QUANTITATIVE ANALYSIS OF MAST CELL IN INVASIVE DUCTAL CARCINOMA OF BREAST

By

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Dissertation submitted to the

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In partial fulfillment of the requirements for the award of the degree of

DOCTOR OF MEDICINE

IN

PATHOLOGY

UNDER THE GUIDANCE OF

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LIST OF ABBREVATIONS USED

NST	– No Special Type
MC	– Mast cell
TNF-α	– Tumor necrosis factor
IL	– Interleukin
ER	– Estrogen Receptor
PR	– Progesterone Receptor
HER 2	– Human Epidermal Growth factor Receptor 2
TDLU	– Terminal duct lobular unit
TEB	– Terminal end buds
NOS	– Not otherwise specified
IHC	– Immunohistochemistry
TB	– Toluidine Blue
H&E	– Haematoxylin and eosin
WHO	– World Health Organisation
SBR	– Scarff-Bloom Richardson

ABSTRACT

BACKGROUND: Breast cancer being the most prevalent, constitutes one third of cancers among women. Invasive carcinoma of no special type (NST) is the most prevalent of all histological types of breast carcinoma with a frequency of about 83%. Tumor microenvironment constitutes immune response caused by neoplastic cells which leads to accumulation of inflammatory cells like mast cells, macrophages, lymphocytes and plasma cells around the tumor tissue. Mast cells secrete several cytokines like tumor necrosis factor α (TNF-α), IL-1, IL-4 and IL-6 which induces apoptosis of malignant cells thus has an inhibitory effect on the tumor growth.

Accumulation of MCs helps in growth of tumor as it facilitates tumor angiogenesis by releasing heparin like molecules. They also secrete histamine, growth factors such as vascular endothelial growth factor and metalloproteases that contribute to tumor invasiveness.

OBJECTIVE: To study the quantitative analysis of mast cells in different grades of invasive ductal carcinoma of breast with respect to estrogen receptor (ER), progesterone receptor (PR) and HER2/neu.

MATERIALS AND METHODS: A retrospective and prospective study was carried on patients diagnosed as invasive carcinoma of breast who underwent total or modified mastectomy procedure fulfilling the inclusion criteria which were received at Department of Pathology from Department of Surgery, BLDE (Deemed to be university) Shri B. M. Patil Medical College, Hospital and Research centre, from 1st January, 2016 to 30th June, 2018.

All total or modified mastectomy specimens were collected in 10% buffered formalin. Histopathologic study was done according to standard protocol. All sections were examined and reviewed for histological grading according to Modified Scarff-Bloom

Richardson. Then representative tissue sections (4-5 μm thickness) were prepared from formalin fixed, paraffin embedded tissues and stained with 0.1% toluidine blue. The mast cells were scanned at low power objectives and counted in 400x for 10 high power fields. Immunohistochemical staining was done for ER, PR and HER2/neu. ER and PR were scored according to the Allred scoring system. HER2 was scored according to American society of clinical oncology (ASCO).

Results: In the present study of 60 cases the mean age was 52 years. Grade II (45.0%) had maximum number of cases followed by Grade I (33.3%), and III (21.7%). Mean MC count was done and the cases falling into Grade I had the highest number of MCs counted with a mean value of 24.05. The mean values of MCs for the Grade II and III were 18.4 and 7.9 respectively with a significant p value. ER positivity was found in 60% of the cases. 55% of the cases showed PR positivity. HER2/neu positivity was noted in 32% of the cases.

Out of 36 cases with ER positivity mean MC count was 23.55 with a p value of <0.0001 indicating statistical significance. Out of 33 cases positive for PR, mean MC count was 24.18, with a p value of 0.0019 which was statistically significant. Twenty eight cases were HER2 positive with a mean MC count of 20.82 which did not show any statistical significance.

Conclusion: Our results indicated that mast cells in breast cancer are inversely proportional to grade of tumor. A higher number of mast cells were seen in tumors of lower grade suggesting a role of mast cells in invasive ductal breast carcinoma. Also there was a positive correlation between ER and PR receptor positivity with presence of MCs in stroma of breast cancer.

Key words: - Breast cancer, Tumor Microenvironment, Mast cell, Invasive ductal carcinoma, Toluidine blue.

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INTRODUCTION

Breast cancer being the most prevalent, constitutes one third of cancers among women which is the second cause of mortality after lung cancer. Invasive carcinoma of no special type (NST) with a frequency of about 83% is the most prevalent of all histological types of breast carcinoma. Increased use of mammography has led to early diagnosis of breast carcinoma and with proper treatment, the morbidity and mortality rates have reduced.[1]

Recently many studies are undertaken on cellular and extra cellular matrix components present in tumor microenvironment which constitutes various innate and adaptive immune cells.[2]

Immune response caused by neoplastic cells leads to accumulation of inflammatory cells like mast cells (MCs), macrophages, lymphocytes and plasma cells around the tumor tissue.[3]

Ehrlich was the first person to report MCs in tumor tissue in 1878.[4] Since then several authors have studied the relation of MCs in breast cancer. Aaltomaa *et al*[5]was the first to observe MCs seen in small groups surrounding the invasive cancer cells which was due to the release of histamine.

MCs are derived from the multipotent hemopoietic bone marrow progenitors cells and migrate while they are still immature from vascular to the peripheral tissue where they mature and are widely distributed throughout the body.[3,6]

MCs which are a part of the innate immune system are recruited and activated in the microenvironment of a developing tumor.[7] Accumulation of MCs helps in growth of tumor as it facilitates tumor angiogenesis by releasing heparin like

molecules. They also secrete histamine, growth factors such as vascular endothelial growth factor, platelet derived growth factor, stem cell factor and nerve growth factor and metalloproteases that contribute to tumor invasiveness.[8,9]

MCs are detrimental to tumor cells by secreting several cytokines like tumor necrosis factor α (TNF-α), IL-1, IL-4 and IL-6 which induces apoptosis of malignant cells thus has an inhibitory effect on the tumor growth.[9]

Mast cell infiltrate apart from breast cancer has also been studied in non small cell lung cancer, basal cell carcinoma, colorectal cancer and pulmonary adenocarcinoma.[7]

The prognosis of breast carcinoma is affected by host and tumor related factors such as patient age, tumor size, histological type and grade, lymph node status, oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2) status. In addition, lymphatic and blood vascular invasion are also significant prognostic factors for poorer survival of breast cancer.[10]

Hence the study was undertaken to know the relationship between histological grades of invasive ductal carcinoma of breast and MCs, as an independent prognostic factor with respect to ER, PR and HER2/neu receptor status.

OBJECTIVE OF THE STUDY:

To study the quantitative analysis of mast cells in different grades of invasive ductal carcinoma of breast with respect to estrogen receptor (ER), progesterone receptor (PR) and HER2/neu.

REVIEW OF LITERATURE

Breast cancer is one of the most common invasive cancer in the world to affect women, and is also the second common cause of cancer deaths after lung cancer in women. It is estimated that worldwide, 1.4 million women in a year are newly diagnosed to have breast cancer, while 458,000 die due to the disease.[11]

Until 1980's the incidence of developing breast cancer was increasing and as a result of early mammographic screening the incidence started declining in developed countries. The incidence decreased since 2005 which is partly attributable to the decreasing use of hormone replacement therapy. Of all mammary carcinomas most common type is invasive carcinoma of NST.[12]

ANATOMY AND PHYSIOLOGY OF BREAST

The breast is a modified sweat gland and is found in both the sexes, but is rudimentary in the males. It is well developed in females after puberty. It forms an important accessory organ of the female reproductive system.[13]

Embryology of Breast:

Breast development starts during 5^{th}or 6^{th} week of fetal life as two thickened ventral bands of ectoderm referred as ectodermal primitive milk streak extending from base of the forelimb (future axilla) to the region of the hind limb (inguinal area). The breast develops initially as primary breast bud, an ingrowth of ectoderm into the mesoderm and later during 12^{th} week initiates the development of 16 to 24 secondary buds. From these secondary buds epithelial cords develop and extend into surrounding

mesenchyme. Mesenchymal cells differentiate into areola and smooth muscles of nipple.[14,15]

At 16 weeks secretory alveoli develops from the tips of buds. The secondary mammary anlage differentiates into sweat gland elements, sebaceous and hair follicles. Breast buds canalize to develop into lactiferous/mammary ducts that open into mammary pit starting at 20^{th} week, and continue until 32^{nd} week. They later develops into nipple areola complex. Before birth, parenchymal differentiation occurs in 32 – 40 week.[14]

Anatomy of Breast:

Breast (mammary gland) lies between 2^{nd} and $6^{th}/7^{th}$ ribs. It is bounded medially by sternal border, laterally mid axillary line and is surrounded by superficial and deep fascia of chest wall.[14]

Components of breast:

1. Skin
2. Superficial fascia: It envelops breast parenchyma along with deep fascia.
3. Breast parenchyma: Comprised principally of three tissue types.
    i. Glandular epithelium
    ii. Fibrous stroma
    iii. Fat and Supporting structures

Glandular epithelium which forms 10-15% of the adult female breast, consists of 15-20 lobes, these are further divided into several lobules called terminal ductules or acini. These represent the secretory portion of gland. The

minor ducts are lined by single layer of cuboidal epithelium while major ducts have double layered epithelium. The myoepithelial cells surround ductal epithelium that helps for propulsion of milk forward. Basement membrane surrounds these cells. Invasive carcinoma and in situ cancer can be distinguished by invasion of the basement membrane.

Fibrous stroma along with supporting structures is commonly called suspensory ligaments of cooper. Involvement by tumor leads to contraction of bands that causes puckering.

4. Nipple-areola complex
5. Deep fascia.[14]

Blood supply

Mammary gland is extremely vascular. It is supplied by the branches of the following arteries:

1. Internal thoracic artery
2. Lateral thoracic, superior thoracic and thoracoacromial branches of the axillary artery
3. Lateral branches of the posterior intercoastal artery

Venous supply:

The superficial veins drain into the internal thoracic and superficial veins of the lower part of the neck.

The deep veins drain into the axillary, internal thoracic and posterior intercoastal veins.

Nerve supply: Breast is supplied by Lateral and Anterior cutaneous branches of the 4^{th} and 6^{th} intercoastal nerves.

Lymphatic drainage: Carcinoma of breast spreads mostly along lymphatics to the regional lymph nodes.

Lymphatic vessels of the breast

The superficial lymphatics drain the skin over the breast except for areola and the nipple. They pass radially to the surrounding lymph nodes (cephalic, axillary, supraclavicular and internal mammary)

Breast parenchyma along with areola and nipple drains into the deep lymphatics. About 75% of lymph from breast drain into axillary nodes, 20% into the internal mammary and 5% into the posterior intercoastal nodes.[13]

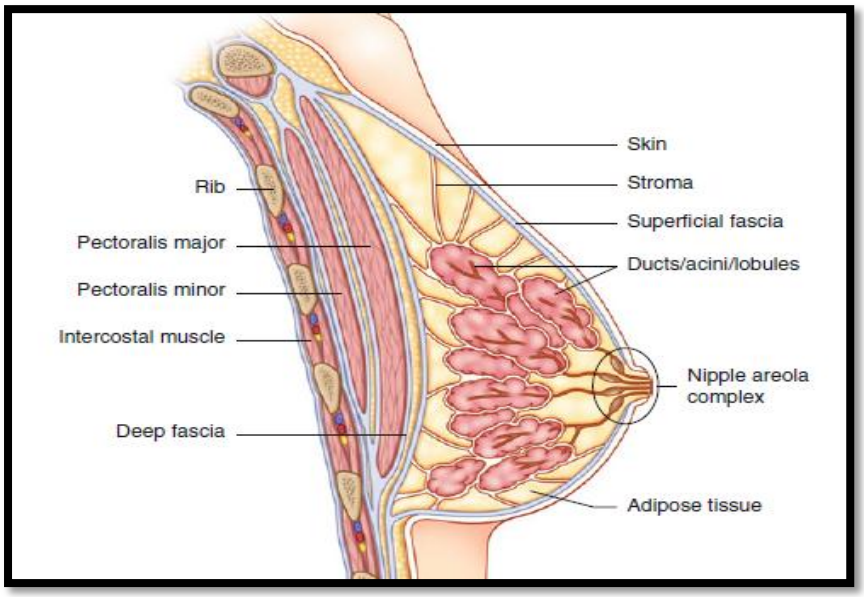


Figure 1 : Anatomy of Mammary Gland[14]

The breasts are highly modified apocrine sweat glands that develop embryologically along two milk lines, extending from axilla to groins. In humans, only one gland develops on each side of the thorax, although accessory breast tissue may be found anywhere along the milk lines.

Each breast consists of 15-25 independent units called breast lobes, each consisting of a compound tubulo-acinar gland. The size of the lobes is quite variable and the bulk of the breast is made up of a few large lobes that connect to the surface. Immediately before opening onto the surface, the duct forms a dilatation called the lactiferous sinus. Smaller lobes end in blind ending ducts that do not reach the nipple surface. The lobes are embedded in a mass of adipose tissue subdivided by collagenous septa.

The nipple contains bands of smooth muscle orientated parallelly at the lactiferous ducts and circularly near the base; contraction of this muscle causes erection of the nipple. Within each lobe of the breast, the main duct branches repeatedly to form a number of terminal ducts, each of which leads to a lobule consisting of multiple acini.

Each terminal duct and its associated lobule are called terminal duct-lobular unit. The lobules are separated by moderately dense collagenous interlobular tissue, whereas the intralobular supporting tissue surrounding the ducts, less collagenous and more vascular. The skin surrounding the nipple and areola, is pigmented and contains sebaceous glands that are not associated with hair follicles.[16,17]

Physiological Breast Development

Mammary gland although it is already present in embryos, but most of the branching morphogenesis required for the development of the ductal tree occurs post-natally around the time of ovarian hormone release at puberty. The main hormones responsible for breast growth and development during puberty are estrogen which stimulates ductal development and progesterone that helps in lobular development and epithelial differentiation.

The distal ends of the mammary ducts develop into bulbous structures composed of multiple layers of epithelial cells, called the Terminal end buds. TEBs which are highly proliferative structures that invade fronts of the ducts extend into the fat pad and branch until the fat pad is completely filled. During pregnancy and lactation the final developmental of the mammary gland occurs, upon stimulation by reproductive hormones. The mammary epithelium differentiates and expands into milk-producing lobular alveoli.

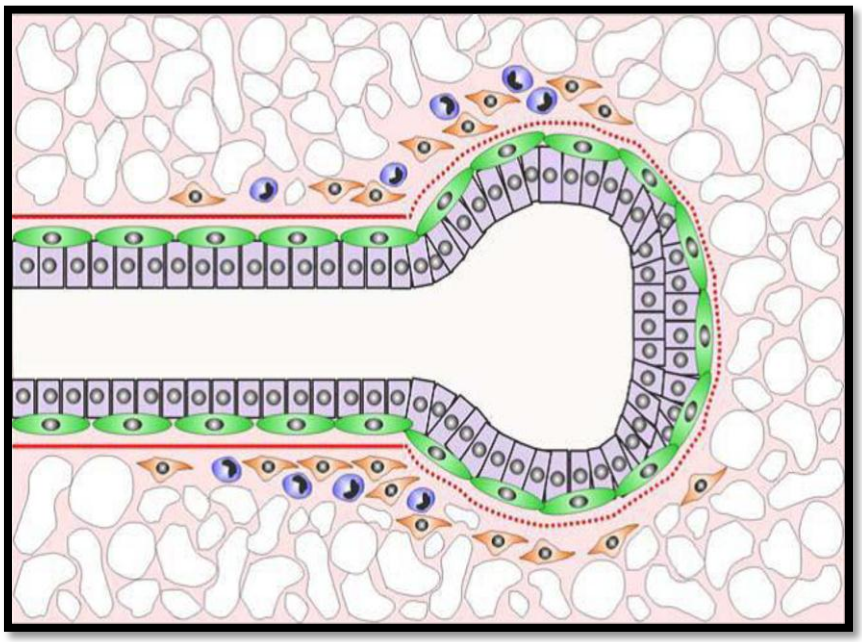


Figure 2 : Schematic Representation of Terminal end bud

Ductal structures contain a layer of luminal epithelial cells (pink) that are surrounded by a layer of myoepithelial cells (green). Terminal end buds as depicted in this diagram contain multiple layers of luminal epithelial cells. The myoepithelial cells are in contact with the laminin-rich basement membrane (red). Fibroblasts and stromal macrophages surround the ducts (orange and blue cells, respectively). The major part of the mammary gland consists of adipocytes (white) (Figure 2).

Histology of Breast:

Mammary glands are compound tubulo-alveolar glands that lie in the superficial connective tissue of the thorax. Glandular elements are arranged in a radial fashion around the nipple, into which ducts from the secretory units empty. The units are separated from each other by connective tissue, adipose cells, and small blood vessels. The glands are lined by cuboidal epithelial cells lying on a basement membrane. Enclosed within the basement membrane are myoepithelial cells in an arrangement similar to that for eccrine sweat glands. Since the mammary glands are epithelial derivatives and can be considered to be modified sweat glands, this similarity of structure is noted.

The connective tissue in the immediate vicinity of the secretory alveoli is highly cellular and contains adipose cells, fibroblasts, lymphocytes, and plasma cells. Elsewhere in the breast, the connective tissue has fewer cells and more collagenous fibers. The ducts are lined by cuboidal or columnar cells and subtle changes in the ducts occur during the ovarian cycle. Early in the cycle, the ducts appear as flattened cords of cells. Under the influence of estrogen, definitive lumens appear in the ducts and the potential secretory cells elongate.

The ducts of the mammary gland merge into 15 to 20 lactiferous ducts that open at the nipple.[16] Several ducts are surrounded by dense connective tissue under the stratified squamous epithelium at the surface. The ducts are lined by flattened epithelial cells. In addition to the dense stromal tissue, the nipple contains circularly arranged smooth muscle fibers that respond to cold or tactile stimulation. Sebaceous glands, without associated hair follicles, are located in the dermis just below the epithelium. The glands open either onto the surface or into the ducts, and their

secretions lubricate the nipple during suckling. Free nerve endings and Meissner's corpuscles are found in the dermal papillae.

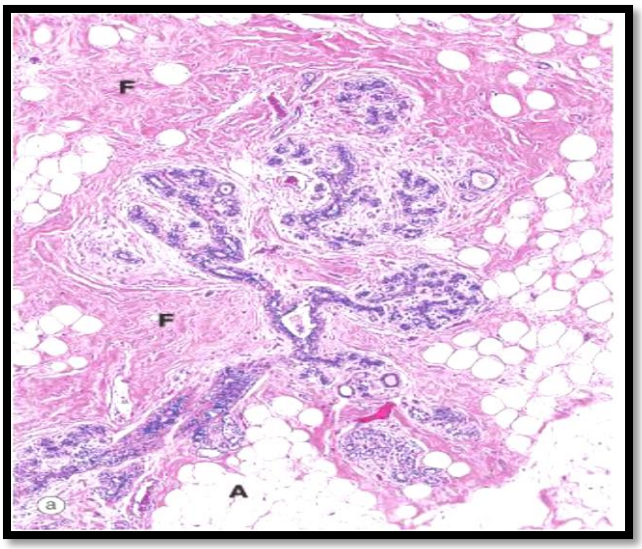


Figure 3 : Histology of Mammary gland (H&E, 100x)[16]

WHO CLASSIFICATION OF TUMOURS OF THE BREAST (2012)

Recently the terminology for breast cancer has been changed from invasive ductal carcinoma, not otherwise specified (NOS) (2003) to invasive carcinoma of NST (2012).

The definition of invasive carcinomas (NST) 2012 is similar to invasive ductal carcinoma (NOS) definition of 2003, only the name 'ductal' has been removed in the new terminology.

This is done because the term "ductal cells" tells unproven histiogenetic assumptions (tumor origin is from the ductal system).[18]

In addition, other specific types of breast cancer are also frequently associated with ductal carcinoma in situ (DCIS), and can therefore, also be regarded as invasive ductal carcinomas, albeit of special type. Thus, the term 'ductal' does not represent a distinguishing pathological feature for breast cancers of no specific or of specific

type, and was considered meaningless. The terms ‘invasive ductal carcinoma’ or ‘ductal NOS’ are accepted as alternative terminology options, but the use of ‘carcinoma of no special type’ is the preferred term. The diagnosis is made by exclusion of recognized specific types of breast cancers.[12]

INVASIVE BREAST CARCINOMA

EPIDEMIOLOGY

Invasive breast cancer is the most common cancer in women which is accounting for 23% of all cancers in women globally.

ETIOLOGY AND RISK FACTORS

Age and gender

Family history of breast cancer

Geographic factors

Race/ Ethnicity

Reproductive history

Ionizing radiation

PATHOGENESIS

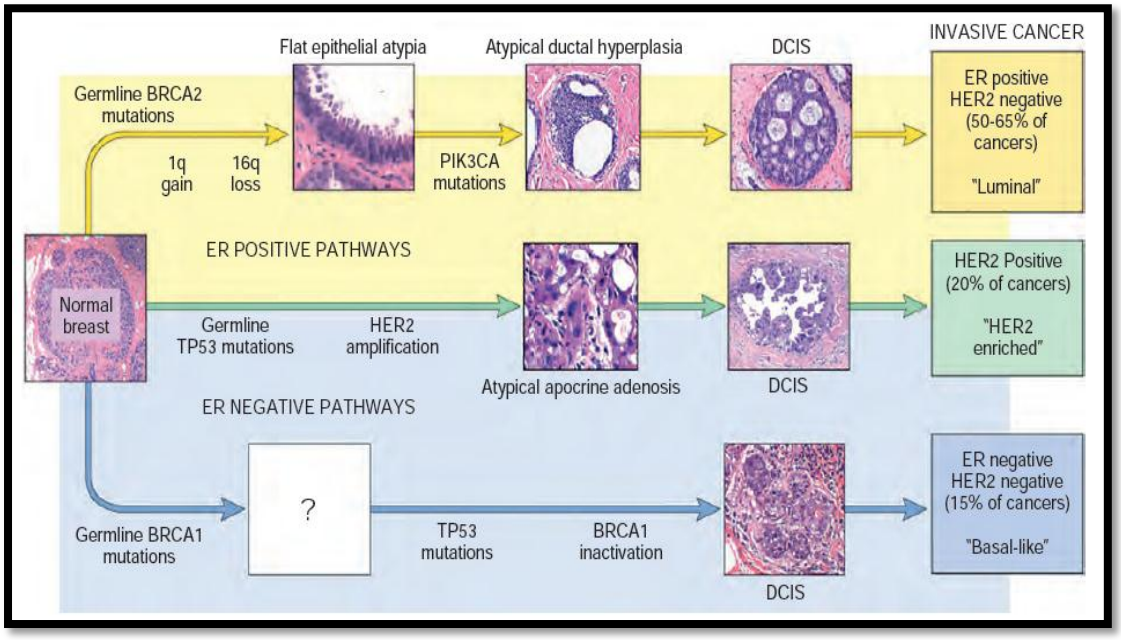


Figure 4 : Major pathways of breast cancer development[19]

Factors that directly contribute for the development of breast cancer are grouped into genetic, hormonal and environmental.

GENETIC:

The major germline mutations in genes that regulate genomic stability or that are involved in progrowth signalling pathways cause susceptibility to breast cancer. BRCA1 and BRCA2 are tumor suppressor genes which encode proteins required for repair of DNA damage. When both the alleles are inactivated or defective leads to cancer. The degree of penetrance, age of onset and susceptibility to other types of cancer differ among the many BRCA1 and BRCA2 germline mutations, but carriers develop breast cancer by the age of 70 years as compared to 12% of the women with risk of breast cancer. BRCA2 mutations are primarily associated with ER positive tumor and strongly associated with triple negative cancers but reasons are unclear.

Genes associated with familial breast cancer include TP53 and PTEN. A clinically common important mutation in breast cancer is HER2 gene amplification.

HORMONAL FACTORS:

Estrogen stimulates the production of growth factors such as platelet derived growth factor, fibroblast growth factor, transforming growth factor, and others. These chemical mediators promote tumor development by autocrine and paracrine mechanisms.

ENVIRONMENTAL FACTORS:

Suggest a variable incidence of breast cancer in genetically homogenous groups.[19]

CLINICAL FEATURES:

Invasive breast carcinoma most common clinical sign is a palpable breast lump, however nipple discharge, skin retraction, nipple inversion may also be seen. Rarely they present as enlargement of the axillary lymph nodes in the absence of any other abnormality in the breast. For definitive diagnosis imaging and histological sampling or fine needle aspiration cytology are indicated because benign breast lesions may also present with similar symptoms.

In imaging mammography is diagnostic. A spiculated mass is the classical appearance of cancer but they can also present as well circumscribed masses.

About 5-15% of palpable cancers which cannot be seen on mammogram are identified with targeted ultrasound.

Clinical examination should be done systematically and the nature of the lump has to be noted. Also look for the presence of any change in contour of the breast or skin dimpling and also assess the axilla.[12]

Localisation

Breast carcinoma arises from the mammary epithelium and most frequently from the epithelial cells of the terminal ductal lobular unit (TDLU). Invasive carcinoma of breast is more common on left side when compared to right with a ratio of approximately 1.07 to 1.

Tumors of the upper quadrant are more common and occur at a frequency of 40 and 50% and frequency is decreased in other quadrants.[20]

INVASIVE CARCINOMA OF NST

DEFINITION

Commonly known as ductal carcinoma NST, comprises the largest group of invasive breast cancers.

GROSS

These tumors show marked variation in size from < 10 mm to > 100 mm. They have an irregular, nodular configuration or ill defined outline. On palpation these tumors are firm to hard or may have a gritty feel when cut with a knife. Cut surface is grey white with yellow streaks.[21]

HISTOPATHOLOGY

Architecturally tumor cells are arranged in cords, clusters and trabeculae while some are predominantly solid or syncytial infiltrative pattern. Few cases show glandular differentiation as tubular structures with central lumina in tumor cell groups. Occasionally areas with single file pattern or targetoid features are seen. Nuclei are highly pleomorphic or regular and uniform with prominent, often multiple nucleoli. Cytoplasm is abundant and eosinophilic. Mitotic activity may be absent or extensive. Ductal carcinoma in situ (DCIS) will be present in upto 80% of cases.[12,22]

GRADING OF INVASIVE CARCINOMA

Invasive carcinoma of NST are graded based on the assessment of tubule/gland formation, nuclear pleomorphism and mitotic activity

Significant association with histological grade and survival has been demonstrated in many studies. Assessment of histological grade is done by Patley & Scarff method first by Bloom and Richardson and recently by Elston and Ellis.

Method of grading: Three tumour characteristics are evaluated:

1. Tubule formation as an expression of glandular differentiation
2. Nuclear pleomorphism and
3. Mitotic counts.

A numerical scoring system of 1 - 3 is used to ensure that each factor is assessed individually. When evaluating tubule and gland formation is assessed over the whole tumour and is a low-power assessment.

Nuclear pleomorphism is assessed for the area showing the worst degree of pleomorphism, and mitotic counting is performed for the area exhibiting the most proliferation. Total number of mitosis per 10 high power fields is counted. The assessment of histological grading is explained in Table: 1 [12]

Molecular testing for Estrogen Receptor, Progesterone Receptor, and HER2

Clinical management of diagnosed patients with invasive breast cancer routinely three molecular biomarkers are used. They are estrogen receptor (ER), progesterone receptor (PR), and HER2. Accurate assessment of all the three biomarkers is essential because they are the targets and indicators of effective treatment therapies towards the invasive carcinoma of breast.[12]

Estrogen Receptor:

ER is a regulator of mammary epithelial growth, differentiation and proliferation whose complex cellular interactions are mediated by a magnitude of ligands, cofactors and other stimuli.[23]

This is a nuclear transcription factor which stimulates the growth, differentiation and proliferation of breast epithelium upon activation by hormone estrogen.[12]

Estrogen which is a steroid hormone synthesized from the ovaries predominantly as oestradiol (Oestradiol -17 β, E2), Oestrone (E1) and Oestriol (E3) are synthesized peripherally. Estrogen directly diffuses through cell membrane and it binds to the nuclear membrane where ER is present. Two isoforms of ER are known: ERα present on chromosome 6 and ER β on chromosome 14. These two receptors are expressed on different tissues like breast, heart, bone, brain, uterus and ovary.[24,25] In breast cancer there is predominance of ERα and it is the hallmark of hormone dependent tumor growth.[26]

Only ER β isoform are associated with gastrointestinal tract, blood vessels and on inflammatory cells.[24]

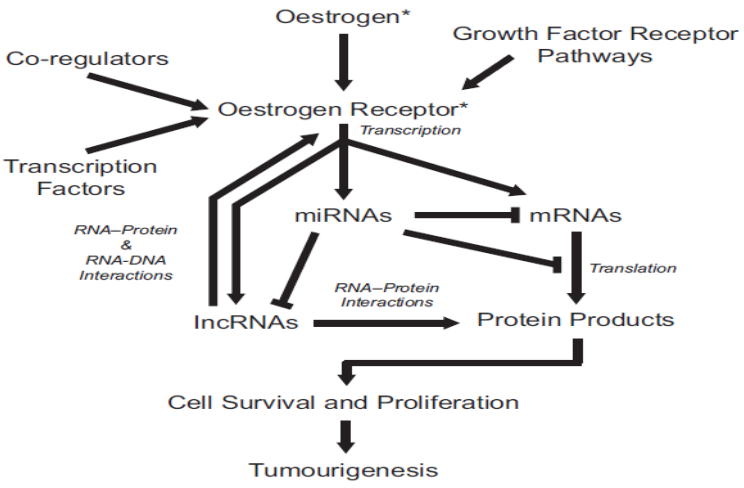


Figure 5 : Mechanism involved in estrogen signalling and ER function[26]

At cellular level estrogen induces or promotes carcinogenesis by various actions like cellular proliferation, evasion of apoptosis, angiogenesis and invasion – metastasis.[24]

Invasion and metastasis:

Series of transformation occur in cancer cells leading to loss of cell adhesion molecules, change in morphology from epithelial to fibroblast known as epithelial – mesenchymal transition (EMT)[27]

Estrogen directly acts on ERα 36 and upregulates Snail 1 expression that has an important role in EMT. It also inhibits E- cadherins and Syndecan 4 which are involved in cell- cell interaction and enhances detachment of cell and metastasis. Estrogen also stimulates a chemotactic receptor CXCR 4 stimulates migration to other sites where ligands of this receptor are found (ex: bone)

Prognosis and ER expression

ER Positive breast cancers without any treatment have better outcome and decreased risk of metastasis. Hormonal therapy can be given for ER positive tumors and they have better prognosis. When the receptor expression is present these are associated with better prognosis and decreased mortality. Tumors which are ER negative do not respond to hormonal therapy and they carry a poorer prognosis.

Anti estrogen treatment

In both pre and post menopausal women who are ER positive adjuvant antioestrogen therapy were given. Adjuvant therapy has proven to reduce the risk of recurrence, decreased mortality and better outcome. Mechanism of action is they inhibit estrogen production. Selective estrogen receptor modulators (SERMs) act as antagonist in breast, uterus and agonists in bone and central nervous system. Commonly used SERMs are Raloxifen and Tamoxifen. [24,28]

PROGESTERONE RECEPTOR

Progesterone is a steroid hormone synthesised from ovary which is essential for development of normal breast during puberty, for lactation and breast feeding. The progesterone effects are mediated by its attachment to its high affinity receptors that are located in the brain, breast and reproductive organs. Progesterone receptors are member of steroid hormone receptor family that act as ligand activated transcription factors which regulate the expression of gene by binding either directly or indirectly at specific sites in the DNA. Upon binding with progesterone or synthetic ligands such as progestins these PR get activated. There are 3 isoforms of PR (PR-A, PR-B and PR-C) which are located as single gene on chromosome 11q 22-23.[29,30]

Clinical findings and treatment

Women's Health Initiative (WHI) and Million Women Study demonstrated that women who were taking hormonal replacement therapy with progesterone and estrogen combination had a greater risk of breast cancer than that with estrogen alone. The tumors were of higher grade and larger in size in women who took combined therapy. They had higher mortality rate, while the reduced use of combined therapy did not cause decline in the incidence of breast cancer.

Several types of progesterone receptor modulators (PRM) have been described but they are not selective and show cross reactivity with GR. Two PRM drugs have been tested in breast carcinoma they are Mifepristone and Onapristone.[29]

HER2

Human epidermal growth factor receptor (HER) is a family of receptors that regulate cell survival, growth and differentiation. Through several signal transduction pathways and play a role in cellular differentiation and proliferation. Four members

are known HER-1, HER-2, HER-3 and HER-4, also called as Erb 1, Erb 2, Erb 3 and Erb 4 respectively. All these receptors contain an extracellular ligand binding site which is cystine rich and has tyrosine kinase catalytic activity. A group of scientist at Massachusetts institute of technology discovered neu oncogene (also called as HER 2, Erb B2 OR P185). Erb B means its origin from Erb b gene which is responsible for ovarian erythroblastosis virus. The Her 2 receptor is a Transmembrane glycoprotein located on long arm of chromosome 17 (17q 12).

HER 2 IN BREAST CANCER

Overexpression of HER 2 is seen in approximately 15-30% of the invasive breast cancers which has its role as prognostic and predictive implication.[31] In some breast cancers P^{95} is found which is an aberrant form of HER 2 which lacks the extracellular domain. P^{95} causes resistance to transtuzumab as the drug requires extracellular domain for binding. In a study done by Seshadri *et al*[32] concluded that 3 fold HER 2 amplification associated with shorter disease free survival. The HER 2 amplification correlates with pathologic stage, number of axillary nodes, histologic type and absence of ER and PR.[33]

HER 2 overexpression is also seen in esophageal cancer, ovarian cancer and endometrial cancer.

TESTING OF HER 2

It is done according to guidelines given from American society of clinical oncology (ASCO) and The college of American pathologist (CAP)

Currently two methods are approved for HER testing they are immunohistochemistry (IHC) and Fluorescence insitu hybridization (FISH)

Pattern of staining of HER 2 in IHC is cell membrane.

Scoring is given in Table: 5.

TRAGETS OF HER 2

1. Transtuzumab: It is a monoclonal antibody which binds to domain IV in the extracellular segments of HER2 receptor.

   Mechanisms are: HER2 shedding is inhibited

   PI3K-AKT pathway is inhibited

   Cell signalling is inhibited

   Antibody mediated cellular cytotoxicity

   Tumor angiogenesis inhibition

2. Lapatinib: This is a dual tyrosine kinase inhibitor and interrupts HER2 and EGFR pathway.
3. Pertuzumab: It is a humanized monoclonal antibody. Inhibits dimerization of HER2 receptors.
4. Neratinib: Irreversible Tyrosine kinase inhibitor of HER2 and EGFR.[34,35]

TUMOR MICROENVIRONMENT

The tumor microenvironment is created by tumor and tumor- induced interactions.

Tumor microenvironment is comprised of proliferating tumor cells, blood vessels tumor stroma and inflammatory cell infiltrate.

There are increasing evidence which tells that unique immune cell, the mast cell, accumulates in the stroma surrounding certain tumors, especially breast and pancreatic adenocarcinoma, as well as melanoma.[36]

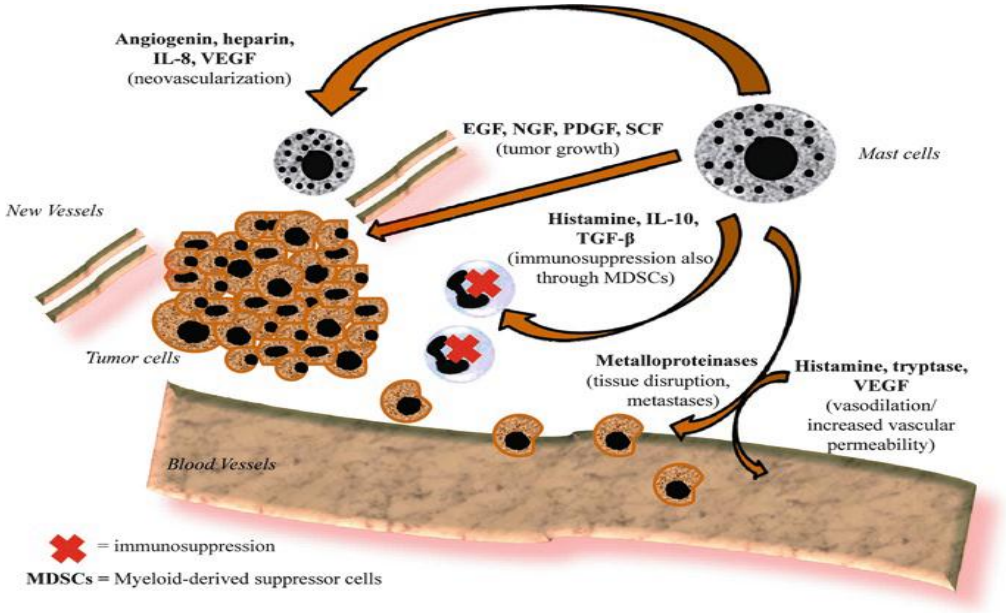


Figure 6 : Possible role of increased number of mast cells in the stroma.[36]

MAST CELL

ORIGIN AND DISTRIBUTION:

MCs are derived from the multipotent hemopoietic progenitors in the bone marrow. They migrate when they are still immature from vascular to the peripheral tissue where they mature and are widely distributed throughout the body. They are commonly found near blood vessels, skin and intestinal mocusa.[3,37]

MCs are part of the innate immune system and they are recruited and activated in the microenvironment of a developing tumor.[8]

The tumor stroma microenvironment could alter the phenotypic behaviour of MCs. For instance, acidity created by rapid cancer cell proliferation inhibits mast cell degranulation, but enhances IL-4 production. Nitric oxide (NO) generated by new vessel growth inhibits mast cell degranulation ,as do oxidized polyamines secreted by the tumor.[8]

MCs are classically viewed as effector cells of IgE-mediated allergic diseases. However, recently their roles have been understood in host defense, innate and adaptive immune responses, and in homeostatic responses, angiogenesis, tissue remodelling, , wound healing, and immunoregulation.[38]

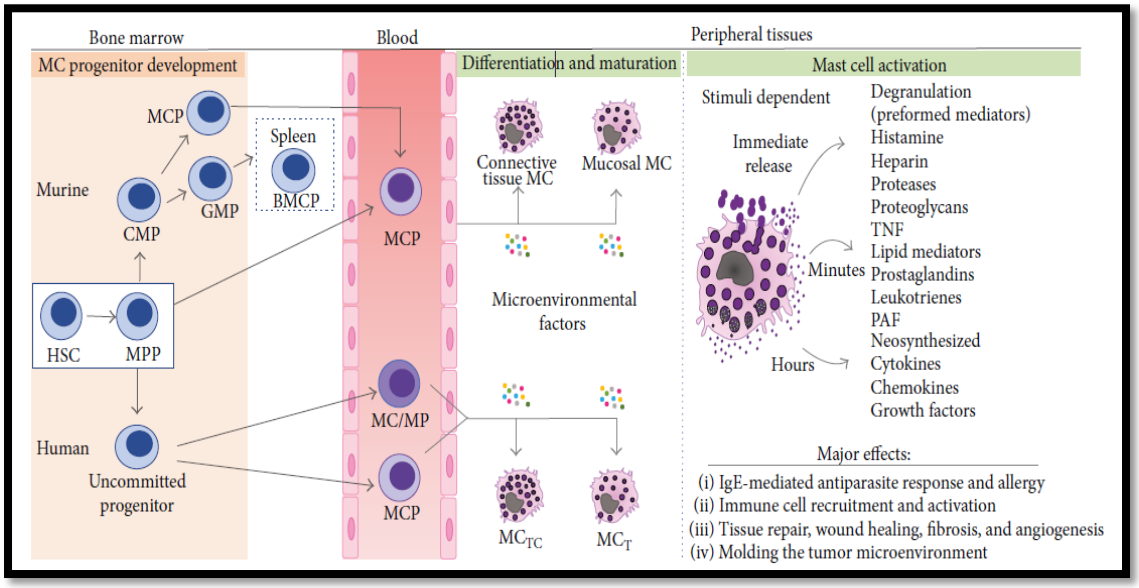


Figure 7 : Over view of MC development and activation[39]

Morphology of mast cell

MCs measure about 20 and 30 µm in diameter and are ovoid, round or spindle shaped with centrally placed round nucleus. Cytoplasm contains numerous (80-300) metachromatic granules, few mitochondria, endoplasmic reticulum and numerous free ribosome's. Metachromasia in granules is due to sulphuric mucoploysaccharide. When activated they undergo degranulation or release solitary granules by a process called piecemeal degranulation that metachromatically stain with thiazine dyes such as toluidine blue. [40]

Granules contain preformed substances, such as histamine, which has a vasodilating action and increases vascular permeability, and heparin, a proteoglycan having an anticoagulant action. [4]

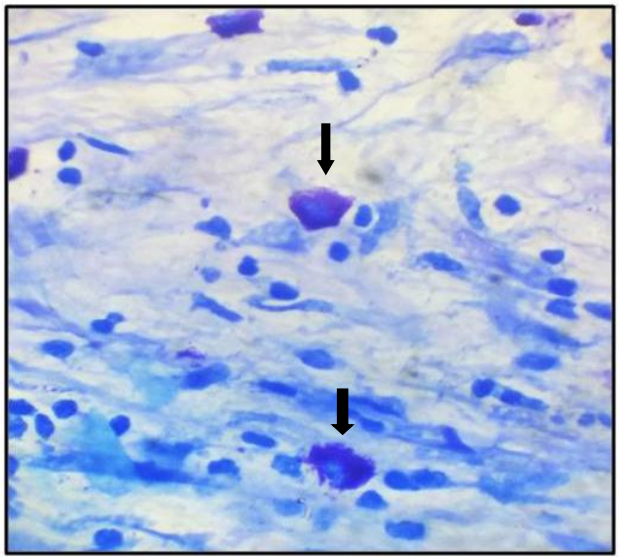


Figure 8 : Microphotograph showing MC (arrow) with metachromatic granules (Toluidine Blue 1000x)

MCs can be divided into two subtypes based on location, histochemical staining, proteolytic enzyme contents, reactivity to secretagogues and dependency on T cells.

1. Connective tissue mast cell $MC_{TC:}$ they contain tryptase , chymase, carboxypeptidase and cathepsin G
2. Mucosal mast cell $MC_{T:}$ conatin only tryptase which is T cell dependent.
3. Depending on the microenvironment conditions MMC can develop into CTMC. [38,40,41]

Mast cell, accumulates in the stroma surrounding certain tumors, especially mammary and pancreatic adenocarcinoma, as well as melanoma.

Many molecules secreted by MCs could benefit the tumor in at least four ways:

(1) Angiogenin, heparin and vascular endothelial growth factor (VEGF), which induce neovascularization

(2) Proteases that disrupt the surrounding matrix and facilitate metastases;

(3) Growth factors such as, epidermal growth factor (EGF), nerve growth factor (NGF), platelet derived growth factor (PDGF) and stem cell factor (SCF);

(4) Histamine, IL-10 and transforming growth factor-b (TGF-b), which are immunosuppressant, along with activation of certain dendritic cells that induce immunologic anergy. These actions could only occur through the unique ability of MCs to release certain mediators selectively without degranulation. Blocking such release of pro-tumor mediators may constitute a novel therapeutic approach.[8,9]

On the other hand, MCs could also be detrimental to tumor growth by secreting several cytokines and proteolytic enzymes participating in inducing apoptosis of the malignant cells, such as IL-4, 11. The dual role of MCs in inhibiting or promoting tumor growth needs to be further investigated.[9]

Aaltomaa *et al*[5] observed that MCs were often situated in small groups around invasive carcinoma cells, were one of the first authors to associate MCs with histamine activity in breast cancer.

MCs have a stimulatory role in angiogenesis and lymphangiogenesis by increasing the release of angiogenic factors [vascular endothelial growth factor (VEGF-A, -C, -D] and endostatin under hypoxic conditions. In breast carcinoma and many other cancers, MC density (MCD) in peritumoral tissue was found to be closely related to angiogenesis.[2]

Mast cell in cancer:

MCs after they are first reported by Paul Ehrlich many studies were done to show MC infiltrate in a variety of hemolytic and solid tumors. So they are termed as Tumor Associated MCs (TAMC). High MC density can be seen intratumoral or peritumoral area in cancers like Prostate, Breast, Thyroid, Pancreas, Melanoma and stomach.[42] Few studies showed that MC when there is increased accumulation associated with poor prognosis in Pancreatic, Gastric and Colorectal tumor, while their function in breast cancer is controversial.[43,44]

MC accumulation occurs due to response of various chemotactic factors which are secreted by immune cells or tumor cells present in tumor microenvironment. The chemotatic factors are VEGF, Angiopoietin 1, SCF, IL-8 and Monocyte chemotatic protein 1.[39]

Role of mast cells in breast cancer

MCs as a role in breast cancer prognosis are still a matter of discussion. MCs contain a variety of bioactive components that has both pro and anti- tumor effects.

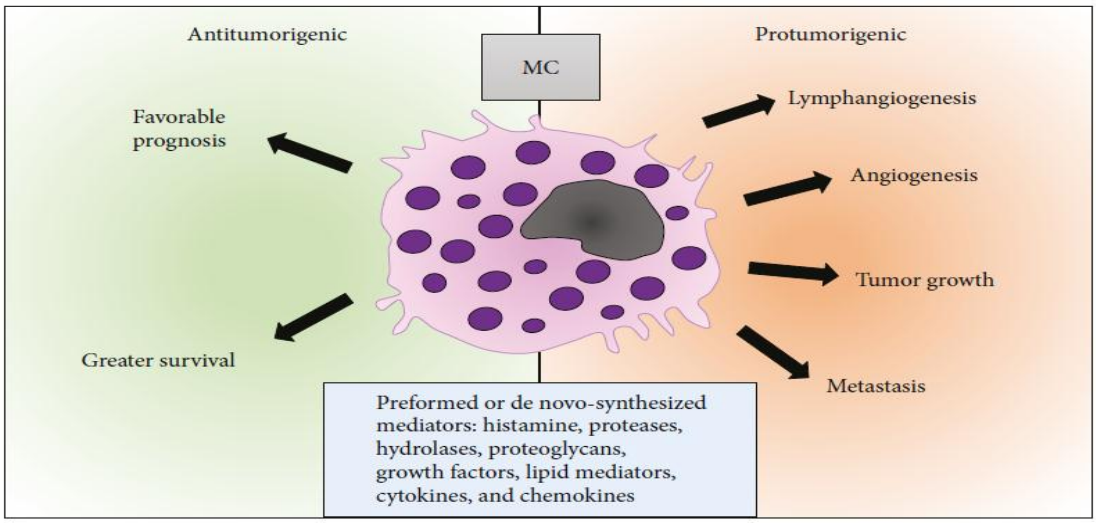


Figure 9 : PRO and ANTI Tumor Effects of MCs in Breast Cancer[39]

The Staining of Mast Cells

The histochemical techniques for subdividing MCs stemmed from the observation by Ehrlich in 1876 that their lysosomal granules have the capacity to take up and stain metachromatically with basic dyes, such as toluidine blue.[45]

STAINING TECHNIQUES

- Haematoxylin and eosin stains
- Metachromatic dye stain
    - Toluidine blue
    - Azure A
    - Giemsa stain
    - Thionin
    - Methylene blue
    - Alcian Blue-Safranin
- Immunohistochemistry markers
    - Tryptase
    - chymotryptase

Metachromasia

Ackrod first used the term "Metachromatic" in 1876 which indicates that the structure being dyed assumes different colour from that of the dye itself.

The granules in MCs can naturally induce metachromatic staining. Metachromatic stains are Romanowsky combination (Wright, Giemsa, May – Grunwald and Leishman), Toludine and Methylene blue.

William Henry Perkin a british chemist in 1856 first prepared toluidine blue. Initially it was known as aniline purple.[40]

Toluidine blue

Toluidine blue (TB) was discovered in 1856 by William henry perkin which was initially used in dye industry. Also known as Tolonium chloride, aminotoluene or methylanaline. Three isoforms of TB are known orthotoluidine, metatoluidine and paratoluidine.

It belongs to basic thiazine group which is partially soluble in alcohol and water. TB is an acidophilic metachromatic dye which stains selectively the acidic components of tissues like DNA and RNA.[46,47]

The extensive use of TB is done for mucosal lesions as vital stain. Two techniques of vital staining are known staining in the living body intravital (invivo) and staining outside the body supravital.

Principle of toluidine staining:

Staining principle of TB is metachromasia. It is due to the stocking of cations present in the dye with the anions in the tissue. This causes shortening of the wavelength of light which has maximum absorption, thus resulting in appearance of red colour instead of blue. Certain subatances like mucins and MCs are stained by this method and they are called chromotopes. In MCs metachromasia is due to heparin which is a heteroglycan rich in half-sulfate esters.

Applicaton of TB

1. Mucins such as acid mucin it stains purple to red.
2. Granules of MCs satin purple is due to heparin and histamine
3. Amyloid is stained blue but bright red birefringence is seen under polarized light
4. Granules in endocrine cells stain purple to red
5. Helicobacter stain dark blue[48]

Bowers H *et al* [49]were the first to study about MCs using toluidine blue . They evaluated the axillary lymph node of 43 breast cancer patients and concluded that increased number of MCs were found in the noninvolved axillary lymph nodes in those women is associated with better survival.

Samoszuk M *et al*[50] conducted study on 35 cases of breast cancer and these were stained with tryptase for MC. They concluded that in preinvasive tumors MCs are accumulated peritumorally and intratumorally in invasive tumors.

A study conducted by Amini RM *et al* [51]on 234 invasive breast cancer and analysed stromal MCs and eosinophils. Total number of MCs were counted using immunostaining for tryptase and concluded that higher numbers of MCs were associated with low tumor grade and estrogen receptor positivity.

Rovera F *et al* [4] studied the role of MCs in invasive ductal breast cancer on 50 cases and MCs were stained with alcian blue, concluded that peritumoral MCs are higher in association with high hormone receptive cancers.

Fakhrjou A *et al* [1]studied the relationship between histological grading of invasive carcinoma of breast ducts and mast cell infiltration on 75 female patients suffering from invasive ductal carcinoma who underwent surgery and concluded that number of MCs around tumor cells increased significantly with an increase in the grade of disease.(1)

Heidarpour M *et al* [9]studied MCs in invasive ductal breast carcinoma and concluded that

a) The presence of MCs in breast cancer is correlated with a much lower grade of tumor.
b) Also stated that there was a positive correlation between ER receptor positivity and the presence of MCs in the stroma of breast cancer.
c) There was no correlation between presence of stromal MCs and PR positivity, HER2/neu positivity.

Divyarani MN *et al* [3]in their study on Quantitative analysis of MCs in invasive ductal carcinoma using toluidine blue technique in 55 cases found that a higher number of MCs were seen in tumors of lower grade suggesting the protective role of MCs in breast cancers.

Rajput AB *et al*[7] studied prognostic significance of MCs in invasive ductal carcinoma of breast and concluded that stromal MCs infiltration in invasive breast cancer is an independent good prognostic marker and reiterates the critical role of local inflammatory responses in breast cancer prognosis.

Kwon GY *et al*[52] conducted a study in 45 cases of invasive breast carcinoma, evaluated MC and macrophage counts with the microvessel density in invasive breast

carcinoma and concluded the maximum number of MCs and macrophages, contributed for angiogenesis

Glajcar *et al*[53] in their study on relationship of MCs with breast cancer molecular subtypes in 108 cases which were evaluated immunohistochemically by tryptase and chymase for MCs and concluded that MCs act as a positive prognostic markers for breast cancer.

Sang J *et al*[54] studied the association of MC infiltrate with clinical features and molecular subtypes of invasive breast cancer on 219 cases and concluded that MCS are beneficial in inhibiting breast cancer.

Keser SH *et al*[2] conducted a study on 104 invasive ductal carcinoma to know the relationship of MC density with lymphangiogenesis and concluded that MC may accelerate spreading of tumor by suppressing its antitumor immunological response.

MATERIALS AND METHODS

Source of data :

All patients presenting with breast lump to the surgery department and with the clinical diagnosis of invasive carcinoma breast who underwent total or modified mastectomy procedure and core needle biopsy specimens fulfilling the inclusion and exclusion criteria which were received at Department of Pathology from Department of Surgery, BLDE (Deemed to be University) Shri. B. M. Patil Medical College, Hospital and Research centre, Vijayapura.

Study period: 1st January 2016 to 30th June, 2018

Inclusion criteria

Diagnosed cases of invasive ductal carcinoma of breast were included.

Core biopsy specimens of invasive ductal carcinoma of breast also were included.

Exclusion Criteria

Patients on radiotherapy/chemotherapy prior to surgery were excluded.

Procedure

All total or modified mastectomy specimens were collected in 10% buffered formalin. Then the specimens were examined for gross characteristics and the specimen borders were inked with different colours to designate the margins and maintain the orientation. Bread – loafing of the specimens was done at an interval of 1 cm each before keeping it for fixation in 10% NBF (Neutral Buffered Formalin). After overnight fixation, representative sections were given from the tumor site, quadrants and extensive search for lymph nodes was done. After processing of the

tissue paraffin embedding was done and blocks were prepared. 3-4 microns thick sections were cut and stained with Haematoxylin and eosin as a part of routine examination. Histopathological examination and grading was done according to Modified Scarff-Bloom Richardson grading system as mentioned in Table : 1.

ROUTINE HAEMATOXYLIN AND EOSIN (H&E) STAINING PROCEDURE

1. Deparaffinize sections, 2 changes of xylene, 10 minutes each
2. Rehydrate in 2 changes of absolute alcohol, 5 minutes each.
3. 95% alcohol for 2 minutes and 75% alcohol for 2 minutes.
4. Immerse in water for 5 minutes
5. Stain in Hematoxylin solution for 8 – 10 minutes
6. Differentiate in 1% acid alcohol for 30 seconds
7. Rinse in running tap water
8. Bluing in 0.2% ammonia water or saturated lithium carbonate solution for 30 seconds to 1 minute
9. Counterstain in eosin-phloxine solution for 30 seconds to 1 minute
10. Dehydrate through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each.
11. Clear in 2 changes of xylene.
12. Mount in DPX.

INTERPRETATION

- Nuclei --- Blue
- Cytoplasm --- Pink to red

Table 1 : Semiquantitative method for assessing histological grade in breast. From ELSTON AND ELLIS (MODIFIED SCARFF-BLOOM RICHARDSON SCORING SYSTEM)[12]

FEATURES	SCORE
1. Tubule and gland formation	
Majority of tumour (> 75%)	1
Moderate degree (10–75%)	2
Little or none (< 10%)	3
2. Nuclear pleomorphism	
Small, regular uniform cells	1
Moderate increase in size and variability	2
Marked variation	3
3. Mitotic counts	
Dependent on microscope field area	1-3

MITOTIC COUNT / 10 high power fields	
1 point	0-9
2 point	10-19
3 point	>20

Final grading

Grade 1	Total score, 3–5	Well differentiated
Grade 2	Total score, 6 or 7	Moderately differentiated
Grade 3	Total score, 8 or9	Poorly differentiated

For the demonstration of MCs representative tissue sections of 3-4 μm thickness were prepared from formalin fixed, paraffin embedded tissues and stained with 0.1% toluidine blue. Then the slides were scanned for MCs in low power objective and MC count was done in 400X per 10 high power fields and results were noted. The counting of MCs was done in Labomed LX300i with a field diameter of 0.45 mm.

Procedure:

1. Deparaffinize and hydrate sections to distilled water.
2. Stain sections in toluidine blue working solution for 1 minute.
3. Wash in distilled water.
4. Dehydrate quickly through 95% and 2 changes of 100% alcohol (10 dips each since stain fades quickly in alcohol).
5. Clear in xylene or xylene substitute, 2 changes, 3 minutes each.
6. Coverslip with resinous mounting medium.

Results:

Mast cells ---------- Violet/red purple.

Background ---------- Blue.

Three additional 3-4 μm thickness sections were taken from the tumor tissue for immunohistochemistry of ER, PR and HER2. ER, PR were scored according to Allred scoring system given in Table: 4.

HER2 scoring was done according to guidelines given from American society of clinical oncology as mentioned in Table: 5.

METHOD OF IMMUNOHISTOCHEMICAL STAINING

Immunohistochemical staining of ER, PR, Her2/neu was done using peroxidase–antiperoxidase method according to the protocol described by DAKO.

- 4 microns thin sections are taken on poly – lysine coated slides.
- Deparaffinization is done by incubating the the slides at 60 ^{0}C for a period of 30 minutes followed by 2 changes of xylene 10 min each (20 min)
- The slides are then kept in three changes in increasing graded alcohols for 5 minutes each (15 min).
- Then slides are washed under running tap water for 5 min.
- Antigen retrieval was done with TRIS EDTA (Tri sodium citrate) buffer (pH 6.0 to 6.2) in pressure cooker 5 minutes in a medium flame.
- Tris buffer (1.21 g of Tris Hydroxy methyl methylamine and 0.37 g of EDTA in 1000 ml distilled water). Ph should be maintained at 8 – 9.
- Slides were allowed to come to room temperature .
- Wash the slides in distilled water for 5 min.
- Slides were then washed with TBS buffer (9.6 g of Tris Hydroxy methyl methylamine and 8.6 g of NaCl in 1000 ml distilled water) pH 7.4-7.6.
- Endogenous peroxidase activity is quenched by covering the slides with 3% H2O2 for 5 min.
- Slides were washed in wash buffer,2 changes for 2 minutes.
- Incubated with Primary antibody (ER, PR, HER 2/neu) which is ready to use, at room temperature in a humidifier chamber for 20 - 30 minutes.
    - The sections were washed again with wash buffer, 2 times for 2 minutes.

- Incubated with secondary antibody - Polymer HRP in a humidifier chamber for 20 minutes
    - The sections were again washed with TBS buffer 2 times for 2 minutes.
- DAB Chromogen was applied for 5 minutes at room temperature for detection of enzymatic activity.
- The sections were again washed with TBS buffer 2 times for 2 minutes.
    - Counter staining was done with Harris Haematoxylin for 30 seconds and then washed with tap water
- Dehydrate in alcohol and cleared in xylene.
- Slides were finally mounted with DPX.

Results:

ER – Nuclear positivity

PR – Nuclear positivity

HER 2- Membrane positivity

Immunohistochemical Scoring System for ER, PR, and HER2/neu [55]

Allred system of scoring for estrogen receptor and progesterone receptor

ER and PR both are nuclear receptors. In Allred system of scoring, depending on the proportion of cells stained, score 0-5 is given (proportion score [PS]) Table 2 and depending on the intensity of staining, score 0-3 is given (intensity score [IS]) Table 3. By adding the PS and IS, we can calculate the final Allred score (PS + IS = AS) Table 4.

Table 2 : Proportion Score

Score	Percentage of stained cells
0	No cells are ER positive
1	≤1% cells are ER positive
2	1-10% cells are ER positive
3	11-33% cells are ER positive
4	34-66% cells are ER positive
5	67-100% cells are ER positive

Table 3 : Intensity Score

Score	Intensity of staining
0	Negative
1	Weak
2	Intermediate
3	Strong

Table 4 : Allred Score (Allred score=Proportion Score + Intensity Score)

Allred score	Effect of hormone therapy
0-1	No effect
2-3	Small (20%) chance of benefit
4-6	Moderate (50%) chance of benefit
7-8	Good (75%) chance of benefit

Table 5 : Immunohistochemistry Scoring Method for HER2[21]

Score	**HER-2 Overexpression**	**Assessment Protein Staining Pattern**
0	Negative	No staining is observed, or membrane staining in fewer than 10% of tumor cells.
1+	Negative	A faint or barely perceptible membrane staining is detected in more than 10% of tumor cells. The cells are only stained in part of the membrane.
2+	Borderline	A weak to moderate complete membrane staining is observed in more than 30% of tumor cells.
3+	Positive	A strong complete membrane staining is observed in more than 30% of the tumor cells.

Sample Size :

Among all the histological types of breast carcinomas, invasive ductal carcinoma is the most prevalent type with frequency of about 83%[1] at 99% confidence interval and 15% of allowable error the sample size worked out was 60.

Using formula:

$$n = \frac{(Z\alpha)2\ p \times q}{L2}$$

Zα= α value at 99%

P = Incidence rate of most prevalent type of invasive ductal carcinoma..

q = 100-p

L = Allowable error =15%

Hence 60 invasive ductal carcinoma cases were included in the study.

Statistical analysis:

Data was analyzed using

1. Diagrams

2. Mean ± S.D

3. ANOVA test was used to describe the association between mast cell density and histological grade.

RESULTS

The purpose of this study was to analyse MCs in different grades of invasive ductal carcinoma with respect to ER, PR and HER2/neu.

The study covered 60 total mastectomy cases which were received in Shri. B. M. Patil Medical Hospital between 1st January, 2016 to 30th June, 2018.

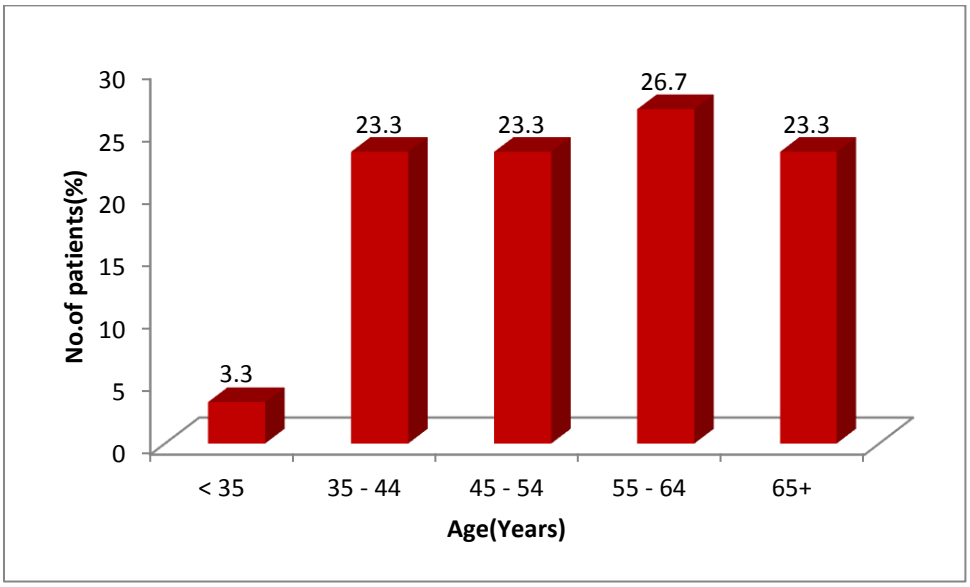
DISTRIBUTION OF CASES ACCORDING TO AGE

Amongst the 60 cases included in the study, age range was from 25 years to 75 years with a mean age of 52 years. Majority of the patients (n=16) were of the age group 55-64 years and there were equal number of cases in 35-44, 45-54 and age more than 65 years, each amounting to 14 cases. The detailed representation of these cases is as follows in the Table 6 and Figure 10.

Table 6 : Distribution of cases according to Age

Age(Years)	No. of Patients	Percentage
< 35	2	3.3
35 - 44	14	23.3
45 - 54	14	23.3
55 - 64	16	26.7
65+	14	23.3
Total	60	100.0

Figure 10 : Distribution of cases according to Age



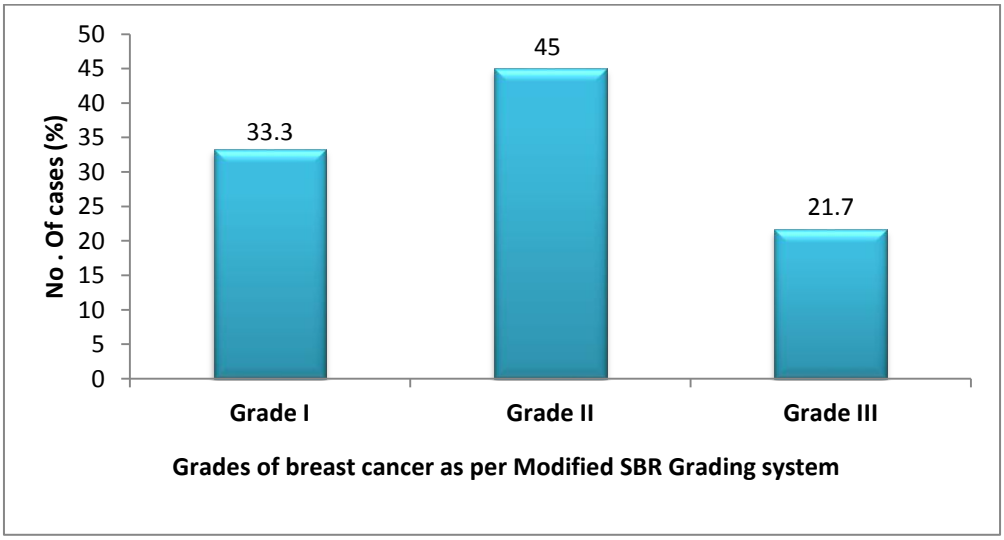
Distribution of cases according to Modified Scarff-Bloom Richardson Grading

The cases were analysed using the modified Scarff-Bloom Richardson Grading as mentioned earlier and the distribution of the cases was represented in the Table 7. With 45% (n= 27), Grade II had the maximum number of cases followed by Grade I and III. The detailed analysis is represented in Table 7 and Figure 11.

Table 7 : Distribution of cases according to Modified SBR Grading

Grade	No. of cases	Percentage (%)
I	20	33.3
II	27	45.0
III	13	21.7
Total	60	100.0

Figure 11 : Distribution of cases according to Modified SBR Grading



Association between mean MCs and Grade of the tumor

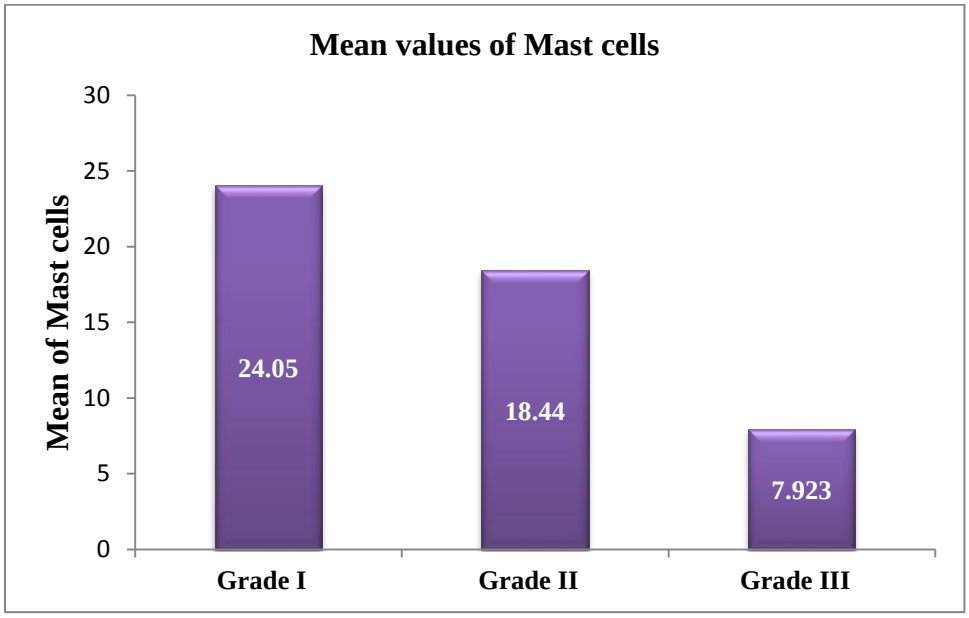
Once the cases were graded as per the Modified Scarff-Bloom Richardson Grading, estimation of MC count was done by counting MCs in 400X magnification in 10 high power fields. The mean mast cell count obtained in such a way was tabulated and the cases falling into Grade I had the highest number of MCs counted with a mean value of 24.05 in 10 high power fields (HPF). The mean values of MCs for the Grade II and III were 18.4 and 7.9 respectively. The range of MCs counted in all the cases was from 0-70 cells per 10 HPF. Using Kruskal Walli's test a p value of 0.0001 was obtained which was statistically significant. The detailed analysis is represented in the Table 8.

Table 8 : Average count of mast cells in IDC samples

Modified SBR Grading	Mean(Median) ± of mast cells	SD	Kruskal walli's test
Grade I (n=20)	24.05(20)	13.92	P=0.0001*
Grade II (n=27)	18.44(11)	18.339	
Grade III (n=13)	7.923(7)	9.15	

Note:-* Indicates Significant

Figure 12 : Average count of mast cells in IDC samples



Distribution of cases according to ER, PR and HER2/neu

As per the protocol outlined (inclusion criteria), immunohistochemistry with ER, PR and HER2/neu was done for all the cases. In evaluating the cases, Allred system was used for scoring ER into positive and negative. ER positivity was found in 60% of the cases. Using the Allred scoring system, PR positivity was in 55% of the cases.

HER2/neu positivity was noted in 46.7% of the cases. The detailed tabulation and graphical representation of the analysis is shown in Tables 9-11 and Figure 13.

Table 9 : Distribution of cases according to ER

ER	No. of cases	Percentage
Negative	24	40
Positive	36	60
Total	60	100.0

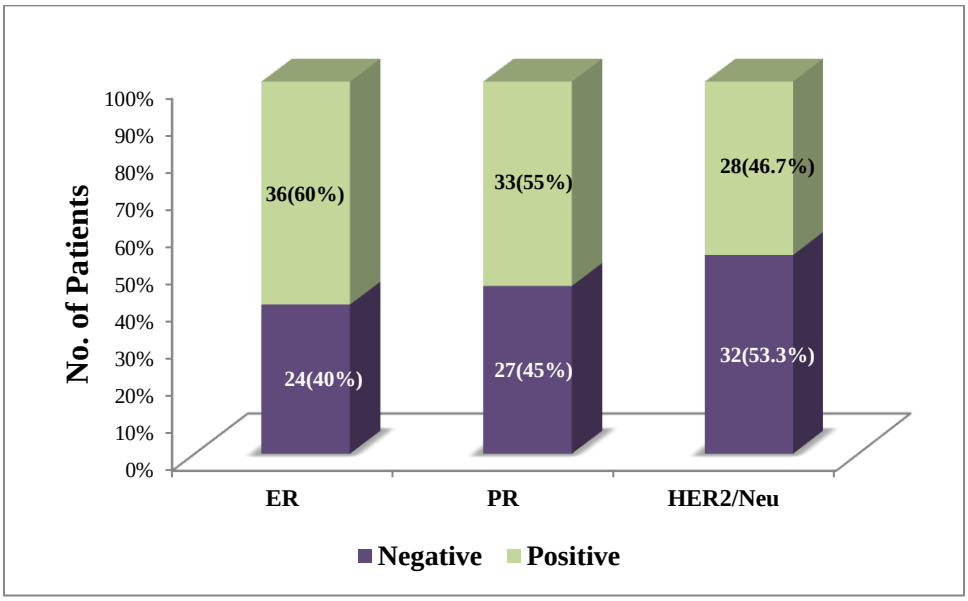
Table 10 : Distribution of cases according to PR

PR	No. of cases	Percentage
Negative	27	45
Positive	33	55
Total	60	100.0

Table 11 : Distribution of cases according to HER2/neu

HER2/neu	No. of cases	Percentage
Negative	32	53.3
Positive	28	46.7
Total	60	100.0

Figure 13 : Distribution of cases according to ER, PR and HER2/neu



LYMPH NODE STATUS:

At the time of diagnosis, lymph node status was evaluated for the presence or absence of metastasis. Just over half of the cases (n=31) had the metastasis detected in lymph nodes and 29 cases were free of tumor deposits. The presence of lymph node metastasis and MC count was analyzed, which revealed that 60% of the patients with out MCs were found to have the metastasis. Further details of which are presented in Table 12, 13 & Figure 14.

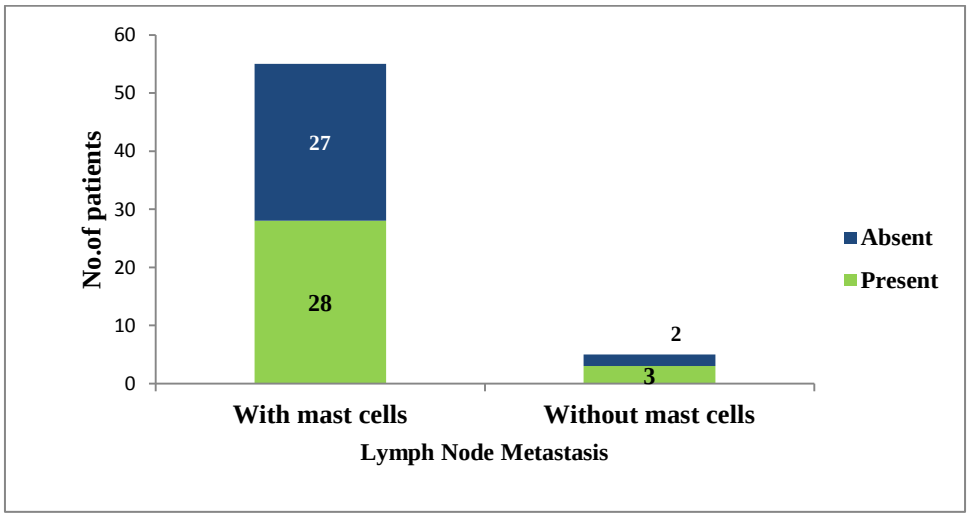
Table 12 : Distribution of cases according to Lymph Node Status

	No. of cases	Percentage
Metastasis present	31	52
Metastasis absent	29	48
Total	60	100.0

Table 13 : Association between MCs and Lymph Node Status

Lymph Nodes / Mast Cell	Positive N(%)	Negative N(%)	Total
With Mast Cell	28(51)	27(49)	55
Without Mast Cell	3(60)	2(40)	5
Total	31	29	60

Figure 14 : Distribution of cases according to Lymph Node Status



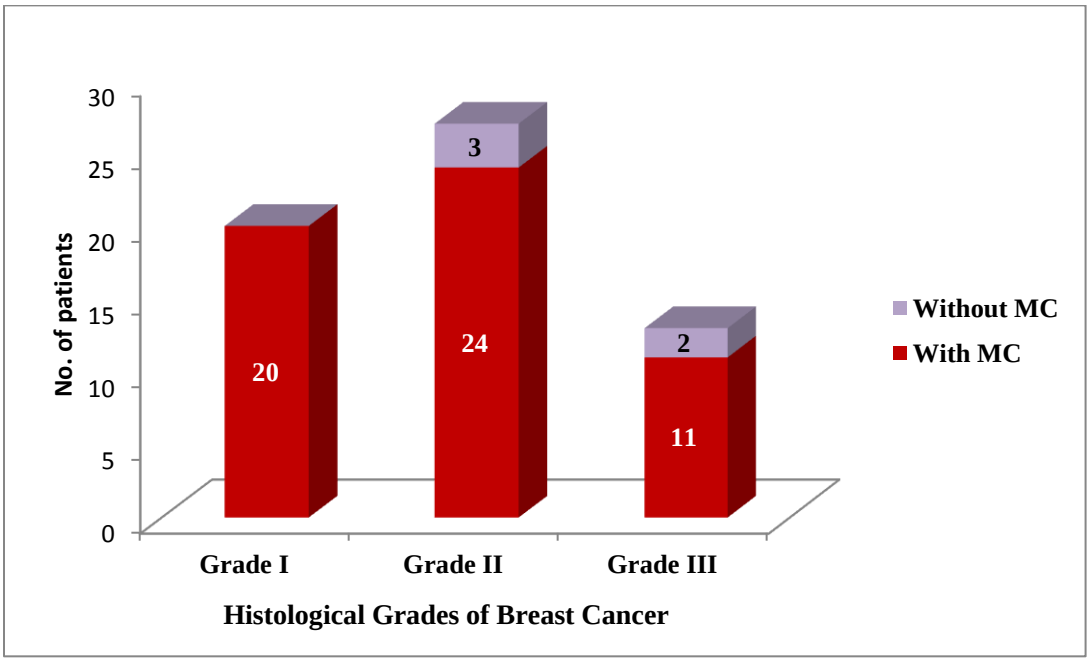
Association Analysis:

In Grade II 3 cases (11%) and in Grade III 2 cases (15%) MCs were found to be absent. In Grade I MCs were found in all the cases. However, p value is not significant.

Table 14 : Association between MCs and histological grade in breast cancer

Grades / Mast Cell	Grade I N(%)	Grade II N(%)	Grade III N(%)	Total	Fisher's Exact test
With Mast Cell	20(100)	24(89)	11(85)	55	P=0.2303 NS
Without Mast Cell	0	3(11)	2(15)	5	
Total	20	27	13	60	

Figure 15 : Association between MCs and histological grade in breast cancer

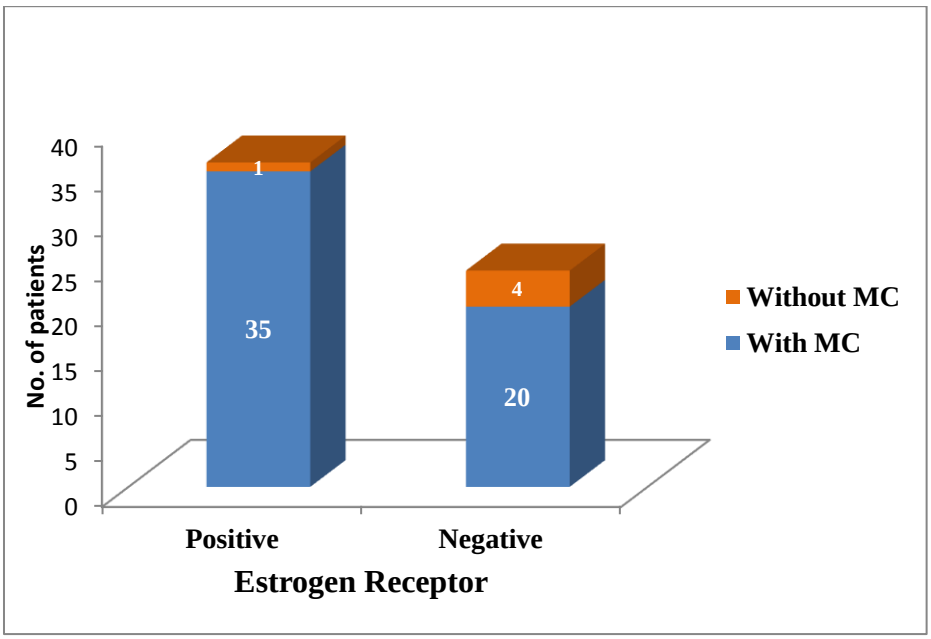


Out of 36 cases with ER positive 35 (97%) cases showed the presence of MCs and in 1 (3%) case MCs was absent. ER negative out of 24 cases , 20 (83%) showed presence of MCs and 4 cases (17%) without MCs. However p value is not significant.

Table 15 : Association between MCs and ER in breast cancer

ER / Mast Cell	Positive N(%)	Negative N(%)	Total	Fisher's Exact test
With Mast Cell	35(97)	20(83)	55	P=0.1469 NS
Without Mast Cell	1(3)	4(17)	5	
Total	36	24	60	

Figure 16 : Association between MCs and ER in breast cancer

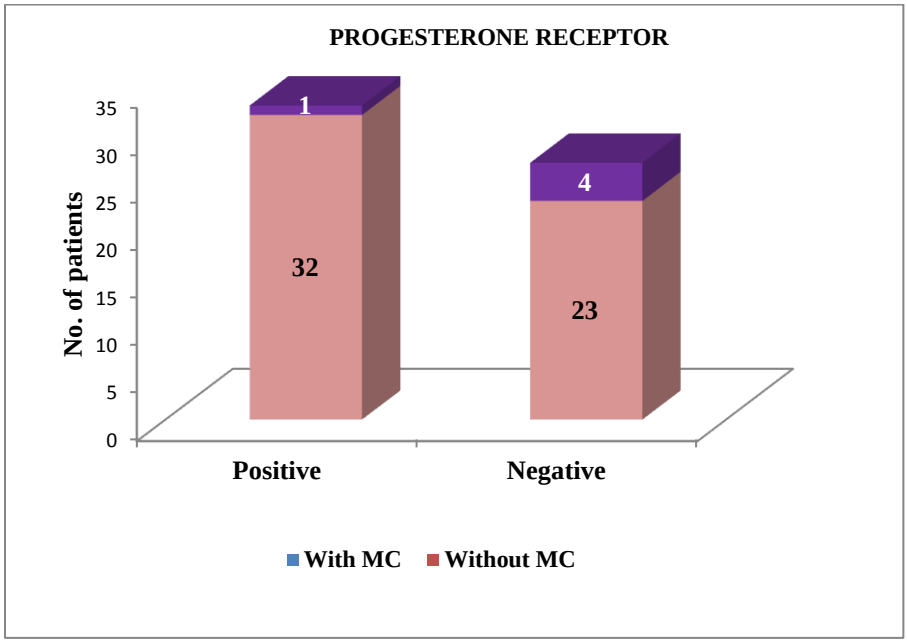


Out of 33 cases with PR positive 32 cases (97%) showed the presence of MCs and in 1 case (03%) MCs were absent. PR negative out of 27 cases , 23 cases (85%) showed presence of MCs and 4 cases (15%) without MCs. However p value is not significant.

Table 16 : Association between MCs and PR in breast cancer

PR / Mast Cell	Positive N(%)	Negative N(%)	Total	Fisher's Exact test
With Mast Cell	32(97)	23(85)	55	P=0.1643 NS
Without Mast Cell	1(3)	4(15)	5	
Total	33	27	60	

Figure 17 : Association between MCs and PR in breast cancer



Out of 28 cases with HER2/neu receptor positive 27 cases (96%) showed the presence of MCs and in 1 case (04%) MCs were absent. HER2/neu receptor negative out of 32 cases , 28 cases (87%) showed presence of MCs and 4 cases (13%) without. However p value is not significant.

Table 17 : Association between MCs and HER2/neu in breast cancer

HER2/neu / Mast Cell	Positive N(%)	Negative N(%)	Total	Fisher's Exact test
With Mast Cell	27(96)	28(87)	55	P=0.3592 NS
Without Mast Cell	1(4)	4(13)	5	
Total	28	32	60	

Figure 18 : Association between MCs and HER2/neu in breast cancer

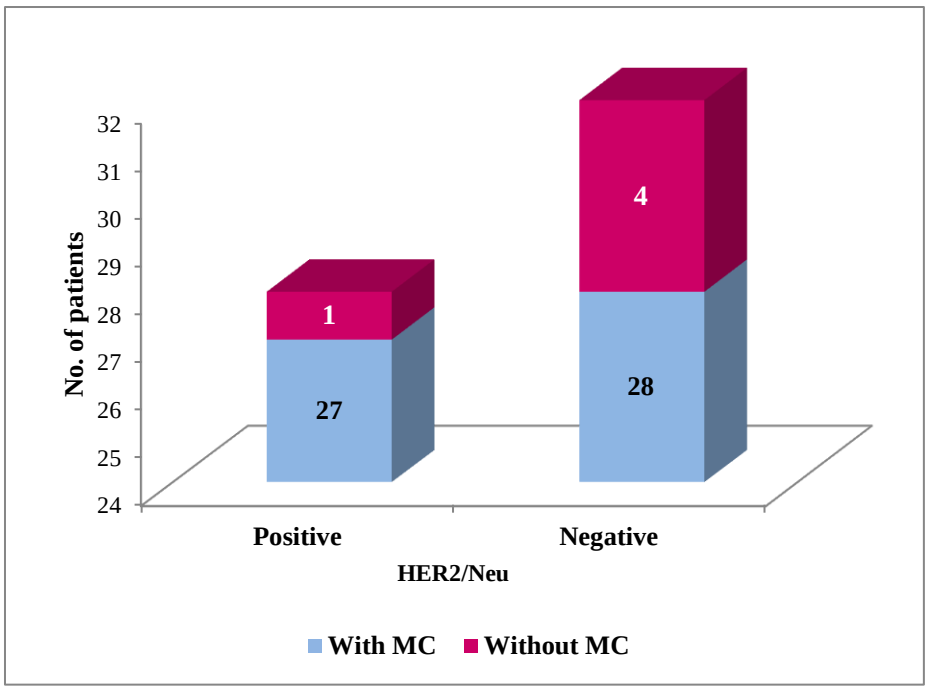


Table 18 : Correlation between ER, PR , HER 2/neu and MCs in breast cancer

Variables	Mean(Median)±SD of mast cells		Mann whitney
	Positive	Negative	U test
ER	23.55(18)±17.02	9.75(8.0)±10.64	U=172.54 p<0.0001*
PR	24.18(18)±18.09	10.52(10)±9.292	U=235.50 p=0.0019*
HER 2/neu	20.82(12)±17.99	15.59(11.5)±14.301	U=378.00 p=0.3030 NS

Note:-* Indicates Significant difference NS:-Indicates No significant difference

Mean of the MCs were correlated with the ER, PR and HER2/neu receptor status. Out of 36 cases with ER positivity mean MCs is 23.55 with a p value of <0.0001 indicating statistical significance. Out of 33 cases positive for PR mean of MCs is 24.18, with a p value of 0.0019 which is statistically significant. For HER2/neu positive cases were 28 with a mean MC count of 20.82 which was not statistically significant.

GRADE 1

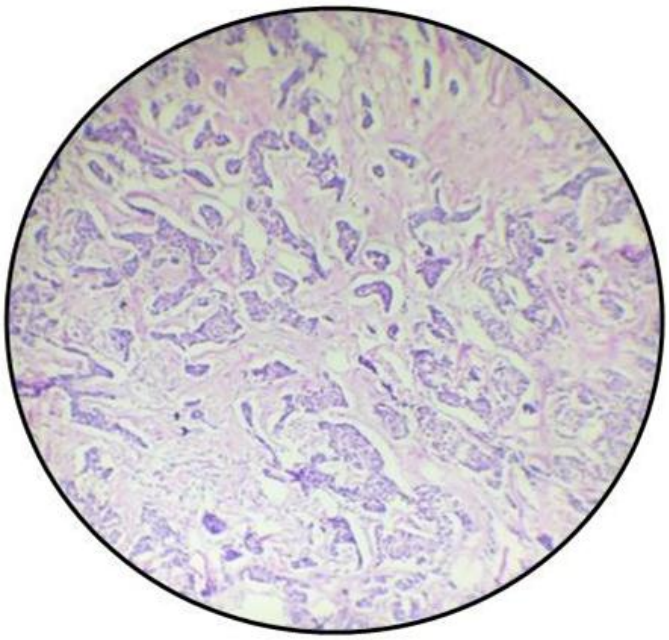


Figure 19 : Microphotograph of Invasive Carcinoma NOS Grade I (H & E, 100X)

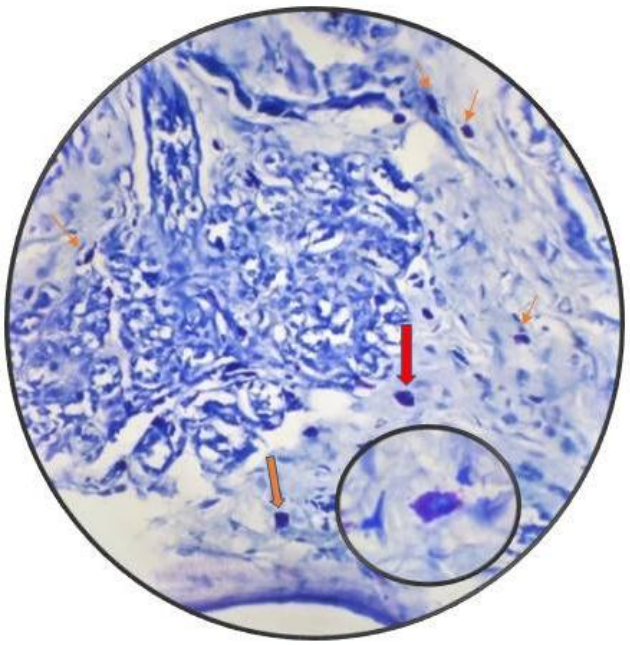


Figure 20 : Microphotograph showing mast cells (arrow marks) in peritumoral area of Grade I with a mean MC count of 58 (Toluidine blue stain, 400X) Inset showing mast cell with purplish pink granules.

GRADE II

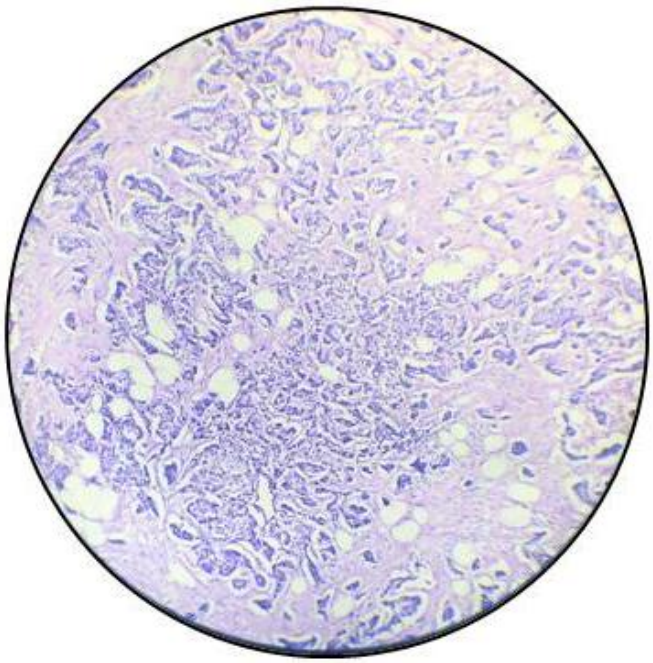


Figure 21 : Microphotograph of Invasive Carcinoma NOS Grade II (H & E, 100X)

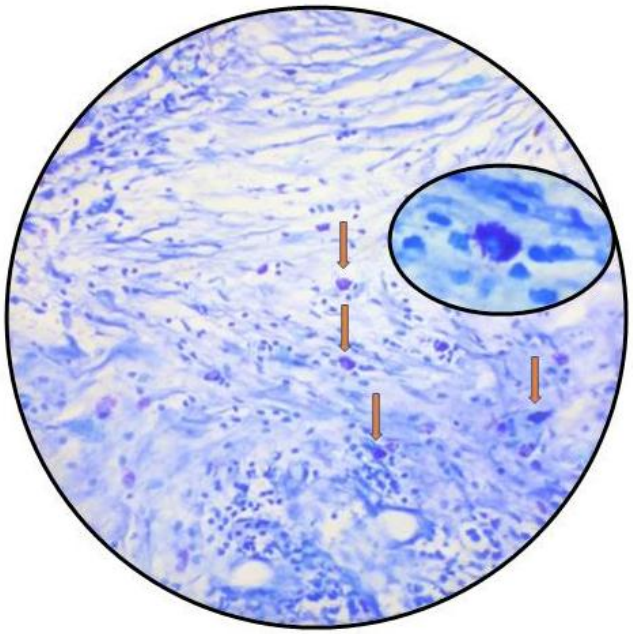


Figure 22 : Microphotograph showing mast cells (arrow marks) in peritumoral area of Grade II with a mean MC count of 36 (Toluidine blue stain, 400X) Inset showing mast cell with purplish pink granules.

GRADE III

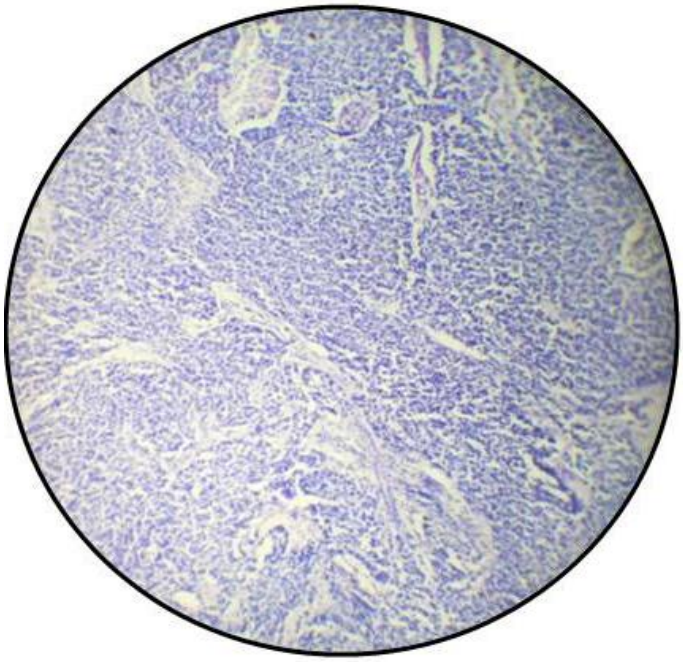


Figure 23 : Microphotograph of Invasive Carcinoma NOS Grade III (H & E, 100X)

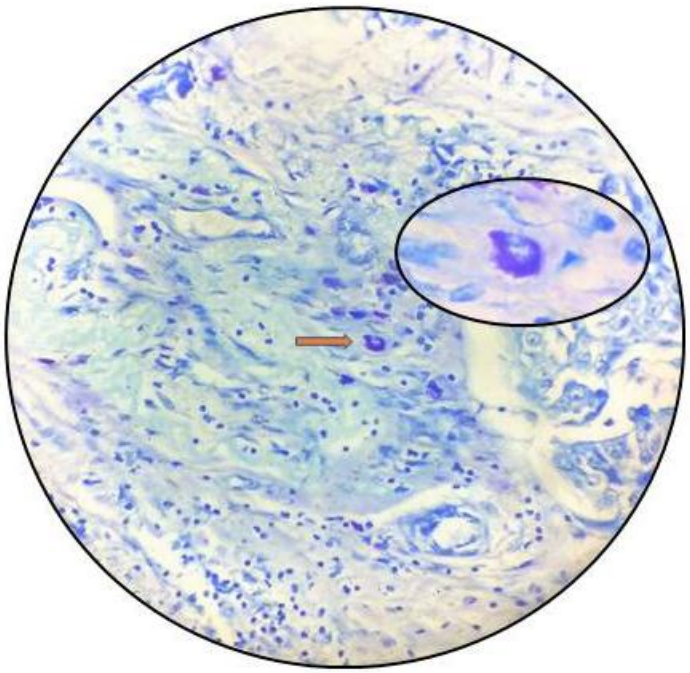


Figure 24 : Microphotograph showing mast cells (arrow marks) in peritumoral area of Grade III with a mean MC count of 09 (Toluidine blue stain, 400X) Inset showing mast cell with purplish pink granules.

ESTROGEN RECEPTOR

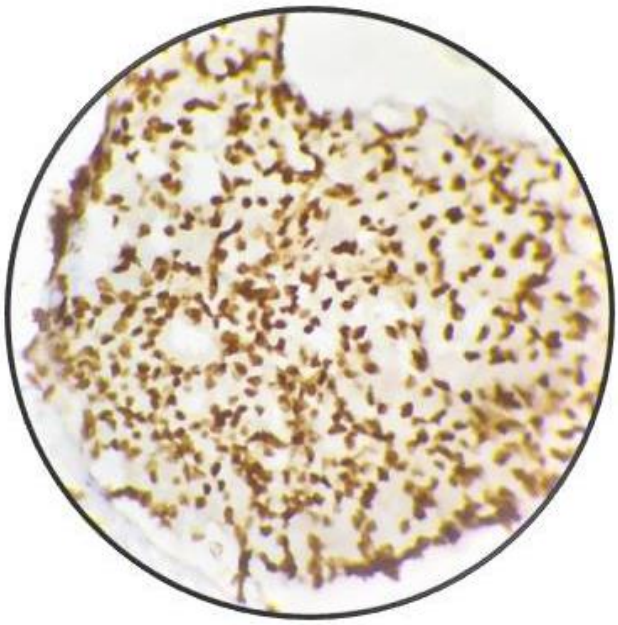


Figure 25 : Microphotograph showing ER Nuclear positivity (100X) with Allred score of 8.

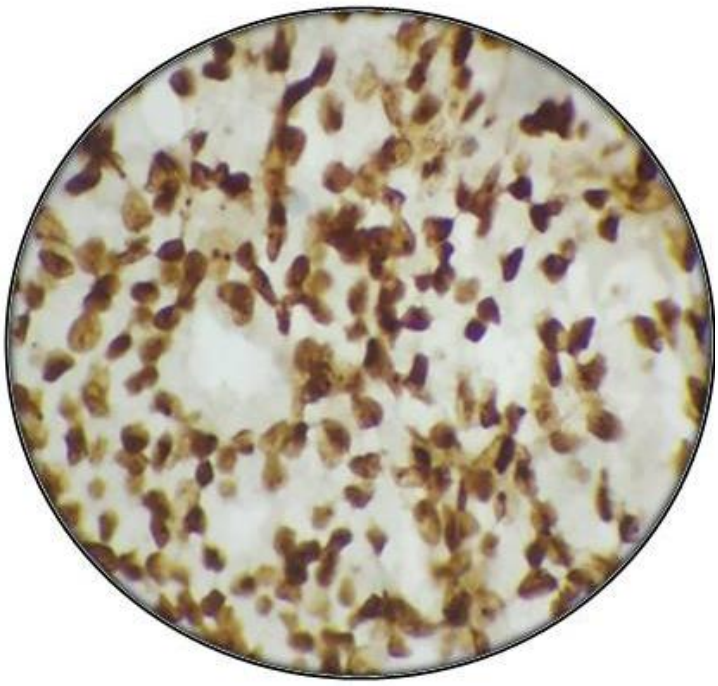


Figure 26 : Microphotograph showing ER Nuclear positivity (400X)

PROGESTERONE RECEPTOR

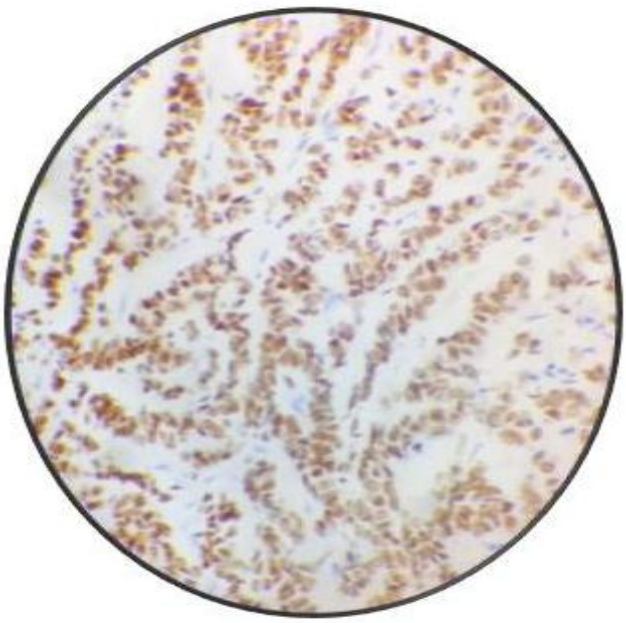


Figure 27 : Microphotograph showing PR Nuclear positivity (100X) with Allred score of 8

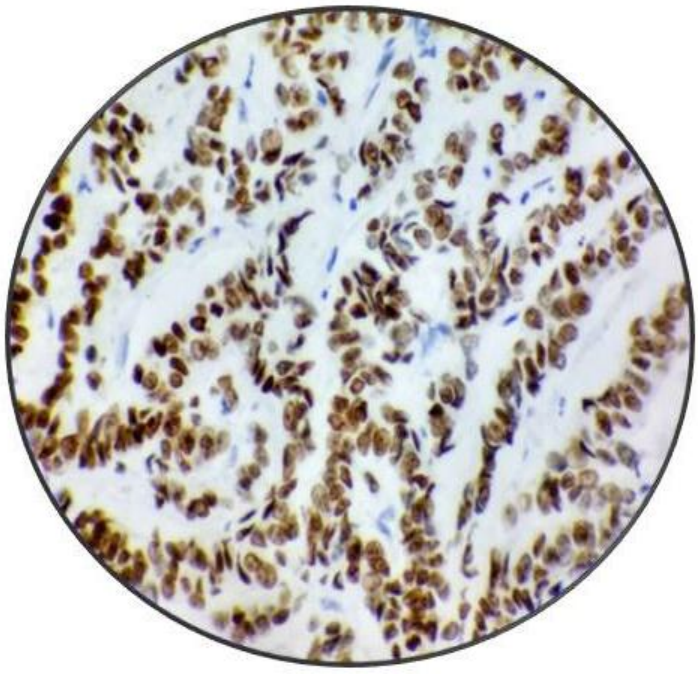


Figure 28 : Microphotograph showing PR Nuclear positivity (400X)

HER 2/ neu

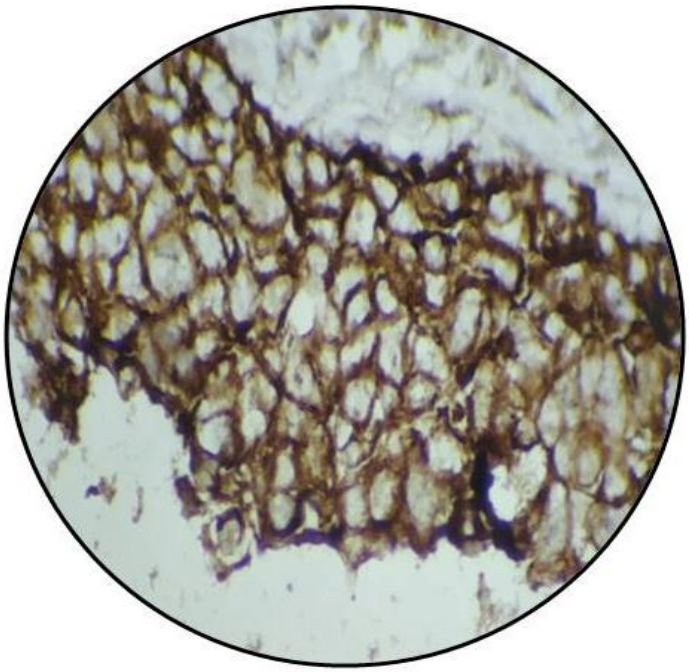


Figure 29 : Microphotograph showing Membrane positivity of HER2/neu (400X) with score of 3+

DISCUSSION

Breast cancer is one of the most common cancer in females. With the advancing investigative modalities in breast cancer, even the management is evolving with a requirement for the precise grading and accurate pathological diagnosis aided by various / molecular techniques. A better understanding of the factors that influence the behavior of tumor and disease course is gaining importance in today's practice.[52]

Thus paving way for several new prognostic markers which are being identified for breast cancer. This has also become a prerequisite currently, as the treatment guidelines recommend adjuvant therapy for management of many sub types of breast cancer.[9]

To better understand the reciprocal relationship between the tumor and role of stromal inflammatory cells like MCs, fibroblasts, macrophages, lymphocytes and T cell subtypes in initiation and progression of cancer many researchers are actively investigating this topic. However, there are very few studies that have been done on invasive breast cancer for demonstration of stromal MCs. The present study has looked into this particular aspect to know the relationship of MCs and invasive carcinoma of breast and correlation of stromal MCs with positivity of ER, PR and HER2/neu.[3,7]

Age distribution for carcinoma breast:

Amongst the 60 cases diagnosed as invasive ductal carcinoma on histomorphological examination with an age range of 55-64 years and with a mean age of 52 years. More or less the age incidence was similar as noted by Fakhrjou *et al* [1] and Divyarani *et al*[3] with cases found between 40-50 years and a mean age of 52 years respectively.

Association of grades in cancer breast:

All invasive ductal carcinoma cases were graded using Modified SBR grading system into Grade I, II and III, with maximum number of cases in Grade II followed by Grade I and III. Similar findings were observed by Divyarani *et al*[3] However, in Heidarpour *et al*[9] Grade I cases and Glajcar *et al*[53] noted that Grade III cases were more. Fakhrjou *et al*[1] has taken equal number of cases in all the three grades. Table 19 represents the comparison of cases in the three grades.

Table 19 : Comparison of number of cases according to Modified SBR grades in various studies

	Present study	**Fakhrjou *et al*[1]**	**Divyarani *et al*[3]**	**Heidarpour *et al*[9]**	**Glajcar *et al*[53]**
Grade I	33.3% (20)	33.3% (25)	20% (11)	50% (54)	15.7% (17)
Grade II	45.0% (27)	33.3% (25)	69% (38)	14.8% (16)	34.2% (37)
Grade III	21.7% (13)	33.3% (25)	10.9% (6)	35.2% (38)	50% (54)
Total	60	75	55	108	108

MCs contain granules that can be stained by metachromatic stains such as toluidine blue and Giemsa stain in which MCs appear as round and purple cells. MCs synthesise factors such as IL-8, heparin and vascular endothelial growth factor which promotes neovascularisation, suppresses immune response by histamine and proteases that helps in metastasis. MCs are detrimental to tumor growth by synthesising cytotoxic substance endogenous peroxidase, inhibit tumor growth by cytokine release like IL-4, IL-1, IL-6 and Tumor necrosis factor α.[7]

Typtase synthesised by MCs promotes fibroblast recruitment leads to tumor fibrosis thus limiting growth of tumor and metastasis.[7]

Stains used for MCs identification:

In the present study MCs were stained with toluidine blue which were identified by the metachromatic reaction, which was also followed in the studies done by Fakhrjou *et al*[1] and Divyarani *et al*[3] Giemsa, Alcian blue and Tryptase were used to demonstrate MCs by Rovere *et al*[4], Amini *et al*[51]and Glajcar *et al*[53]. c-KIT (CD-117), Tryptase and Chymase were also used by some of the authors as represented in Table 20.

Table 20 : Various stains used for MC count in various studies

STUDIES BY	Mast cell stain
Present study	Toluidine blue
Fakhrjou *et al*[1], Divyarani *et al*[3]	Toluidine blue
Rovere *et al*[4]	Giemsa and alcian blue stains
Heidarpour *et al*[9]	Giemsa
Rajput *et al*[7]	c- Kit (CD-117)
Amini *et al*[51]	Tryptase
Glajcar *et al*[53]	Tryptase and chymase

Association of mean MCs count in different grades of invasive carcinoma breast:

The relationship of the number of MCs and grades of disease was investigated. MCs were found mainly in tumor stroma adjacent to neoplastic cells and also seen infiltrated within the islands of tumor cells.

In the present study MCs were counted in 400x, in 10 high power fields and mean MC count was seen to be highest in Grade I, followed by Grade II and Grade III. These results correlated with the studies done by Divyarani *et al*(3), Jana S *et al*[56]and Sang J *et al*[54].

In a study conducted by Heidarpour *et al*[9] also showed that the presence of stromal MCs were correlated with the low grade of the tumor.

Maximum number of MCs were seen in low grade and lesser number of MCs were seen as the grade of the tumor increased, suggesting that MCs may have an inhibitory role in development of breast cancer.

Whereas, studies conducted by Fakhrjou *et al*[1] and Kwon *et al*[52] showed that higher number of MCs were seen in Grade III, as increase in number of MCs which are recruited by the tumor cells contributes to angiogenesis that may facilitate in the expansion of primary tumor leading to increase in its proliferative rate.[52]

Table 21 : Comparison of mean MC count with tumor grade in various studies

Tumor grade	**Mean mast cell count**					
	Present study	**Divyarani *et al*(3)**	**Jana s *et al*(56)**	**Glajcar *et al*(53)**	**Fakhrjou *et al*(1)**	**Kwon *et al*(52)**
Grade I	24.05	16.64	13.03	24.41	15.92	25.13
Grade II	18.44	15.79	6.3	23.46	45.32	39.43
Grade III	7.923	12.50	4.44	15.87	67.80	43.07
Total	16.80	15.22	7.92	21.14	43.01	35.87
p value	0.0001	0.69	-	<0.002	-	>0.05

ER, PR and HER2/neu status in invasive carcinoma breast:

This table is showing the number of ER, PR and HER2/neu positive and negative cases in various studies. In the present study Allred score was used for scoring ER and PR positive and negative cases. In our study ER Positive cases are 36(60%) and negative are24 (40%). PR positive are 33 (55%) and negative are27 (45%). HER2 positive and negative cases 28(32%) and 32(53%) respectively. The results of various other studies are given in Table 22.

Table 22 : Comparison of ER, PR and HER 2 with various studies

		Present study	**Keser s *et al*(2)**	**Sang J *et al*(54)**	**Mohammed *et al*(10)**	**Heidarpour *et al*(9)**	**Amini *et al*(51)**
ER	**Positive**	60%(36)	67.3%(70)	67.1%(147)	61%(283)	49.1% (53)	76% (179)
	Negative	40%(24)	32.7%(34)	32.9%(63)	34%(159)	50.9% (55)	19% (44)
PR	**Positive**	55%(33)	67.3%(70)	65.3%(143)	43%(202)	44.4% (48)	68% (159)
	Negative	45%(27)	32.7%(34)	34.7%(67)	52%(243)	55.6% (60)	28% (65)
HER 2	**Positive**	32%(28)	63.6%(66)	23.7%(52)	16%(73)	73.1% (73.1)	-
	Negative	53.3%(32)	36.5%(38)	76.3%(158)	79%(370)	26.9% (26.9)	-

Lymph node status in invasive carcinoma of breast:

In the present study lymph node metastasis was seen in 52% (31 cases) at the time of diagnosis and negative in 48% (29 cases) which was comparable to Sang J *et al*[54] and Amini *et al*[51] . The results of various other studies are given in Table 23.

Table 23 : Comparison of lymph node metastasis with other study

		Present study	Sang J *et al*[54]	Keser *et al*[2]	Amini *et al*[51]	Heidarpour *et al*[9]
Lymph node metastasis	**Positive**	52% (31)	58.4 %(128)	67.3 %(70)	50% (118)	36.1% (39)
	Negative	48% (29)	41.6% (91)	32.6% (34)	49% (114)	63.9 (69)

Association of Mean MC count with ER, PR and HER2

The mean values of MCs are compared with the ER, PR and HER2 status of carcinoma breast in our study. The mean MC count is more in ER, PR positive cases when compared to ER, PR negative cases and the p value is < 0.0001 which is statistically significant.

Table 24 : Comparison of mean MC count with hormonal profiles

		Present study	Kwon *et al*[52]
ER	**Positive**	23.55	27.19
	Negative	9.75	44.35
	P value	<0.0001	0.010
PR	**Positive**	24.18	29.83
	Negative	10.52	48.92
	P value	0.0019	0.005
HER 2	**Positive**	20.82	30.92
	Negative	15.59	25.00
	P value	0.3030	> 0.05

In a study done by Sang J *et al*[54], the mean MC density was higher in ER and PR positive cases when compared to ER and PR negative cases.

Heidarpour *et al*[9] has compared the MC percentage with ER and PR status. According to them MC percentage was more in ER positive cases and there was no correlation between MC percentage with PR and HER2 status.

As ER and PR positivity is associated with good prognosis, more number of MCs among these cases indicates a better prognosis.

Study done by Kwon *et al*[52] showed that the mean MCs are more in ER and PR negative cases. According to them MC contributes in angiogenesis and helps in invasion of tumor cells. So MC count was inversely related to the hormonal status.

In present study mean MC count was higher in HER2 positive cases when compared to negative however the p value is not statistically significant. It is similar to kwon *et al*[52] study.

CONCLUSION

- In our study mean age of the patients was 52 years and maximum number of cases belonged to Grade II, according to modified SBR grading system.
- Our results indicated that, mast cells in breast cancer were inversely proportional to the grade of tumor. A higher number of mast cells were seen in tumors of lower grade suggesting a beneficial role of mast cells in breast carcinoma and therefore, can be used as markers for risk stratification in invasive breast cancer.
- Increased mast cell index in ER and PR receptor positive breast cancer indicates positive correlation between them.
- Mast cells might act as a new target for the adjuvant treatment of breast cancer through the selective inhibition of angiogenesis, and tumor promoting molecules.

SUMMARY

The present study was conducted as a retrospective and prospective study from 1st January 2016 to 30th June 2018 at Department of Pathology in BLDE (Deemed to be University) Shri B.M.Patil Medical College, Hospital and Research centre, Vijayapura.

1. Sixty cases with total/modified radical mastectomy specimens which were diagnosed as invasive ductal carcinoma were included in the study. These total mastectomy specimens were processed after overnight fixation, sections of (4-5 μm) thickness were prepared and stained with H&E.
2. Subsequently they were studied by light microscopy for histologic type, grade and lymph node status. Grading was done according to Modified Scarff-Bloom Richardson grading system into Grade I, II and III.
3. Majority of the cases were in the age group of 55-64 years.
4. Maximum number of cases were in Grade II (45.0%) followed by Grade I (33.3%), and Grade III (21.7%).
5. For the demonstration of Mast Cells representative tissue sections of 3-4 μm thickness were prepared from formalin fixed, paraffin embedded tissues and stained with 0.1% toluidine blue.
6. MCs were counted in the peritumoral area under 400X IN 10 high power fields.
7. Mean MC count was done and the cases with Grade I had the highest number of MCs with a mean value of 24.05. The mean values of MCs for the Grade II and III were 18.4 and 7.9 respectively, with a significant p value.
8. Immunohistochemistry for various prognostic markers like ER, PR and HER2/neu status was done for all the cases. Allred system of scoring was used

for interpretation of ER and PR markers. ER positivity was found in 60% of the cases. PR positivity was in 55% of the cases and 32% of the cases were HER2/neu positive.

9. Out of 36 cases with ER positivity, mean MC count was 23.55 with a p value of <0.0001 indicating statistical significance. Out of 33 cases positive for PR, mean MC count was 24.18, with a statistically significant p value of 0.0019. Where as in 28 HER2 positive cases, the mean MC count was 20.82 with a p value was not statistically significant.

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ANNEXURES

ETHICAL CLEARANCE

B.L.D.E. UNIVERSITY'S

SHRI.B.M.PATIL MEDICAL COLLEGE, BIJAPUR-586 103

INSTITUTIONAL ETHICAL COMMITTEE

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this college met on 04/10/2016 *at* 3-00pm *to scrutinize the Synopsis of Postgraduate Students of this college from Ethical Clearance point of view. After scrutiny the following original/corrected & revised version synopsis of the Thesis has been accorded Ethical Clearance.*

Title Quantitative analysis of mast cell in invasive ductal carcinoma of Breast

Name of P.G. student Ramadevi Pyla

Dept of Pathology

Name of Guide/Co-investigator Dr R.M. Potekar

Professor of Pathology.

DR.TEJASWINI. VALLABHA
CHAIRMAN
INSTITUTIONAL ETHICAL COMMITTEE
BLDEU'S, SHRI.B.M.PATIL
MEDICAL COLLEGE, BIJAPUR.

Following documents were placed before E.C. for Scrutinization
1) Copy of Synopsis/Research project.
2) Copy of informed consent form
3) Any other relevant documents.

B.L.D.E (DEEMED TO BE UNIVERSITY),

SHRI B.M.PATIL MEDICAL COLLEGE HOSPITAL AND RESEARCH CENTER, VIJAYAPURA-586103

INFORMED CONSENT FOR PARTICIPATION IN DISSERTATION/RESEARCH

I, the undersigned, S/O D/O W/O---------- , aged -- years, ordinarily resident of ---------- do hereby state/declare that Dr Rama Devi Pyla of Shri B M Patil Medical College, Hospital has examined me thoroughly on at (place) and it has been explained to me in my own language that I am suffering from ____disease (condition) and this disease/condition mimic following diseases . Further Doctor informed me that she is conducting dissertation/research titled "Quantitative analysis of mast cell in invasive ductal carcinoma of breast" under the guidance of Dr. R M Potekar requesting my participation in the study. Apart from routine treatment procedure the pre-operative, operative, post-operative and follow-up observations will be utilized for the study as reference data.

Doctor has also informed me that during conduct of this procedure _____like adverse results may be encountered. Among the procedure related complications most of them are treatable but are not anticipated. Further Doctor has informed me that my participation in this study help in evaluation of the results of the study which is useful reference to treatment of other similar cases in near future, and also I may be benefited in getting relieved of suffering or cure of the disease I am suffering.

The Doctor has also informed me that information given by me, observations made/ photographs/ video graphs taken upon me by the investigator will be kept secret and not accessed by the person other than me or my legal hirer except for academic purposes.

The Doctor did inform me that though my participation is purely voluntary, based on information given by me, I can ask any clarification during the course of treatment / study related to diagnosis, procedure of treatment, result of treatment or prognosis. At the same time I have been informed that I can withdraw from this study at any time if I want or the investigator can terminate me from the study at any time from the study but not the procedure of treatment and follow-up unless I request to be discharged.

After understanding the nature of dissertation or research, diagnosis made, mode of treatment, I the undersigned Shri/Smt _____under my full conscious state of mind agree to participate in the said research/dissertation.

Signature of patient:

Signature of doctor:

Witness: 1.

2.

Date:

Place

PROFORMA

• **Demographic Details:** **Date:**

Name: Age :

Religion : Occupation :

Residence :

• **OPD / IPD No. :**

• **Lab. No. /Sample No. :**

• **Chief complaints:**

• **History of present illness:**

• **Past history:**

• **Family history:**

• **General physical examination:**

• Pallor : Icterus :

• Built : Nourishment :

• **VITALS:** PR: RR:

BP: TEMPERATURE:

Systemic examination:

1. Cardiovascular system

2. Respiratory system

3. Central Nervous System

4. Per Abdomen Examination

- Local examination:
- Location :
- Size :
- Shape :
- Surface :
- Consistency :
- Mobility:
- Fixity to underlying structures:
- Lymph node involvement:
- Clinical diagnosis:

• **Investigations:**

FNAC:

MAMMOGRAPHY:

• **Histopathological Diagnosis**:

Grade

Mast cell index

ER positivity

PR positivity

HER2/neu positivity

KEY TO MASTER CHART

Sl.no	– Serial Number
OP	– Out Patient
IP	– In patient
F	– Female
HPR	– Histopathology Report
SBR	– Scarff-Bloom-Richardson
MC	– Mast Cell
ER	– Estrogen Receptor
PR	– Progesterone Receptor
LN	– Lymph Node
P	– Positive
N	– Negative
Pr	– Present
Ab	– Absent

MASTER CHART

Sl.no	OP/IPNo	Age (Y)	Gender	HPR NO	SBR Grading system	MC count	ER	PR	HER 2 /neu	LN status
1	IP/268963/16	60	F	4612/16	Grade I	13	N	N	N	Ab
2	IP/27304/16	60	F	5202/16	Grade II	8	N	P	P	Ab
3	IP/24927/16	50	F	4773/16	Grade I	17	P	P	N	Ab
4	IP/255427/16	60	F	4333/16	Grade I	18	P	N	N	Ab
5	IP/226375/16	38	F	3882/16	Grade III	0	N	N	N	Pr
6	IP/15287/16	68	F	3253/16	Grade II	2	P	P	P	Ab
7	IP/15503/16	65	F	3041/16	Grade I	24	P	P	P	Ab
8	IP/14994/16	25	F	2880/16	Grade II	23	P	P	N	Pr
9	IP/14828/16	45	F	2798/16	Grade III	7	N	N	P	Pr
10	IP/149621/16	55	F	2565/16	Grade I	8	P	P	P	Ab
11	IP/2016/16	40	F	2440/16	Grade II	70	P	P	P	Pr
12	IP/10981/16	54	F	2371/16	Grade II	12	P	P	P	Pr

13	IP/8949/16	45	F	1865/16	Grade I	12	P	P	P	Pr
14	IP/9087/16	66	F	1818/16	Grade I	23	P	P	P	Pr
15	IP/66285/16	54	F	1084/16	Grade III	2	P	P	P	Pr
16	IP/3632/16	50	F	791/16	Grade I	40	P	N	P	Ab
17	IP/4313/16	40	F	314/16	Grade I	12	P	P	N	Ab
18	IP/257/16	55	F	139/16	Grade II	10	P	P	P	Pr
19	IP/41822/15	65	F	168/17	Grade I	14	P	N	N	Pr
20	IP/12790/17	50	F	187/17	Grade II	10	N	N	P	Ab
21	IP/3879/17	40	F	857/17	Grade II	48	N	P	N	Pr
22	IP/5665/17	75	F	1246/17	Grade II	38	P	P	P	Pr
23	IP/65615/17	55	F	1442/17	Grade I	11	P	P	N	Pr
24	IP/7274/17	45	F	1575/17	Grade III	6	N	N	N	Pr
25	IP/114593/17	65	F	2162/17	Grade III	9	N	N	N	Pr
26	IP/10778/17	55	F	2330/17	Grade II	58	P	P	P	Pr
27	IP/10963/17	28	F	2358/17	Grade II	4	N	N	N	Pr
28	IP/133874/17	55	F	2591/17	Grade I	7	N	N	N	Pr
29	IP/2017/158496	57	F	3036/17	Grade III	8	P	P	P	Pr

30	IP/2017/15433	38	F	3221/17	Grade II	0	P	P	N	Ab
31	IP/41567/15	60	F	47/16	Grade II	10	N	N	P	Ab
32	IP/10566/16	70	F	154/16	Grade I	40	P	P	P	Pr
33	IP/2017/25567	65	F	5158/17	Grade I	52	P	P	P	Ab
34	IP/2016/4313	44	F	3521/16	Grade II	14	P	N	N	Ab
35	IP/2017/496	50	F	877/17	Grade II	11	P	N	N	Ab
36	IP/2017/93860	40	F	1792/17	Grade I	32	P	P	P	Ab
37	IP/2017/27587	66	F	5524/17	Grade I	17	N	P	P	Ab
38	IP/2017/27587	66	F	5670/17	Grade II	42	P	P	N	Pr
39	IP/2017/30375	60	F	6340/17	Grade III	0	N	N	N	Ab
40	IP/2017/31402	68	F	6342/17	Grade II	8	N	P	N	Ab
41	IP/2017/33281	45	F	6451/17	Grade II	11	P	P	N	Pr
42	IP/2017/34402	45	F	6569/17	Grade II	18	P	P	P	Pr

43	IP/2017/37210	56	F	6995/17	Grade II	0	N	N	P	Pr
44	IP/2017/37670	58	F	7096/17	Grade I	22	P	N	P	Pr
45	OP/2017/375763	43	F	7168/17	Grade II	0	N	N	N	Pr
46	OP/2017/417008	38	F	7915/17	Grade III	12	N	N	N	Pr
47	IP/2017/A/385	65	F	4751/17	Grade II	10	N	N	N	Ab
48	IP/2017/3098	35	F	652/17	Grade II	12	P	N	N	Ab
49	IP/2018/3344	35	F	822/18	Grade III	3	N	N	N	Pr
50	OP/2018/47398	42	F	853/18	Grade II	32	N	N	N	Ab
51	IP/2018/6457	60	F	1183/18	Grade III	12	N	N	P	Pr
52	OP/2018/72795	60	F	1208/18	Grade III	1	N	N	N	Ab
53	OP/2018/84704	40	F	1363/18	Grade I	30	P	P	P	Ab
54	IP/2018/10095	51	F	1885/18	Grade III	8	N	N	N	Ab

55	IP/2018/11810	65	F	2303/18	Grade II	9	P	P	P	Ab
56	IP/2018/12050	60	F	2410/18	Grade I	38	P	P	N	Ab
57	IP/2018/16367	50	F	2946/18	Grade I	51	P	P	N	Ab
58	IP/2018/17166	43	F	3065/18	Grade II	29	P	P	N	Ab
59	OP/2018/181505	68	F	3106/18	Grade II	9	N	N	P	Pr
60	OP/2018/195827	45	F	3386/18	Grade III	35	P	P	N	Pr