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IN
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UNDER THE GUIDANCE OF
DR. MANJULA K, MD
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DEPARTMENT OF PATHOLOGY
SRI DEVARAJ URS MEDICAL COLLEGE, KOLAR
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"A STUDY OF TUMOR ASSOCIATED MACROPHAGES AND THEIR SUBPOPULATION M1 AND M2 BY IMMUNOHISTOCHEMISTRY IN PRIMARY BREAST CARCINOMA"

IN SRI DEVARAJ URS MEDICAL COLLEGE, KOLAR

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List of Abbreviations

IARC – International Agency for Research on Cancer

ER – Estrogen Receptor

PR – Progesterone Receptor

Her 2 – Human epidermal growth factor receptor 2

TAM – Tumor-associated macrophages

VEGF – Vascular endothelial growth factor

CD68 – Cluster of differentiation 68

CD163 – Cluster of differentiation 163

IL - Interleukin

TDLU – Terminal duct lobular unit

WHO – World Health Organisation

DCIS – Ductal carcinoma in situ

GCDFP15 - Gross Cystic Disease Fluid Protein-15

MC – Medullary carcinoma

AJCC – American Joint Committee on Cancer

MBR – Modified Bloom Richardson

TIL – Tumor-infiltrating lymphocytes

LAMP – Lysosomal associated membrane protein

HIF – Hypoxia-Inducible factor

MRD – Medical Records Department

IHC – Immunohistochemistry

H&E – hematoxylin and eosin

NPI – Nottingham Prognostic index

TABLE OF CONTENTS

LIST OF TABLES

16.	Comparison of density of expression of intratumoral and peritumoral CD163 macrophages with the pathological stage.	
17.	Comparison of density of expression of intratumoral and peritumoral CD163 macrophages with grade.	
18.	Comparison of density of expression of intratumoral and peritumoral CD163 macrophages with the tumor size.	
19.	Comparison of density of expression of intratumoral and peritumoral CD163 macrophages with pathological lymph node status.	
20.	Comparison of age distribution with other studies.	
21.	Comparison of the size of the tumor with other studies.	
22.	Comparison of tumor grade with other studies.	
23.	Comparison of T category with other studies.	
24.	Comparison of N staging with other studies.	
25.	Comparison of density of CD68 TAMs with other studies.	
26.	Comparison of density of CD163 TAMs with other studies.	

LIST OF CHARTS

15.	Comparison of density of expression of intratumoral and peritumoral CD163 macrophages with grade.	
16.	Comparison of density of expression of intratumoral and peritumoral CD163 macrophages with the tumor size.	
17.	Comparison of density of expression of intratumoral and peritumoral CD163 macrophages with pathological lymph node status.	

LIST OF FIGURES

17.	Showing high intratumoral distribution of CD68 macrophages. Biopsy No. –B/228/20. H&E stain. 40X	

ABSTRACT

BACKGROUND: One of several major causes of cancer-related deaths in women is breast carcinoma. The macrophage is the primary immune cell present in the tumor microenvironment. They are, therefore, also called tumor-associated macrophages (TAMs). CD68 has a pro-inflammatory and antitumor response. CD163 has an anti-inflammatory response. Collection of intratumoral TAMs correlates with adverse clinical outcomes. Tumor microenvironment targeting helps in reducing tumor burden and improve prognosis.

AIMS AND OBJECTIVES: To analyze the density of expression of CD68 TAMs and CD163 TAMs with intratumoral and peritumoral distribution in primary breast carcinoma and study the association of CD68 TAMs and CD163 TAMs with stage and grade of primary breast carcinoma.

MATERIALS AND METHODS: This is a laboratory-based exploratory study. Data of 55 primary breast carcinoma cases were included in the study, and cases with metastatic tumors from other sites, recurrent lesions, and patients subjected to chemotherapy and radiotherapy were excluded. H&E slides were reviewed. Immunostaining for CD68 and CD163 was performed. The cases were distributed into low and high groups based on cut-off points according to the median. P-value of <0.05 considered statistically significant.

RESULTS: The study demonstrated that the density of CD68 macrophages in the peritumoral area increases as the pathological stage increases and the density of CD68 macrophages in the intratumoral area decreases as the tumor grade increases and is

statistically significant ($p<0.05$). Cancer tissue showed higher CD163 TAMs density than those in normal tissues, but the correlation with pathological stage, grade, and lymph node metastasis is not significant.

CONCLUSION: The density of CD68 macrophages in the peritumoral area increases as the pathological stage increases, and the density of expression of CD68 macrophages in the intratumoral area decreases as the grade of tumor increases. A high density of CD68 macrophages was seen in the peritumoral area if lymph node metastasis is present in the study. A high density of CD68 macrophages expression was seen in the peritumoral area if lymph node metastasis is present and is statistically associated.

KEYWORDS: Breast carcinoma, CD68, CD163, TAMs.

INTRODUCTION

One of several major causes of cancer-related deaths in women is breast carcinoma.[1] Worldwide incidence of breast malignancy varies from 19.3 to 89.7/ 100,000 populations.[2] According to the International Agency for Research on Cancer (IARC), 55.4 percent of all breast carcinomas were invasive breast carcinomas during 2017.[3,4] In India, the mortality rate of breast malignancy in 2015 was 5,69,832. In the Kolar region, in 2010, breast cancer incidence was 6.41%.[5]

The tumor microenvironment comprises both malignant and non-malignant populations of cells. Non-malignant populations include leukocyte infiltrate, proliferating blood vessels, and stromal cells. Stromal cells of the tumor microenvironment are cancer-associated fibroblasts, adipocytes, pericytes, lymphatic, and vascular endothelial cells. Leukocytes comprising the tumor microenvironment are Macrophages, T-lymphocytes, B-Lymphocytes, Natural killer cells. The macrophage is the primary immune cell present in the tumor microenvironment. They are, therefore, also called Tumor-associated macrophages (TAMs). TAMs classify as M1 and M2 depending on their activation mechanism, i.e., Classical and Alternative activation.[6]

CD68 is a pan macrophage marker and plays a crucial role in pro-inflammatory and antitumor response by activating type1 T cell response and by producing free radicals that can damage DNA it has tumoricidal activity.[7] CD163 is highly specific for M2 macrophages. It has an anti-inflammatory response and leads to hypoxia-induced angiogenesis upregulated as the carcinoma progresses to promote metastasis and proliferation.[8] TAMs have been studied thoroughly in Hepatocellular carcinoma, Lung carcinoma, and gastric carcinoma.[9,10,11]

Many studies have postulated that the collection of intratumoral TAMs correlates with adverse clinical outcomes.[12] But still, the prognostic importance of localization and densities of both the TAMs is not well evaluated. Previous studies concluded that TAMs are associated with ER, PR, Her2neu status, stage, grade, and lymph node status. So in breast cancer, TAMs in different locations and densities may have different prognostic values.[12,13,14]

Therapies for breast cancer targets tumor cells themselves. Many studies concluded that tumor microenvironment targeting helps in reducing tumor burden and improve prognosis.[6,15,16]

In breast cancer, TAMs may increase invasion, modulate tumorigenesis by stimulating tumor angiogenesis through vascular endothelial growth factor(VEGF), degrade the extracellular matrix by generating proteases, and leads to repression of the function of CD8+ T cells, which inhibits the tumor growth resulting in poor prognosis.[17]

Results of studies done on breast carcinoma using TAMS are quite variable. They used various markers to assess macrophages. Some of them used only CD68, while others combined both CD68 and CD163.[8,18] Many studies did on stroma and the nest, while others only counted the total TAMs within the tumor proper.[19]

The presence of total tumor-infiltrating lymphocytes and specific CD8+ cytotoxic T cells associates with a successful response to chemotherapy and a significant reduction in the relative risk of death. However, the ability of TAMs to suppress T-cell responses at the interface between tumor and stroma represents a significant obstacle to successful immunotherapy. Macrophages have emerged as an independent co-factor in breast cancer progression and represent an attractive target for breast cancer therapy. Besides, inhibition of tumorigenic factors and mechanisms promoted by TAMs, such as Epithelial growth

factor(EGF) mediated metastasis and cancer stem cells(CSC) support, provides a novel mechanism to treat breast cancer.

This study evaluated the density of CD163 and CD68 in intratumor and peritumor locations and found out the importance of the TAMs location with the grade and stage.

Objectives

- To analyze the density of expression of CD68 TAMs and CD163 TAMs with intratumoral and peritumoral distribution in primary breast carcinoma.

- To study the association of CD68 TAMs and CD163 TAMs with stage and grade of primary breast carcinoma.

REVIEW OF LITERATURE

Normal anatomy

Breast is a modified, specialized apocrine sweat gland that develops along two milk lines extending from the bilateral armpit to the groin. It covers the area from the second to the seventh rib and from the midaxillary line's anterior margin to the sternum's lateral margin. A hyperpigmented, slight extension from the top of the breast is the nipple. It is skin covered and comprises dense fibrous tissue, smooth muscle fiber bundle to make it "erectile" and aid in milk expression. The areola is the hyperpigmented skin surrounding the nipple, and pigmentation increases during pregnancy.[20]

The breast parenchyma contains ducts of 15 to 20 lobes, all of which enter the nipple and dilate to form milk sinuses, bonded together by septa of connective tissue balled as interlobular connective tissue. Each lobe has a pyramidal shape with a base away from the nipple. The suspensory ligaments, connective tissue band reach out from interlobular connective tissue and gets attached to the dermis.[21]

There is a separate main duct, the lactiferous duct, to drain each ductal system. Lactiferous duct ends at the nipple as a secretory pore. Just beneath the areola, the lactiferous duct forms a sinus called lactiferous sinus to accumulate milk. Beyond the sinus, the duct extends as

successive branches of decreasing size and is known as large and interlobular (intermediate). The ducts, called the terminal duct lobular units (TDLU), are the breast's functioning units.[22] The TDLU comprises an intralobular duct with protruding blunt end or round saccules known as ductules. These ductules form secretory units called acini during lactation. Individual lobes vary in size, and generally, about half of them increase in size and start functioning during lactation. Intralobular stroma, which responses to hormones, also embeds the TDLU.[23,24]

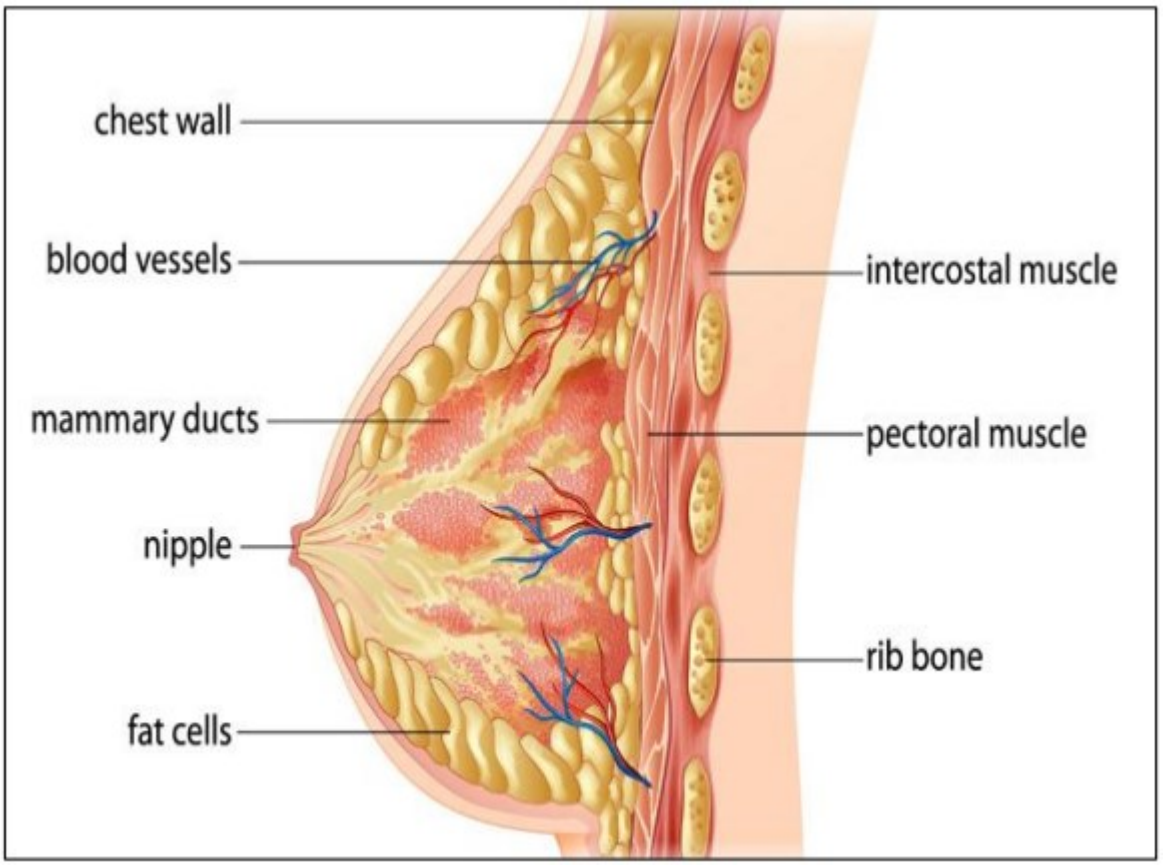

Figure 1: Normal breast

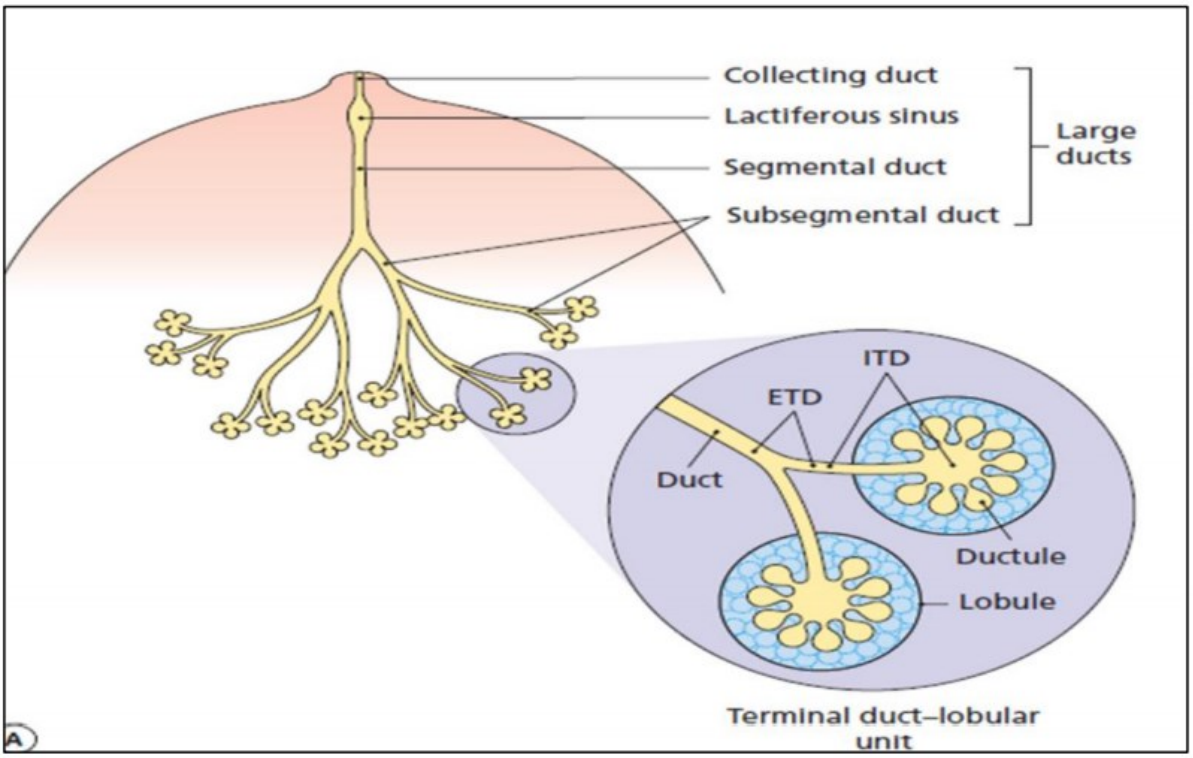

Figure 2: TDLU

Normal histology

Stromal components and epithelial components are the two main components of the breast. The duct and lobular system's dual-layered epithelium lining rested on a basement membrane and enveloped by a stroma. The Inner Layer of ducts are lined by cuboidal to columnar type of epithelial cells, and myoepithelial cells line the outer layer. The basement membrane surrounds both the ducts, ductules as well as the acini.[25]

As earlier mentioned, two types of stroma are there, interlobular stroma and intralobular stroma. The interlobular stroma surrounds TDLUs and large ducts. It has less cellularity and more collagen as compared to intralobular stroma and is composed of fibro-adipose tissue. Within the TDLU, stroma envelops acini. The stroma is loose and comprises fibroblasts with a background of acid mucin and collagen, with occasional lymphocytes and histiocytes. It is

hormonally responsive. Nipple & areola complex is composed of lactiferous sinus lined by similar cells in the ducts until close to the surface, where the epithelium becomes stratified squamous epithelium.[26]

Physiology

Mammary glandular development and functions are instigated predominantly by hormonal stimuli, including estrogen, progesterone, prolactin, oxytocin. Estrogen and prolactin signal trophic changes to the breast and play an integral part in mammary glands' normal development and functioning.[27] Estrogen initiates the breast's ductal development, whereas progesterone primarily increases the epithelial cell differentiation and lobular development. Progesterone also reduces the estrogen binding in mammary epithelium and limits the proliferation of tubular units. Prolactin is the chief hormonal stimulus for lactogenesis in late pregnancy and the postpartum period. Prolactin increases ER and encourages epithelial cells to act in harmony with ductular and lobulo-alveolar growth.[28] Growth hormone and glucocorticoids stimulate ductal growth. Insulin and growth hormone are involved in lobulo-alveolar differentiation and growth. Lobules form by the cells lying at the end of the terminal duct. Hypothalamus secretes a GnRH, which stimulates the release of FSH and LH from the anterior pituitary. LH and FSH further stimulate the ovary to secrete estrogen and progesterone. Estrogen and progesterone, apart from the usual physiologic functions, also serve as negative feedback control for LH, FSH, and GnRH.[29,30]

WHO classification of breast carcinoma[31]

Epithelial tumors	Micro invasive carcinoma
Invasive breast carcinoma	Invasive breast carcinoma of no special type Pleomorphic carcinoma Carcinoma with osteoclast-like stromal giant cells

	Carcinoma with choriocarcinomatous features Carcinoma with melanotic features Invasive lobular carcinoma Classic lobular carcinoma Solid lobular carcinoma Alveolar lobular carcinoma Pleomorphic lobular carcinoma Tubuloalveolar carcinoma Mixed lobular carcinoma Tubular carcinoma Cribriform carcinoma Mucinous carcinoma Carcinoma with medullary features Medullary carcinoma Atypical medullary carcinoma Invasive carcinoma with NST with medullary features Carcinoma with apocrine differentiation Carcinoma with signet ring cell differentiation Invasive micropapillary carcinoma Metaplastic carcinoma of no special type Low-grade adenosquamous carcinoma Fibromatosis-like metaplastic carcinoma Squamous cell carcinoma Spindle cell carcinoma Metaplastic carcinoma with mesenchymal differentiation

	Chondroid differentiation Osseous differentiation Other types of mesenchymal differentiation Mixed metaplastic carcinoma Myoepithelial carcinoma
Rare types	Carcinoma with neuroendocrine features Neuroendocrine tumor, well-differentiated Neuroendocrine carcinoma, poorly differentiated Carcinoma with neuroendocrine differentiation Secretory carcinoma Invasive papillary carcinoma Acinic cell carcinoma Mucoepidermoid carcinoma Polymorphous carcinoma Oncocytic carcinoma Lipid rich carcinoma Glycogen rich, clear cell carcinoma Sebaceous carcinoma Salivary gland/skin adnexal type tumors Cylindroma Clear cell hidradenoma
Epithelial-myoepithelial tumors	Pleomorphic adenoma Adenomyoepithelioma Adenomyoepothelioma with carcinoma Adenoid cystic carcinoma

Precursor lesions	Ductal carcinoma in situ Lobular neoplasia Lobular carcinoma in situ Classic lobular carcinoma in situ Pleomorphic lobular carcinoma in situ Atypical lobular hyperplasia
Intraductal proliferative lesions	Usual ductal hyperplasia
Papillary lesions	Intraductal papilloma Intraductal papilloma with atypical hyperplasia Intraductal papilloma with ductal carcinoma in situ Intraductal papilloma with lobular carcinoma in situ Intraductal papillary carcinoma Encapsulated papillary carcinoma Encapsulated papillary carcinoma in situ
Benign epithelial proliferation	Sclerosing adenosis Apocrine adenosis Microglandular adenosis Radial scar/complex sclerosing lesion
Adenomas	Tubular adenoma Lactating adenoma Apocrine adenoma Ductal adenoma
Mesenchymal tumors	Nodular fasciitis Myofibroblastoma

	Desmoid-type fibromatosis Inflammatory myofibroblastic tumor Benign vascular lesions Haemangioma Angiomatosis Atypical vascular lesions Pseuodoangiomatous stromal hyperplasia Granular cell tumor Benign peripheral nerve sheath tumors Neurofibroma Schwannoma Lipoma Angiolipoma Liposarcoma Angiosarcoma Rhabdomyosarcoma Osteosarcoma Leiomyosarcoma
Fibroepithelial tumors	Fibroadenoma Phyllodes tumor Benign Borderline Malignant Periductal stromal tumor, low grade Hamartoma

Tumors of nipple	Nipple adenoma Syringomatous tumor Paget's disease of the nipple
Malignant lymphoma	Diffuse large B cell lymphoma Burkitt's lymphoma T-cell lymphoma Anaplastic large cell lymphoma, ALK-negative Extranodal marginal zone B cell lymphoma of MALT type Follicular lymphoma
Metastatic tumors	
Tumors of the male breast	Gynaecomastia Carcinoma Invasive carcinoma In situ carcinoma
Clinical patterns	Inflammatory carcinoma Bilateral breast carcinoma

Invasive breast carcinoma

The WHO classification of invasive breast carcinoma has evolved over a long period and, as a result, has had incorporated into it a wide range of criteria, based on cell type (e.g., apocrine carcinoma), based on type and amount of secretion (e.g., mucinous carcinoma), based on the pattern of arrangement (as in papillary carcinoma), and based on the pattern of spread (as in inflammatory carcinoma, which strictly speaking is a clinical and not a pathologic definition).

Epidemiology

It accounts for about 23% of all malignancy in women globally and 27% in developed countries. The incidence of tumors of the breast increases with age.[31] The international incidence of breast carcinoma varies markedly, with the highest incidence in Northern Europe and America, followed by Southern and Eastern Europe, and South America, and lowest in Asia.[32,33] The prevalence of breast cancer has increased due to better management and increased incidence.

India has reported epidemiologic transition and marked an increase in the incidence of carcinoma of the breast due to pathophysiological changes in reproductive factors, dietary habits, and increasing life expectancy.[33] Approximately 75,000 cases occur every year in Indian women.[34] Urban areas show a high incidence of breast carcinomas and constitute 30.5% of all tumors, and in rural areas, it is 20-25%.[35] The results found in a randomized controlled trial done in Kolar on "Clinical Breast Examination as a Screening Method" revealed that the breast carcinoma incidence ranges from 29.8 per 100000 in women not clinically examined or screened (control group) to 38.4 per 100000 women who underwent clinical examination or screening (interventional group). In the 14 intervention groups and control groups, the age-standardized prevalence rates for early breast carcinoma were 19.8 and 8.1 per 100,000 people, and for advanced breast carcinoma, 19.6 and 21.7 per 100,000 women.[36] In the early stages, the lesion can also be identified by clinical evaluation and screening.

Risk Factors

The cause of breast carcinoma is dependent on many variables, including diet, reproductive factors, and hormones. The "western lifestyle" characterized by a high-calorie diet rich in animal fat and proteins, combined with a lack of physical exercise, leads to breast carcinoma.

Alcohol is also described to be another cause for breast cancer, but of low risk. Compared to other neoplasms, breast carcinoma shows familial clustering.

The high penetrance gene found on chromosome 17q21 is BRCA1, and on chromosome 13q12.3, BRCA2 has a high-risk correlation of developing breast carcinoma when affected by germline mutation.[10] Epidemiological trials have found that both endogenous hormone production and exogenous hormone therapy positively correlate with breast carcinoma.[37] Most of the breast carcinomas are detected during the reproductive years. It occurs more commonly in women who have early menarche and remain nulliparous or if parous have the first child delivered at a late age—owing to a reduction of breastfeeding often raises the possibility of breast carcinoma. The incidence starts rising at puberty, then increases to a peak at menopause.[38]

Clinical features

The most prevalent complaint is a palpable mass with rapid onset and progression, sometimes associated with pain, discovered accidentally or during screening programs. Other symptoms include nipple abnormalities like nipple discharge, tethering, puckering, dimpling, distortion, retraction, and eczema over at least one-third of the breast. Nipple discharge is bloody in ductal carcinoma. Paget's disease is crusty, flaking skin associated with underlying malignancy. Edema can be present involving more than two-thirds of the breast or skin thickening with peau d' orange (pitted edema) with dilated veins. Ulceration of overlying skin with the fungating lesion is seen in advanced disease. Rarely presenting symptoms can be hard and fixed axillary or supraclavicular lymphadenopathy without any abnormality in

the breast. Metastatic symptoms include hemoptysis, dyspnea, chest pain, back pain, and jaundice, depending on the metastasis site. Bone metastasis is common in lumbar vertebrae followed by the femur, thoracic vertebrae, and skull; these are usually osteolytic and leads to pathological fracture. Transcoelomic spread causes malignant ascites and deposits in the pouch of Douglas. Bilateral ovaries are involved, known as Krukenberg's tumor. All these symptoms can occur in benign breast diseases. Breast lump should be evaluated with mammography in females less than 35 years and ultrasonography in more than 35 years of age, followed by histological sampling with core biopsy or fine-needle aspiration cytology.

Gross

Tumor size varies from 0.5 cm to 10 cm or more in advanced cases. Some lesions are firm; some are hard in consistency. Consistency is related to the stromal or malignant cell to mass ratio and the composition of associated stromal elements. They have irregular, stellate, ill-defined margins.[38] Some may have a gritty feel on cutting due to calcification. On the cut surface, it is usually grey white with yellow streaks.

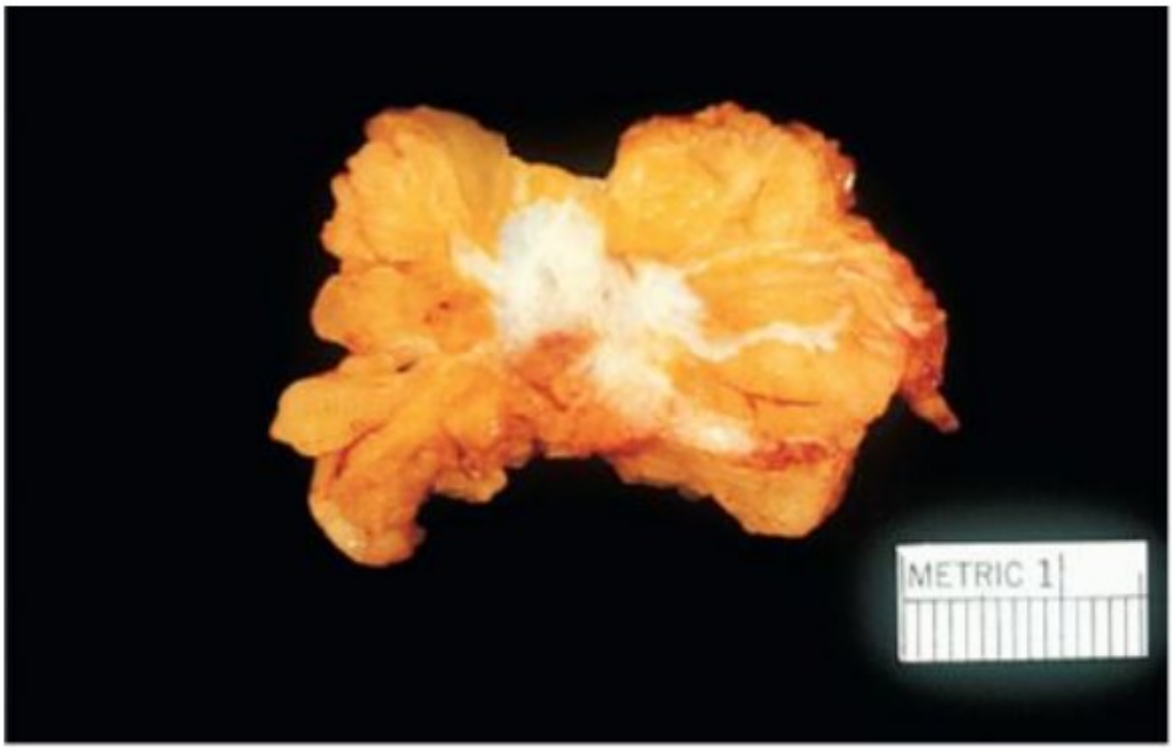

Figure 3: Gross cut section of invasive carcinoma breast.

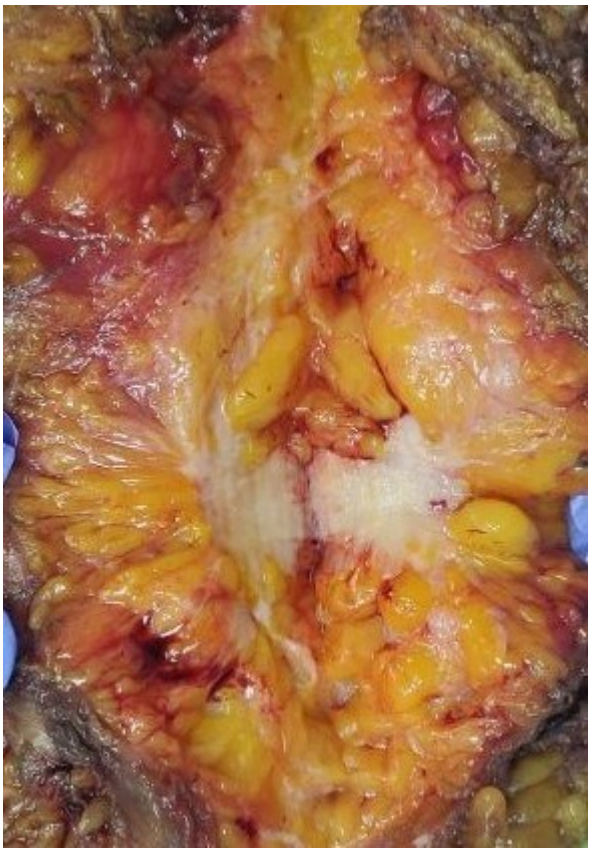

Figure 4- Gross cut section of invasive carcinoma breast.

Histopathology

Invasive carcinoma - no special type represents the prototypic expression of breast carcinoma and consists of a diverse group of tumors that fail to exhibit adequate characteristics to classify it as any specific histological type, diagnosed through a process of exclusion of the recognized special types. The tumor can have highly infiltrating and permeated margins. The neoplastic cell arrangement could be in trabeculae, clusters, and cords and have abundant eosinophilic cytoplasm and highly pleomorphic nuclei with prominent nucleoli. Mitotic activity may be scanty to extensive10. In up to 80% of cases, foci of ductal carcinoma in-situ (DCIS) are seen. The stroma is immensely variable. Foci of squamous metaplasia, clear cell changes, apocrine metaplasia calcification, and necrosis can be seen. Lymphocytes and

macrophages infiltrate typically seen at the interphase between tumor and stroma. The tumor cells show positivity for low molecular weight keratin (particularly types 8, 18, and 19) and epithelial membrane antigen(EMA). Two other markers are mammaglobin, which is more sensitive and Gross Cystic Disease Fluid Protein-15 (GCDFP-15) is a more specific marker.

Special subtypes

Invasive lobular carcinoma

Tumor cells are small to moderately sized cells that lack cohesion. Individual cells are seen dispersed through fibrous tissue or exhibit single file pattern or linear cords that invade the stroma, usually with little reaction from the host or without disturbance to the background tissue architecture. Cords and strands have one to two layers of cells. Few other rare variants are there, like solid, alveolar, tubulo-lobular, pleomorphic & mixed.[39,40]

Tubular carcinoma

Tubular carcinomas are a subset of invasive carcinoma that has a better prognosis. It comprises tubular structures with a lining of one layer of cells and has an open lumen. It accounts for about 2% of total invasive carcinomas of the breast. Tubules should compose > 90% of the tumor cells. Mixed tubular carcinoma has ≥ 75% tubular arrangement. Due to the low frequency of lymph node metastasis, a lower risk of recurrence, and an excellent overall survival rate, tubular carcinomas have an excellent prognosis.[41]

Cribriform carcinoma

A type of invasive carcinoma with an intraductal cribriform pattern is called invasive cribriform carcinoma (ICC). 50% of the tumor may show a tubular pattern. It accounts for 0.3–0.8% of breast carcinomas and consists of a cribriform pattern in >90% of the lesion. The tumor has angulated islands, in which bridges of cells form a well-defined sieve-like pattern.

The tumor, which has a majority of cribriform patterns and few tubular patterns, is also an invasive cribriform carcinoma. A mixed type of invasive cribriform carcinoma is a tumor composing of <50% of other types of patterns other than tubular carcinoma. It metastasizes very rarely to the axillary lymph nodes and carries a good prognosis.41,[42]

Carcinoma with medullary features

It is a broad category that includes medullary carcinomas (MC), atypical medullary carcinoma, and no special type (NST) subset of invasive carcinomas. Common features are circumscribed or pushing border, a syncytial growth pattern, cells with high-grade nuclei, and prominent lymphoid infiltration. They represent about <1% of all breast carcinomas.[43]

Metaplastic carcinoma

The incidence of metaplastic carcinomas is just 0.3% of all of the invasive carcinoma. They are composed of other cellular components apart from the glandular component. The sarcomatous components vary from spindle cell component, myxoid, bone, and cartilage. Gross features vary from well-defined lesions to irregular masses with speculated margins. Microscopically there are two main subtypes: monophasic "sarcomatoid," also known as spindle cell carcinoma with squamous component or without squamous components, and the other one is biphasic "sarcomatoid" carcinoma.[44] The tumor probably is derived from myoepithelial cells. Based on the myoepithelial cell's presence or absence, metaplastic carcinoma differentiates into epithelial and mesenchymal elements.[45]

Molecular subtypes

Despite all the interest surrounding the molecular classification, the clinical importance of subtyping invasive breast cancers beyond routine histologic type, grading, and ER, PR, or HER2 neu status has not been universally established, and it is these very characteristics that

determine therapy currently. Thus assignment of cases to a specific molecular subtype is not a requirement at present.[46]

Prognostic and predictive factors[46]

1. Tumor size- It is the largest measured diameter of the tumor. An increase in tumor size is associated with more chances of distant metastasis rate and poor survival.
2. Histological type - Infiltrating ductal carcinoma is the commonest breast carcinoma constituting 22%. Inflammatory carcinoma has lower survival rates among different histological types, but with systemic chemotherapy, the prognosis is better, with 25 to 50% survival rates.
3. Presence of necrosis – Necrosis is an independent prognostic factor. Central necrosis and fibrosis were observed in large tumors with higher T stage and negligible in early breast cancers. They significantly lack hormone receptors and are associated with a higher grade.
4. Inflammatory cell infiltrates – The presence of intratumor and peritumor mononuclear inflammatory cell infiltrate reflects the host defense mechanism against the tumor cells and is associated with better prognosis irrespective of their hormone receptor status, grade, and other clinic-pathological characteristics. Macrophages proved to be beneficial in fighting cancer cells.
5. Lymphatic invasion – This is associated with higher chances of lymph node metastasis and a higher tumor stage and guides the clinician in considering adjuvant treatment decisions in chemotherapy contraindicated patients.
6. Vascular invasion – Defined as "penetration by the tumor cells into the lumen of an artery or vein." It is associated with distant metastasis, larger tumor size, higher grade, and lower survival. The patients who have a systemic disease or metastatic disease will have a vascular invasion.

7. Perineural invasion – This is associated with lymphovascular invasion and a higher grade of the tumor.
8. Stromal characteristics – Tumors with minimal stromal reaction usually have a higher histological grade and higher nuclear grade. In contrast, tumors with an excellent stromal response like fibrosis and desmoplasia are stellate shaped, circumscribed, low grade, and are likely to be hormone receptor-positive.
9. Axillary node status is commonly associated with disease-free and overall survival rate. Tumors with higher grade, histological type, stage, and lymphovascular invasion have increased risk of axillary lymph node metastasis.

The American Joint committee on cancer (AJCC) stages for breast cancer[46]

T – Primary tumor

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
Tis	(DCIS) Ductal carcinoma in situ
Tis	(LCIS) Lobular carcinoma in situ

Tis	(Paget) Paget disease of the nipple not associated with invasive carcinoma and/or carcinoma in situ (DCIS and/or LCIS) in the underlying breast parenchyma.
T1	T1mi Micro invasion 0.1 cm or less in greatest dimension
T1a	More than 0.1 cm but not more than 0.5 cm in greatest dimension
	More than 0.5 cm but not more than 1 cm in greatest dimension
T1b	More than 1 cm but not more than 2 cm in greatest dimension
T1c	Tumour more than 2 cm but not more than 5 cm in greatest dimension
T2	Tumour more than 5 cm in greatest dimension
T3	Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules)
T4a	Extension to the chest wall (does not include pectoralis muscle invasion only)
T4b	Ulceration, ipsilateral satellite skin nodules, or skin edema (including peau d'orange)
T4c	Both 4a and 4b, above
T4d	Inflammatory carcinoma

N – Regional lymph nodes (pN)

pNX	cannot be assessed
pN0	No regional lymph node metastasis histologically
pN0(i-)	no regional lymph node metastasis by histology or immunohistochemistry
pN0(mol+)	pN0(i+) : isolated tumor cells (cluster ≤ 0.2 mm and < 200 cells)
pN1mi	RT-PCR positive but negative by light microscopy

pN1a	micrometastasis (tumor deposit > 0.2 mm and ≤ 2.0 mm or ≤ 0.2 mm and > 200 cells)
pN1b	metastasis in 1 - 3 axillary lymph nodes with at least 1 tumor deposit > 2.0 mm
pN1c	metastasis in internal mammary sentinel lymph node with tumor deposit > 2.0 mm
pN2a	pN1a and pN1b
pN2b	metastasis in 4 - 9 axillary lymph nodes with at least one tumor deposit > 2.0 mm
pN3a	metastasis in clinically detected internal mammary nodes with pathologically negative axillary nodes
pN3b	metastasis in ≥ 10 axillary lymph nodes with at least one tumor deposit > 2.0 mm or metastasis to infraclavicular lymph node
pN3c	positive internal mammary node by imaging with pN1a or pN1b
pNX	metastasis in ipsilateral supraclavicular lymph node

Distant metastasis (M)

M0	No distant metastases
M1	Distant metastases

Stage grouping

Stage 0	Tis	N0	M0

Stage IA	T1	N0	Mo
Stage IB	T0, T1	N1mi	M0
Stage IIA	T0, T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	Mo
	T3	N0	M0
Stage IIIA	T0,T1,T2	N2	M0
	T3	N1, N2	M0
Stage IIIB	T4	N0,N1,N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Microscopic grade

Considering both architecture and cytology have been found to correlate with prognosis, Elston and Ellis modified the original Bloom and Richardson and Bansal et al.[47] grading schemes based on tubule formation and nuclear degree atypia. This is the Modified Bloom-Richardson grading system (MBR) (Annexure - 3). It also incorporates the mitotic activity to the previous classification. The grade is calculated by adding the scores designated for tubule formation, pleomorphism of nucleus, and mitotic count.[48]

Nottingham Modified Bloom Richardson Grading of the tumor.[48]

Criteria	**Score 1**	**Score 2**	**Score 3**
Tubule formation	> 75%	10 to 75%	< 10%
Nuclear	Minimal variation in	Moderate variation in	Marked variation in

pleomorphism	nuclear size and shape	nuclear size and shape	nuclear size and shape
Mitotic counts per 10 HPF	0-5	5-10	More than 11

Overall grade

- Grade 1(score 3,4 or 5)
- Grade 2(score 6 or 7)
- Grade 3(score 8 or 9)

Grading is advocated for all, regardless of morphological type, as it serves to prognosticate the metastasis and survival, independent of the lymph node's status, and predicts chemotherapy response.

Tumor-infiltrating lymphocytes (TILs)

Carcinomas demonstrating a prominent lymphocytic reaction, particularly triple-negative and HER2-positive tumors, better respond to neoadjuvant chemotherapy as indicated by emerging data.[49]

Skin invasion

The invasion of skin is correlated with a decreased survival rate. Invasion of dermal lymphatic vessels in the "inflammatory carcinoma" picture is a particularly ominous prognostic sign.[17,23]

Tumor stroma

Carcinoma breast is a heterogeneous disease. Clinically, it has been classified by the expression level of ER, PR, and HER2 neu. Recently molecular classification with different

subtypes has been introduced with a greater understanding of disease characteristics and outcomes. Cancers evolve in a complex tissue microenvironment, which leads to sustained growth, invasion, and metastasis. The development of cancer is co-mediated by the tumor microenvironment and cancer cells rather than being a cell-autonomous process.[50,51]

Evolution of breast cancer

Breast cancer evolves from intermediate hyperplastic lesions with and without atypia (usual ductal hyperplasia, atypical lobular hyperplasia, atypical ductal hyperplasia) to in situ carcinoma. In atypical hyperplasia, the breast cells have abnormal size, shape, number, growth pattern of cells that appear as immoderate growth of the ducts (atypical ductal hyperplasia) or the cells of the lobules (atypical lobular hyperplasia). If breast tissue has an increased number of benign cells inside the duct, it is called usual ductal hyperplasia.[52] In DCIS, tumor cells are confined to the mammary duct lumen. LCIS comprises non-invasive hyperplasia of the breast lobules. In situ carcinoma means myoepithelial layer and basement membrane are intact, but epithelial cells proliferate. When the myoepithelial layer and basement membrane disruption occurs, tumor cells invade surrounding tissues and distant organs, ultimately leading to metastasis.[53,54] Genetic and epigenetic modifications are repeatedly seen within normal breast stroma, benign breast conditions, DCIS, and invasive carcinoma. The tumor stroma ratio and the stroma type of primary breast cancer are connected with recurrence, distant metastasis, and death.

Cluster of differentiation 68(CD68)

Also known as Macrosialin, the molecular size of CD68 is 110 kD. It is a glycoprotein containing 354 amino acids generally in association with the endosomal/lysosomal compartment. The CD68 gene encodes it on chromosome 17. Typically it stains cells of macrophage, lineage including kupffer cells and osteoclasts. It has the same properties as

lysosomal-associated membrane proteins (LAMPs) and grouped with LAMP glycoproteins. CD68 is also known as LAMP-4.[20]

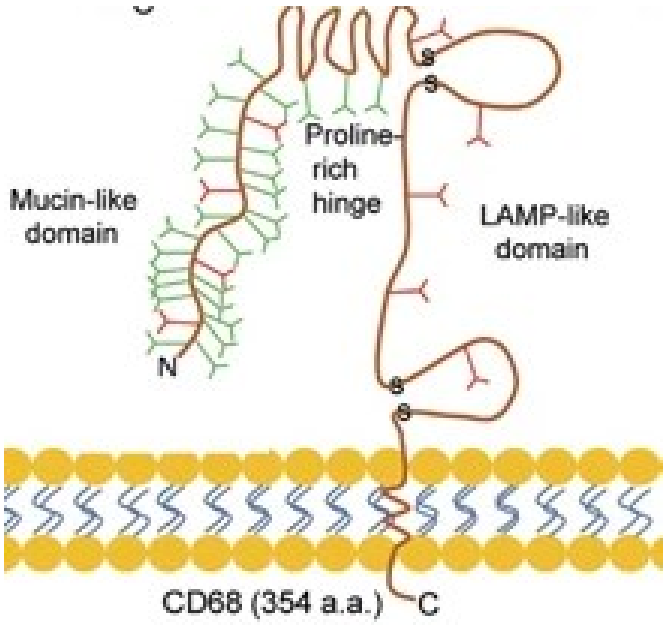

Figure 5: General features of the domain organization of CD68

The domain folds into a compact β-sheet sandwich. It is essential to note that the LAMP domain does not reach far into the vesicle lumen but is a compactly folded unit directly adjacent to the membrane. As shown in the scheme, each molecule contains the short C-terminal cytoplasmic tail and the transmembrane domain. The central portion of the molecule is located in the lumen of lysosomes/endosomes. The length of the amino acid (a.a) sequence of each protein is shown in brackets. The glycosylated extracellular domain mediates the binding of CD68 to selectins and organ-specific lectins on its surface. Its functions include activation and recruitment of macrophages in a specific site, engulf dead cells (phagocytosis), and foreign bodies.[20]

CD68 is broadly used as a cancer-associated diagnostic and prognostic marker. It is used to identify neoplasms with macrophage lineage and also may be expressed by tumor cells from other lineages. The finding of CD68 expression by tumor cells is not surprising since metastatic tumor cells widely express immune markers to escape macrophage-mediated phagocytosis and cell-damaging effects from cytotoxic $CD8^+$ T cells during the invasion of a normal, non-tumor tissue environment. The macrophages lysosomes and late endosomes express CD68 antigen in the granules, thus giving cytoplasmic staining. CD68 positive TAMs present in the tumor microenvironment show high serum and stromal levels of VEGF.[55] In this way, altering the tumor microenvironment facilitates angiogenesis in the tumor and reduces cancer to radiotherapy. High grade and high macrophage index are adversely correlated with decreased relapse-free survival and reduced overall survival and indicate poor prognosis.[55]

Contrary to this, many studies have shown a correlation of M1 macrophages with a lesser grade of tumor and better survival in breast cancer by releasing pro-inflammatory cytokines.

Cluster of differentiation 163 (CD163)

It functions as a receptor for the Hb-haptoglobin complex and plays an essential function in the body's immune system in reaction to intravascular and extravascular hemolysis and against bacterial infections.

It has a molecular size of 130 kDa and has 1048 amino acid residues in extracellular domains. A dissolved form of CD163 is seen in cerebrospinal fluid and is called sCD163 represents receptor shedding and structural and functional modulation of CD163.[56]

It mediates cell-cell interactions between erythroblasts and macrophages. CD163 expression is controlled by inflammatory mediators-IFN γ, IL6, IL-10, glucocorticoids. CD163 is of particular significance because its expression can be shown by immunofluorescence, immunohistochemically, Insitu hybridization techniques. In contrast, CD68 lacks specificity for the monocyte/macrophage system and are detected in numerous ranges of normal and neoplastic cell types. Interfollicular macrophages and histiocytes express CD163 in lymph nodes, splenic red pulp macrophages, alveolar 32 macrophages in the lung, macrophages in bone marrow, kupffer cells of the liver, dermal macrophages of skin. CD163 is expressed in hematologic malignancies such as histiocytic sarcoma, CMML.[56]

sCD163 is upregulated in diseases like diabetes, Gaucher disease, rheumatoid arthritis, and Hodgkin's lymphoma. Many studies have concluded that more density of expression of CD163 molecule in breast cancer is associated with higher chances of lymph node metastasis, higher grade, increased tumor size, and higher chances of tumor progression.[57]

Shabo I et al.[58] studied CD163 expression, D2-40, CD31, S-phase fraction in75 colorectal cancer patients (46 colonic and 29 rectal) operated during 1982-1986. CD163 expression was analyzed in five grades. CD31 and D2-40 density were studied along with the S-phase fraction. Tumor-associated macrophage infiltration was assessed in four grades. CD163 was positive in 20 percent of colorectal cancer patients and linked to advanced tumor levels and poor prognosis. Dense macrophage infiltration has been associated with positive CD163 expression in tumor cells. They concluded that Tumor-associated macrophage infiltration is an independent prognostic marker independent of CD163 expression in tumor cells.

An analysis of CD163 expression in rectal carcinoma was also performed in 163 patients with rectal cancer and followed up for 71 months. In pre-treatment biopsies of 101 patients, 10 had CD163 positive tumor cells, and local recurrence and decreased survival were observed

during the follow-up period compared to CD163 negative tumors. Out of the preoperative Randomized irradiated patients, 31% positivity of CD163, and in non-irradiated patients, 17% were positive. There was increased positivity in irradiated patients. They concluded that a high density of expression of CD163 in rectal carcinoma is linked to early local recurrence and reduced survival. Manieki et al.[59] studied the density of expression of CD163 in bladder tumor biopsies and concluded that CD163 mRNA expression is closely linked with muscle-invasive, aggressive cancers, and poor prognosis. CD163 expression was not limited to TAMs but also by a large number of tumor cells. They also confirmed the dense Tumor-associated macrophage infiltration association with advanced-stage tumors. Shabo I and Stal O et al.[60] studied the density of CD163 expression in breast carcinoma tumor cells.

Immunostaining for CD163, MAC387, CD68 was done in tissue microarray from 127 patients. CD163 expression is 48%, MAC387 is 14%, while CD68 expression was not seen. The density of CD163 expression in cancer cells was compared with DNA ploidy, TNM stage, Nottingham histological grade, node state, distant metastasis, and survival. Study subjects were followed up for 13 years. They concluded that CD163 has a significant effect on metastases and decreased overall survival.

Tumor microenvironment and tumor-associated macrophages

The tumor microenvironment comprises both malignant and non-malignant populations of cells. Non-malignant populations include leukocyte infiltrate, proliferating blood vessels, and stromal cells. Rudolf Virchow first suggested in 1863 the link between systemic inflammation and tumorigenesis. He observed that infiltrating leukocytes were a hallmark of the tumor. Leukocytes comprising the tumor microenvironment are Macrophages, T-lymphocytes, B-Lymphocytes, Natural killer cells. Quatromoni et al.[9] considered TAMs as

the seventh hallmark of cancer. Stromal cells of the tumor microenvironment are cancer-associated Fibroblasts, adipocytes, pericytes, lymphatic, and vascular endothelial cells.

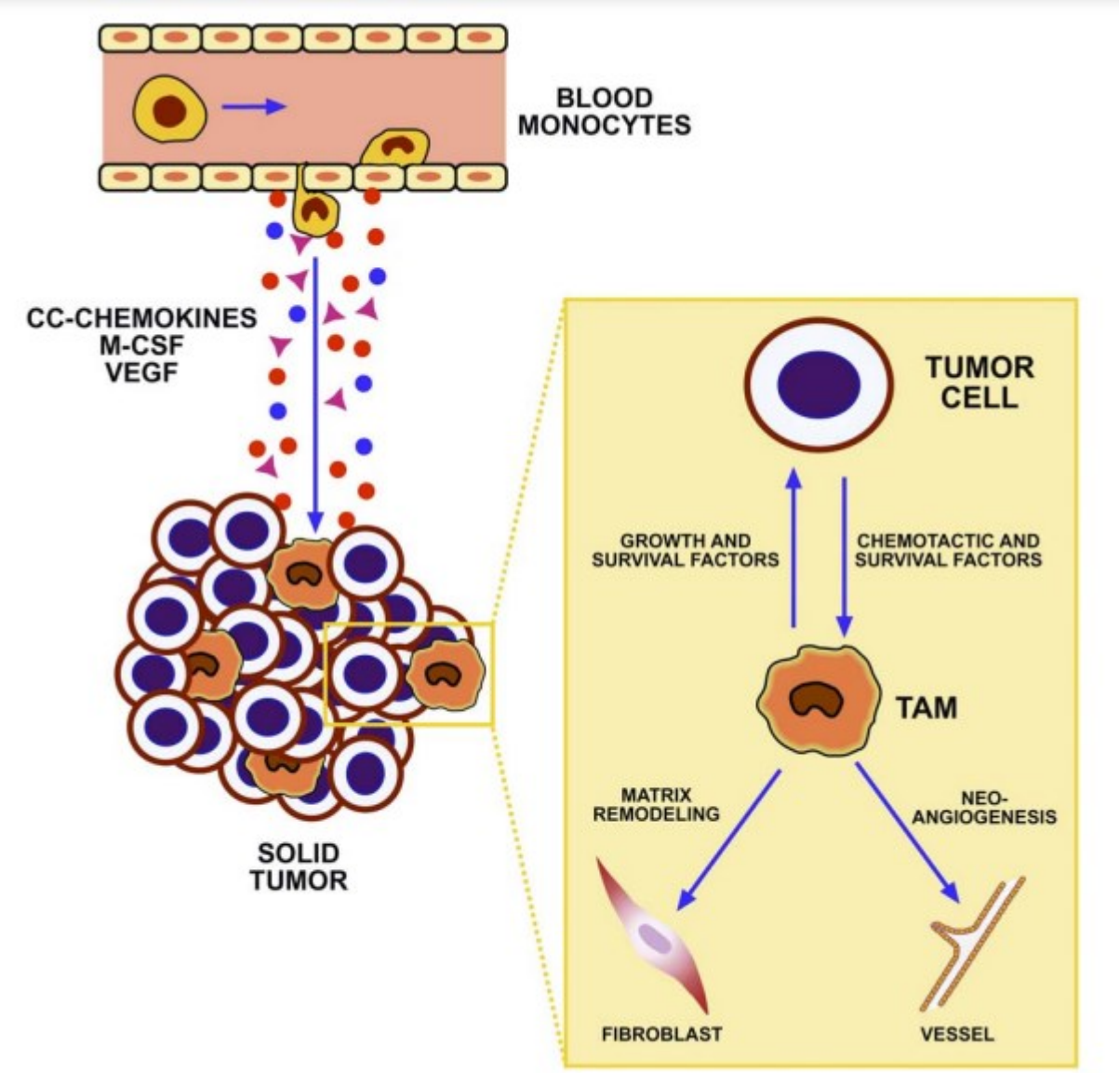

Figure 6: TAMs and tumor microenvironment.

Polarization of macrophages

Bacterial lipopolysaccharide(LPS), Interferon-γ(IFN-γ), Toll-like receptor agonism polarizes the macrophages into the M1 phenotype. Activated M1 macrophages destroy microbes, eliminate the tumor, presents antigen to T-cells causing an adaptive immune response. IL-4,

IL-10, IL-13, TGF-β, PGE2 polarises macrophages to the M2 phenotype. Activated M2 macrophages cause angiogenesis via VEGF production, Tissue remodeling via MMPs (Matrix Metalloproteinases) causing digestion of Extracellular matrix helping tumor development.

Sica et al.[61] studied various forms of M2 polarization. They are classified as M2a, M2b, M2c.

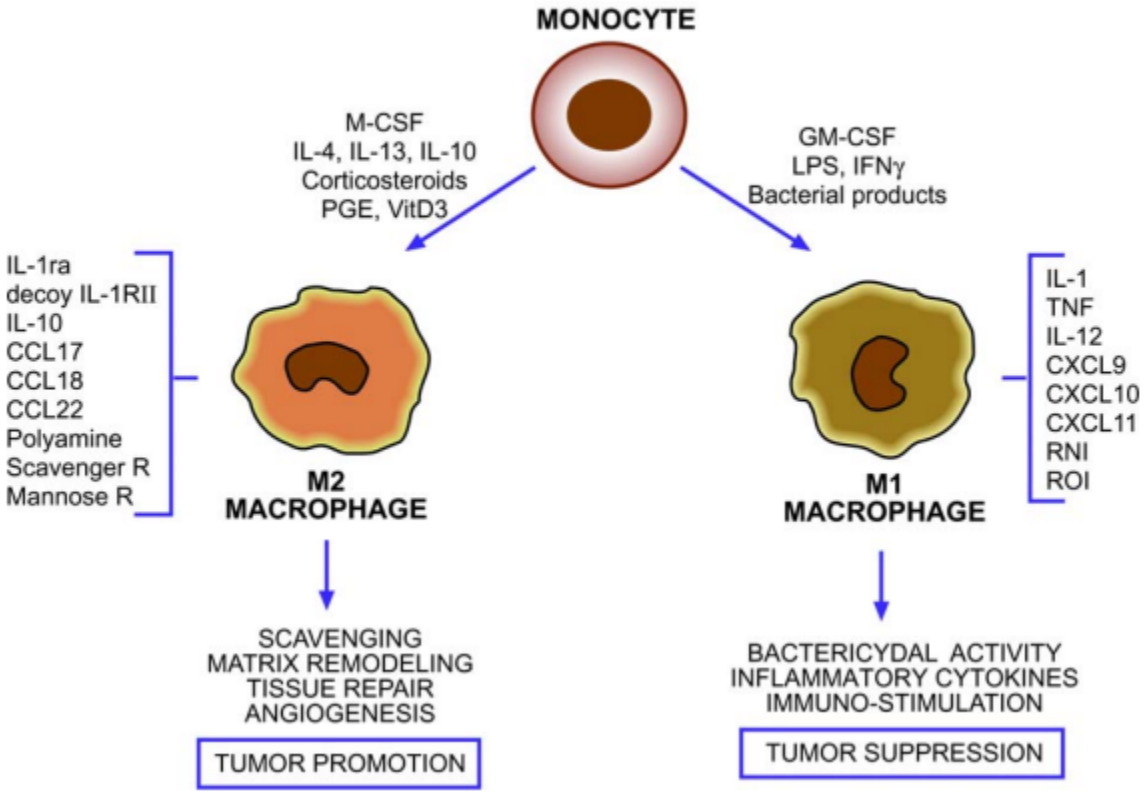

Figure 7: Differentiation of macrophages into M1 and M2.

Functions of tumor-associated macrophages

TAMs are M2 phenotype macrophages with tumor-promoting functions. They help in angiogenesis, releasing growth factors for tumor cell proliferation, releasing cytokines to suppress immunity, and destroying extracellular matrix components, causing tumor progression, invasion, and metastasis. Exposure to poorly vascularized regions in tumor

upregulates hypoxia-inducible factors (HIF)-1α and HIF-2α in macrophages causing metabolic adaptation to an oxygen-poor environment and enabling immunosuppressive function.

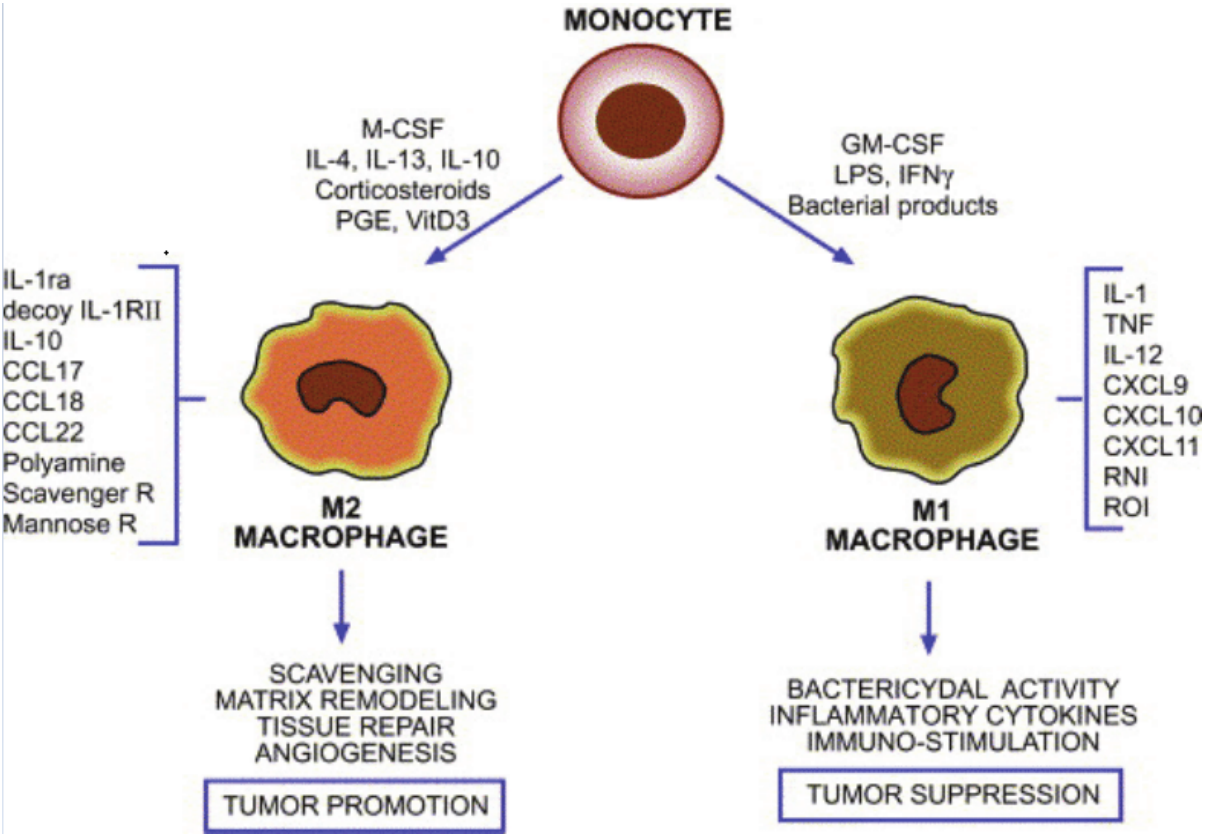

Figure 8: Functions of TAMs

Medrek C et al.[12] studied the presence of TAMs in 144 Breast cancer patients. They studied the density of CD163 and CD68 expression in tissue microarrays of breast carcinomas. They concluded that CD163 positive TAMs in the peritumoral area is positively correlated with an advanced grade, tumor size, ER negativity, Triple negativity, and Ki67 positivity. CD68 positivity is associated only with larger tumor size. They also found that some CD163 positive areas lack CD68 positivity, indicating that CD163 could be used as a TAMs marker with prognostic significance. Bacman et al.[62] studied TAMs infiltration in 310 colorectal carcinoma tissue microarrays. TGFβ signaling was analyzed. Higher grade tumors and patients with lymph node metastases showed dense TAM infiltration and lower TGF-β1.

They concluded that decreased TGFβ receptors 38 and increased TAMs infiltration significantly associated with higher-stage tumors and reduced survival. Forssell et al.[63] studied the role of TAMs in the survival of patients. They studied 446 colorectal cancer specimens and stained them with CD68. Subjects with more CD68 hotspot scores had a better prognosis. Lackner C et al.[64], in a study about TAMs and vWF positive vessels in colorectal cancer. CD68 and CD34 were studied in 70 colorectal patients. They concluded that increased TAMs, vWF positive vessels are a separate marker for prognosis in higher-stage tumors. Steidi et al.[65], in a study regarding TAMs in Hodgkin's Lymphoma. Their results suggest that elevated TAMs in Classic Hodgkin's Lymphoma was closely linked to decreased survival.

Target for therapy

The presence of total tumor-infiltrating lymphocytes and specific CD8+ cytotoxic T cells associates with a successful response to chemotherapy and a significant reduction in the relative risk of death. However, the ability of TAMs to suppress T-cell responses at the interface between tumor and stroma represents a significant obstacle to successful immunotherapy. Macrophages have emerged as an independent co-factor in breast cancer progression and represent an attractive target for breast cancer therapy. Besides, inhibition of tumorigenic factors and mechanisms promoted by TAMs, such as Epithelial growth factor(EGF) mediated metastasis and cancer stem cells(CSC) support, provides a novel mechanism to treat breast cancer.

Materials and Methods

STUDY DESIGN – Laboratory-based exploratory study.

SOURCE OF DATA- Primary breast carcinoma specimens received in the Pathology department of R.L. Jalappa Hospital and Research Centre affiliated to SDU Medical College, Kolar from December 2018 to May 2020, and also the paraffin blocks were taken retrieved from Archives of Pathology department from the year January 2016 to November 2018 will be included in the study.

DURATION OF STUDY – One and half years.

METHOD OF COLLECTION- All Primary Breast Carcinoma Specimens confirmed by histopathological examination were included in the study. Data regarding the clinical details (age, sex, histological grading) was collected from the MRD (Medical Records Department). H&E slides were reviewed for Histopathological types and grading and staging of the tumor. Immunostaining for CD68 and CD163 was performed on all breast carcinoma cases using appropriate positive and negative controls by peroxidase and anti-peroxidase method.

Table 1: Details of IHC marker.

Antigen	Clone	Species	Producer	Control	Dilution	Stain
CD68	KP1	Mouse	PathnSitu	Tonsil	Pre-diluted	Membranous/ Cytoplasmic staining
CD163	EP324	Rabbit	PathnSitu	Spleen	Pre-diluted	Membranous/ Cytoplasmic

PROTOCOL

1) **Section Cutting-** Sections are cut at approximately 3-4 µ m, floated on to positively charged slides and incubated at 37^{o} C for one day, and further incubated at 58° C overnight.

2) **Deparaffinization** - Xylene – I and xylene II - 15 min each

3) **Dexylenisation** - Absolute alcohol – Ist and IInd - 1 min each

90% Alcohol and 70% alcohol – 1 min each

4) Tap water – 10 min washing

5) Distilled water – 5 min rinsing

5) **Antigen Retrieval technique**- Microwave at power 10 for 6 minutes in Tris EDTA buffer of PH 9.0 for three cycles. Slides cooled to room temperature.

6) Transfer to TBS buffer (Tris buffer solution – Ph.- 7.6). Three times wash for 5 minutes.

7) **Peroxidase block** – for 30 minutes to inhibit the peroxidase enzyme. TBS for 5minute x 3 times-wash.

8) Drain and cover section with primary antibody

9) TBS buffer was 5minute x 3 times.

10) Super sensitive polymer horseradish peroxidase(HRP) for 30 minutes. TBS buffer washes 5minute x 3 times.

11) Apply enzyme-conjugated secondary antibody to the slide and incubate at room temperature for 1 hour. TBS buffer washes 5minute x 3 times.

12) DAB Colour development for 30 minutes. TBS buffer washes 5minute x 3 times.

14) TBS buffer

15) Rinse in tap water for 5minute

16) Haematoxylin counterstain

17) Dehydrate, clear, and mount with DPX.

SAMPLE SIZE: Sample size was estimated by using the proportion of CD163 marker positivity in primary breast carcinomas, which was 9%[15] by using the formulae –

$$\text{Sample size} = \frac{Z_{1-\alpha/2}{}^2 \, p(1-p)}{d^2}$$

Here

$Z_{1-\alpha/2}$ = Is standard normal variate (at 5% type 1 error ($P<0.05$) it is 1.96 and at 1% type 1 error ($P<0.01$) it is 2.58). As in majority of studies P values are considered significant below 0.05 hence 1.96 is used in formula.

p = Expected proportion in population based on previous studies or pilot studies.

d = Absolute error or precision – Has to be decided by researcher.

P = 9 or 0.09

q = 91 or 0.91

d = 8% or 0.8

Using the above values at a Confidence level of 95%, a sample size of 55 subjects with primary breast cancer were selected for the study.

STATISTICAL ANALYSIS:

Microsoft Excel datasheet was used for data collection and entry and then analyzed using the software's SPSS 22 version. Categorical data were represented in frequencies and proportions, and chi-square was calculated as a test of significance. Continuous data are represented as a mean and standard deviation, and an independent t-test was used as a test of significance to identify the mean difference. P-value of <0.05 considered statistically significant.

INCLUSION CRITERIA:

- All Primary breast carcinoma cases.

EXCLUSION CRITERIA:

- Metastatic tumor from other sites.
- Recurrent lesion.
- Patients subjected to chemotherapy and Radiotherapy.

Nottingham Prognostic Index

NPI	**Score**	**5-year survival**	**Prognosis**
I	≤ 2.4	96%	Excellent
II	≥ 2.4 but ≤ 3.4	93%	Good
III	≥ 3.4 but ≤ 5.4	78%	Moderate
IV	≥ 5.4	44%	Poor

Nottingham Prognostic index = (0.2 X S) + N + G

S = Size of tumor in centimetre

N = Number of lymph nodes, 0 = 1, 1-3 = 2, >3 = 3

G= Grade of tumor

According to the definition, intratumoral macrophages means intraepithelial tumor-infiltrating macrophages. Peritumoral macrophages are macrophages in the stromal tissue surrounding the tumor nest. A hotspot is an area with the highest level of TAMs**.**

CD68 and CD163 were identified by their macrophage morphology and cytoplasmic staining with strong cell membrane positivity. For screening, low magnification(10x) was used, and ten hotspot areas were selected with the maximum density of cells showing positivity. At 40x total number of positively stained cells were counted in both peritumoral and intratumoral areas separately without access to any clinical information.[67]

For statistical analysis, positive cells were categorized into two groups of low and high, based on cut-off points according to the median. The cases were distributed into low and high groups using cut-off values of 3/HPF for CD68 TAMs intratumoral distribution, 10/HPF for CD68 TAMs peritumoral distribution, and 3/hpf for CD163 TAMs for intratumoral distribution, and 8/HPF for CD163 TAMs for peritumoral distribution as shown in the following table.

Table 2: Distribution of CD68 and CD163 macrophages.

Variable	Mean	Median	Range
CD68 TAMs Intratumoral	3.05	3.00	2-5

Peritumoral	10.47	10.00	2-15
CD163 TAMs Intratumoral Peritumoral	3.69 10.8	3.00 8.00	3-8 3-20
The ratio of CD163 and CD68 Intratumoral Peritumoral	1.2 1.03	1 0.8	1-1.11 1-1.25

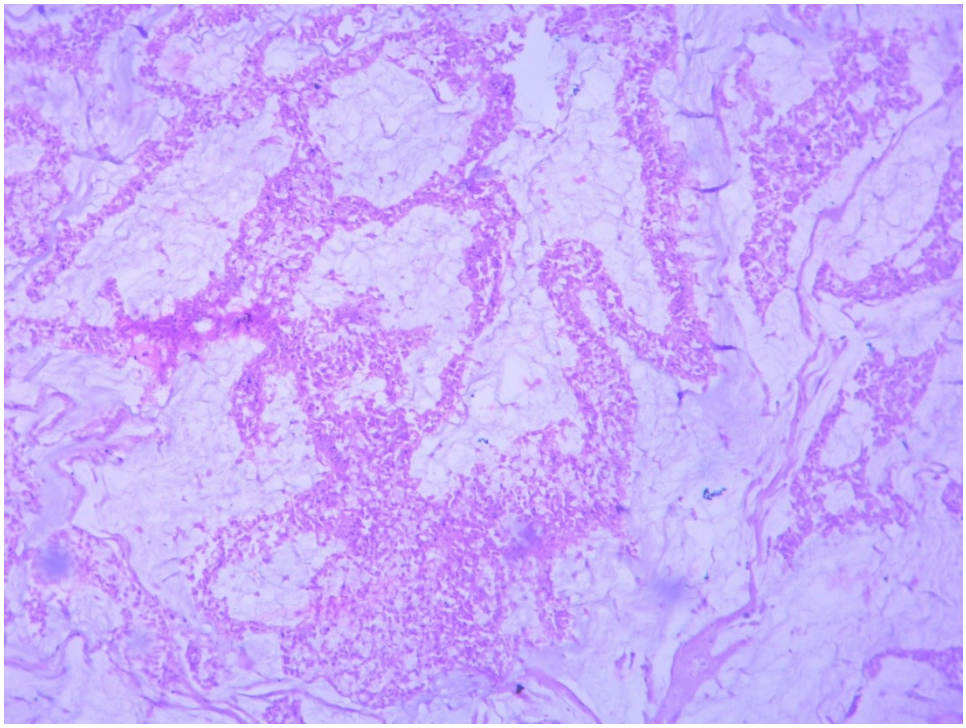

Figure 9: H&E section showing Mucinous carcinoma. (x 10X)

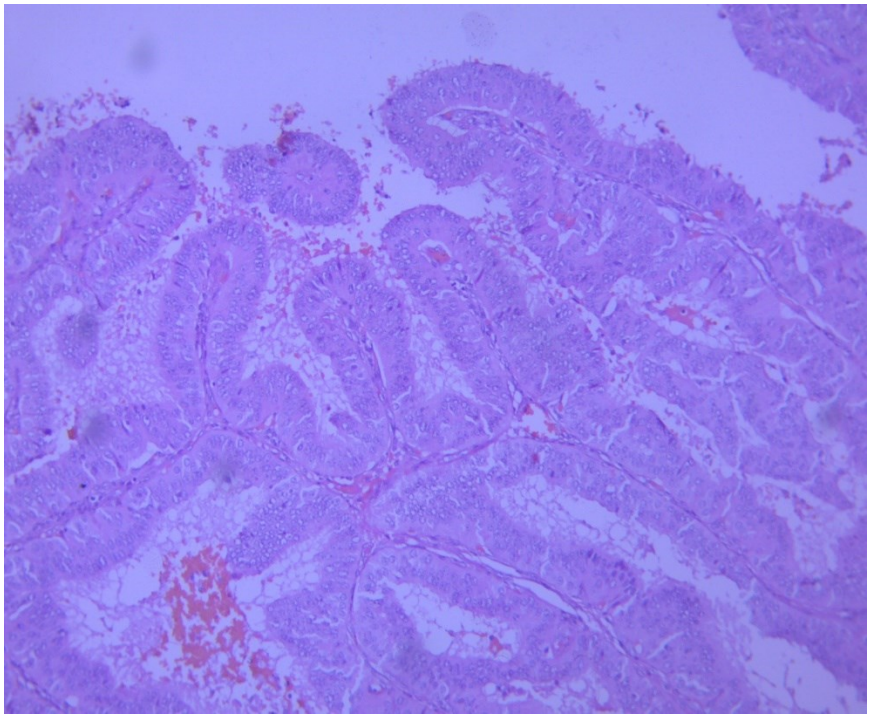

Figure 10: H&E stain section showing Papillary carcinoma. (x 10 X)

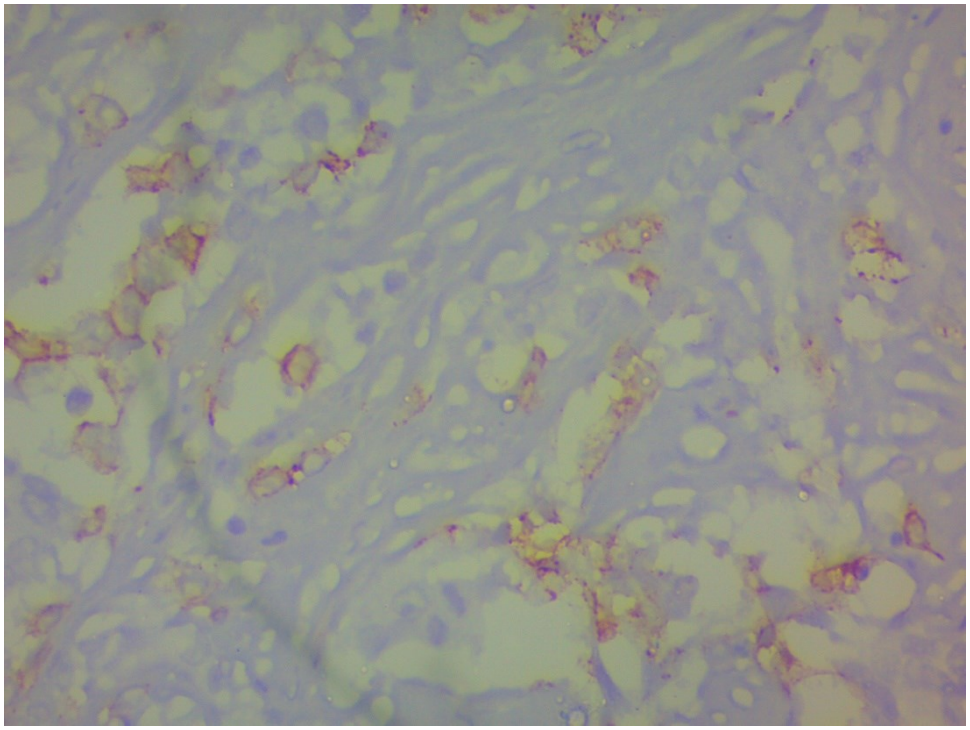

Figure 11: IHC staining with CD68 showing strong membrane and cytoplasmic staining (x 40X)

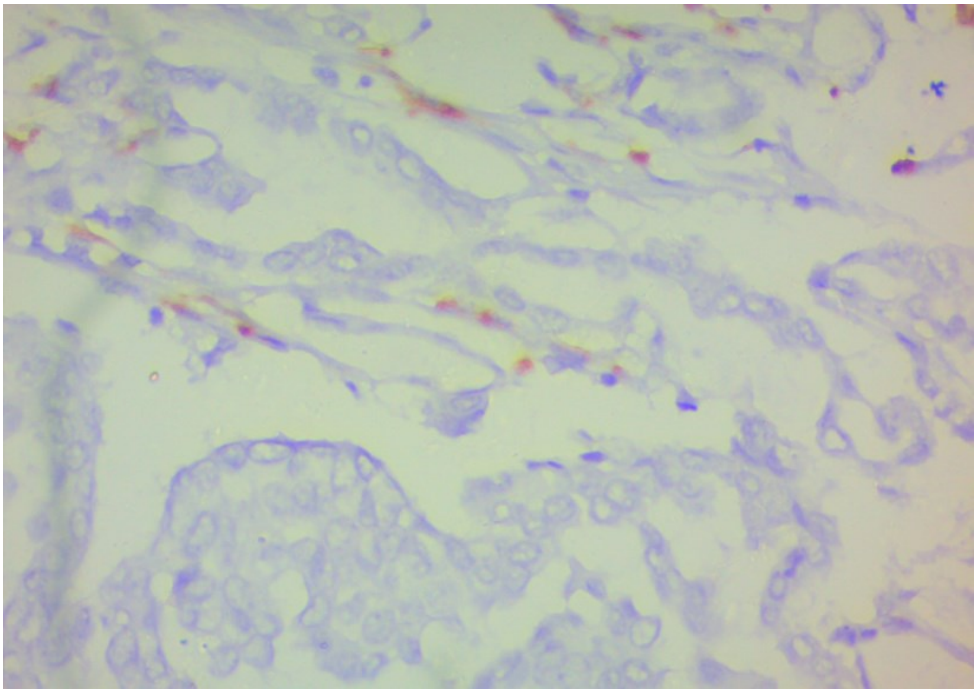

Figure 12: IHC staining with CD163 showing high (>8/hpf) peritumoral distribution. (x 40X)

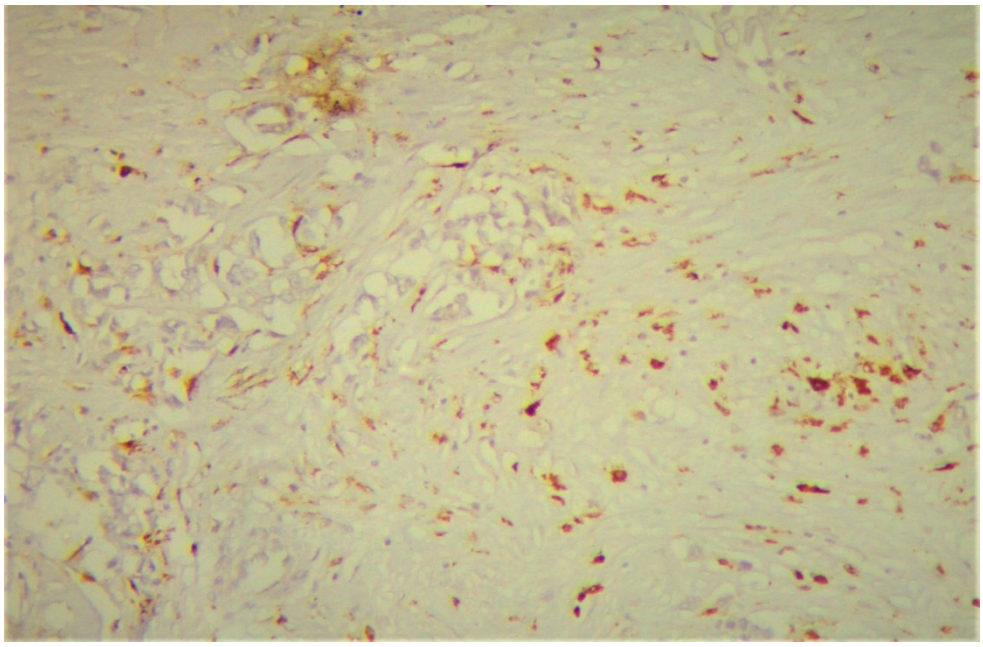

Figure 13: IHC staining with CD163 showing high (>8/hpf) peritumoral distribution (x 40X)

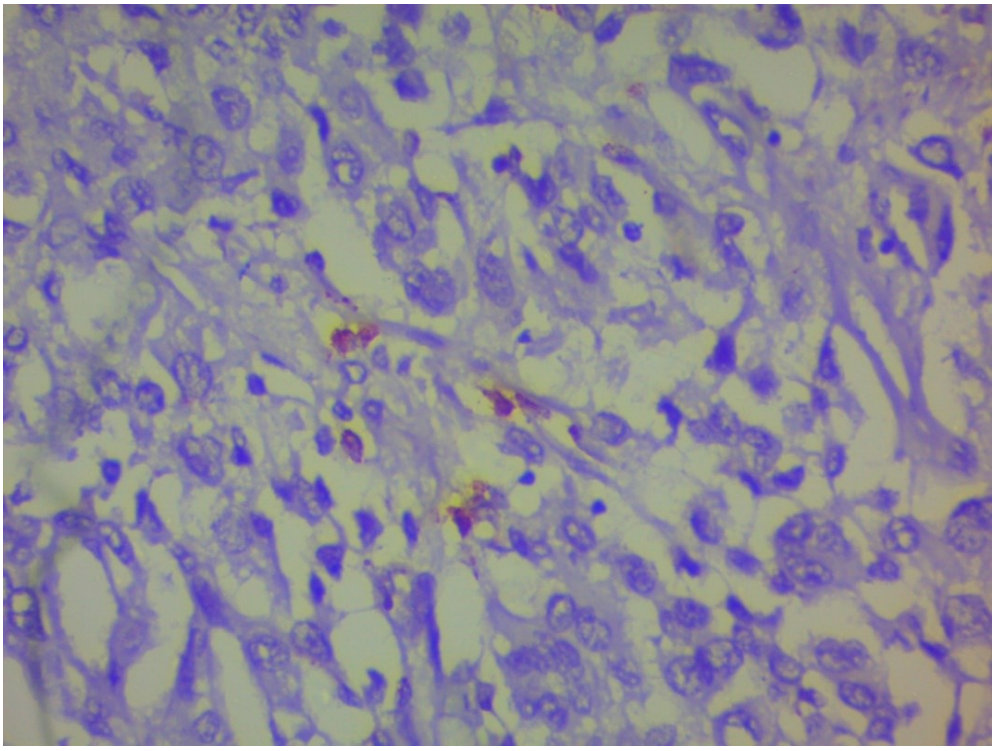

Figure 14: IHC staining showing low (<3/hpf) peritumoral distribution of CD163 (x 40X)

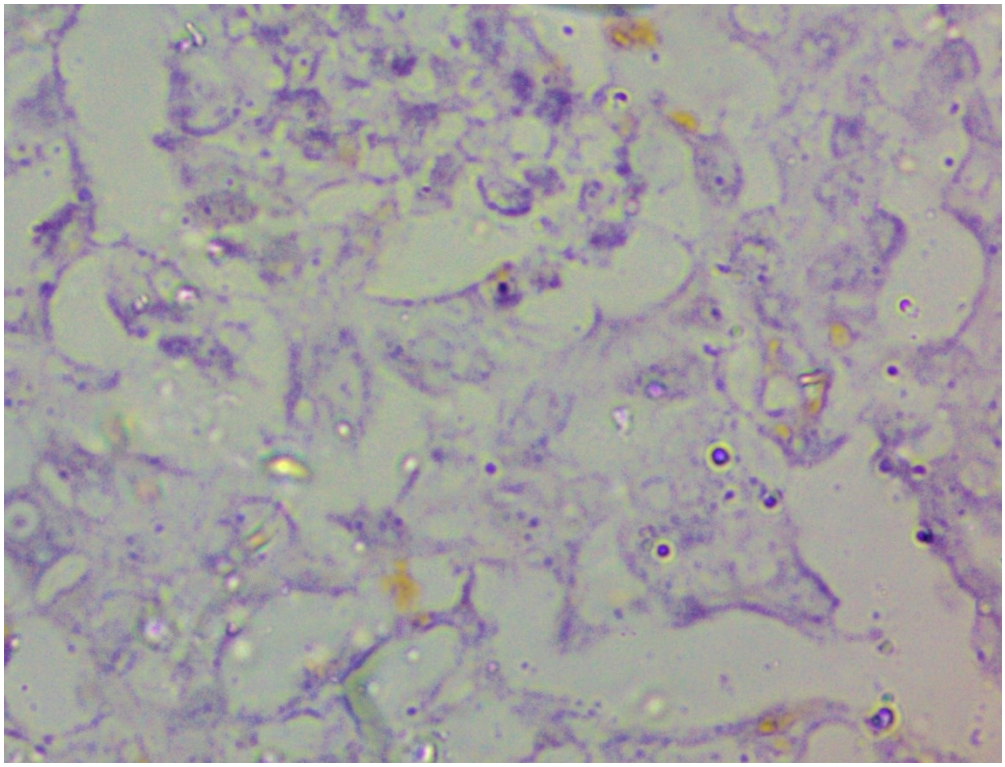

Figure 15: IHC staining with CD163 showing low (<3/hpf) peritumoral distribution (x 40X)

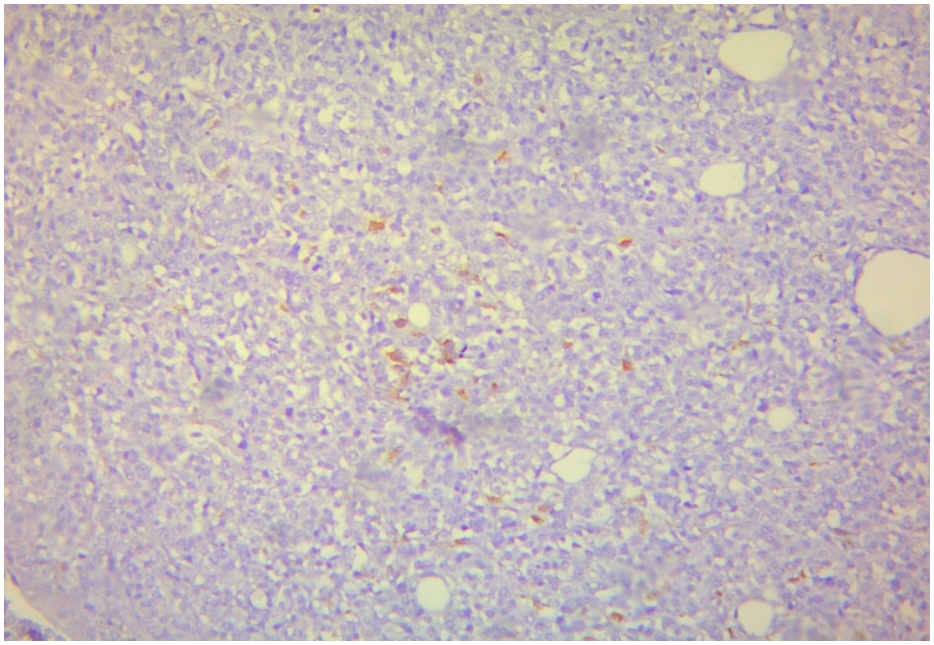

Figure 16: IHC staining with CD68 showing high intratumoral distribution (x 40X)

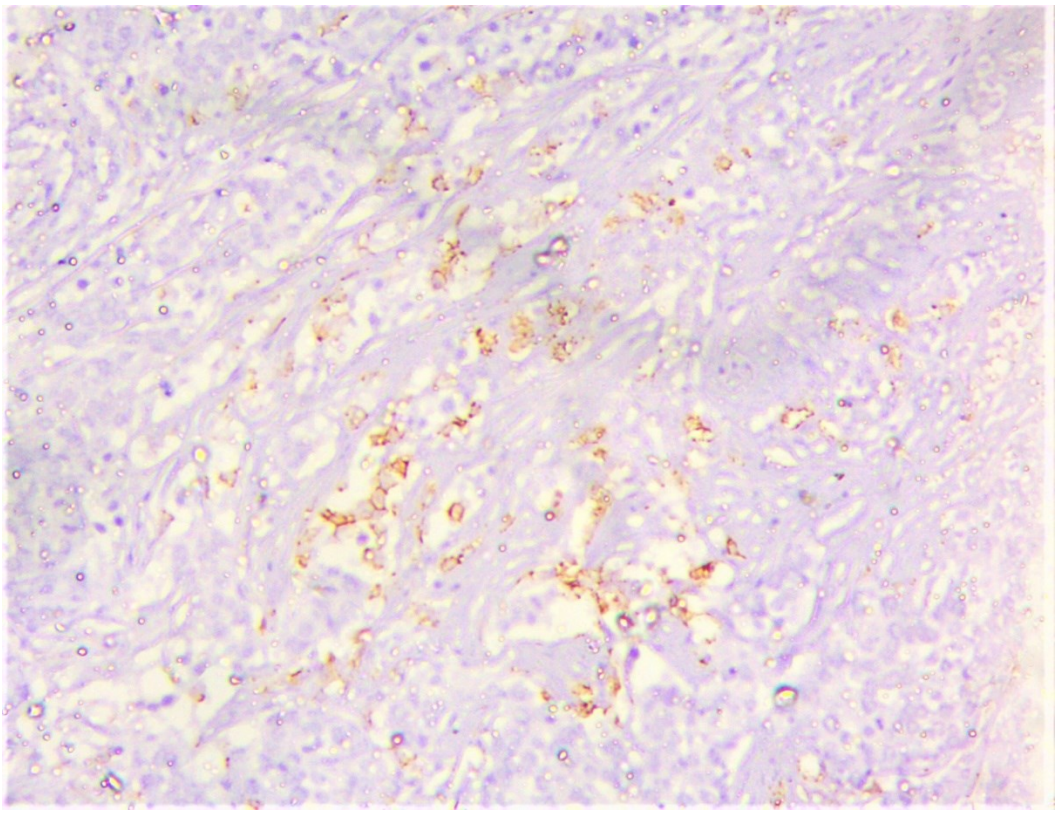

Figure 17: IHC staining with CD68 showing high intratumoral distribution (x 40X)

Results

The maximum number of cases were between 50- 59 years, i.e., 19 cases, followed by 11 cases in the 40-49-year age group and 10 cases in the 60-69 years of age group. 30-39-year age group has 6 cases, 8 cases of > 70 years, 1 case in between 20-29 years.

Table 3: Age distribution of subjects in the study group.

		Count	Percentage (%)
Age	<50 years	21	38.1
	>50 years	34	61.9
	Total	55	100

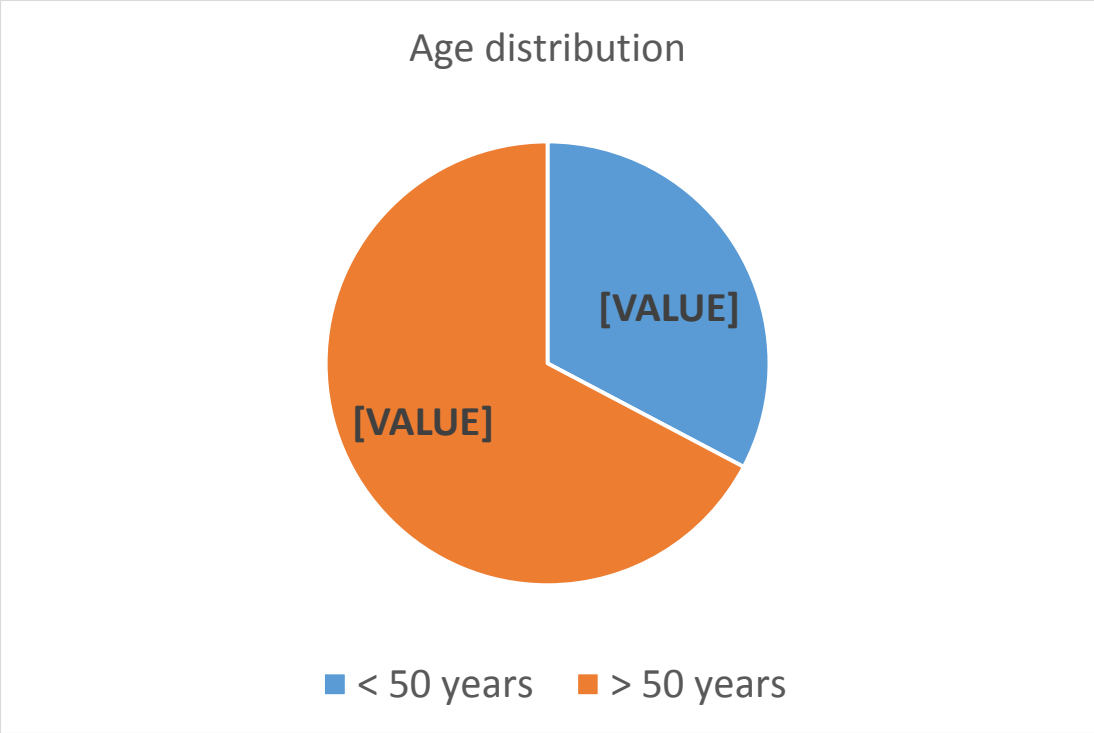

Chart 1: Bar diagram showing the age distribution of subjects in the study.

Table 4: Tumor size distribution in the study group.

		No. of cases	Percentage (%)
Tumor size	< 5 cm	34	61.8
	> 5 cm	21	38.2

Most of the patients lumped size 2-5 cm, i.e., 27 cases. In 21 cases, the lump size was > 5 cm, and in only 7 cases, the lump was < 2cm.

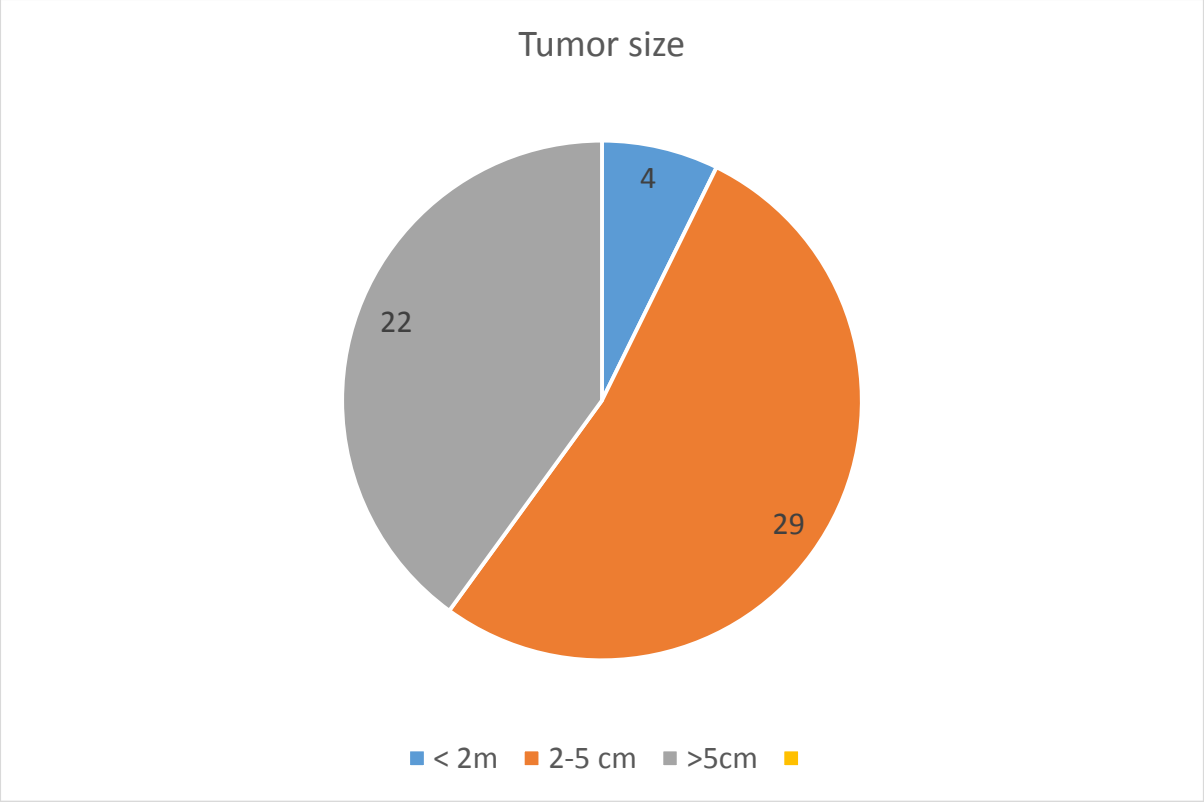

Chart 2: Pie chart showing the frequency of tumor size distribution in the study group.

Table 5: Distribution of histopathological diagnosis in the study subjects.

		Count	Percentage (%)
Histopathological diagnosis	Infiltrating ductal carcinoma	51	92.72
	Mucinous carcinoma + Infiltrating ductal carcinoma	1	1.82
	Papillary carcinoma	1	1.82
	Squamous cell carcinoma	1	1.82
	Mucinous carcinoma	1	1.82

Infiltrating ductal carcinoma was the most prevalent form seen in 51 participants. One case had both mucinous carcinoma and infiltrating ductal carcinoma features. Papillary carcinoma, Squamous carcinoma, mucinous carcinoma cases were only one each.

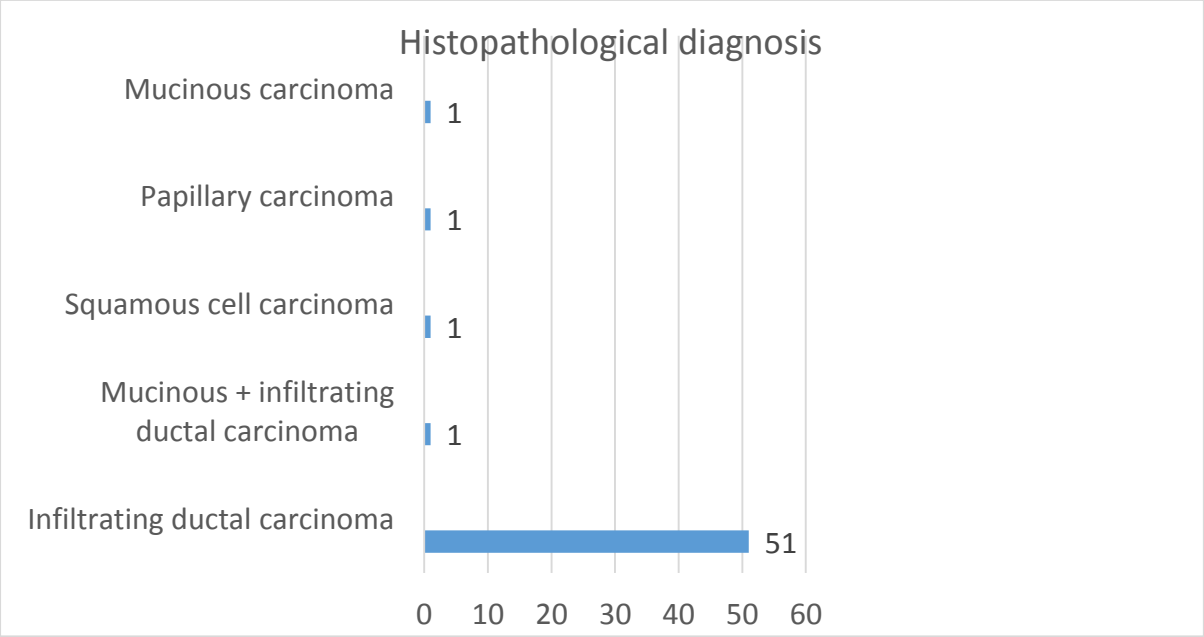

Chart 3: Bar diagram showing the frequency of distribution of histopathological diagnosis in the study subjects.

Table 6: T staging in the study group.

		Count	Percentage (%)
T category	T1	7	12.73
	T2	27	49.09
	T3	14	25.45
	T4	7	12.73

T2 and T3 category was seen in 27 and 14 cases respectively, and T1 and T4 category was seen in 7 cases each.

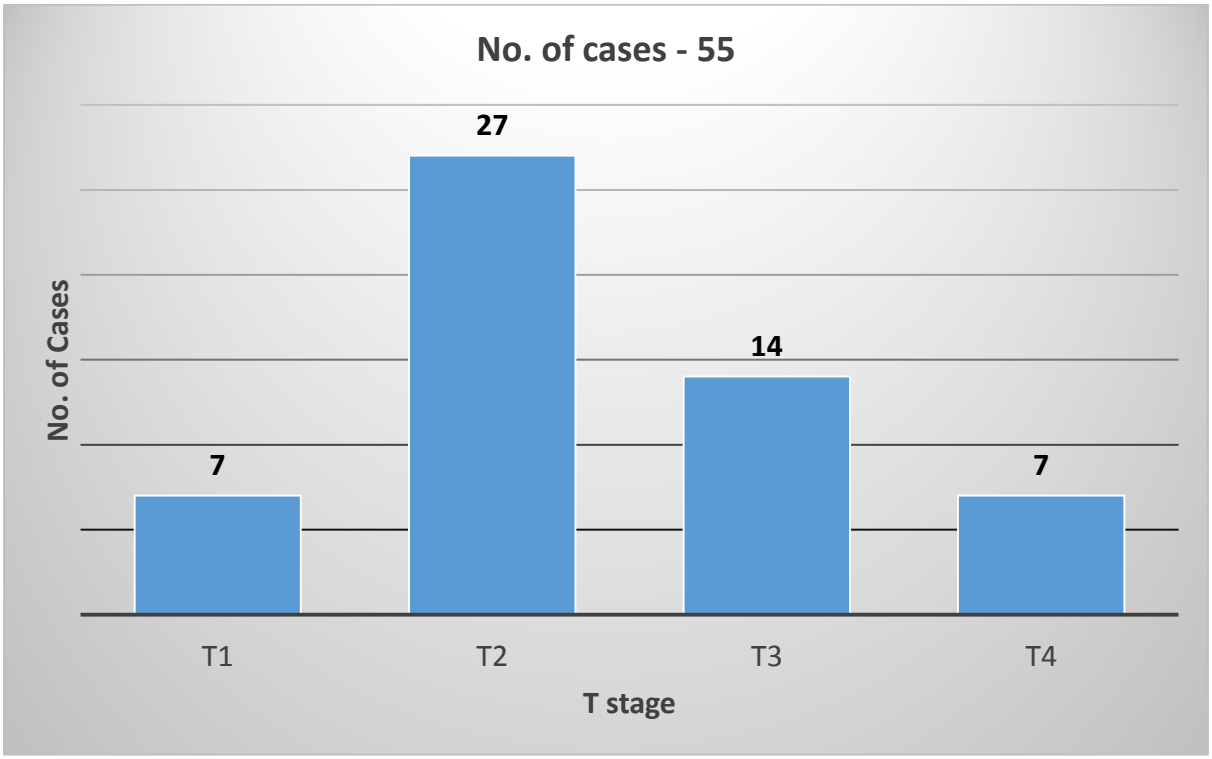

Chart 4: Bar diagram showing the frequency of T staging in the study group.

Table 7: N category distribution

		Count	Percentage (%)
N category	N0	22	40
	N1	15	27.2
	N2	11	20
	N3	5	9
	Nx	2	3.8

In 2 cases, no lymph nodes were retrieved grossly as well as microscopically. Twenty-two cases had no lymph node metastasis. However, 15 cases were in the N2 category, 11 cases in the N2 category, and 5 cases were in the N3 category.

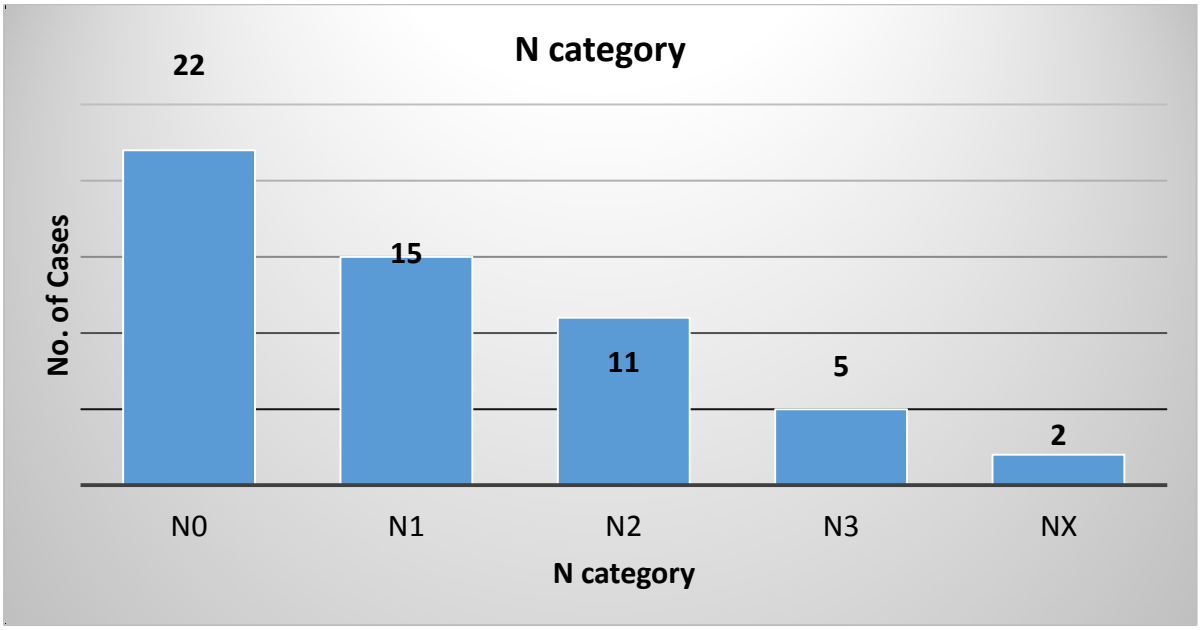

Chart 5: Bar diagram showing the frequency of N category distribution among study subjects.

Table 8: Tumor grade distribution.

		Count	Percentage (%)
Grade	Grade 1	32	58.2
	Grade 2	15	27.3
	Grade 3	8	14.5

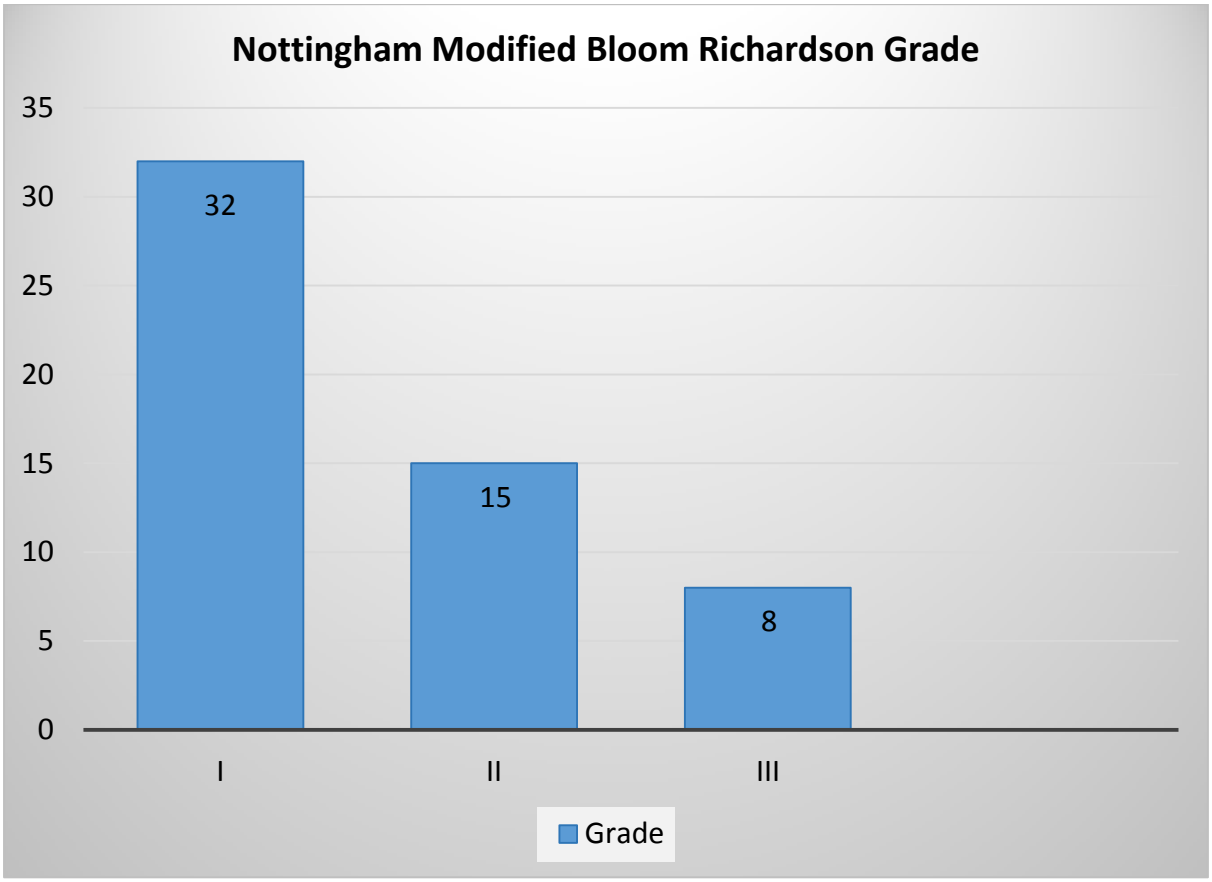

Chart 6: Bar diagram showing the frequency of tumor grade distribution.

Table 9: Lymph node status of the study group.

	Positive	Negative
No. of cases	31	24

In 31 patients, metastasis was found in lymph nodes, and in 24 patients, lymph nodes were negative.

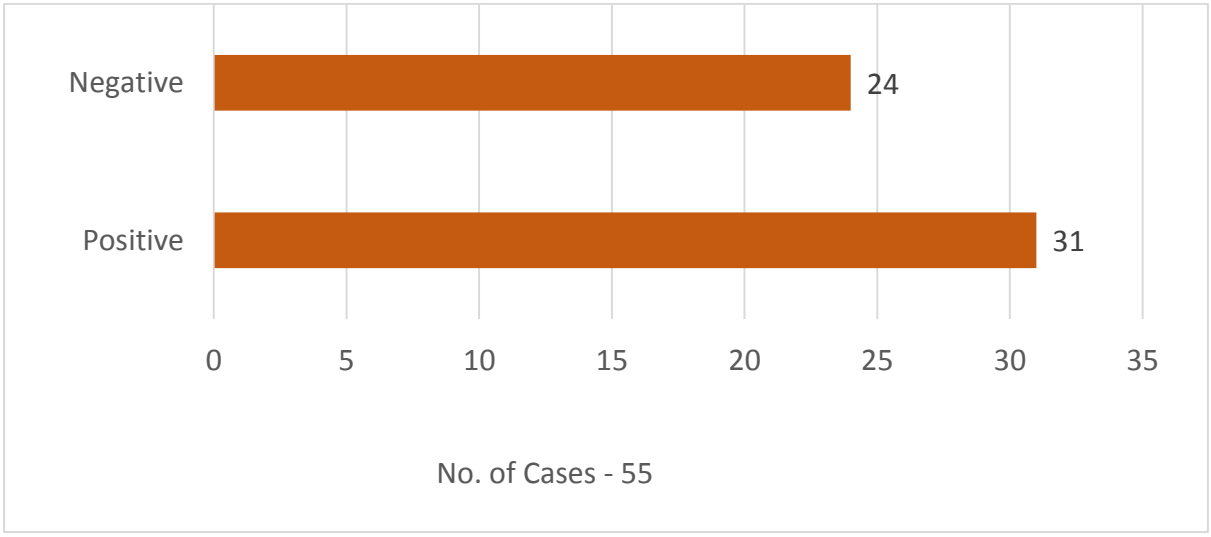

Chart 7: Bar diagram showing lymph node status of the study group.

Table 10: Stage of tumor in various cases in the study subjects.

		Count	Percentage (%)
Stage	I	3	5.4
	II	29	52.7
	III	23	41.9

Only 3 cases were at stage 2 at the time of presentation, and more cases were in stage 2, followed by 23 cases in stage 3.

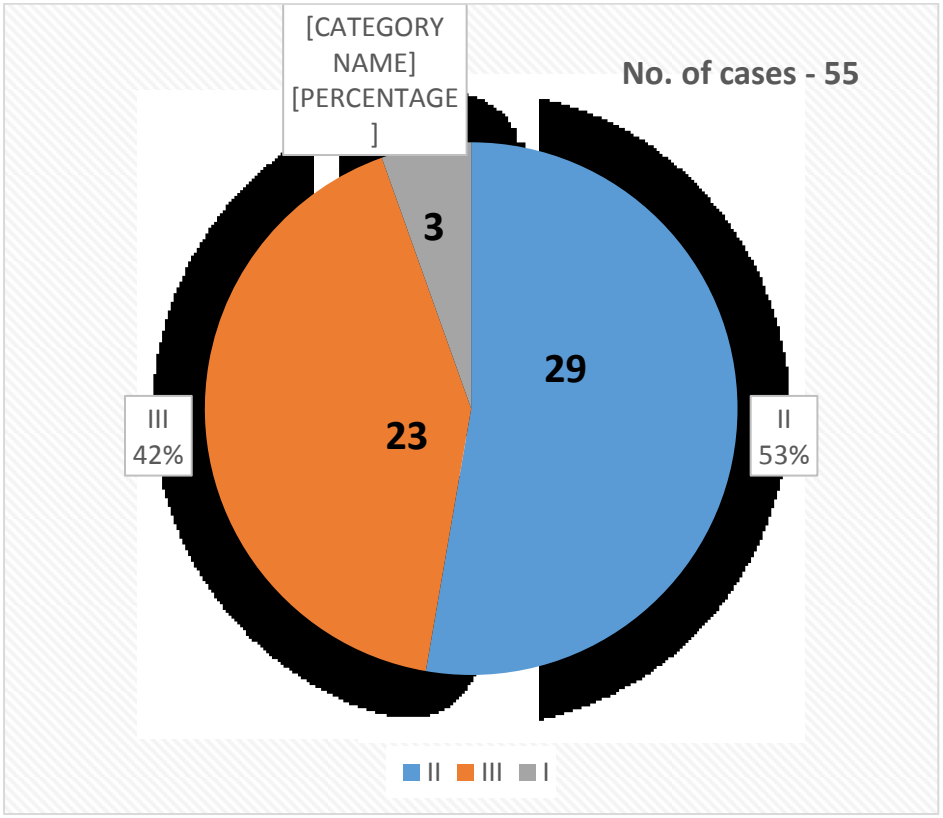

Chart 8: Pie chart showing the stage of tumor in various cases in the study subjects.

Table 11: Nottingham prognostic index among the study subjects.

		No. of cases	Percentage (%)
Nottingham prognostic index	2-2.4	4	7.3
	2.4-3.4	17	30.7
	3.4-5.4	20	36.4
	>5.4	14	25.6

According to NPI, 4 cases had an excellent prognosis, 17 had a good prognosis. A moderate prognosis was observed in 20 cases, and unfortunately, 14 cases had a poor prognosis.

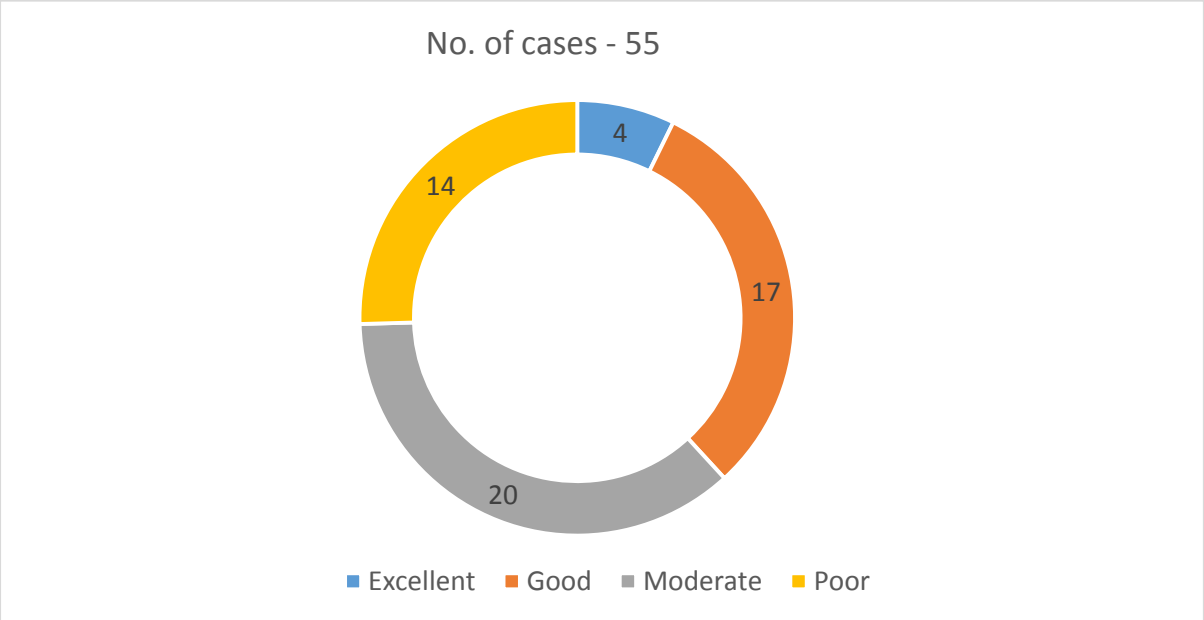

Chart 9: Nottingham prognostic index among the study subjects.

The density CD68 and CD163 expression were determined for all 55 cases. CD68 and CD163 macrophages were detected in intratumoral and peritumoral areas.

Table 12: Comparison of density of expression of intratumoral and peritumoral CD68 macrophages with the pathological stage.

Intratumoral CD68 macrophages				Peritumoral CD68 macrophages		
Pathological stage	Low(≤3/hpf)	High (>3/hpf)	p value	Low (≤10/hpf)	High (>10/hpf)	p value
I	2(3.6%)	1(1.8%)	0.402	1(1.8%)	2(3.6%)	0.037*
II	11(20%)	15(27.2%)		5(9.1%)	21(38.1%)	
III	8(14.5%)	18(32.7%)		0(0%)	26(47.2%)	
Chi square = 1.824, df= 2				Chi square = 6.588, df= 2		

The study demonstrated that the density of CD68 macrophages in the peritumoral area increases as the pathological stage increases and is statistically significant.

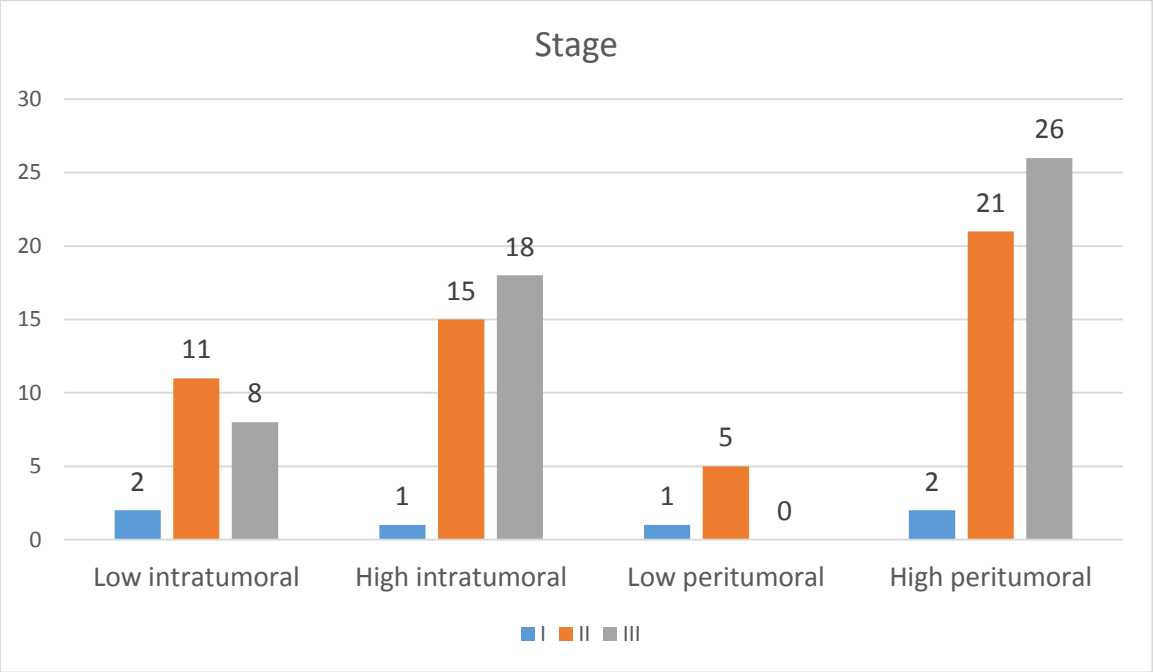

Chart 10: Comparison of density of expression of intratumoral and peritumoral CD68 macrophages with the pathological stage.

Table 13: Comparison of density of expression of intratumoral and peritumoral CD68 macrophages with pathological grade.

Intratumoral CD68 macrophages				Peritumoral CD68 macrophages		
Grade	Low(≤3/hpf)	High (>3/hpf)	p value	Low (≤10/hpf)	High (>10/hpf)	p value
I	16(29.1%)	16(29.1%)	0.023*	4(7.2%)	28(51%)	0.826
II	4(7.2%)	11(20%)		1(1.8%)	14(25.4%)	
III	1(1.8%)	7(12.7%)		1(1.8%)	7(12.7%)	

Chi square =4.972, df=2 Chi square =0.382, df=2

In the study, the density of expression of CD68 macrophages in the intratumoral area decreases as the grade increases and is statistically significant (p=0.023).

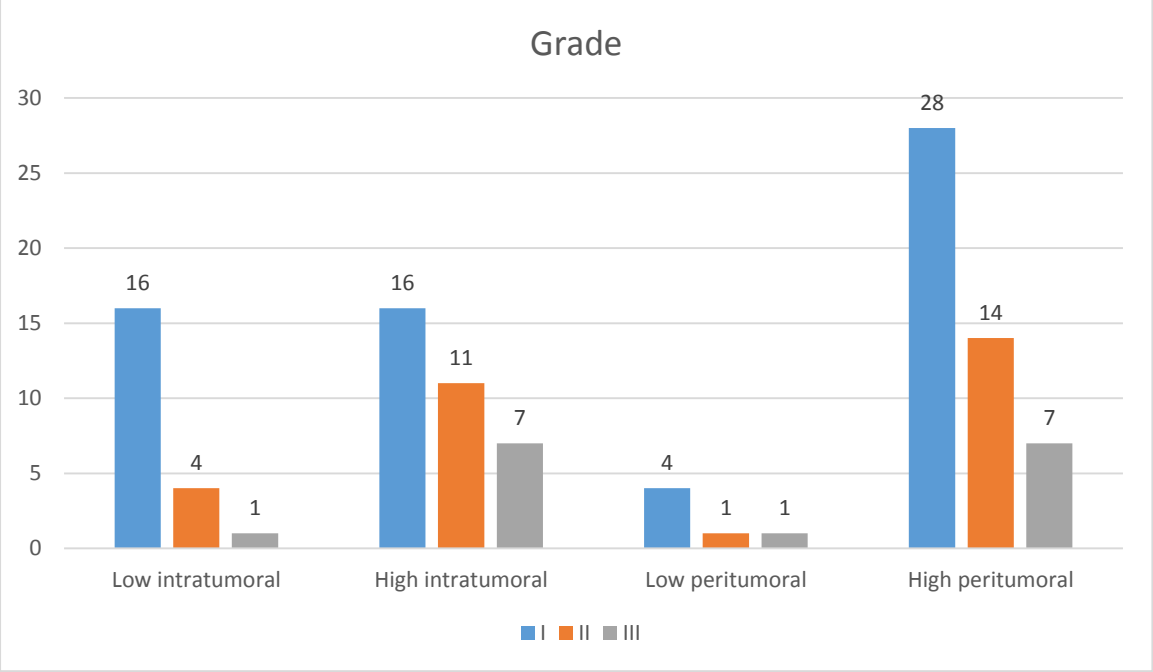

Chart 11: Comparison of density of expression of intratumoral and peritumoral CD68 macrophages with pathological grade.

Table 14: Comparison of density of expression of intratumoral and peritumoral CD68 macrophages with the tumor's size.

Intratumoral CD68 macrophages				Peritumoral CD68 macrophages		
Size	Low(≤3/hpf)	High (>3/hpf)	p value	Low (≤10/hpf)	High (>10/hpf)	p value
< 5 cm	17(31%)	22(40%)	0.197	5(9.1%)	34(61.8%)	0.478
> 5 cm	4(7.2%)	12(21.8%)		1(1.8%)	15(27.2%)	

Chi square = 1.661, df=1 Chi square =0.504, df=1

More density of expression of CD68 macrophages is seen in both intratumor and peritumor area if the tumor size is < 5 cm compared to the tumor size of > 5 cm, but statistically, this is not significant.

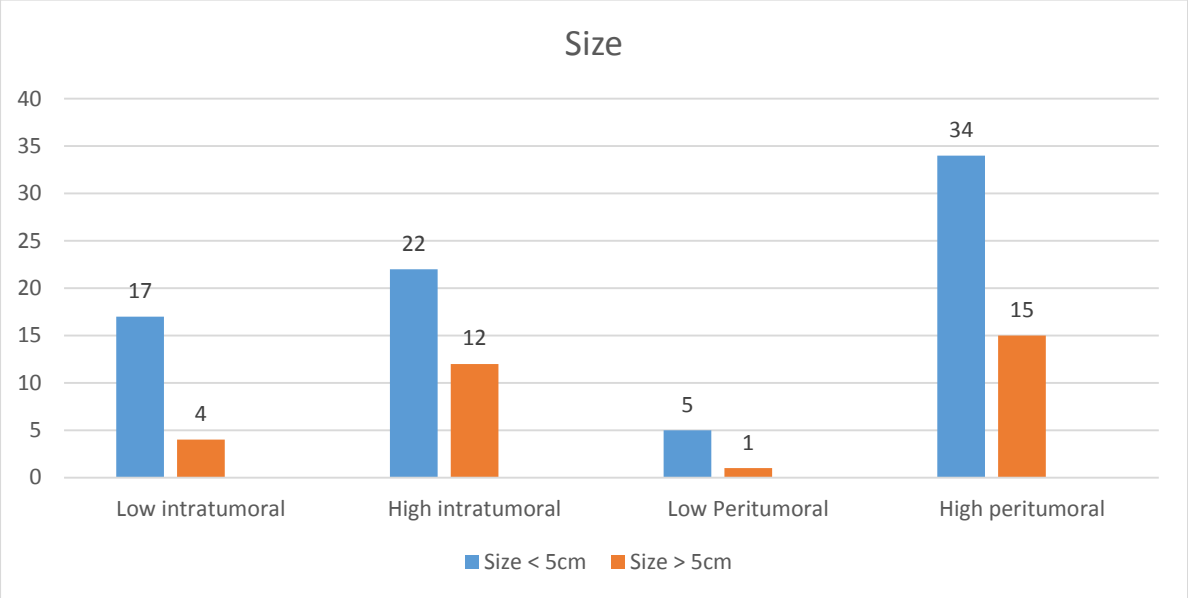

Chart 12: Comparison of density of expression of intratumoral and peritumoral CD68 macrophages with the tumor's size.

Table 15: Comparison of density of intratumoral and peritumoral CD68 macrophages with lymph node metastasis.

Intratumoral CD68 macrophages				Peritumoral CD68 macrophages		
Node Metastasis	Low (≤3/hpf)	High (>3/hpf)	p value	Low (≤10/hpf)	High (>10/hpf)	p value
Absent	10(18%)	14(25.4%)	0.640	5(9.1%)	19(34.5%)	0.038 *
Present	11(20%)	20(36.3%)		1(1.8%)	30(54.5%)	
Chi square =0.219, df=1				Chi square =4.315, df=1		

In the study, a high density of CD68 macrophage expression was seen peritumoral if lymph node metastasis is present and is statistically associated (p=0.038).

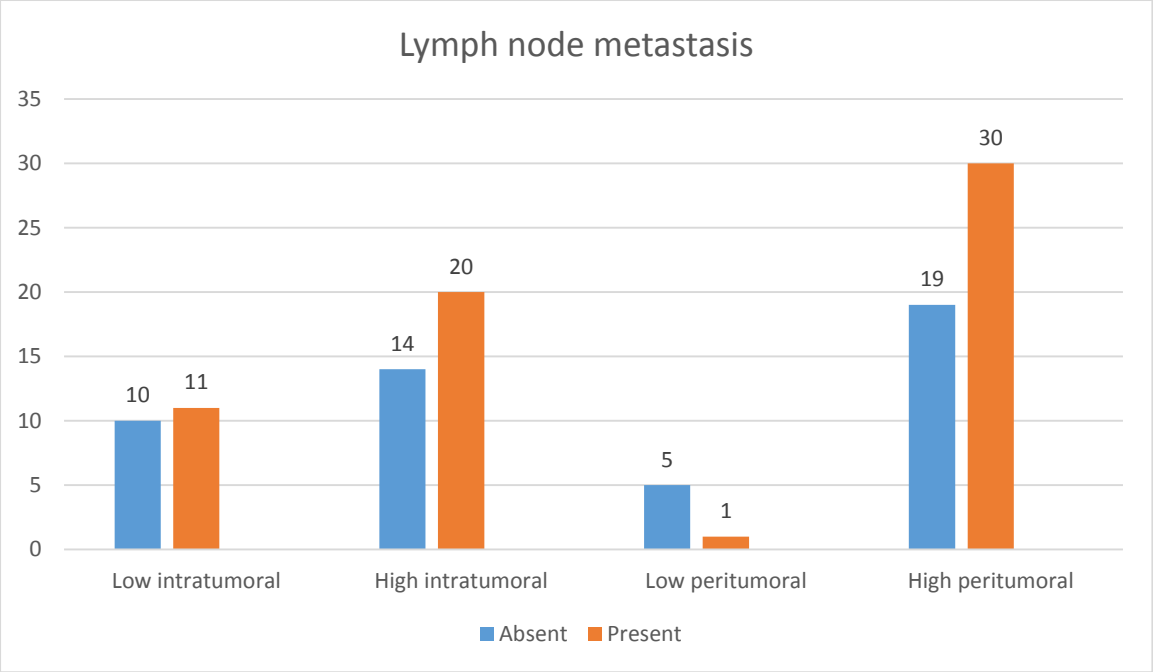

Chart 13: Comparison of density of intratumoral and peritumoral CD68 macrophages with lymph node metastasis.

Table 16: Comparison of density of expression of intratumoral and peritumoral CD163 macrophages with the pathological stage.

Intratumoral CD163 macrophages				Peritumoral CD163 macrophages		
Pathological stage	Low(≤3/hpf)	High (>3/hpf)	P value	Low (≤8/hpf)	High (>8/hpf)	p value
I	1(1.8%)	2(3.6%)	0.338	0(0%)	3(5.4%)	0.883
II	19(34.5%)	7(12.7%)		2(3.6%)	24(43.6%)	
III	19(34.5%)	7(12.7%)		2(3.6%)	24(43.6%)	

Chi square =2.172, df=2 Chi square = 0.249, df= 2

More density of expression of CD68 macrophages was seen in pathological stage II and stage III compared to stage I in intratumoral and peritumoral areas.

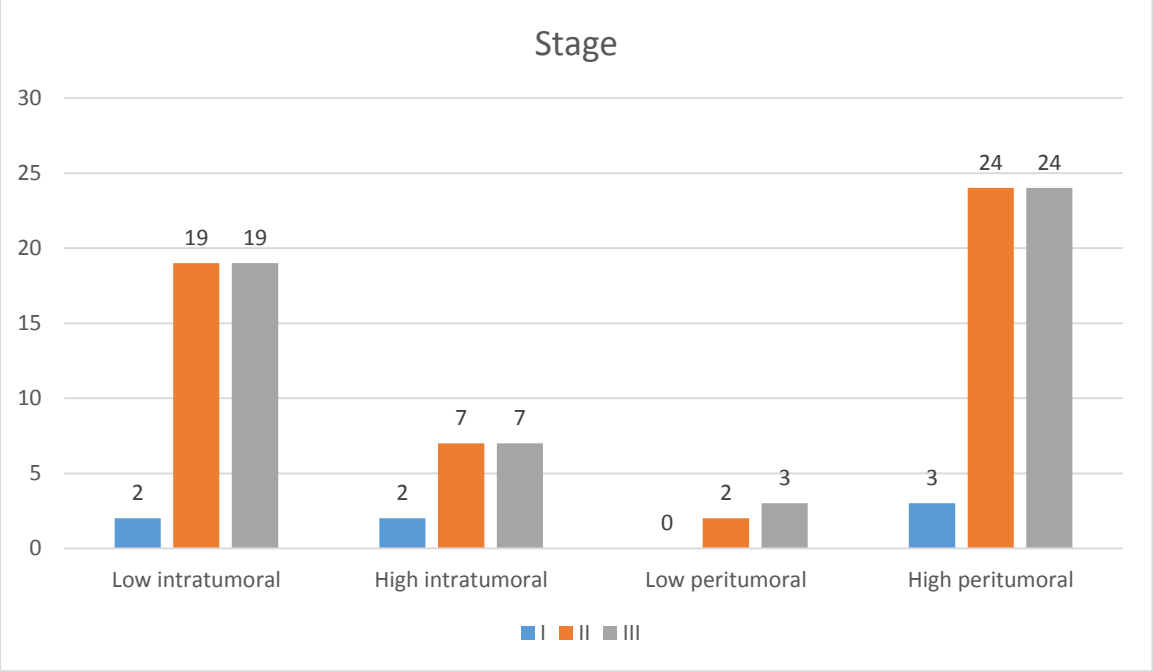

Chart 14: Comparison of density of expression of intratumoral and peritumoral CD163 macrophages with the pathological stage.

Table 17: Comparison of density of expression of intratumoral and peritumoral CD163 macrophages with grade.

Intratumoral CD163 macrophages				Peritumoral CD163 macrophages		
Grade	Low(≤3/hpf)	High (>3/hpf)	p value	Low (≤8/hpf)	High (>8/hpf)	p value
I	22(40%)	10(18.1%)	0.914	2(3.6%)	30(54.5%)	0.474
II	11(20%)	4(7.2%)		2(3.6%)	13(23.6%)	
III	6(11%)	2(3.6%)		0(0%)	8(14.5%)	
Chi square =0.180, df=2				Chi square =1.494, df=2		

In the study, the density of expression of CD68 macrophages in the intratumoral area decreases as the tumor grade increases, but this is not statistically significant.

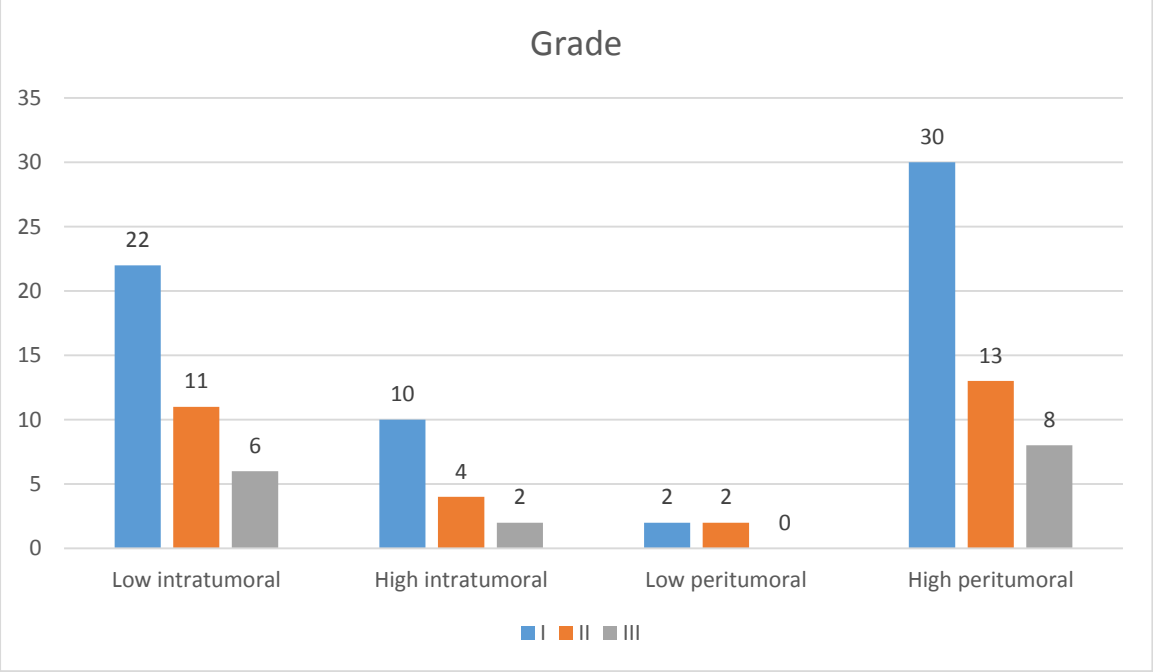

Chart 15: Comparison of density of expression of intratumoral and peritumoral CD163 macrophages with grade.

Table 18: Comparison of density of expression of intratumoral and peritumoral CD163 macrophages with the tumor size.

Intratumoral CD163 macrophages				Peritumoral CD163 macrophages		
Size	Low(≤3/hpf)	High (>3/hpf)	p value	Low (≤8/hpf)	High (> 8/hpf)	p value
< 5 cm	27(49.1%)	12(21.8%)	0.669	4(7.2%)	35(63.6%)	0.183
> 5 cm	12(21.8%)	4(7.2%)		0(0%)	16(29.1%)	

Chi square =0.183, df=1 Chi square =1.770, df=1

More density of expression of CD68 macrophages is seen in both intratumor and peritumor area if the tumor size is < 5 cm compared to the tumor size of >5 cm, but statistically, this is not significant.

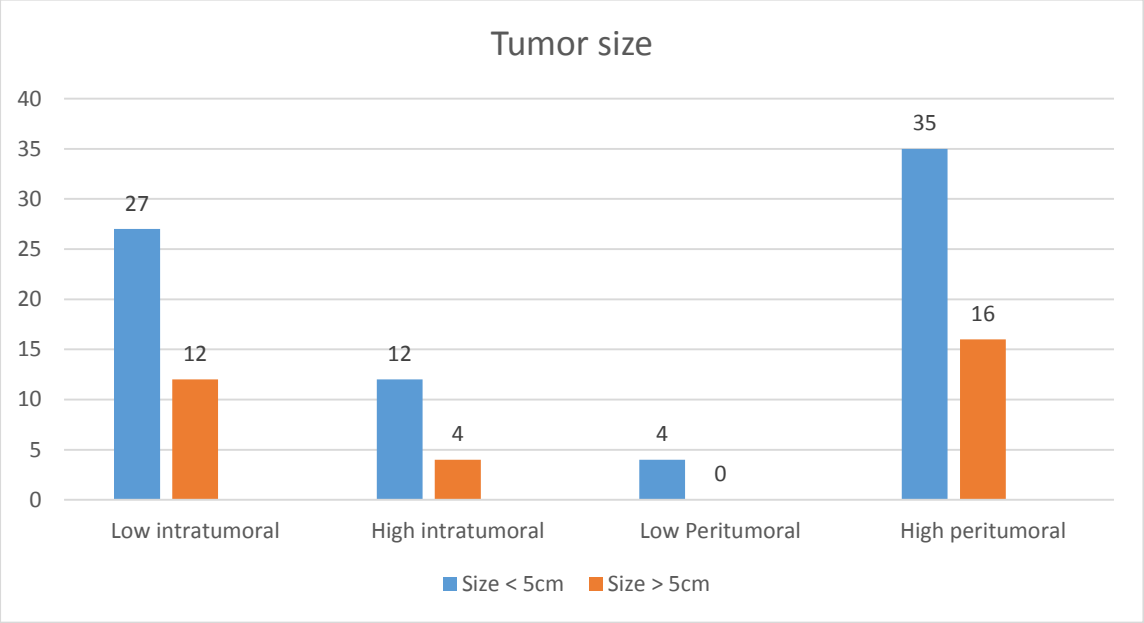

Chart 16: Comparison of density of expression of intratumoral and peritumoral CD163 macrophages with the tumor size.

Table 19: Comparison of density of expression of intratumoral and peritumoral CD163 macrophages with pathological lymph node status.

Intratumoral CD163 macrophages				Peritumoral CD163 macrophages		
Node Metastasis	Low(≤3/hpf)	High (>3/hpf)	p value	Low (≤8/hpf)	High (>8/hpf)	p value
Negative	17(31%)	7(12.7%)	0.991	2(3.6%)	22(40%)	0.790
Positive	22(40%)	9(16.3%)		2(3.6%)	29(52.7%)	

Chi square =0.00, df=1 Chi square =0.071, df= 1

In the study, a low density of expression of CD68 macrophages is seen in the intratumoral area, and a high density of expression of CD68 macrophages was seen in the peritumoral area but is not statistically significant.

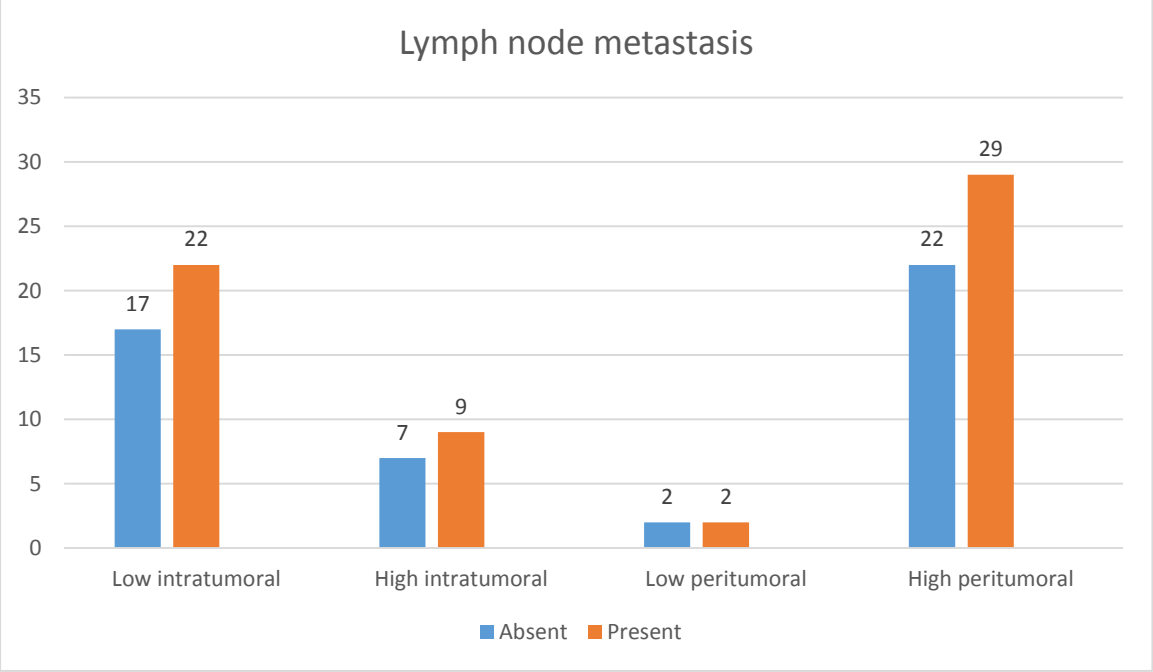

Chart 17: Comparison of density of expression of intratumoral and peritumoral CD163 macrophages with pathological lymph node status.

Discussion

Breast carcinoma is one of the major causes of cancer-related mortality in women worldwide. Increased incidence of breast carcinoma is also found in India. TAMs are a part of the tumor microenvironment and can elicit tumor cell transformation, induce destructive tumor reactions, and positively or negatively affect tumor progression depending on the subset, i.e., CD68 or CD163. Areas of hypoxia occur in tumors more than 2 mm, and factors like Monocyte chemotactic protein (MCP-1) and granulocyte-macrophage-colony stimulating factors are produced to recruit monocytes within the tumor microenvironment. Studies are currently concentrating on targeting the tumor microenvironment to improve prognosis and decrease the resistance against the treatment.

CD68 has M1 and M2 subtypes of macrophages. M1 is tumoricidal, and M2 has protumor activity, but studies show that CD68 macrophages increase vascularity and lymph node metastasis. Meanwhile, CD163, a specific biomarker for M2 macrophages have been associated with poor clinicopathological characters in some study. TAMs were observed in all the cases in the present study, and more TAMs infiltration was seen in intratumoral and peritumoral areas.

In the study, the maximum cases were between 50-59 years, followed by 40-49 years, the age group ranged from 28 years to 82 years, and the mean age was 51.4 years. The highest incidence was found in elderly patients, which is the same as literature facts.

Table 20: Comparison of age distribution with other studies.

Age	Jamiyan T et al[66]	Morita Y et al[67]	Sousa et al[8]	Present study
< 50 years	32(29.9%)	20(37.7%)	213(37.9%)	21(38.1%)
> 50 years	75(70.1%)	33(62.3%)	349(62.1%)	34(61.9%)

In this study, more number of patients had tumor size of > 2 cm grossly, which is in concordance with other studies done by Jeong H et al.[20] with 60.8% of cases, Ahmed et al.[68] with 76.6% cases, Kwon GY et al.[69] with 75.5% of cases having tumor size of > 2 cm. In the present study, more density of CD68 macrophages is seen in both intratumor and peritumor area if the tumor size is < 5 cm compared to the tumor size of > 5 cm, but statistically, this is not significant.

Table 21: Comparison of the size of the tumor with other studies.

Tumor Size	Jeong H et al.[20]	Ahmed I et al.[68]	Kwon GY et al.[69]	Present study
< 2cm	144(39.2%)	21(23.3%)	11(24.4%)	7(12.7%)
> 2cm	223(60.8%)	69(76.7%)	34(75.5%)	47(87.3%)

The majority of the present study cases were in grade I (58.1%), followed by grade II (27.2%). However, in the study done by Sousa et al.[8], Kwon GY et al.[69], Ahmed I et al.[68] more number of cases were found to be of grade II, whereas Wiratama et al.[71] study has the maximum number of cases in grade III, which is inconsistent with the present study. The density of expression of CD68 macrophages in the intratumoral area decreases as the grade increases and is statistically significant.

Table 22: Comparison of tumor grade with other studies.

Tumor grade	Sousa et al.[8]	Kwon GY et al.[69]	Ahmed I et al.[68]	Wiratama PA et al.[71]	Present study
I	46(8.3%)	8(17.8%)	13(14.6%)	3(0.7%)	32(58.1%)
II	264(47.1%)	23(51.1%)	53(58.8%)	14(31.8%)	15(27.2%)

III	250(44.6%)	14(31.1%)	24(26.6%)	27(61.3%)	8(14.5%)

In the present study, the majority were in the T2 stage, which is in concordance with the study done by Wiratama PA et al.[71] On the contrary, a study done by Ahmed et al.[68] and Ni C et al.[70] has more cases in the T1 stage because more early breast carcinoma subjects were included in their study.

Table 23: Comparison of T category with other studies.

T stage	Ni C et al.[70]	Wiratama PA et al.[71]	Ahmed I et al.[68]	Present study
T1	729(78.8%)	6(39.6%)	35(39%)	7(12.7%)
T2	145(15.6%)	20(45.5%)	19(21%)	27(49.2%)
T3	76(8.2%)	8(18.2%)	23(26%)	14(25.4%)
T4	24(2.5%)	10(22.7%)	13(14%)	7(12.7%)

Ni C et al.[70], Jamiyan et al.[66], and Kwon et al.[69] studies have more cases with no lymph node metastasis. Whereas the present study, a study by Shourouk E et al.[72] and Ahmed et al.[68], have more cases with tumor cell metastasis to lymph nodes. In the study, a high density of CD68 macrophage expression was seen peritumoral if lymph node metastasis is present and is statistically associated.

Table 24: Comparison of N staging with other studies.

Lymph node metastasis	Ni C et al.[70]	Jamiyan T et al.[66]	Shourouk E et al.[72]	Ahmed I et al.[68]	Kwon GY et al.[69]	Present study
Absent	526(53.4%)	65(62%)	26(43.4%)	35(38.8%)	24(53.3%)	22(40%)

Present	458(46.6%)	40(38%)	34(56.7%)	55(62.2%)	21(46.6%)	33(60%)

The study demonstrated that the density of CD68 macrophages in the peritumoral area increases as the pathological stage increases and is statistically significant. No correlation was found in a study done by Jamiyan T et al.[66] between high density of CD68 TAMs infiltration with any clinicopathological parameters; this may be due to CD68 express both M1 and M2, which have opposite effects.

Gwak et al.[73] and Jeong et al.[20] observed that the high density of TAMs was related to high tumor grade in both locations (intratumor and peritumor). Similarly, Ni C et al.[70] and Sousa et al.[8] showed high CD68 TAMs with a high histological grade but did not specify the location. Ch'ng et al.[74] and Yang et al.[75] observed that increased TAMs in the peritumoral area and not within the tumor were associated with high tumor grades. On the contrary, Yuan et al.[76] did not find any correlation between CD68 TAMs and tumor grade. Medrek et al.[12] revealed that the high density of CD68 TAMs in both intratumoral and peritumoral locations was related to poor prognostic factors.

Table 25: Comparison of density of CD68 TAMs with other studies.

	Intratumoral				Peritumoral			
	Jamiyan T et al.[66] / Shourouk et al.[72]		Present study		Jamiyan T et al.[66]/ Shourouk et al.[72]		Present study	
Age	Low	High	Low	High	Low	High	Low	High
<50 years	14 (13.1%)	18 (16.8%)	1 (1.8%)	18 (32.7%)	17 (15.8%)	15 (14%)	6 (11%)	13 (23.6%)
>50 years	40 (37.3%)	35 (32.7%)	5 (9.1%)	31 (56.3%)	37 (34.5%)	38 (35.1%)	15 (27.2%)	21 (38.1%)
Size < 5cm	18 (30%)	14 (23.3%)	5 (9.1%)	34 (61.8%)	23 (38.3%)	16 (21.6%)	17 (30.1%)	22 (40%)
> 5cm	12 (20%)	16 (26.6%)	1 (1.8%)	15 (27.2%)	8 (13.3%)	13 (26.6%)	4 (7.2%)	12 (21.8%)
Grade I	20 (18.6%)	11 (10.2%)	4 (7.2%)	28 (51%)	17 (15.8%)	14 (13.1%)	16 (29.1%)	16 (29.1%)
Grade II and III	34 (31.7%)	42 (39.2%)	2 (3.6%)	21 (38.1%)	37 (35.5%)	37 (35.5%)	5 (9.1%)	8 (14.5%)
LN not involved	17 (28.3%)	9 (15%)	5 (9.1%)	19 (34.5%)	17 (28.3%)	9 (15%)	10 (18.1%)	14 (25.4%)
LN involved	13 (21.6%)	21 (35%)	1 (1.8%)	30 (54.5%)	14 (23.3%)	20 (33.3%)	11 (20%)	20 (36.3%)
Stage 1	3 (5%)	0 (0%)	2 (3.6%)	1 (1.8%)	2 (3.3%)	1 (1.6%)	1 (1.8%)	2 (3.6%)

Stage 2	17 (28.3%)	15 (25%)	11 (20%)	15 (27.2%)	20 (33.3%)	12 (20%)	5 (9.1%)	21 (38.1%)
Stage 3	10 (16.6%)	15 (25%)	8 (14.5%)	18 (32.7%)	9 (15%)	16 (26.6%)	0 (0%)	26 (47.2%)

In comparison with a study done by Jamiyan et al.[66], it was found that the density of intratumoral expression of CD68 TAMs in the age group of patients less than 50 years in agreement with the findings of the present study.

On analysis of the density of intratumoral expression of CD68 TAMs in age group less than 50 years, Jamiyan T et al.[66] show low expression, which was in disagreement with the findings of the present study in which more than 50% that is 56.3% belonging to the age group of 50 years or more showed higher intratumoral expression of CD68 TAMs.

On comparison of the density of peritumoral expression of CD68 TAMs in the age group less than 50 years, it was found that there is a disagreement between the findings of the present study with a study done by Jamiyan T et al.[66], which showed low peritumoral expression of CD68 TAMs.

On the evaluation of the density of peritumoral expression of CD68 TAMS in more than 50 years, it was found that the findings of the study by Jamiyan T et al.[66] are as per the findings of the present study.

On comparison of the density of expression of CD68 TAMs in the tumor of less than 5 cm, it was found that the findings of the present study showed complete discordance with the study done by Shourouk E et al.[72] with a higher intratumor expression of CD68 TAMs in the present study but a low intratumor expression of CD68 TAMs in the study done by Shourouk E et al.[72]

On comparison of intratumoral expression of CD68 macrophages in patients with tumor size more than 5 cm, both the studies showed high expression.

There was a complete discordance comparing the density of peritumor CD68 macrophages in tumor size less than 5 cm. Shourouk et al.[72] showed low expression and high expression in the present study.

On the analysis of the density of peritumoral CD68 TAMs in less than 5 cm tumor size, there was complete concordance with the study done by Shourouk et al.[72] and the present study, showing higher expression in both the studies.

On comparison of the intratumoral density of CD68 TAMs in patients with grade 1 tumor, there was a complete concordance between the findings of the study done by Jamiyan T et al.[66] and the present study with the lower expression in the study done by Jamiyan T et al.[66] and higher expression in the present study.

On evaluating the density of expression of intratumoral CD68 TAMs in patients with grade II and III tumors, there was a complete concordance between both studies, with both showing high expression.

The density of expression on peritumoral macrophages CD68 TAMs in patients with grade I tumor, study done by Jamiyan T et al.[66] shows low expression whereas present study showed no difference in the percentage of distribution patients with low and high expression. Whereas density of peritumoral expression of peritumoral CD68 TAMs in grade II and III, a study was done by Jamiyan T et al.[66] showed no difference in the percentage of expression showing low and high expression, but the present study showed high expression.

On analysis of the density of expression of intratumoral CD68 TAMs, there was complete discordance in studies done by Jamiyan T et al.[66] and the present study in patients with no lymph node metastasis. In contrast, in patients with lymph node metastasis, there was a

complete concordance in the study done by Jamiyan T et al.[66] and the present study, showing a higher density of peritumoral expression CD68 TAMs.

On comparing the density of expression of intratumoral CD68 TAMs, there was complete discordance between the present study and study done by Shourouk E et al.[72], which shows low expression, but the present study shows high expression.

On the evaluation of the density of expression of intratumoral CD68 TAMs in cases with lymph node metastasis, both the study showed complete concordance with high expression in the present study as well as the study done by Shourouk E et al.[72]

On analysis of the density of expression of peritumoral CD68 Tams in patients with no lymph node metastasis, there was complete discordance between the study done by Shourouk E et al.[72] and the present study with lower expression in the study done by Shourouk E et al.[72] and higher expression in the present study. On analysis of the density of expression of peritumoral CD68 TAMs on patients with lymph node metastasis, both the study showed high expression.

On comparing the density of expression of intratumoral expression of CD68 TAMs in patients with stage I disease, there was a low expression in the study done by Shourouk E et al.[72] and present study. Whereas on stage II disease, there was complete discordance between the study done by Shourouk E et al.[72] and the present study showing low expression and high expression in the present study. Nevertheless, in patients with stage III tumors, complete concordance between the study done by Shourouk E et al.[76] and the present study showing high expression in both the studies.

On analysis of the density of peritumoral expression of CD163 TAMs in patients with stage I disease, there was complete discordance between the present study and the study done by Shourouk E et al.[72] low expression, but the present study showed high expression. Whereas in

patients with stage II disease, there was complete discordance between the study done by Shourouk E et al.[72] and the present study. Shourouk E et al.[72] studies showed low expression, and the present study showed high expression. Analysis of peritumoral expression of CD68 TAMs in patients with stage III disease, there was complete concordance showing higher expression in both the studies.

Table 26: Comparison of density of CD163 TAMs with other studies.

	Intratumoral				Peritumoral			
	Jamiyan T et al.[66]/ Shourouk et al.[72]		Present study		Jamiyan T et al.[66] / Shourouk et al.[72]		Present study	
Age	Low	High	Low	High	Low	High	Low	High
<50 years	13 (12.1%)	19 (17.7%)	2 (3.6%)	17 (31%)	15 (14%)	17 (15.8%)	14 (25.4%)	5 (9.1%)
>50 years	41 (38.3%)	34 (31.7%)	2 (3.6%)	34 (61.8%)	40 (37.3%)	35 (32.7%)	25 (45.4%)	11 (20%)
Size 0< 5cm	25 (41.6%)	14 (23.3%)	27(49.1%)	12(21.8%)	21 (35%)	18 (30%)	4 (7.2%)	30 (54.5%)
> 5cm	7 (11.6%)	14 (23.3%)	12(21.8%)	4(7.2%)	10 (16.6%)	11 (18.3%)	0 (0%)	16 (29.1%)
Grade I	25 (23.3%)	6 (5.6%)	2 (3.6%)	30 (54.5%)	22 (20.5%)	9 (8.4%)	22 (40%)	10 (18.1%)
Grade II and III	29 (27.1%)	47 (43.9%)	2 (3.6%)	21 (38.1%)	33 (30.8%)	43 (40.1%)	17 (31%)	6 (11%)
LN not involved	21 (35%)	5 (8.3%)	2 (3.6%)	22 (40%)	15 (25%)	11 (18.3%)	17 (30.9%)	7 (12.7%)
LN involved	11 (18.3%)	23 (38.3%)	2 (3.6%)	29 (52.7%)	16 (26.6%)	18 (30%)	22 (40%)	9 (16.3%)
Stage 1	3	0	1	2	3	0	0	3

	(5%)	(0%)	(1.8%)	(36.6%)	(5%)	(0%)	(0%)	(5.4%)
Stage 2	22 (36.6%)	10 (16.6%)	19 (34.5%)	7 (12.7%)	16 (26.6%)	16 (26.6%)	2 (3.6%)	24 (43.6%)
Stage 3	7 (11.6%)	10 (16.6%)	19 (34.5%)	7 (12.7%)	12 (20%)	13 (23.6%)	0 (0%)	24 (43.6%)

On comparison of the density of intratumoral expression of CD163 TAMs in the age group less than 50 years, it was found that the findings of the present study and a study done by Jamiyan T et al.[66] showed complete concordance as both the studies showed high expression.

Whereas on the evaluation of the density of intratumoral expression of CD163 TAMs in the age group more than 50 years, the findings of the present study showed disagreement with the findings of a study done by Jamiyan T et al.[66], the present study showing higher intratumoral expression of CD163 TAMs in contrast to the low intratumoral expression of CD163 TAMs by Jamiyan T et al.[66]

On analysis of the density of peritumoral expression of CD163 TAMs in the age group less than 50 years of a study done by Jamiyan T et al.[66] with the present study's findings showed discordance. Present study showing low expression in this age group, whereas a study done by Jamiyan T et al.[66] showed high expression.

On analysis of the density of intramural expression of CD163 tumor-associated in more than 50 years, there was a complete concordance with a study done by Jamiyan T et al.[66] with present study as both showing low expression.

In the analysis of intratumoral expression of CD163 TAMS with tumor size less than 5 cm, there was complete discordance between the two studies showing low expression in the study done by Shourouk et al.[72] and higher expression in the present study.

On analysis of comparison of the density of peritumoral expression of CD163 TAMs in patients with tumor size less than 5 cm, there was complete concordance between the studies done by Shourouk et al.[72] and the present study. Whereas in patients with a tumor size of more than 5 cm, there was complete discordance in peritumoral expression in studies done by Shourouk et al.[72] and the present study.

Comparing the density of expression of intratumoral CD163 TAMs in patients with grade I tumor showed discordant result with the study done by Jamiyan T et al.[66] and the present study, showing low expression in the study done by Jamiyan t et al.[66] and higher in the present study. In contrast, the same comparison of the density of expression of intratumoral CD163 TAMS in grade II and III results are concordant with Jamiyan T et al.[66] studies showing higher expression in both. On analysis of the density of expression of peritumoral expression of CD163 TAMs, a study done by Jamiyan T et al.[66] and present study showed low expression in grade I cases whereas cases with grade II and III there was complete discordance between both the studies.

On comparing the density of intratumoral CD163 TAMs in patients with no lymph node metastasis, there was complete discordance between the present study and the study done by Jamiyan T et al.[66], showing low expression in patients with no lymph node metastasis and higher expression in the present study.

On analysis of the density of expression of intratumoral CD163 TAMs in cases with lymph node metastasis, there was complete concordance between the study done by Jamiyan T et al.[66]

Furthermore, the present study both showing high expression. On evaluating the peritumoral expression of CD163 TAMs in patients with no lymph node metastasis, there was complete

concordance between the study done by Jamiyan T et al.[66] and the present study, both showing low peritumoral expression.

On analysis of the density of expression of peritumoral CD163 TAMs in cases with no lymph node metastasis, there was complete discordance concordance between the present study and study done by Jamiyan T et al.[66], which is showing high expression in the present whereas the present study showing low expression.

On comparing the density of expression of CD163 TAMs in cases with no lymph node metastasis, there was complete discordance between the present study and study done by Shourouk E et al.[72], showing low, but the present study showed high expression.

On evaluating the density of intratumoral expression of CD163 TAMs in patients with positive lymph node metastasis, there was complete concordance between the present study and the study done by Shourouk E et al.[72] with higher expression in both the studies.

On analysis of the density of peritumoral expression of CD163 TAMs in patients with no lymph node involvement by tumor cells, there was complete concordance between the present study and study done by Shourouk E et al.[72], with both showing low expression. On the evaluation of peritumoral expression of CD163 TAMs in patients with positive lymph nodes, there was complete discordance between a study done by Shourouk E et al.[72] and the present study with high expression in the study done by Shourouk E et al.[72] and low expression in the present study.

On comparison of the density of expression of CD163 TAMs in cases with stage I disease, there was complete discordance between the present study and study done by Shourouk E et al. [72], whereas in patients with stage II disease, there was concordance between study done by Shourouk E et al.[72] and the present study. Both the studies showed low expression. In patients with stage III tumor, there was complete discordance between the study done by Shourouk E

et al.[72] and the present study showing lower expression in the present study and higher expression in the study done by Shourouk E et al.[72]

On comparison of peritumoral expression of CD163 in stage I disease, there was a complete discordance between the study done by Shourouk E et al.[72] and the present study showing low expression in the study done by Shourouk E et al.[72] and higher expression in the present study. Whereas in patients with stage II disease, there was no difference in the percentage of expression in the studies done by Shourouk E et al.[72], but it showed higher expression in the present study. While inpatients with stage III disease, both the studies showed complete concordance having higher expression in the study done by Shourouk E et al.[76] and present study.

In a study done by Jeong H et al.[20] and Ahmed et al.[68], the higher density of TAMs showed statistical association with more tumor size, a higher grade, which can be explained by the protumoral M2 phenotype of TAM. TAMs have been shown to enhance the growth of breast cancer cells. These genes are responsible for enhanced tumorigenicity and resistance to chemotherapy in breast cancer cells. Factors involved are TAMs function in the inhibition of antitumor response by releasing vascular endothelial growth factor, epidermal growth factor, platelet-derived growth factor, Tumor necrosis factor – α, Transforming growth factor –β, Interleukin -1β, Interleukin -8. They also showed that higher nodal metastasis is statistically associated with a higher density of TAMs. The epithelial-mesenchymal transition of tumor cells is necessary to initiate the invasion promoted by TAMs release by downregulating β-catenin and E-cadherins.

Epidermal growth factor released by TAMs and colony-stimulating factor-1 released by adenocarcinoma cells leads to the production of podosomes and invadopodia by TAMs adenocarcinoma cells to extravasation and degradation of the extracellular matrix.

In the present study, more density of expression of CD163 macrophages was seen in pathological stage II and stage III compared to stage I in intratumoral and peritumoral areas. The density of CD163 macrophages decreases as the tumor grade increases, but this is not statistically significant.

In tumor size, less than 5 cm, low density of CD163 TAMs is observed in the intratumoral area, but high density is observed in the peritumoral area, but statistically, this is not significant. In the study, a low density of expression of CD163 macrophages is seen in the intratumoral area, and a high density of expression of CD68 macrophages was seen in the peritumoral area is not statistically significant.

Jung KY et al.[77] found no significant difference in CD163 TAMs density among TNM stages in lung, breast, or thyroid cancer. However, cancer tissue showed higher CD163 TAMs density than those in normal tissues, similar to the present study.

Medrek et al.[12] concluded that dense infiltration of CD163 TAMs in the peritumoral area was associated with ER and PR negativity, grade, tumor size, but there was no such association in the intratumor area. Several studies have reported that TAMs densities are associated with a good prognosis; such contradictory results might be due to differences in grade, the number of cases, or tumor size.

Studies were done on relapse-free survival, and overall survival by Sousa et al.[8] revealed that CD163 cells in primary breast tumors are associated with poor prognosis, correlated with ER negativity, poor differentiation (grade 3), and ductal type. Yang et al.[75] also found that increased CD163 TAMs in the peritumoral area was correlated with poor prognostic factors, but they also did not find any statistical difference in the intratumoral area.

Jamiyan et al.[66] included only triple-negative cancer and observed that CD163 does not affect prognosis statistically, but more TAMs density was found, especially CD163. Shourouk et

al.[72] reported a high density of CD163 TAMs was associated with larger tumor size, vascular invasion, nodal metastasis, and stage in both intratumoral and peritumoral areas.

Ni C et al.[70] also concluded that CD163 TAMs are significantly associated with poor prognosis and advanced histologic grades in early breast cancer. However, they included only Basal-like breast cancer cells because they are more likely to express a broader range of receptors for macrophage type of cytokines, which could recruit macrophages into the microenvironment and promote monocyte differentiation into M2-like macrophages.

There are a few limitations in this study; firstly sample size is less; secondly, like other studies, as immunohistochemistry can only measure one or two markers per sample, it may not fully reflect the complex factors involved. More advanced studies using different technologies are expected, and further studies are required to determine the cross-interaction between diverse TAMs and the tumor microenvironment.

Conclusion

- One of the study's objectives is to determine the density of CD68 TAMs and CD163 TAMs concerning primary breast carcinoma.
- Our study conclusively demonstrates the density of expression of CD68 TAMs progressively increases in concordance with the pathological stage of breast cancers.
- Simultaneously, the density of CD68 TAMs in the intratumoral area exhibits progressive reduction as the grade of breast cancer increases.
- Also, breast carcinomas with lymph node metastasis exhibited a high density of CD68 Tams in the peritumoral area. However, more such studies are needed along with molecular subtyping to study the role of CD68 and CD163 TAMs expression in primary breast carcinoma.

Summary

- The study was undertaken in the Pathology department, SDU Medical College, Kolar, from December 2018 to May 2020.
- Prospective cases were collected from January 2019 to May 2020, and retrospective cases were collected from June 2018 to December 2018.
- Fifty-five cases were collected, out of which the majority belonged to 51-59 years (34.5%).
- The majority of cases had a tumor size of less than 5 cm.
- The majority of cases were of grade I tumors (58.2%).
- Tumor stage T2 had the maximum number of cases (49.1%)
- Lymph node stage N0 had the majority of cases (40%).
- The majority of cases were Nottingham Prognostic index category III (36.4%).
- The study demonstrated that the density of CD68+ macrophages in the peritumoral area increases as the pathological stage increases and is statistically significant.
- In the study, the density of expression of CD68+ macrophages in the intratumoral area decreases as the tumor grade increases and is statistically significant.
- In the study, more density of expression of CD68+ macrophages seen in both intratumor and peritumor area if the tumor size is < 5 cm as compared to tumor size of > 5 cm
- In the study, a high density of CD68+ macrophages expression was seen peritumoral, if lymph node metastasis is present and is statistically associated.

- More density of CD163+ macrophages was seen in pathological stage II and stage III compared to stage I in intratumoral and peritumoral areas.

- In the study, the density of CD163+ macrophages in the intratumoral area decreases as the tumor grade increases, but this is not statistically significant.
- More density of CD163+ macrophages is seen in both intratumor and peritumor area if the tumor size is < 5 cm compared to >5 cm.
- In the study, a low density of CD163+ macrophages was seen in the intratumoral area, and a high density of CD163+ macrophages was seen in the peritumoral area.
- Further studies are required with a large sample size to prove the role of CD68 and CD163 expression in primary breast carcinoma and its relation with stage and grade. Nevertheless, this study can be a pilot study to improve the result of a meta-analysis.

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ANNEXURES

PROFORMA

Case No:

Name:

Age:

IP No:

Biopsy No:

Presenting complaints:

Past history:

Family History:

Menstrual history:

Neoadjuvant therapy received: YES/ NO

Clinical diagnosis with TNM staging:

Type of surgery:

Side: Right/ left

GROSS FEATURES

Specimen

Partial breast

Total breast (including nipple and skin)

Others:

Specimen Size: **Specimen**

Laterality:

Tumor Focality:

Measurements: Skin:

CUT SURFACE

Size: Appearance:

Consistency: Margins:

Histopathological diagnosis: Associated with DCIS: YES/ NO

Closest margin to the tumor: Skin involvement:

Histological grading**:** Vascular invasion:

MICROSCOPY

Tumor Site: Invasive Carcinoma **Ductal Carcinoma In Situ:**

Macroscopic and Microscopic Extent of Tumor: *Size (Extent) of DCIS:*

Skin: *Architectural Patterns:*

Nipple: *Nuclear Grade:*

Skeletal Muscle: *Necrosis:*

Lobular Carcinoma In Situ:

Histologic Type of Invasive Carcinoma:

Histologic Grade: Nottingham Histologic Score:

Glandular (Acinar)/Tubular Differentiation:

Nuclear Pleomorphism

Mitotic Count

Overall Grade

Margins:

Lymph-Vascular Invasion:

Extranodal Extension

Method of Evaluation of Sentinel Lymph Nodes:

Pathologic Staging (pTNM):

Primary Tumor (Invasive Carcinoma)

Regional Lymph Nodes

Distant Metastasis (M)

LYMPH NODE

Total No. examined: Total No. positive:

Lymph Node Sampling

Lymph nodes

Sentinel lymph node

Axillary dissection (partial or complete dissection)

Lymph nodes present within the breast specimen

Other lymph nodes

Specimen Integrity: Additional Pathologic Findings:

Immunohistochemical staining :

CD68

CD163

KEYS TO MASTER CHART

Age	1- <50 years 2- >50 years
Laterality	1 - Left 2- Right
Histopathological diagnosis	1- Infiltrating ductal carcinoma 2- Squamous cell carcinoma 3- Mucinous carcinoma 4- Papillary carcinoma
Size	1- < 5cm 2- > 5cm
Malignancy grading	1- Well-differentiated 2- Moderately differentiated 3- Poorly differentiated
Lymph node involvement	1- Present 2- Absent
Pathological T	1- T1 2- T2 3- T3 4- T4

Pathological N	0- N0 1- N1 2- N2
Stage	1. I 2. II 3. III
Nottingham prognostic index(NPI)	1- Excellent prognosis 2- Good prognosis 3- Moderate prognosis 4- Poor prognosis
CD163 intratumor	1. Low expression 2. High expression
CD163 peritumor	1. Low expression 2. High expression
CD68 intratumor	3. Low expression 4. High expression
CD68 peritumor	3. Low expression 4. High expression