HER2) has been used as predictive markers for identifying a high-risk phenotype and for selection of the most efficient therapies [19].

The usual surgical procedure for carcinoma breast is radical mastectomy. The outcome after surgery varies widely. Prognostic information is important in counseling patients about the likely outcome of their disease and planning further management.[20] Apart from clinical parameters like age, menopausal status and disease presentation, important prognostic indicators in histopathology are tumour size and extent, histologic type, histologic grade and lymph node status.[21] Receptor status is the other most important prognostic and predictive marker for breast cancer. ER positivity is strongly associated with age at diagnosis, being more prevalent among post-menopausal women. [22] There are factors which not only are predictive of outcome, but also direct therapies against particular molecular targets. [22]

Some of these factors are:

1) Estrogen and progesterone receptors (ER, PR) -The presence of these nuclear hormone receptors is correlated with a better outcome and is an important predictor of response to hormonal (anti- oestrogen) therapy. About 80% of carcinomas that are ER and PR positive respond to hormonal manipulation, whereas only about 40% of those with either ER or PR alone respond. Conversely cancers that fail to express ER or PR have a less than 10% likelihood of responding to hormonal therapy but are more likely to respond to chemotherapy [2].
2) HER2/neu (c-erbB2)- HER2/neu over expression is associated with poorer survival, but its main importance is as a predictor of response to agents that target this transmembrane protein (eg. Trastuzumab or herceptin) [2].
3) Proliferative rate- In addition to mitotic counts as part of histologic grading, proliferation can be measured by immunohistochemical detection of cellular proteins produced during the cell cycle, eg.Ki-67. Carcinomas with high proliferation rates have a poorer prognosis but may respond better to chemotherapy. [2]

Thus current therapeutic approaches for breast carcinoma consist of combinations of surgery, postoperative radiation, hormonal treatment, chemotherapy and trastuzumab. The choice between hormonal therapy which has minimal side effects and chemotherapy with well-known morbidity and risks is a major responsibility of the clinician. Accurate and reliable assessment of the ER, PR and HER2/neu status of breast cancers by the pathologist is therefore crucial. Hence the present study is being undertaken to establish a correlation between ER and PR status, HER2/neu over expression, the proliferative activity, clinical features and tumour histopathology, and to effectively use these parameters to prognosticate and treat breast cancer patients.

Immunohistochemical markers provide early and accurate information on long-term outcome and help in prediction of the response to treatment in breast carcinoma. Generally ER concentrations are lower in tumours in premenopausal women than in postmenopausal women. It has been found that presence of ER is significantly associated with high grades, absence of tumour necrosis, presence of marked tumour elastosis and older age group[1].In one study, ER and PR positivity was observed in 75% and 55% of invasive carcinomas, respectively. All pure tubular, colloid and infiltrating lobular carcinomas were ER positive, while all medullary, apocrine and metaplastic, and most high nuclear grade carcinomas were ER negative. [23] HER2/neu overexpression is found in nearly all cases of high grade ductal carcinoma in situ (DCIS), in 20-30% of invasive ductal carcinoma and in a smaller percentage of invasive lobular carcinoma.[1] High Ki-67 levels usually correlate with a poor prognosis in breast cancer. Ki-67 levels are higher in women with ER and PR negativity, HER2/neu overexpression, larger tumour diameters, axillary node involvement, lymphovascular invasion and grade 2-3 tumours [24]

Molecular subtypes of breast cancer

Analysis of gene expression arrays has resulted in the recognition of several fundamentally different subtypes of breast cancer [4]. Because it is not always feasible to obtain gene expression array information, mainly due to financial reasons, a simplified classification, closely following that proposed by Cheang *et al.* [9] has been adopted as useful shorthand. Subtypes defined by clinico-pathological criteria are similar to but not identical to intrinsic subtypes and represent a convenient approximation. This approach uses immunohistochemical detection of ER and PR, the detection of overexpression of HER2 protein and/ or amplification of the corresponding gene – *HER2/neu* oncogene, and Ki-67 labelling index, a marker of cell proliferation, as the means of identifying tumour subtypes. Ki-67 labelling index presents more substantial challenges, but important guidelines for this test are under development. Initially, Ki-67 was not included between markers by which breast cancer molecular subtypes were determined [9, 10].

Luminal-like breast carcinoma

Luminal breast cancer is characterized by the expression of ER and/or PR in the background of high, low or any Ki-67 and positive or negative HER2. Additional markers like GATA3, BCL2 oncoprotein (BCL2) and cytokeratin (CK) 8/18 were previously searched for in the luminal type[25].At present, the definition of the luminal subtype is independent on other markers like the CK 5/6 and epidermal growth factor receptor (EGFR), but the expression of these markers may be found in some cases. According to positivity or negativity of HER2 and the degree of cellular proliferation, luminal breast cancers can be divided in two distinct groups: luminal A and luminal B [26].

Luminal A

The typical immunohistochemical profile of luminal type breast cancer is ER positive and/or PR positive, and HER2 negative. Based on the molecular profile, all cases with pure lobular carcinoma *in situ* represent luminal A tumours [27]. Consecutively, the large majority of invasive lobular carcinomas have a profile characteristic for luminal A. Depending on literature, luminal A subtype comprises 56-61% of cases and tend to have the most favourable long-term survival [28]. Many of the genes found in luminal A breast carcinoma are typically expressed in the luminal epithelium that lines the ducts [29].

Luminal B

Previously, luminal B molecular subtype included all breast cancer cases, which immunohistochemically co expressed hormone receptors (ER and/or PR) and HER2. This group comprises 9-16% of all cases and is associated with more aggressive nature than luminal A. Luminal B breast cancers include high grade tumours and are associated with lower long-term survival [28]. Initially, Ki-67 was not included in the criteria defining this subtype [29].

According to recent modifications in the surrogate classification of intrinsic breast cancer subtypes, luminal B group is divided in two parts: luminal B (HER2 negative) and luminal B (HER2 positive). Luminal B (HER2 negative) subtype includes all cases with ER and/or PR positivity, HER2 negativity and high Ki-67, but luminal B (HER2 positive) subtype includes breast cancer cases with positive ER and/or PR in connection with positive HER2 and any Ki-67 level [30].

HER2 type (non luminal)

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

The frequency of HER2 positive subtype is 8-16%. The HER2 positive subtype includes two distinct subtypes based on the expression of ER: ER-negative that cluster near the basal-like tumours (HER2 positive ER negative subtype), and ER (may also express PR) positive as in luminal B subtype [26]. In the majority of the cases, p53 is not expressed, and the expression of CK 8/18 is heterogeneous and moderate. If positive, reaction for EGFR is focal and restricted to less than 5% of tumour cell population. HER2 type is frequently associated with ductal carcinoma *insitu* (DCIS), many cases have high grade and are characterized by poor prognosis [26-28].

Normal breast-like type/unclassified breast cancer

The frequency of normal breast-like type/unclassified breast cancer is 6-10%. Basal cells in the normal breast duct

immunohistochemically stain with CK 5/6, but luminal cells express CK 8/18 [29]. Basal cells represent a mixture of different cell types with high proliferative potential, but luminal cells are more differentiated. Whether these cell types include a stem cell population capable of self-renewal is still unknown.

Normal breast-like cancer mainly is a triple negative tumour and is close to basal-like carcinoma in terms of the molecular profile. Regarding the immunohistochemical profile, outcome and survival, these tumours also are close to the basal-like breast cancer. Nuclear grade is higher than in luminal breast cancer types, as is the mitotic index. The unclassified type is negative for all five markers: ER, PR, HER2, CK 5 and EGFR. It has a slightly better prognosis than basal-like type, and does not respond to neoadjuvant therapy. It is important to point out that the term 'unclassified' within the frames of this classification is not synonymous with 'not otherwise specified' [28].

Basal-like breast carcinoma

Basal-like breast cancer (8 to 20% of breast cancer cases) lacks ER, PR and HER2 expression, but express CK 5/6 and/or EGFR [31] in gene microarray analyses or by immunohistochemistry. The term "basal-like cancer" describes a molecular phenotype initially defined using complementary deoxyribonucleic acid (DNA) microarrays, whereas "triple negative" is a term based on clinical assays for ER, PR, and HER2 [4, 6]. Although most triple negative breast tumors cluster within the basal-like subgroup, these terms are not synonymous there is up to 30% discordance between the two groups [5]. There are no specific hallmark features on routine histopathological slides that help to identify these tumours, although some common morphological traits are described. The basal-like cancer is frequently associated with solid architecture, pushing borders, prominent lymphocyte infiltration, scant stroma, high grade, high nuclear/ cytoplasmic ratio, high mitotic index and presence of necrosis, especially *comedo* type necrosis [32, 33]. It is more frequent in premenopausal patients [34]. Basal-like cancer shows a high rate of p53 mutations and is common among *BRCA1* mutation carriers [26]. A high proportion (90.8%) of basal-like tumours presents with metaplastic features [35]. The metaplastic breast cancer shows positive reaction for EGFR, CK 5/6, CK 14, CK 17, and p63 in the majority of cases. By immunohistochemical panel, 93.8% metaplastic breast cancer can be classified as basal-like tumours [36].

Majority of medullary cancer cases fall into this subtype as well [35]. Based on genetic and immunohistochemical analysis, medullary carcinoma seems to be a subtype of basal-like type, based on the triple negative character and CK 5/6 expression [36].

Many but not all basal-like tumours stain for both CK 5/6 and CK 8/18. Almost half of basal-like tumours consist of a mixture of CK 5/14 positive and negative tumour cells [26]. Specific markers of the myoepithelial cells (smooth muscle actin, p63, cluster of differentiation (CD) 10) are not frequent and not substantial to characterize this subtype of tumour [32].

The basal-like cancers less frequently disseminate in axillary lymph nodes, liver and bones, and develop metastatic deposits in the brain and lungs [37, 38]. Basal-like carcinoma is associated with higher rate of recurrence and of cancer-related death, independently of lymph node status and tumour size [39]. Adjuvant anthracyclin based chemotherapy is less effective in case of basal-like carcinoma

Triple negative breast cancer phenotype

Triple negative phenotype includes all breast cancers that lack ER, PR, HER2, CK 5/6 and EGFR expression by gene and immunohistochemical analyses. Triple negative breast cancer represents 10 to 17% of all breast cancers [40, 41].The prevalence of triple negative tumours is 15-23% in patients under the age of 40, 16-30% for patients aged 40-49, and 11-54% for patients over 50 years [42]. The evaluation of the molecular profile in large series has demonstrated that triple negative tumours fall into the basal-like and unclassified tumours. The diagnosis of these tumours has the advantage that these three stains (ER, PR and HER2) are already routinely used in immunohistochemistry to guide the therapeutic strategy. The aggressive character of this type of tumour is demonstrated by the recurrences that occur between 1 and 3 years, and the majority of deaths occur in the first 5 years, following therapy [28].The unfavourable prognosis is also supported by the fact that the majority of triple negative cases are predominantly of histological grade 3, up to 77%-96.8% of cases [28, 40-42]. Triple negative tumours form a heterogeneous group, and 56 to 84% of them express CK 5/6 and EGFR.

Approximately 80% overlap between triple negative and intrinsic basal-like subtype but triple negative breast cancer also includes some special histological types such as (typical) medullary and adenoid cystic carcinoma with low risk of distant recurrence [30].

Basal differentiation by cytokeratin 5/6

Cytokeratin 5/6 has been employed as a marker of basal differentiation resulting in association with triple negative molecular subtype that, in turn, has been related to younger age, high tumour grade, mitoses, high nuclear grade and p53 expression [43-45]. However, the relationships between different basal cytokeratins and the basal-like or triple negative differentiation are complex [45].

In a study done by Hyuna Sung & Jonine et al, it was found that tumour characteristics and known risk factors were generally similar in basal-positive and basal-negative luminal A tumours. The small differences in tumour features and family history between the two luminal A subtypes warrant further investigations in future studies with larger number of subjects and detailed annotation of subtype and risk factor information.[46]. Tumour characteristics and risk factors did not vary significantly by the expression of basal markers, although results suggested that basal-positive luminal tumours tended to be smaller and node negative, and were more common in women with a positive family history and lower body mass index [47].

3. Materials and Methods

Patients

Patients with primary, invasive breast carcinoma, diagnosed and routinely operated between May 2017 and April 2018 at Lourdes Hospital, Kochi were enrolled in the study. Patients without invasive component in tumour and those who have been treated with neoadjuvant chemotherapy before operation were excluded from study. All patients were women. It's a prospective, observational, hospital based study. All biopsy samples requested for immunohistochemical studies primarily for further treatment are included in the study.

Method of sampling

Complete enumeration method will be used for selecting samples for the study.

Inclusion criteria

Surgical specimens of patients of any age operated for breast carcinoma.

Exclusion criteria

- Cases where only trucut biopsy has been done, as all required parameters will not be available for assessment.
- Cases where there is extensive tumour necrosis with insufficient viable tumour cells for the accurate evaluation of the immunohistochemical markers.

Sample size

Sample size is determined by the formula 'n > z^2 P Q/ D^2'; where n is the sample size, z is the confidence coefficient, P the rate of incidence, Q = 1-P and D is the error of estimate. As per published records, P works out to be 0.02%.

By taking 95% confidence and an error of estimate of 0.15%, the minimum sample size worked out for the study to be statistically significant is 34

Immunohistochemistry

The formalin-fixed, paraffin-embedded tissues, cut at 3-4 micron thick sections on four electrostatic slides (Starfrost, Waldemar, Braunschweig, Germany) coated with adhesive (poly-L-lysine) were investigated by immunohistochemistry(IHC) to detect ER, PR, CK5/6, HER2/neu overexpression and Ki-67 proliferative index.

The technique for IHC will include antigen retrieval in tris buffer in a retriever using heat-induced epitope retrieval in TEG buffer at pH 9.0, blocking endogenous peroxidase with 3% hydrogen peroxide, incubating with primary mouse monoclonal antibody, developing chromogenicity with diaminobenzidine (DAB) and counterstaining with haematoxylin. The slides were rinsed with distilled water and covered by cover glass (Prestige) using automated cover slipper (Dako Coverslipper). All IHC reagents were produced by Dako, Glostrup, Denmark. HER2 protein overexpression was detected by HercepTestTM according to manufacturer's (Dako) instructions. Appropriate positive and negative controls were performedThe immunostained slides will be examined for nuclear staining in case of ER, PR and Ki-67, and membrane staining in case of HER2/neu and CK5/6. In each case, the proportion of positive staining tumor cells (expressed in percentage) and the average intensity of staining will be evaluated respectively.The evaluation of ER alpha and PR status was carried out according to the American Society of Clinical Oncology/ College of American Pathologists (ASCO/ CAP) guideline recommendations for IHC testing of ER and PR. The breast cancer case was considered positive if at least 1% of tumour cells showed positive nuclear staining of any intensity [76]. Classification, closely following that proposed by Cheang *et al*. [9] has been adopted

- .Luminal A (ER+, HER2-, Ki67<15%)
- Luminal B (ER+, HER2 +/-, Ki67>/= 15%)
- HER2 positive. (ER-, HER2+)
- Basal-like (ER-, HER2-, Basal markers +)[9]

For the assessment of cytokeratin 5/6, the immunohistochemically stained slides were examined for staining pattern (cytoplasmic or membrane) and proportion and intensity of staining of tumour cells. IHC staining intensity, score and staining proportion score were calculated as below [11]. Any cytoplasmic staining with the cytokeratin 5/6 in cancer cells was scored as positive [77].

Intensity score	Proportion score
0= No staining.	1=<10% Positive Staining
1= Weak staining.	2=10-25% Positive Staining
2= Moderate staining.	3=26-33% Positive Staining
3= Strong staining	4=34-66% Positive Staining
	5= 66-100% Positive Staining

Guidelines for interpretation of ER by Allred Method

Proportion Score	Observation	Intensity Score	Observation
	0%	0	None
1	1%	1	Weak
2	1-10%	2	Intermediate
3	11-33%	3	Strong
4	33-66%		
5	66-100%		
Total Score			Interpretation
0-2			Negative
3-8			Positive

Guidelines for reporting HER2

Result	Criteria
Negative (Score 0)	No staining observed or incomplete, faint/barely percerptible membrane staining in <10% of invasive tumour cells
Negative (Score 1)	Incomplete, faint/barely percerptible membrane staining in >10% of invasive tumour cells
Equivocal (Score 2)	Incomplete and /or weak to moderate circumferential membrane staining in >10% of invasive tumour cells or complete intense circumferential membrane staining in <10% of invasive tumour cell
Positive (Score 3)	Complete intense circumferential membrane staining in >10% of invasive tumour cell

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 [30].

Five breast cancer molecular subtypes were defined based on ER, PR, HER2 and Ki-67 levels determined by IHC.

Positive ER and/or PR, negative HER2, low Ki-67 (<14%) corresponded to the luminal A subtype. Luminal B subtype was divided in two groups – luminal B (HER2 negative) and luminal B (HER2 positive). Luminal B (HER2 negative) was recognised by positive ER and/ or PR, negative HER2 and high Ki-67 (≥14%), but luminal B (HER2 positive) was identified by positive ER and/ or PR, positive HER2 or amplified *HER2/neu* and any level of Ki-67. HER2 positive breast cancer subtype was recognised by positive (3+) HER2 or amplified *HER2/neu*, in the absence of ER and PR. Absent ER, PR and HER2 defined triple negative breast cancer subgroup [30].

The routine microscopy was viewed in Clinical Microscope LABOMED vision 2000 microsope, IHC assessment and cell counting was made by Axiolab microscope (Carl Zeiss AG, Oberkochen, Germany).

Data analysis

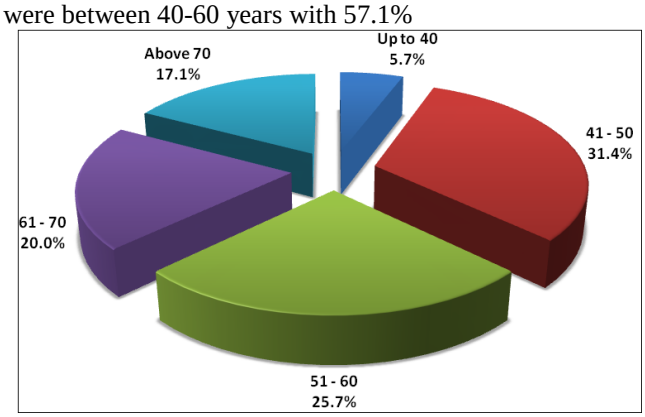
Computer software Statistical Package for Social Sciences (SPSS version 11.0, for Microsoft Windows) and Microsoft Word and Excel 2010 are used for the statistical analysis in this study. Data were analysed using mean ± standard deviation, descriptive statistic methods as descriptive and cross tabulation 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 significan

4. Observation and Results

Table 1: Distribution of Age (Years)

Age (Years)	Frequency	Percent
Up to 40	2	5.7%
41 - 50	11	31.4%
51 - 60	9	25.7%
61 - 70	7	20.0%
Above 70	6	17.1%

In the present study, regarding the distribution of age and correlating with molecular subtypes, predominant population were between 40-60 years with 57.1%

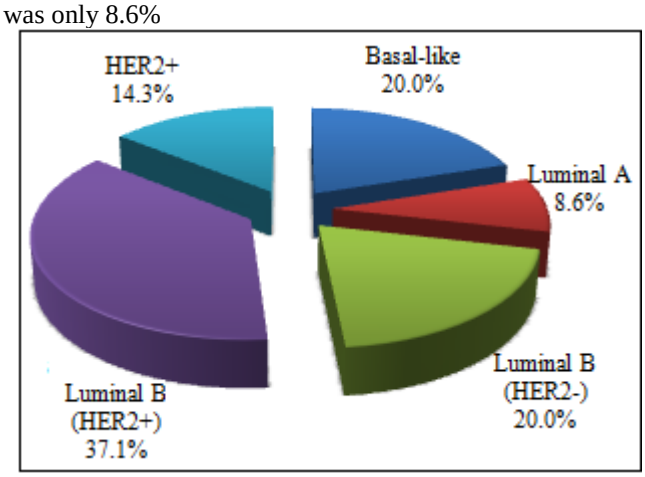


Graph 1: Pie chart showing distribution of Age (Years)

Table 2: Distribution of Molecular Type

Molecular Type	Frequency	Percent
Basal-like	7	20.0%
Luminal A	4	8.6%
Luminal B (HER2-)	7	20.0%
Luminal B (HER2+)	13	37.1%
HER2+	5	14.3%

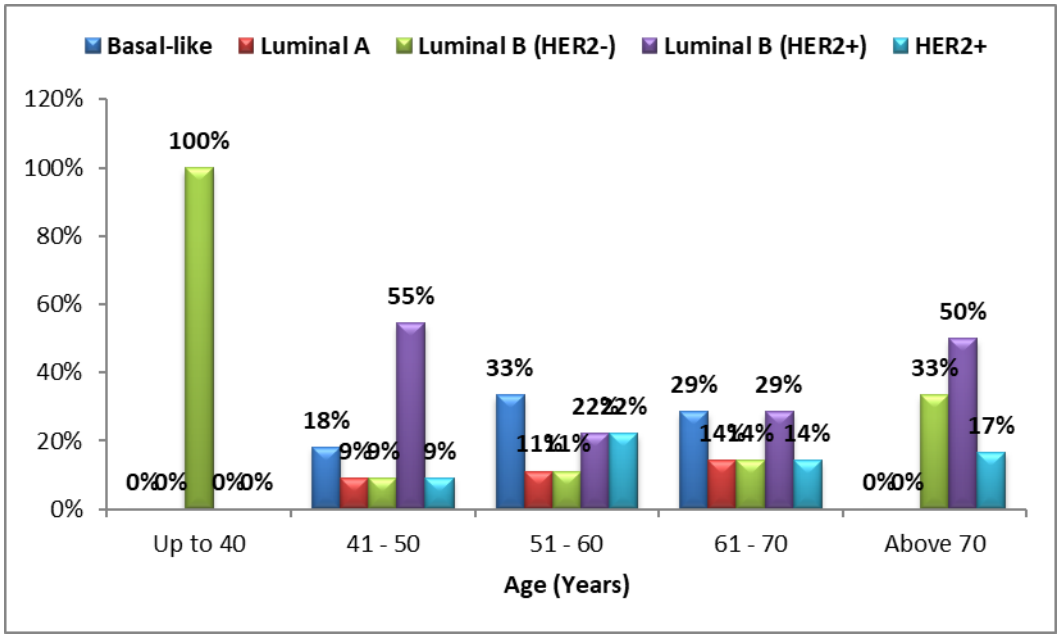
The predominant group is in luminal B molecular subtype(57.1%), followed by basal like(20%). Luminal A was only 8.6%



Graph 2: Pie chart showing Molecular Type

Table 3: Relationship between Molecular Type and Age

Age (Years)	Molecular Type					Total	p - value
	Basal-like	Luminal A	Luminal B (HER2-)	Luminal B (HER2+)	HER2+		
Up to 40	0 (0.0%)	0 (0.0%)	2 (100.0%)	0 (0.0%)	0 (0.0%)	2	0.719
41 - 50	2 (18.2%)	1 (9.1%)	1 (9.1%)	6 (54.5%)	1 (9.1%)	11	
51 - 60	3 (33.3%)	1 (11.1%)	1 (11.1%)	2 (22.2%)	2 (22.2%)	9	
61 - 70	2 (28.6%)	1 (14.3%)	1 (14.3%)	2 (28.6%)	1 (14.3%)	7	
Above 70	0 (0.0%)	0 (0.0%)	2 (33.3%)	3 (50.0%)	1 (16.7%)	6	
Total	7 (20.0%)	3 (8.6%)	7 (20.0%)	13 (37.1%)	5 (14.3%)	35	



Graph 3: Relationship between Molecular Type and Age

Table 4: Distribution of Histologic Type

Histologic Type	Frequency	Percent
IDC	28	80.0%
ILC	3	8.6%
IMC	2	5.7%
IPC	1	2.9%
MC	1	2.9%

28 (80 %) -Invasive ductal carcinomas, 3 (8.57 %) invasive lobular carcinomas, 2 (5.72%) medullary carcinoma and 1 (2.86 %) represented mucinous carcinoma

Graph 4: Pie chart showing Histologic Type

28 (80 %) -Invasive ductal carcinomas, 3 (8.57 %) invasive lobular carcinomas, 2 (5.72%) medullary carcinoma and 1 (2.86 %) represented mucinous carcinoma

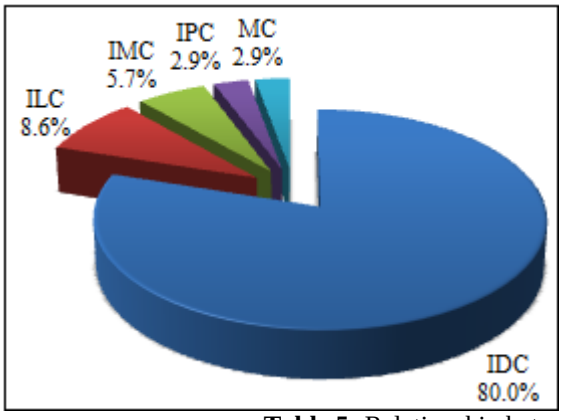
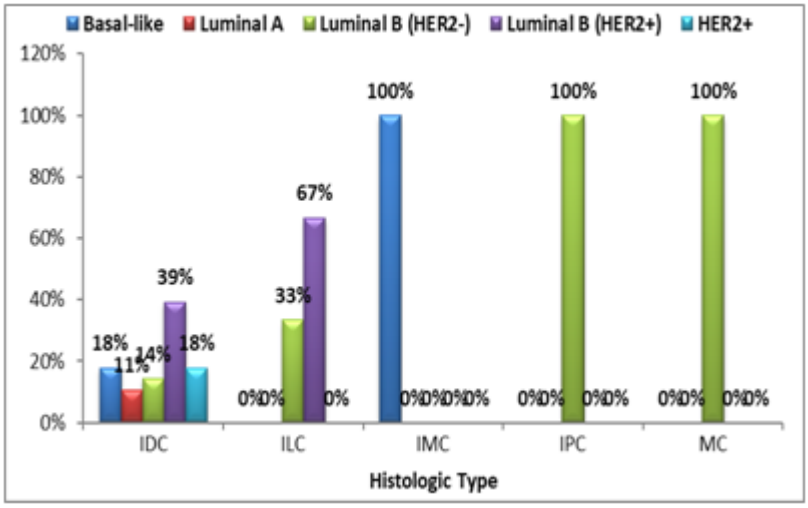


Table 5: Relationship between Molecular Type and Histologic Type

Histologic Type	Molecular Type					Total	p - value
	Basal-like	Luminal A	Luminal B (HER2-)	Luminal B (HER2+)	HER2+		
IDC	5 (17.9%)	3 (10.7%)	4 (14.3%)	11 (39.3%)	5 (17.9%)	28	
ILC	0 (0.0%)	0 (0.0%)	1 (33.3%)	2 (66.7%)	0 (0.0%)	3	
IMC	2 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2	
IPC	0 (0.0%)	0 (0.0%)	1 (100.0%)	0 (0.0%)	0 (0.0%)	1	
MC	0 (0.0%)	0 (0.0%)	1 (100.0%)	0 (0.0%)	0 (0.0%)	1	
Total	7 (20.0%)	3 (8.6%)	7 (20.0%)	13 (37.1%)	5 (14.3%)	35	0.32

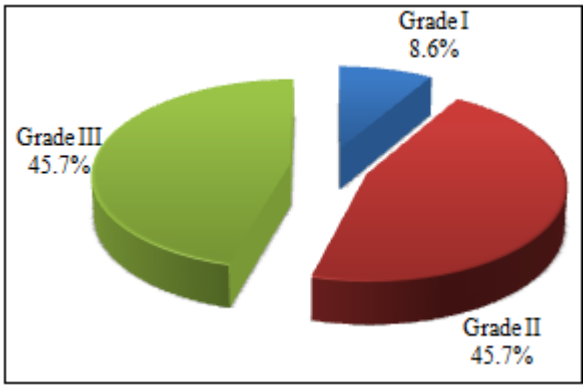
The Luminal A molecular subtype comprised ductal breast cancer (75%) and other tumours (25%). Luminal B molecular subtype included more ductal breast cancer cases (75%), but fewer lobular breast cancer cases (10%) and rest by papillary carcinoma (6.25%)



Graph 5: Relationship between Molecular Type and Histologic Type

Table 6: Distribution of Histologic Grade

Histologic Grade	Frequency	Percent
Grade I	3	8.60%
Grade II	16	45.70%
Grade III	16	45.70%



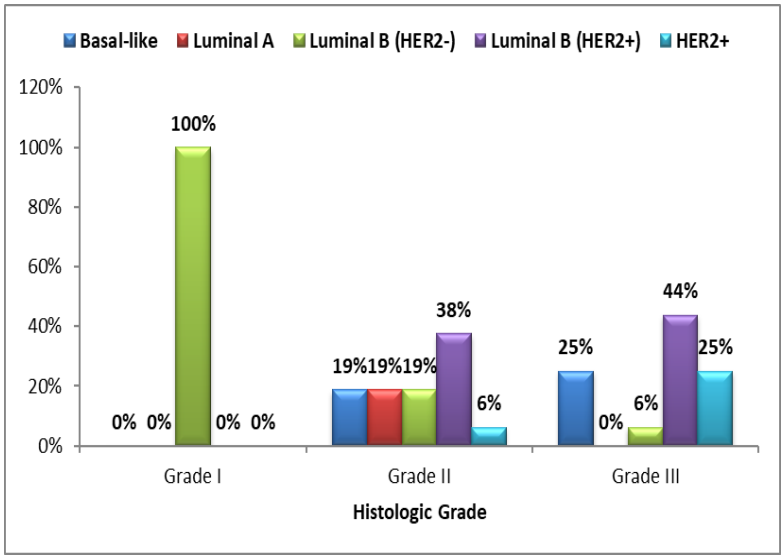
Graph 6: Pie chart showing Histologic Grade

All cases in the presented research work were classified as follows: G1 –3(8.57%) G2 – 16 (45.71%) and G3 -16 (45.71%).

Table 7: Relationship between Molecular Type and Histologic Grade

Histologic Grade	Molecular Type					Total	p - value
	Basal-like	Luminal A	Luminal B (HER2-)	Luminal B (HER2+)	HER2+		
Grade I	0 (0.0%)	0 (0.0%)	3 (100.0%)	0 (0.0%)	0 (0.0%)	3	0.043
Grade II	3 (18.8%)	3 (18.8%)	3 (18.8%)	6 (37.5%)	1 (6.3%)	16	
Grade III	4 (25.0%)	0 (0.0%)	1 (6.3%)	7 (43.8%)	4 (25.0%)	16	
Total	7 (20.0%)	3 (8.6%)	7 (20.0%)	13 (37.1%)	5 (14.3%)	35	

High grade tumours are more common than grade 1 tumours.



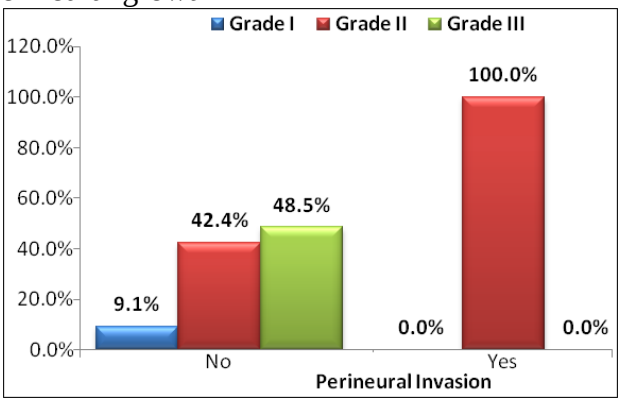
Graph 7: Relationship between Molecular Type and Histologic Grade

High grade tumours are more common than grade 1 tumours.

Table 8: Crosstab between Histologic Grade and Perineural Invasion

Perineural Invasion	Histologic Grade			Total
	Grade I	Grade II	Grade III	
No	3 (9.1%)	14 (42.4%)	16 (48.5%)	33
Yes	0 (0.0%)	2 (100.0%)	0 (0.0%)	2
Total	3 (8.6%)	16 (45.7%)	16 (45.7%)	35

No statistically significant association was observed between breast cancer grade and perineural growth



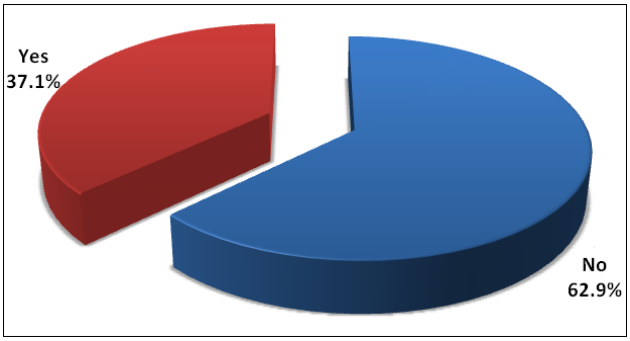
Graph 8: Relation between Histologic Grade and Perineural Invasion

No statistically significant association was observed between breast cancer grade and perineural growth

Table 9: Distribution of Lymphovascular Invasion

Lymphovas Invasion	Frequency	Percent
No	22	62.90%
Yes	13	37.10%

Out of 35 cases 13 cases (37.1%) showed lymphovascular invasion



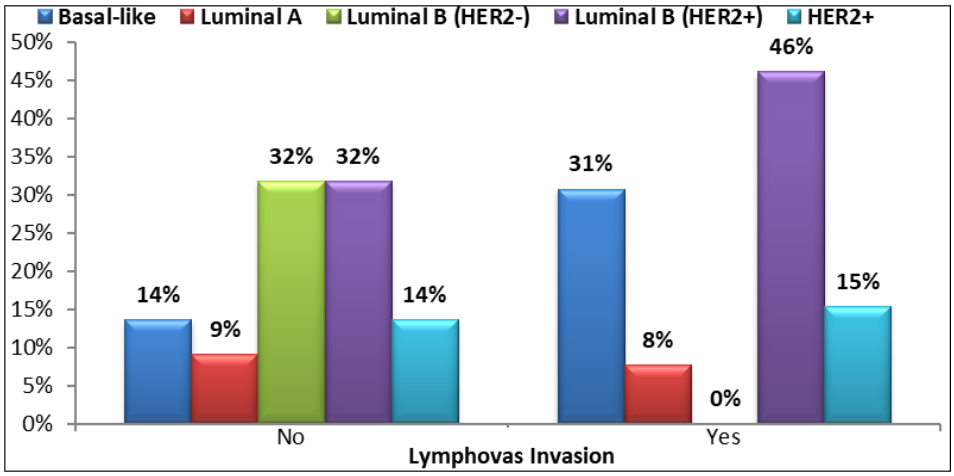
Graph 9: Pie chart showing Lymphovascular Invasion

Lymphovascular invasion is detected in 37.1% of the total cases studied and in 30.8% in triple negative cases

Table 10: Relationship between Molecular Type and Lymphovascular Invasion

Lymphovas Invasion	Molecular Type					Total	p - value
	Basal-like	Luminal A	Luminal B (HER2-)	Luminal B (HER2+)	HER2+		
No	3 (13.6%)	2 (9.1%)	7 (31.8%)	7 (31.8%)	3 (13.6%)	22	0.172
Yes	4 (30.8%)	1 (7.7%)	0 (0.0%)	6 (46.2%)	2 (15.4%)	13	
Total	7 (20.0%)	3 (8.6%)	7 (20.0%)	13 (37.1%)	5 (14.3%)	35	

Among the positive cases it is more in Luminal B & Her2 positive cases. The invasion identified with higher frequency in high grade breast cancer cases in comparison to intermediate or low grade breast cancers.

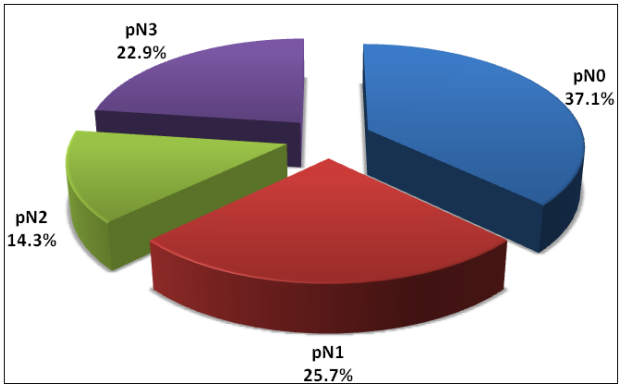


Graph 12: Relationship between Molecular Type and Lymphovascular Invasion

Table 11: Distribution of Lymph Node Status

Lymph Node Status	Frequency	Percent
pN0	13	37.10%
pN1	9	25.70%
pN2	5	14.30%
pN3	8	22.90%

pN0 was observed in 22 cases (62.2%), pN1 – 9 cases (25.6%) pN2 – 8 (22.8%) and pN3 – 5 cases (1.43 %)



Graph 9: Pie chart showing Lymph Node Status

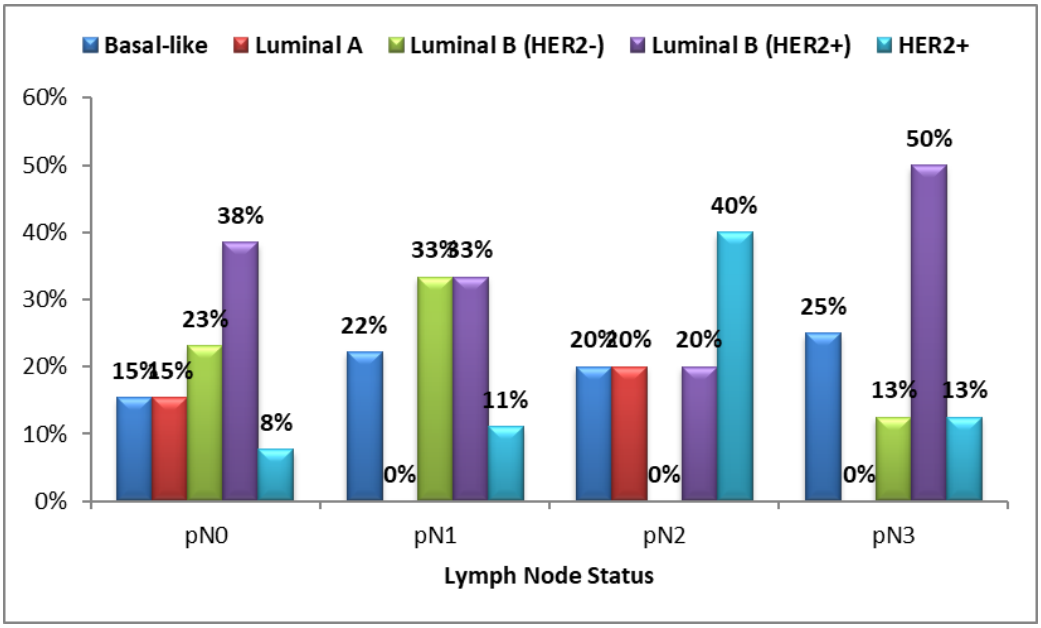
pN0 was observed in 22 cases (62.2%), pN1 – 9 cases (25.6%) pN2 – 8 (22.8%) and pN3 – 5 cases (1.43 %)

Table 12: Relationship between Molecular Type and Lymph Node Status

Lymph Node Status	Molecular Type					Total	p - value
	Basal-like	Luminal A	Luminal B (HER2-)	Luminal B (HER2+)	HER2+		
pN0	2 (15.4%)	2 (15.4%)	3 (23.1%)	5 (38.5%)	1 (7.7%)	13	
pN1	2 (22.2%)	0 (0.0%)	3 (33.3%)	3 (33.3%)	1 (11.1%)	9	
pN2	1 (20.0%)	1 (20.0%)	0 (0.0%)	1 (20.0%)	2 (40.0%)	5	
pN3	2 (25.0%)	0 (0.0%)	1 (12.5%)	4 (50.0%)	1 (12.5%)	8	
Total	7 (20.0%)	3 (8.6%)	7 (20.0%)	13 (37.1%)	5 (14.3%)	35	0.846

Negative lymph node status was predominant in luminal B (61.6%). Low in HER 2 positive cases (7.7%).

No statistically significant differences in lymph node status in other molecular subtypes were found



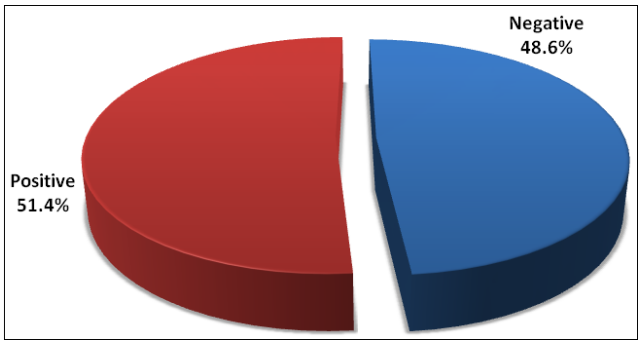
Graph 10: Relationship between Molecular Type and Lymph Node Status

Negative lymph node status was predominant in luminal B (61.6%). Low in HER 2 positive cases (7.7%). No statistically significant differences in lymph node status in other molecular subtypes were found

Table 13: Distribution of HER2 Status

HER2 Status	Frequency	Percent
Negative	17	48.60%
Positive	18	51.40%

HER2 positivity in the present study is shown in 51.4% of cases



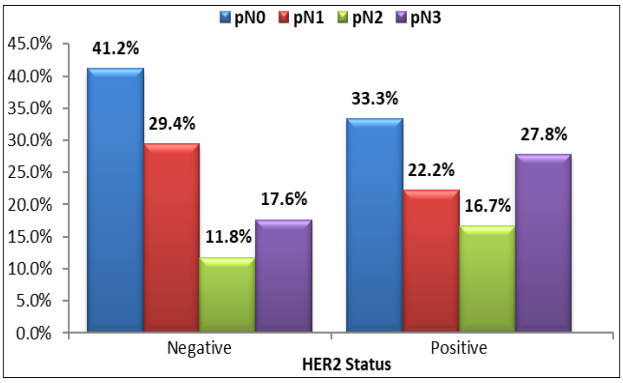
Graph 13: Pie chart showing HER2 Status

HER2 positivity in the present study is shown in 51.4% of cases

Table 14: Crosstab between Lymph Node Status and HER2 Status

HER2 Status	Lymph Node Status				Total
	pN0	pN1	pN2	pN3	
Negative	7 (41.2%)	5 (29.4%)	2 (11.8%)	3 (17.6%)	17
Positive	6 (33.3%)	4 (22.2%)	3 (16.7%)	5 (27.8%)	18
Total	13 (37.1%)	9 (25.7%)	5 (14.3%)	8 (22.9%)	35

No correlation was found between pN parameter and HER2 over expression



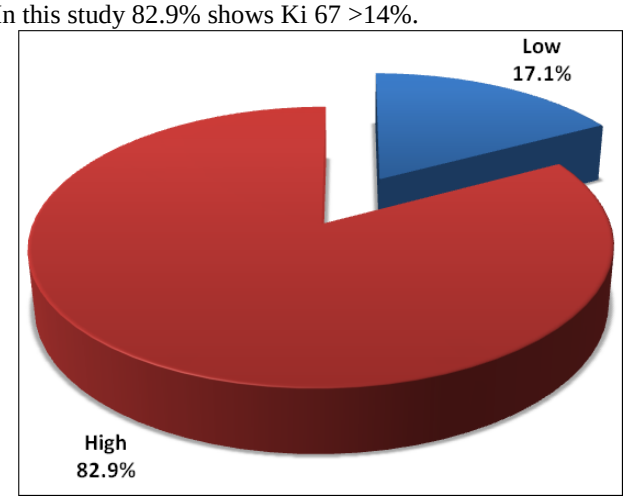
Graph 14: Relation between Lymph Node Status and HER2 Status

No correlation was found between pN parameter and HER2 overexpression

Table 15: Distribution of Ki-67

Low	6	17.10%
High	29	82.90%

In this study 82.9% shows Ki 67 >14%.



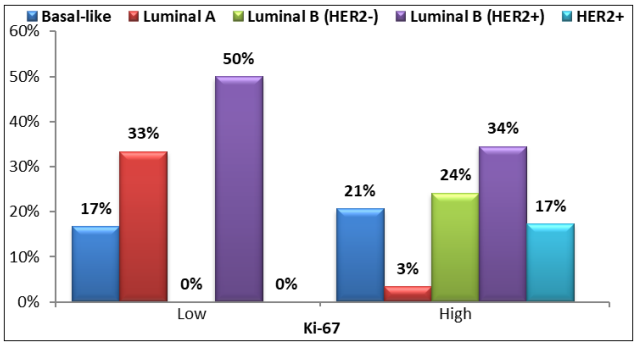
Graph 15: Pie chart showing Ki-67

In this study 82.9% shows Ki 67 >14%.

Table 16: Relationship between Molecular Type and Ki-67

Ki-67	Molecular Type					Total	p – value
	Basal -like	Luminal A	Luminal B (HER2-)	Luminal B (HER2+)	HER2+		
Low	1 (16.7%)	2 (33.3%)	0 (0.0%)	3 (50.0%)	0 (0.0%)	6	0.119
High	6 (20.7%)	1 (3.4%)	7 (24.1%)	10 (34.5%)	5 (17.2%)	29	
Total	7 (20.0%)	3 (8.6%)	7 (20.0%)	13 (37.1%)	5 (14.3%)	35	

High Ki 67 in Luminal B and Basal like Breast carcinoma



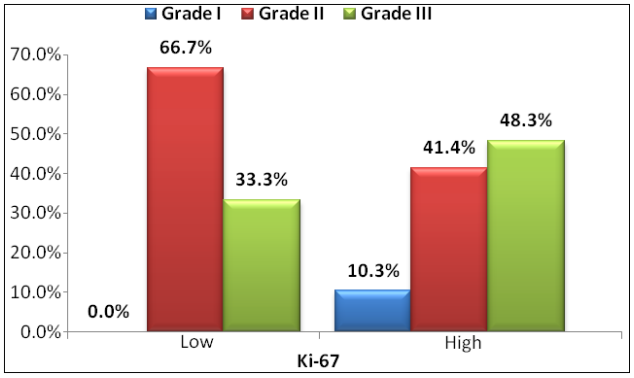
Graph 16: Relationship between Molecular Type and Ki-67

High Ki 67 in Luminal B and Basal like Breast carcinoma

Table 17: Crosstab between Histologic Grade and Ki-67

Ki-67	Histologic Grade			Total
	Grade I	Grade II	Grade III	
Low	0 (0.0%)	4 (66.7%)	2 (33.3%)	6
High	3 (10.3%)	12 (41.4%)	14 (48.3%)	29
Total	3 (8.6%)	16 (45.7%)	16 (45.7%)	35

High grade tumours show high Ki 67 values. Ki-67 labelling index



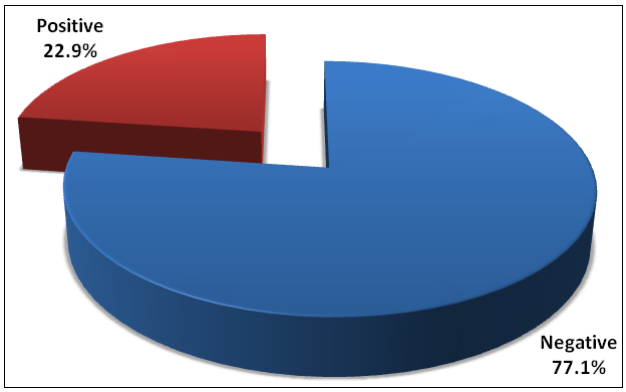
Graph 17: Relation between Histologic Grade and Ki-67

High grade tumours show high Ki 67 values. Ki-67 labelling index

Table 18: Distribution of CK 5/6

CK 5/6	Frequency	Percent
Negative	27	77.10%
Positive	8	22.90%

The expression of CK 5/6 was found in 22.0% invasive breast carcinoma cases.



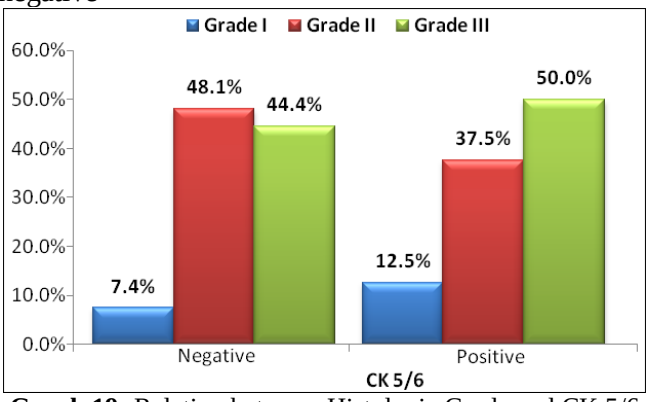
Graph 18: Pie chart showing CK 5/6

The expression of CK 5/6 was found in 22.0% invasive breast carcinoma cases.

Table 19: Crosstab between Histologic Grade and CK 5/6

CK 5/6	Histologic Grade			Total
	Grade I	Grade II	Grade III	
Negative	2 (7.4%)	13 (48.1%)	12 (44.4%)	27
Positive	1 (12.5%)	3 (37.5%)	4 (50.0%)	8
Total	3 (8.6%)	16 (45.7%)	16 (45.7%)	35

In our study most of the high grade tumors are CK 5/6 negative



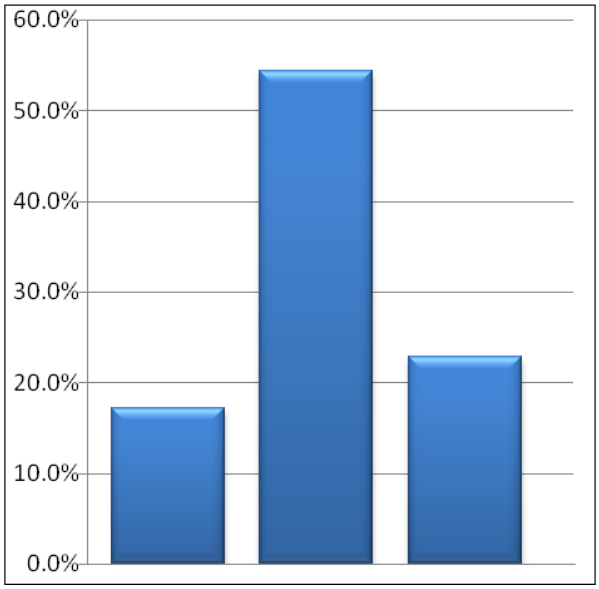
Graph 19: Relation between Histologic Grade and CK 5/6

In our study most of the high grade tumors are CK 5/6 negative

Table 20: Distribution of STAGE

Stage	Frequency	Percent
Stage 1	4	11.4%
Stage 2	16	45.7%
Stage 3	15	42.8%
Stage 4	0	0

Stage 1– 4 tumours (11.4%); Stage 2 – 16 tumours (45.7%); Stage 3– 15tumours (42.8%) and Stage 4 – 0 tumours (0%).



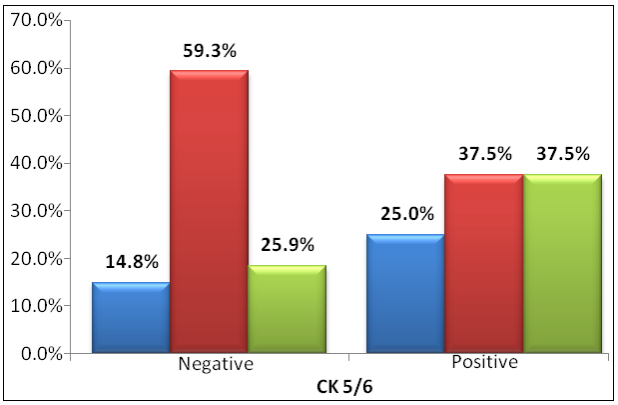
Graph 20: Graph showing STAGE

Stage 1– 4 tumours (11.4%); Stage 2 – 16 tumours (45.7%); Stage 3– 15tumours (42.8%) and Stage 4 – 0 tumours (0%).

Table 21: Crosstab between Stage and CK 5/6

CK 5/6	Stage				Total
	I	II	III	IV	
Negative	4 (14.8%)	16 (59.3%)	7 (18.5%)	0 (0.0%)	27
Positive	2 (25.0%)	3 (37.5%)	3 (37.5%)	0 (0.0%)	8
Total	6 (17.1%)	19 (54.3%)	8 (22.9%)	0 (0.0%)	35

CK 5/6 positivity is equally distributed in grade II and III tumors, 37.5%

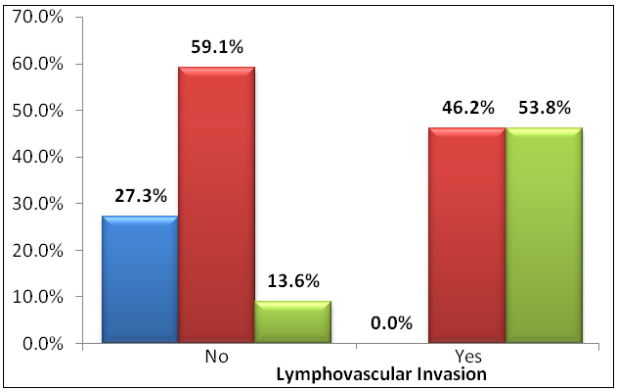


Graph 21: Relation between Stage and CK 5/6

CK 5/6 positivity is equally distributed in grade II and III tumors, 37.5%

Table 22: Stage III tumors show more lymphovascular invasion 53.8%

Crosstab between Stage and Lymphovascular Invasion					
Lymphovas Invasion	Stage				Total
	I	II	III	IV	
No	6 (27.3%)	13 (59.1%)	3 (13.6%)	0 (0.0%)	22
Yes	0 (0.0%)	6 (46.2%)	7(53.8%)	0 (0.0%)	13
Total	6 (17.1%)	19 (54.3%)	10(28.6%)	0 (0.0%)	35

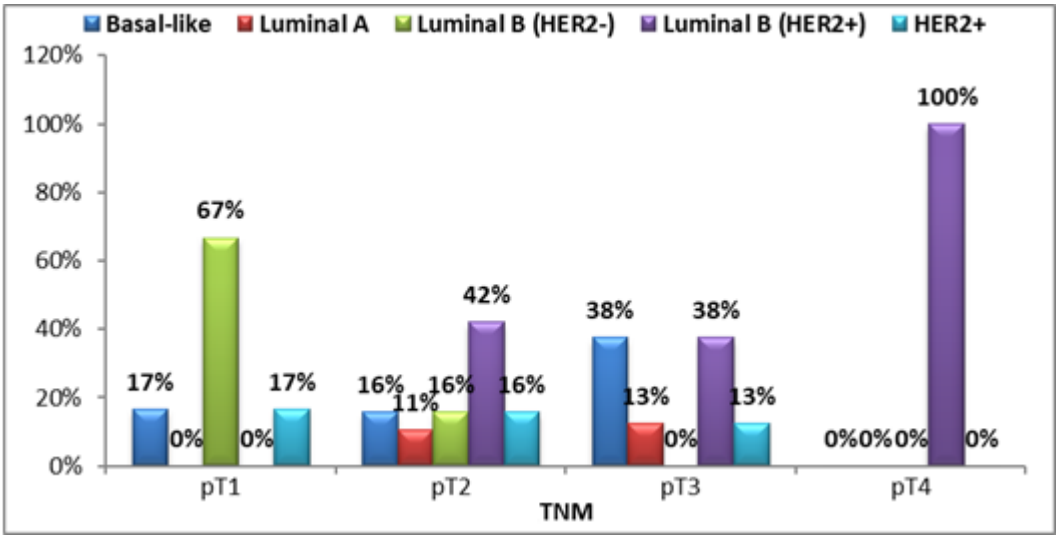


Graph 22: Relation between Stage and Lymphovascular Invasion

Stage III tumors show more lymphovascular invasion 53.8%

Table 23: Relationship between Molecular Type and Stage

TNM	Molecular Type					Total	p - value
	Basal-like	Luminal A	Luminal B (HER2-)	Luminal B (HER2+)	HER2+		
Stage I	1 (16.7%)	0 (0.0%)	4 (66.7%)	0 (0.0%)	1 (16.7%)	6	0.186
Stage II	3 (15.8%)	2 (10.5%)	3 (15.8%)	8 (42.1%)	3 (15.8%)	19	
Stage III	3 (37.5%)	1 (12.5%)	0 (0.0%)	5 (37.5%)	1 (12.5%)	10	
Stage IV	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0	
Total	7 (20.0%)	3 (8.6%)	7 (20.0%)	13 (37.1%)	5 (14.3%)	35	

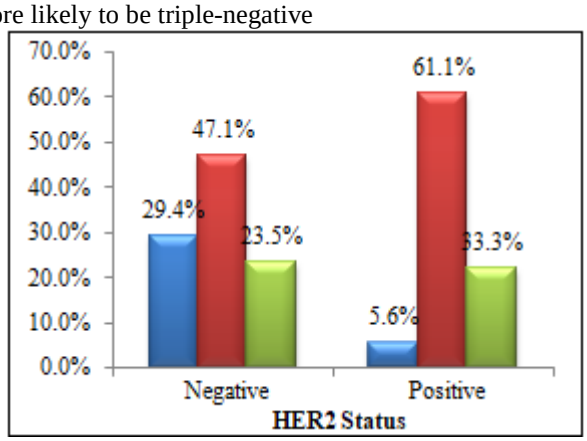


Graph 24: Relationship between Molecular Type and Stage

Table 24: Crosstab between Stage and HER2 Status

HER2 Status	Stage				Total
	I	II	III	IV	
Negative	5 (29.4%)	8 (47.1%)	4 (23.5%)	0 (0.0%)	17
Positive	1 (5.6%)	11 (61.1%)	6 (33.3%)	0 (0.0%)	18
Total	6 (17.1%)	19 (54.3%)	10 (28.6%)	0 (0.0%)	35

Patients with late-stage (stage III and stage IV) tumors were more likely to be triple-negative



Graph 24: Relation between Stage and HER2 Status

5. Discussion

As breast cancer represents a heterogenous group of tumours with variable biological and clinical characteristics, the identification of prognostic and predictive markers is clinically important. ER and PR, determined by IHC, are widely used both as predictive markers for hormonal therapy and as prognostic factors. HER2 status, as determined by IHC or FISH, indicates poorer survival. Possible benefits may be derived by therapeutically targeting these molecules. Recently, gene expression microarray studies have shown a strong prognostic power[78] but immunohistochemistry remains a convenient and powerful means of prognostic evaluation in a clinical setting as it is less expensive and easier to perform[79].

The prognostic or predictive factors that currently are in use do not provide sufficient information to allow accurate individual risk assessment and treatment planning, emphasizing the need for additional prognostic and therapeutic factors [79].

Approximately half of all new breast cancers are diagnosed in the developing world, where the analysis of prognostic factors needs to be inexpensive and easy to replicate. Even in the developed world, microarray analysis has yet to fully replace classical IHC. Thus, in the absence of routine gene-expression profiling, surrogate IHC markers for molecular breast cancer subtypes have emerged as a more practical means of characterising breast cancer types according to prognosis and/or differential response to specific agents [80].For example, a five-marker method, which examines ER, PR, HER2, CK 5/6, and Ki67 have been proposed as a surrogate system for identifying basal-like breast cancer [9]. Such an approach could have practical benefits. Moreover, it is becoming increasingly apparent that the success of new anticancer therapies is likely to be dependent upon the use of new biomarkers to detect patients who will benefit from a particular treatment [81].

In the present study, regarding the distribution of age and correlating with molecular subtypes, predominant population were between 40-60 years with 57.1%.In the published studies, age ranges are from 46 to 62 years, which correlates with our observation.(Table 1&3). In a study of breast cancer molecular subtypes and response to different preoperative chemotherapy, Rouzier *et al.* [96] included 82 females whose mean age was 52 years (range 29-79 years). In another study evaluating 151 breast cancer cases, the mean age was 46 years ranging 28-70 years. Among them, 73.5% of patients were below 60years [79]. with invasive ductal carcinoma being the most common tumour. The above observations are in agreement with other studies.

In the present study, 28 (80 %) of 35 primary breast tumours were invasive ductal carcinomas, 3 (8.57 %) invasive lobular carcinomas, 2 (5.72%) medullary carcinoma and 1 (2.86 %) represented mucinous carcinoma(Table 4). The predominance of invasive carcinoma, (ductal NOS) is recognised by other researchers as well, e.g., this histological type comprised 91.4% of breast cancers in the series of Lee, Im *et al*., 2007. Bennis *et al*. described the rate of invasive ductal carcinoma as 87.4% while invasive

lobular carcinomas comprised only 4% of breast cancers, followed by metaplastic carcinoma (3%), medullary carcinoma (2%) and few cancers of rare histology (3%), summarized as other types [82]. The data regarding the presence and type of breast cancer can be characterised as highly reliable. Within the present research work, Invasive ductal carcinoma is the most common histological type and this correlates with the previous studies.

In our study, the predominant group is in luminal B molecular subtype(57.1%), followed by basal like(20%). Luminal A was only 8.6% (Table 2). Study by Yang etal showed a distribution of Lum inal A tumours comprising 69% of the tumours followed by basal like 12%, Her 2 8% and luminal B 6%.[83]High prevalence of luminal B correlates with the data from Columbia and North Africa. There is a low prevalence of luminal A subtype in our study, which may be explained by a high Ki67 index noticed in our study group, which would have caused a classification of luminal A into luminal B subtype. A study done by Turkoz FP etal done on 1884 invasive breast cancer cases, found increased risk for post menopausal women with her 2 over expressing and luminal A breast cancers [84] In our study, Her 2 molecular subtype is not observed in less than 40 years.

In Spitale *et al*. study, evaluating 1214 breast cancer cases, the mean age of patients was 62.7±14.0 years. After classification of breast cancer by molecular subtypes, the mean age in the basal cell like group was 58.5 years and in Her 2 positive cases 62.3 years. [29]. Onitilo *et al*. included in study 1134 patients, whose mean age was 62.7 years. By molecular subtype, luminal B group contained patients with highest age 64.4 years. HER2 molecular group included patients with the age 59.9 years [38]. In our study, evaluating 35 cases, the mean age of the patients was 53.48 years. By molecular subtype, luminal B group contained patients with mean age 52.7 years. The mean age for basal like phenotype group is 54.4 yrs and that of Her 2 positive cases is 60.8 yrs correlating well with the published data. The age range of basal cell-like or triple negative phenotype group was 50-60 years and in HER2 positive breast cancer group is 40-60 years in our study.

The Luminal A molecular subtype comprised ductal breast cancer (75%) and other tumours (25%). Luminal B molecular subtype included more ductal breast cancer cases (75%), but fewer lobular breast cancer cases (10%) and rest by papillary carcinoma (6.25%) (Table 5).HER2 positive molecular subtype shows similar relationships between histological types as luminal B subtype, but with even more marked predominance of ductal carcinoma – ductal breast cancer constituted most of it. Basal-like breast cancer also was characterised by high percentage of ductal breast cancer (80%) The obtained data are in aggrement with other published studies. The association between the histological types and molecular subtypes of breast cancer has been analysed by Yang *et al*. He found that luminal A tumors included a higher percentage of lobular carcinomas, the lowest frequency of poorly differentiated carcinomas, and the highest frequency of small tumors in this relatively infrequently screened population[83] In a study done by Jenna Lynn Senger etal, Invasive lobular carcinoma is more likely to be estrogen and progesterone receptor positive compared to IDC and are usually her 2 negative [85] correlating with this data [86] But in our study 2 of three invasive lobular carcinoma cases(66.7%), were Her 2positive.(Table 5) An extensive sampling of the tumour may have revealed a mixed phenotype in these cases, further emphasizing the significance of extensive morphological evaluation. Invasive medullary carcinoma display a basal prolfile, but a favourable prognosis [87].Two of the invasive medullary phenotype identified in our study showed basal phenotype. One case of Mucinous carcinoma phenotype in our study showed ER and PR positivity with lack of Her 2 neu expression in agreement with other series published in literature.[88]. Recent publications have shown that molecular classification of breast cancer also has important prognostic value [19]. Luminal A tumours were shown to be associated with good prognosis and a less aggressive behaviour if compared with the basal-cell like or *HER2/neu* groups [19]. Basal-cell like subtype has been associated with aggressive behaviour, poor clinical outcomes and lack of response to the usual endocrine therapies, shorter survival and presence of *BRCA1* mutations [29]. Several studies have shown that breast carcinomas may be stratified in subtypes similar to those defined by expression profiling using a panel of IHC markers [29]. Subtyping breast cancer using microarrays for gene expression analysis is the best way to perform molecular classification, but it is not always feasible to obtain gene expression array information according to high costs or inaccessibility of fresh tissues, therefore simplified classification has been adopted as useful shorthand [9].

By histological grade all cases in the presented research work were classified as follows: G1 –3(8.57%) G2 – 16 (45.71%) and G3 -16 (45.71%). (Table 6 &7). High grade tumours are more common than grade 1 tumours.Very similar data are reported by Bertolo *et al*.: G1, 8%; G2, 45%; and G3, 47% of cases [89]. Lee *et al*. classified 19.2% of cases as G1, 35.9% as G2 and 44.9% as G3 [79]. Callagy *et al*. reported slightly different composition by grade showing statistically significantly lower rate of low grade cancers and higher – of high grade cancers: G1, 9%; G2, 32% and G3, 59%. In contrast, Nottingham series showed similar results: G1 – 19%, G2 – 33%, G3 – 48% of cases, respectively, without statistically significant differences from the presented study [77]. Histological grade in Yamashita *et al*. study was following: G1 in 17%, G2 in 59%, but G3 in 24% of cases. Le *et al*. describe breast cancer grade subsequently: G1 – 7.9%, G2 – 53.7% and G3 – 39%. In both these studies statistically significant excess of G2 cancers was found [79].

High grade cancers constituted the largest part of luminal B molecular subtypes (91.45%). Luminal B breast cancers are reported to have lower expression of hormone receptors, higher expression of proliferation markers and higher histological grade than luminal A[89]. Onitilo *et al*. analysed breast cancer histological grades by three-tiered system. Their study group comprised G3 tumours (35.9%), G2 tumours (38.4%) as well as relatively small proportion of G1 tumours (21.2%). Data were missing in few cases (4.6%). Luminal A molecular subtype group contained more G2 breast cancers (44.9%) followed by well differentiated (28.9%) and poorly differentiated (21.5%) breast cancers. In our study 3 cases of Luminal A subtype are of grade

II(100%). Luminal B subtype breast cancers were less differentiated containing more poorly differentiated tumours G3-(49.1%), the moderately differentiated G2 – 41.4%, followed with few cases of well differentiated breast cancers – 6%. In our study grade III luminal B subtype comprised 8 cases(40%), grade II of 9 cases(45%) and grade 1of 3 cases(15%). The study result shows a lower prevalence of grade I or low grade tumour among luminal B. ER positive cases are found to be more challenging to correlate with Ki 67[90] which may explain this disparity between grade and molecular phenotype.

In study done by Onitilo et al, HER2 positive and triple negative molecular subtypes, poorly differentiated breast cancers were frequently observed (77.7% and 76.3%, respectively), followed by moderately differentiated (20.0% and 12.5%, respectively) and well differentiated (1.2% and 4%, respectively) cancers [38]. In our study, HER2 positive and basal-like cancers were predominantly(80%, 57% respectively) Grade 3. None of the grade 1 tumours are Her 2 neu positive correlating with the published literature[90]. As an adverse prognostic factor, Her 2 neu positivity has been associated with poorly differentiated high grade tumours, high proliferation rate, metastasis to lymph node and resistance to certain types of chemotherapy (Immunohistochmeical detection of her 2 neu over expression in breast carcinoma in nigerians: a 5 year retrospective study [91].

IHC subtypes were significantly different by histological grade (*P*=0.0053) in Bennis *et al*. study. The unclassified, basal-like and HER2 positive subtypes showed higher percentage of cases with histological grade 3 (53%; 47.6% and 42.2% respectively), and a very low percentage of tumours with histological grade 1: 0%, 4.8% and 13.3%, respectively [82]. Similar tendency is shown in the present study data where triple negative molecular subtype group of breast cancer practically contain poorly differentiated breast cancers (92% of G3 vs. 8% of G1/G2 group). The same situation is in HER2 positive molecular subtype group and Luminal B (HER2 positive) group. If G1 and G2 group cases are counted together then amount is greater than single G3 group in luminal A breast cancer subtype like in Spitale *et al.* study [29]. The differences that were identified within the frames of the present research are statistically significant. No statistically significant association was observed between breast cancer grade and perineural growth (*P*=0.2).(Table 8)

Lymphovascular invasion is detected in 37.1% of the total cases studied and in 30.8% in triple negative cases and the detection rate is comparable with published literature (22-48%, 24-45% respectively).) [109]

Among the positive cases it is more in Luminal B(46.2%) & basal like(30.8%) cases. Luminal B cases showed 70% of cases with lymphovascular tumour enboli. The proportion among luminal B cases was 71.2% in the study done on 390 patients from North Africa by Hinde El Fatemi etal [112].The invasion was identified with higher frequency in high grade breast cancer cases(50%) in comparison to intermediate (41.3%) or low grade breast cancers(0%), data comparable with literature. (Table10). [109]

In a study done by Guo-Shiou Liao et al the highest incidence of LVI positivity (26.4% vs. 26.9%, respectively) and lymph node involvement (39.7% vs. 36.4%, respectively) occurred in the luminal B and luminal HER2 subtypes. Among Luminal B cases lymphovascular invasion and lymph node status(0%, 42.9%), but in luminal B her 2 subtype(46.2%, 61.5%), the LVI positivity rate and lymph node involvement is found to be higher in luminal B her 2 subtype compared to Luminal B her 2 neu cases. [113]

In another study done by Sonal Agarwal et al LVI was associated with younger age (P = 0.001), greater tumor size (P = 0.007), higher Nottingham grade (P = 0.001), Negative ER Status (P = 0.001), Negative PR Status (P = 0.002), Positive HER2/*neu*status (P = 0.021) [111]The variation in the proportion of LVI positive cases may be due to difficulty in assessment due to stromal artefact, necessitating routine use of immunohistochemistry which is not practical in our study circumstances increasing the economic burden.[109].

In the present study, pN0 was observed in 22 cases (62.2%), pN1 – 9 cases (25.6%) pN2 – 8 (22.8%) and pN3 – 5 cases (1.43 %).(Table 7&8).The general distribution of pN is within the published range although the available data show some diversity.
In the research article published by Lee *et al*., the following lymph node status was described: N0, 51.3% of the evaluated 80 patients; pN1, 22.5%, pN2, 11.2% and pN3, 15% of cases [79].

Yamashita *et al.* analysed 503 cases. In this group, metastases have not been found in 57% of cases, from 1 to 3 positive lymph nodes were identified in 24% cases, but more than 3 positive lymph nodes were found in 19% [87]. Spitale *et al*. divided breast cancer by metastases in lymph nodes as positive or negative cases. Positive lymph node status was in 39.6% of cases, but negative in 60.4% of cases [29]., In the University of British Colombia study of 800 cases, lymph nodes were free of metastases in 30%, N1 was observed in 41% and more than 3 nodes were positive in 29% cases. The Nottingham case series used for the validation study consisted of 1, 961 cases of primary operable breast carcinoma patients, nodal status of 1, 938 cases was negative in 64%, positive in 1 to 3 nodes – 28%, and positive in more than 3 nodes in 8% [77].

Similarly, Onitilo *et al*. classified the lymph node status into negative (61.2%) or positive (31%). In addition, lymph node investigation had not been done in 7.8% of cases [38].

Carey *et al*. reported absence of lymph node metastases in approximately 2/3 of investigated lymph nodes (61%) whereas 39% of cases presented with breast cancer metastases. Negative lymph node status was predominant in luminal A (66%), luminal B (53%), basal-like (61%) and unclassified (71%) breast cancer molecular subtypes. Positive lymph node status was more frequent among HER2 positive cases [34]. In our study, absence of lymph node metastases is seen in 37.1% cases. Negative lymph node status is prevalent in luminal A(66.7%), followed by luminal B(40%), basal like (28%) and Her 2 positive cases(20%) (Table 10). Lee *et al.* performed complete lymph node investigation and found that N0 group comprised largest part

of all investigated cases – 40.5%. There were 33.7% of cases corresponding to N1, 15.4% – N2 and 10.4% – N3 [79]. Study done by Chengshuai Si, et al Luminal B type (Luminal HER2-, Luminal HER2+) shows significant higher Lymph Node involvement. Their inference was LN involvement is an intrinsic characteristic for molecular subtype of breast cancer. Triple positive and triple negative breast cancer accounts the most and least possibility of LN involvement.[114] But in our study, Her 2 subtype is found to have a higher lymph node involvement(80%), followed by triple negative(71.4%). Triple positive cases has got 61.5% of lymph node metastases.(table 10). Study done by Nicholas K. Howland, in 375 patients on univariate analysis, age (<50), higher tumor grade, HER2+ status, tumor size, and molecular subtype were significant for lymph node positivity. Their conclusion was that Luminal A tumors have the lowest risk of LN metastasis, whereas luminal HER2 subtype has the highest risk of LN metastasis. Immunohistochemical-based molecular classification can be readily performed and knowledge of the factors that affect LN status may help with treatment decisions.[115]. Our data shows the lowest risk for luminal A in case of lymph node metastases as observed in the above study.

HER2 positivity in the present study is shown in 51.4% of cases (Table 12 &13) which is more than other studies [29]. As an adverse prognostic factor, Her 2 neu positivity has been associated with poorly differentiated high grade tumours, high proliferation rate, metastasis to lymph node and resistance to certain types of chemotherapy [99]. It must be emphasized that the cut-off levels for HER2 positivity have changed over years. This might have led to increased Her 2 positivity results in the current study. Lee *et al. has* found correlation between the overexpression of HER2 and larger tumour size (P=0.03) and more extensive axillary lymph node involvement as characterised by P=0.02 [79].

High tumour proliferation activity recognised by high levels of Ki-67 expression is associated with worse outcomes [56]. 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.In present study 82.9% shows Ki 67 >14%..(Table 15-18). Intermediate and high grade tumours show high Ki 67 values(75%, 87.5%). Ki-67 labelling index presents substantial challenges, as important guidelines for this test are still under development.In a retrospective study, 260 women by Seyed-Hamid Madani, there was significant correlation between Ki-67 with nuclear grade, human epidermal growth factor receptor 2 (HER2). Based on this result, more patients with Ki-67 ≥20% have higher nuclear grade, and HER2-positive. There was correlation between Ki-67 with type of tumor (P = 0.009. His conclusion was that the higher Ki-67 has a direct significant correlation with higher nuclear grade, p53-positive, and HER2-positive. Furthermore, triple negative patients have higher Ki-67 compared to other subtypes[116]. Spitale *et al*. found that Basal cell-like and HER2 molecular subtypes were associated with high Ki-67 labelling index. The current study is in concordance with the above observation with 100 % of her 2 and basal like subtype tumours having high Ki 67 proliferative index(>14%). Mean Ki67 percentage was highest in her 2 subtype(56.8%), followed by basal like(56.3%) and luminal B(30.8%). Ki 67 is expected to be very high in high grade, triple negative cancers(predomiunantly ranging from 30-80% and her 2 positive/ ER negative cancers(predominantly ranging from 20-60%) and results significantly lower than 20 % in these case types are not expected.[29] Other factors influencing Ki 67 results like longer tissue ischemic time and reparative changes after core biopsy should be kept in mild while assessing the rat of Ki67 index. [90].

The expression of CK 5/6 was found in 22.0% of consecutive invasive breast cancer cases. The frequency of CK 5/6 presence (20% in the current study) is within the published range [107, 108]. 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. In our study, CK 5/6 was found positive in triple negative basal like tumours only. Statistically significant associations between the presence of CK 5/6 and lack of oestrogen and progesterone receptors were identified. The CK 5/6 positive cases were significantly associated with higher proliferation. These findings are in agreement with the published evidence [107, 108].

According to Staging, all 35 tumours were classified subsequently: Stage 1– 4 tumours (11.4%); Stage 2 – 16 tumours (45.7%); Stage 3– 15tumours (42.8%) and Stage 4 – 0 tumours (0%). Stage III tumors are 37.5% each in basal like and Luminal B tumors and 12.5% each in Her2 and Luminal A tumors. A study done on a total of 5044 patients by Tingting Zuo et al. Patients with late-stage (stage III and stage IV) tumors were more likely to be triple-negative. Survival varied by stage and molecular subtype. The 5-year OS rates for patients with stage I, II, III, and IV diseases were 96.5%, 91.6%, 74.8%, and 40.7%, respectively. The 5-year OS rates for patients with luminal A, luminal B, HER2, and triple-negative subtypes of breast cancer were 92.6%, 88.4%, 83.6%, and 82.9%, respectively. Assessment of overall survival of different molecular subtype is beyond scope of this study. Multivariate analysis showed that stage at diagnosis and molecular subtype were important prognostic factors for breast cancer [117]

The study shows a predominance of T2 tumours(54.3%), followed by T3(22.9%), T1(17.1%) and T4(5.7%). In another large study, comprising 1134 breast cancer cases, 71.4% of tumours were T1. T2 cancers, measuring 2.1-5 cm formed 23.1% of the study group. The frequency of T3 was 4.7%, and of Tx – 0.8%, showed highest percentages of tumours measuring ≤ 2 cm (78.9%, 62.1%, 47.1% and 54.0%, respectively), followed by T2 [38]. Our results not matching for the above results. Spitale *et al*. classified breast cancers by TNM and resulted in the following distribution: T1, 62.1%; T2, 35.2% and T3, 2.7% of all cases. By molecular subtypes, luminal A group consisted of T1, 65.9%; T2, 31.4% and T3, 2.7%. Luminal B group comprised T1, 58.3% and T2, 41.7% cancers. HER2 positive molecular subtype group showed opposite data with dominance of relatively larger tumours measuring 2-5 cm: T2, 66.0% and T1, 34.0%. Basal-like breast cancer (7% from all cases) comprised slightly higher number of T1 (48.1%) than T2 cancers (42.0%), and some cases (9.9%)

were more than 5 cm large [29]. Our study also showed a dominance of larger tumours in her 2 subtype with T2(2-5cms size) amounting 60%, T3 for 20% and T1 for 20%. In basal like subtype, we had 14.3% of T1, 42.9 % each of T2 and T3, showing a higher prevalence of larger tumours. Luminal A showed 75% of T2 and 25% of T3. Luminal B showed 50% of T2 tumours, 20% each of T4 and T3 tumours and 10% of T1. The discordance with Tumour size prevalence and its correlation with molecular subtype may be due to increased prevalence of luminal B and her 2 subtype in the population, variation in the socioeconomic status, public awareness and quality of health care systems prevailing in different populations. Irigoyen *et al*. describe more frequent occurrence of pT1 in luminal A and luminal B molecular subtype than in basal, HER2 positive or normal molecular subtypes that showed predominance of pT2. In this study, pT3 and pT4 composed only small fraction [88]. But our results not matching with the data.

6. Conclusions

1) Though the common histologic type is invasive ductal carcinoma as seen in published data, our study population showed a predominance of luminal B subtype associated with higher recurrence rate, poor prognosis and unfavourable clinicopathological characteristics, may benefit from more aggressive treatment.
2) Her 2 neu overexpression associated with poorly differentiated high grade tumours, high proliferation rate, metastasis to lymph node and resistance to certain types of chemotherapy, noted in our study group, the result may be confirmed by larger studies to be conducted in larger institutions with a larger sample size.
3) Our study showed a predominance of larger tumour size and higher tumour grades, may be due to predominance of luminal B subtype, which is alarming and highlights the importance of early screening and the urgent need to improve women's awareness of breast cancer in our region.
4) Variation in the distribution of molecular phenotypes seen in our study, emphasises the need for a similar study to be done on a larger sample size in Indian population.

7. Illustrated Images

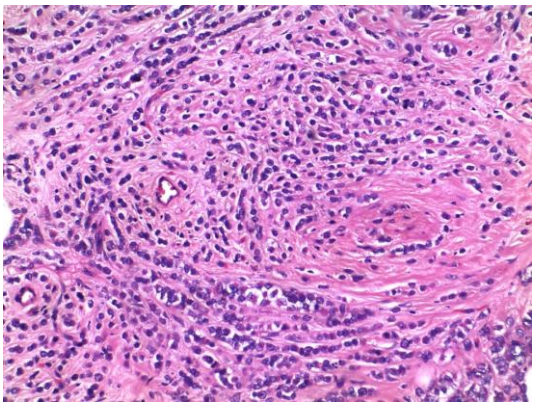


Figure 1: Iinvasive Lobular breast carcinoma. Haematoxylin eosin, 100 x.

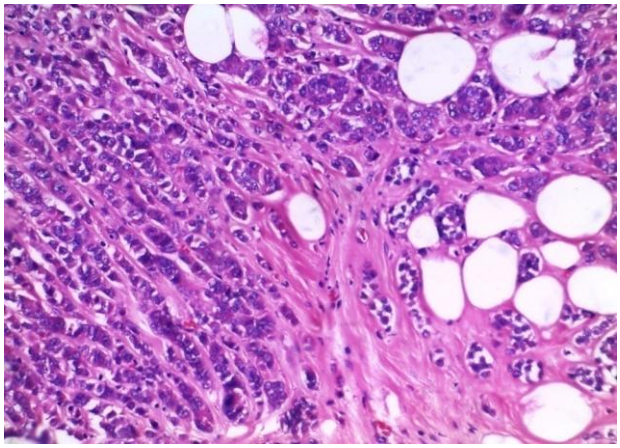


Figure 2: Iinvasive Lobular breast carcinoma. Haematoxylin eosin, 100 x.

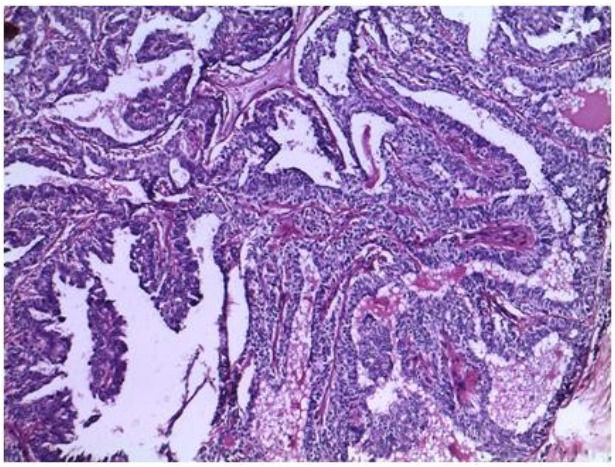


Figure 3: Iinvasive Papillary breast carcinoma. Haematoxylin eosin, 100 x

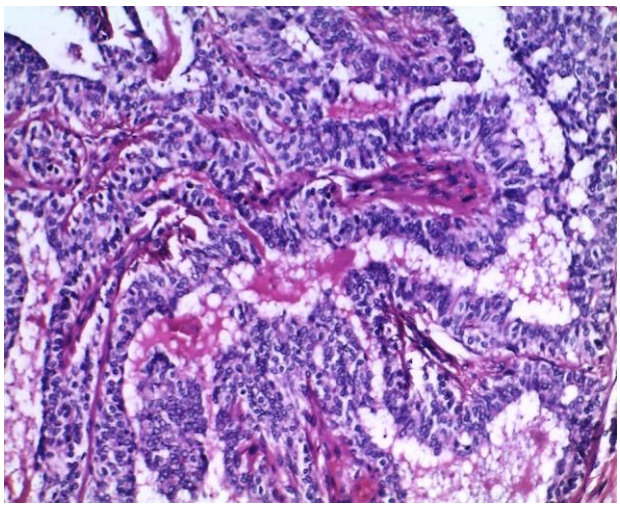


Figure 4: Iinvasive Papillary breast carcinoma. Haematoxylin eosin, 400 x.

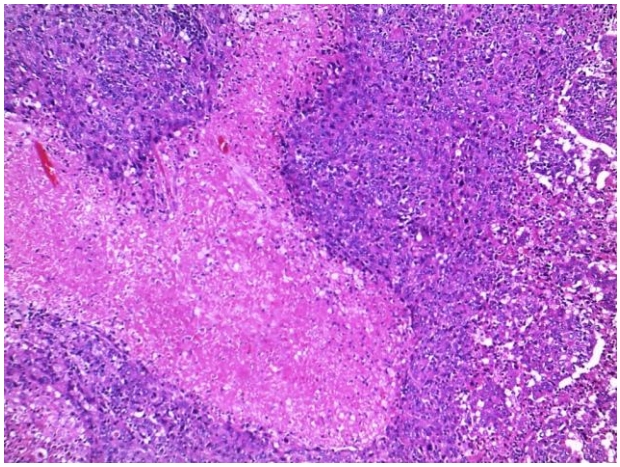


Figure 5: Iinvasive Medullary breast carcinoma. Haematoxylin eosin, 400 x.

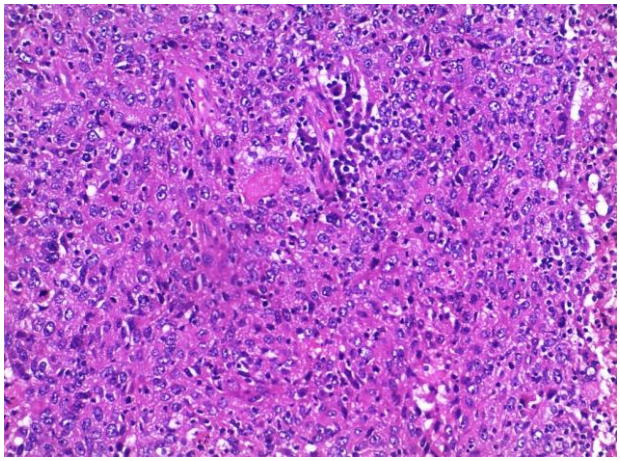


Figure 6: Iinvasive Medullary breast carcinoma. Haematoxylin eosin, 100 x.

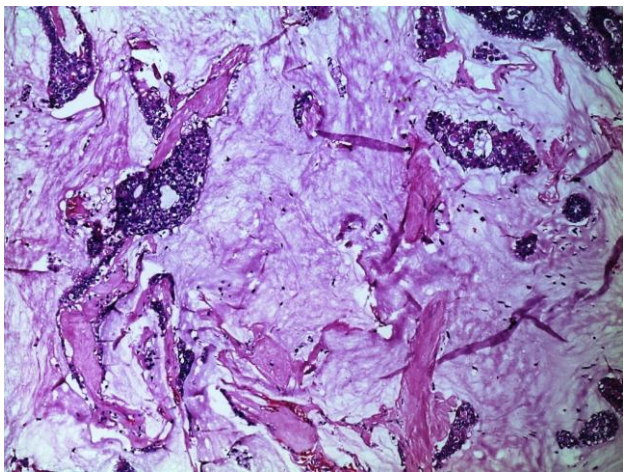


Figure 7: Iinvasive Mucinous breast carcinoma. Haematoxylin eosin, 100 x.

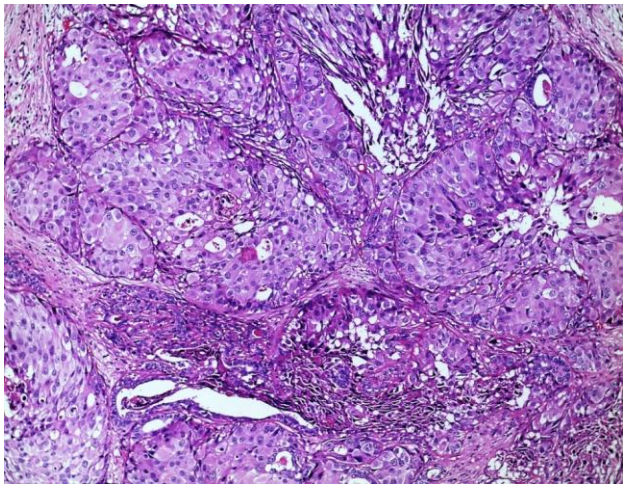


Figure 8: Iinvasive ductal breast carcinoma. Haematoxylin eosin, 100 x

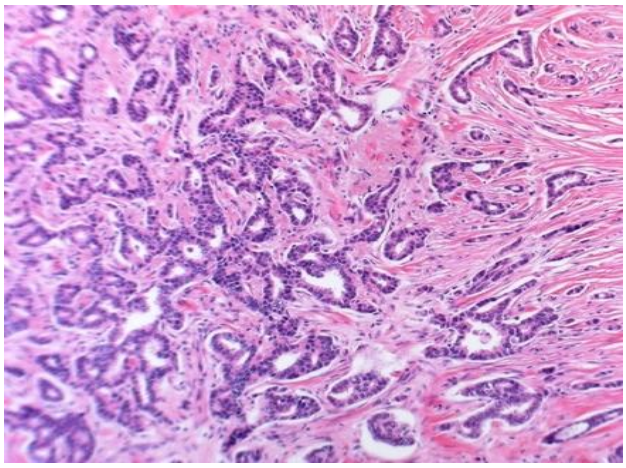


Figure 9: Iinvasive ductal breast carcinoma. Haematoxylin eosin, 100 x

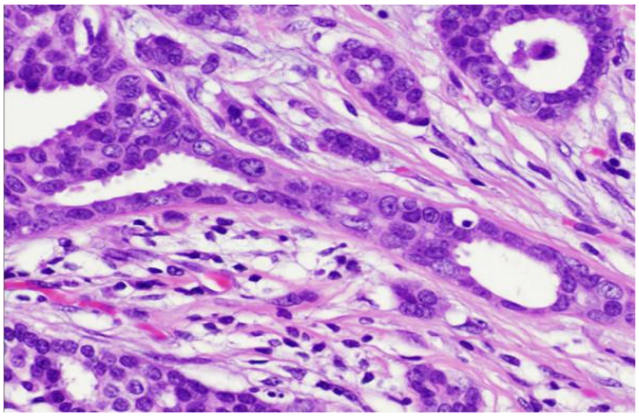
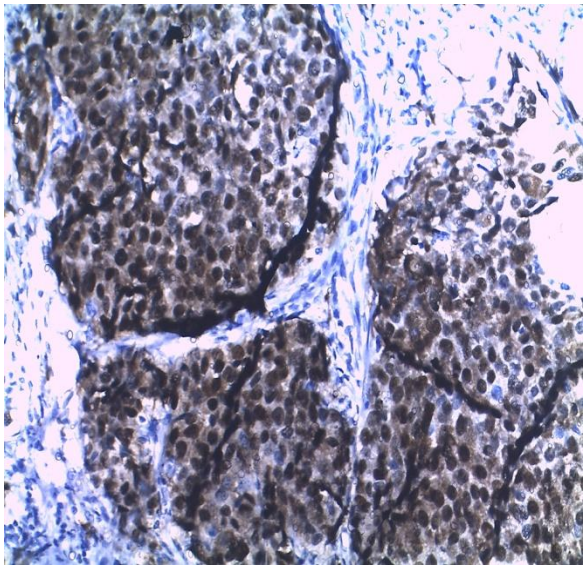
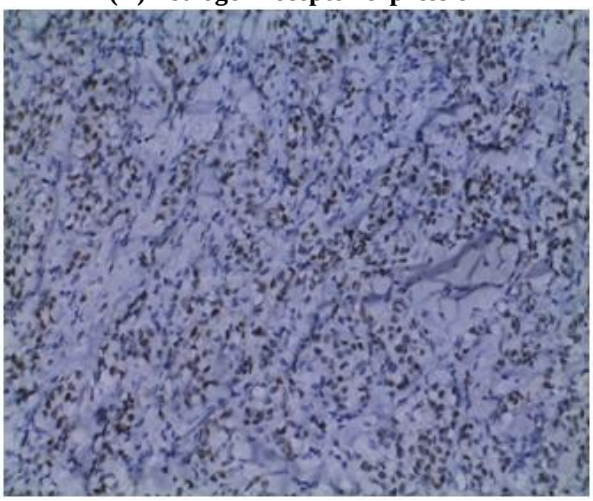


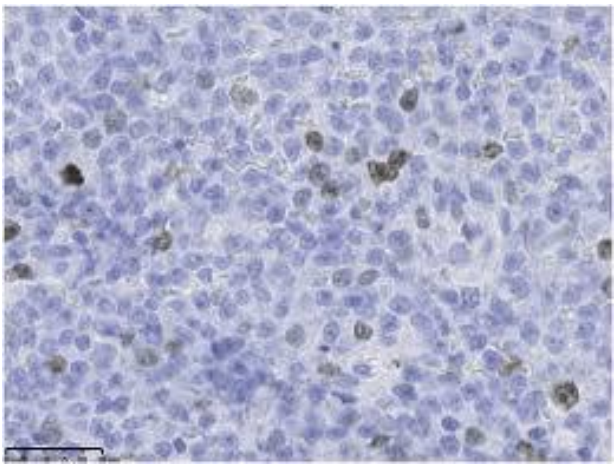
Figure 10: Iinvasive ductal breast carcinoma. Haematoxylin eosin, 100 x.



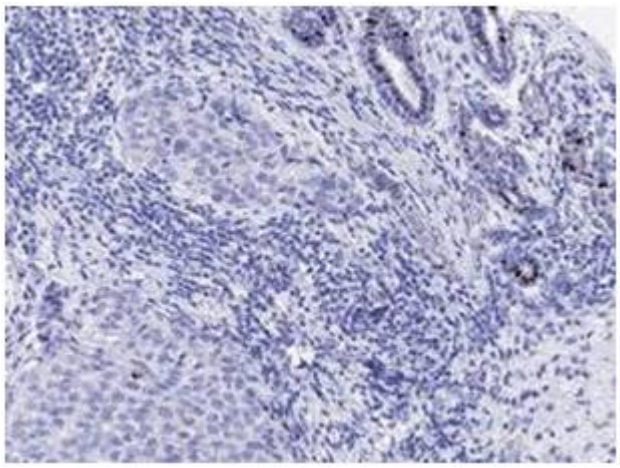
(A) Estrogen receptor expression



(B) Progesterone receptor expression

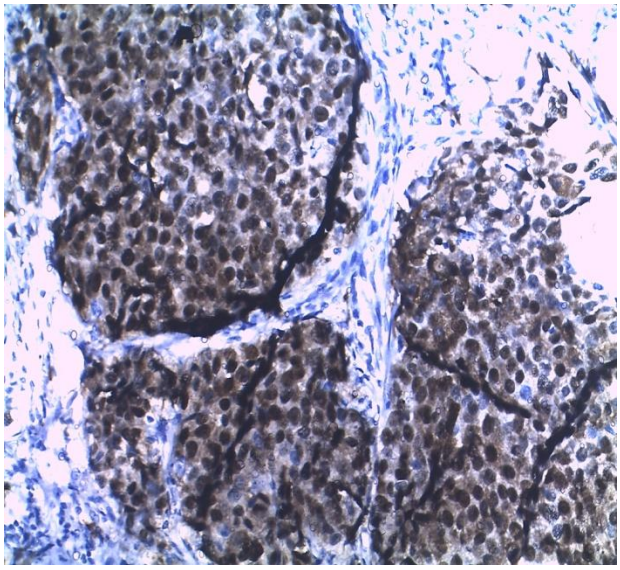


(C) Lack of HER2 protein

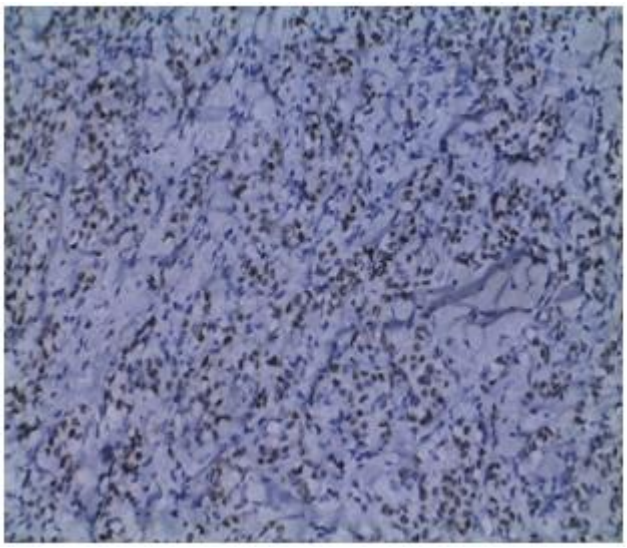


(D) Low proliferation fraction.

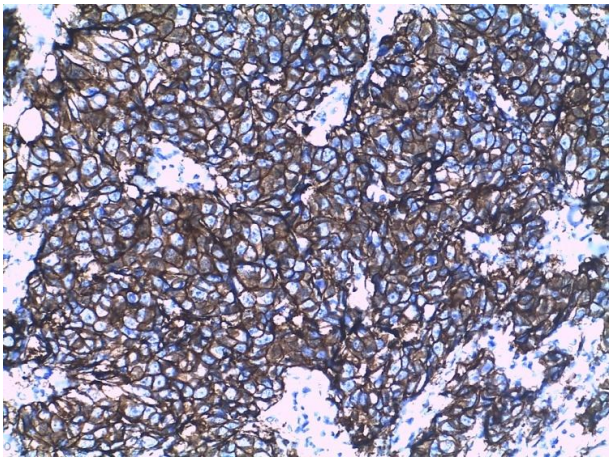
Figure 11: MOLECULAR SUBTYPE – LUMINAL A



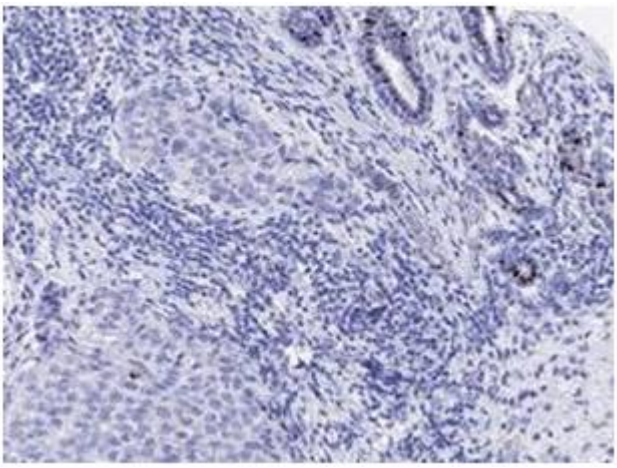
(A) Estrogen receptor expression



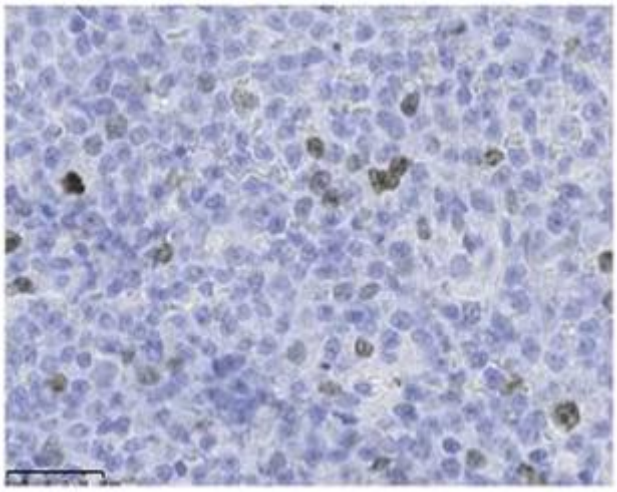
(B) Progesterone receptor expression



(C) HER2 protein expression

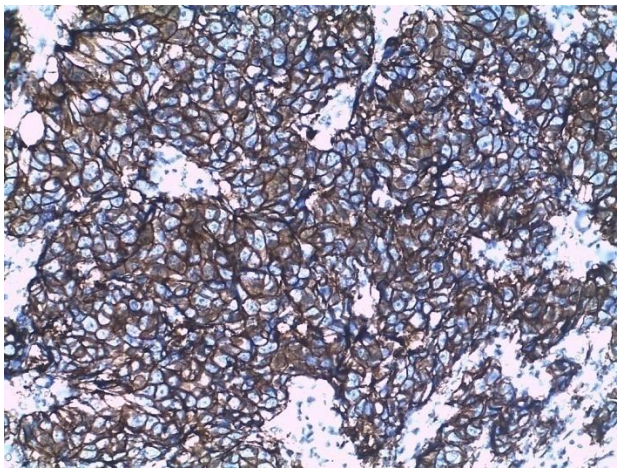


(B) Progesterone receptor negative

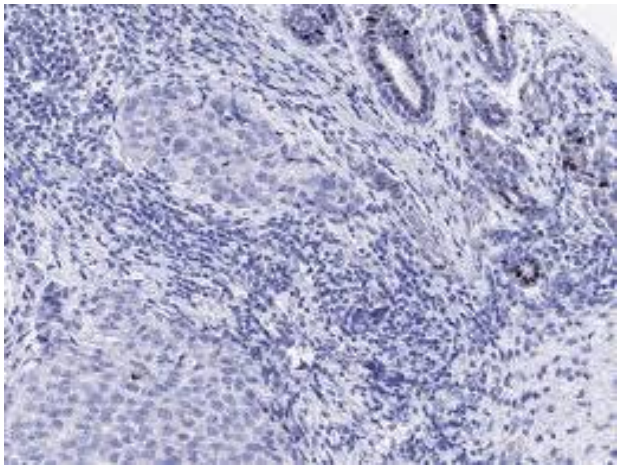


(D) Low proliferation fraction

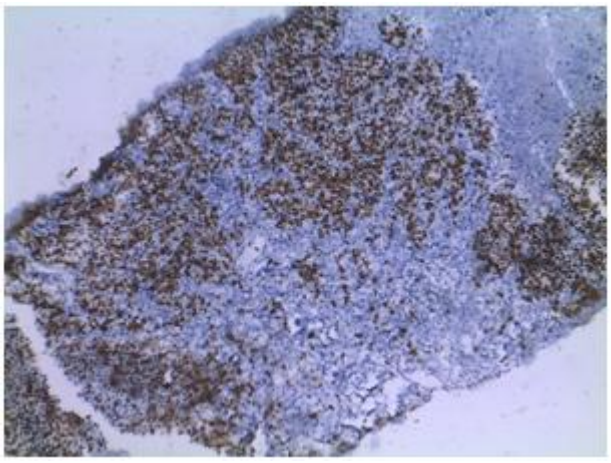
Figure 12: MOLECULAR SUBTYPE – LUMINAL B



(C) HER2 protein overexpression

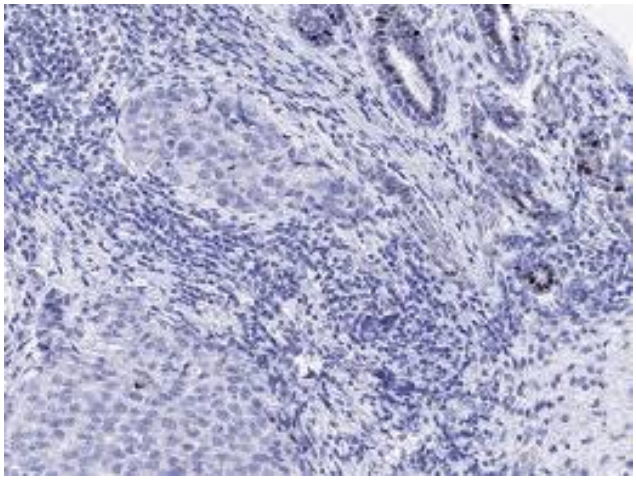


(A) Estrogen receptor negative

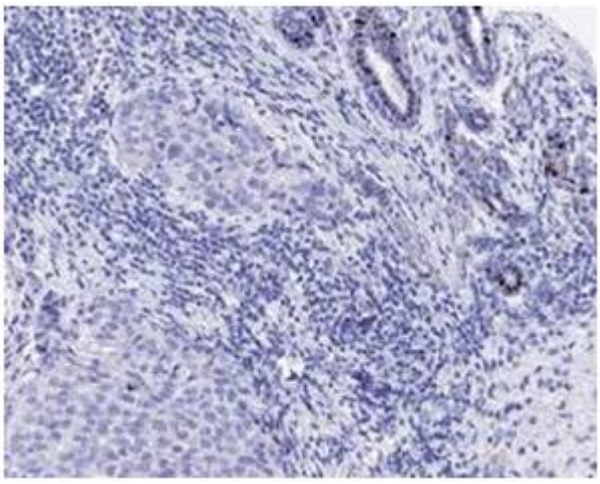


(D) Moderate proliferation fraction

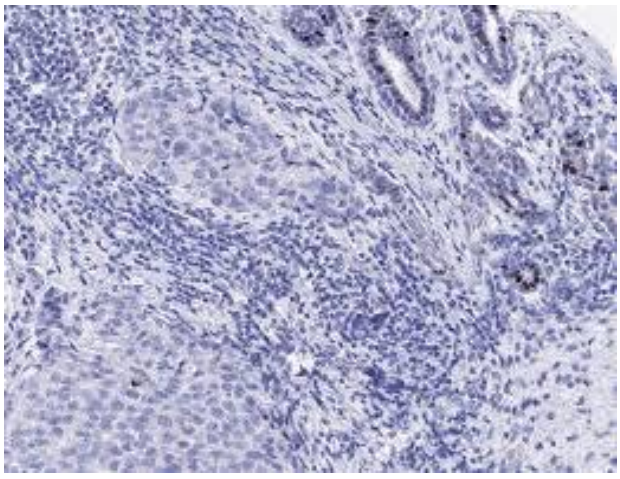
Figure 13: MOLECULAR SUBTYPE – HER 2 POSITIVE



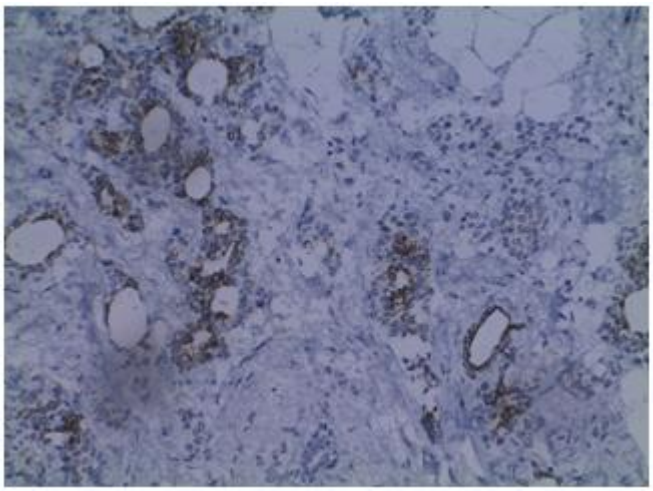
(A) Estrogen receptor negative



(B) Progesterone receptor negative



(C) HER2 protein NEGATIVE



(D) CK5/6 POSITIVE

Figure 14: MOLECULAR SUBTYPE – BASAL LIKE (TRIPLE NEGATIVE)

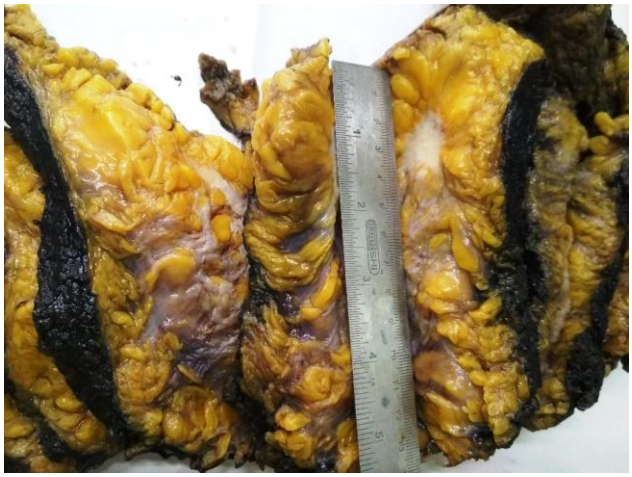


Figure 15: pT2) by gross examination after segmental breast tissue excision

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