CORRELATION OF CLINICOPATHOLOGICAL FINDINGS AND C5AR2 EXPRESSION IN BREAST CANCER

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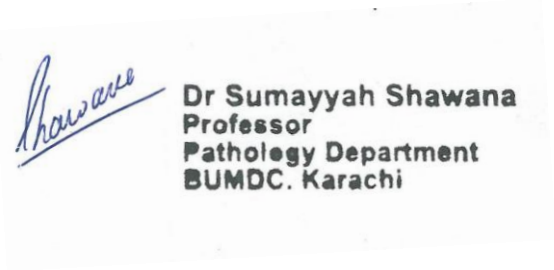

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Dedicated to my beloved mother Hameeda Khaliq,

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ABSTRACT

The breast cancer is the most prevalent cancer among females worldwide. According to Shaukat Khanum Memorial Hospital statistics during the year 2022, breast cancer was the leading cancer among the top ten malignancies in adult females accounting for almost 1/2 of the cancer cases alone (51.1%) (Shahid, et al.,2023). The tumor microenvironment plays a central role in tumor proliferation and invasion via cancer-associated fibroblasts, macrophages, and various other mediators like complement, chemokines. Complement component C5a is a potent anaphylatoxin and chemotactic agent. Its receptor C5aR2-mediated inflammatory response is partly responsible for tumorigenesis. The C5aR2 mRNA is detected in human peripheral blood leukocytes, platelets, bone marrow, spleen, and other organs of which neutrophils are the most abundant source of C5aR2 (Ting, Malgorzata, Ke li, 2017). Objectives were to evaluate the expression of C5aR2 (GPR77) expression in stromal and epithelial cells of breast carcinoma and to determine the correlation of C5aR2 (GPR77) expression in breast carcinoma with clinicopathological parameters. It was a cross-sectional study carried out at PNS Shifa Hospital, Karachi, for a period of six months. 128 formalin-fixed paraffin-embedded tumor specimens of breast cancer patients were selected and examined for immunohistochemical expression of C5aR2, using Rabbit polyclonal C5aR2 antibodies IgG type, in breast cancer cells and stromal cells. Clinical and pathological records were reviewed for data collection. The results of immunohistochemistry were correlated with clinicopathological parameters of breast cancer. This study was conducted to determine the correlation of complement receptor C5aR2 with clinicopathological parameters of breast cancer (age, receptor status, stage, grade, proliferation marker Ki-67), using a simplified protocol. The results revealed increased C5aR2 expression in tumor cells as compared to stromal cells. An upregulation of C5aR2 expression was observed in old age. The expression of C5aR2

increased with the increasing grade and advancing stage. This experiment showed a strong association of C5aR2 with hormone receptors and HER2. In tumor cells, Luminal B showed the highest C5aR2 expression whereas in stromal cells C5aR2 expression was frequently linked with TNBC. Increased C5aR2 expression was identified in tumors with poor treatment response while decreased C5aR2 expression was observed in definite tumor response. High levels of Ki-67 were frequently associated with elevated C5aR2 expression in tumor cells.C5aR2 was also found to be correlated with the infiltration of immune cells, especially macrophages. The current study also revealed overexpression of C5aR2 in the endothelium of blood vessels found in the stroma of breast cancer. In conclusion, our study highlights the significance of C5aR2 expression in tumor and stromal cells of breast cancer, its involvement in chemoresistance, and immune infiltration. Thereby indicating its potential as a valuable prognostic marker and therapeutic target in breast cancer management.

Key words: Breast cancer, C5aR2, Cancer Associated Fibroblasts, Immunohistochemistry, Ki-67.

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LIST OF ABBREVIATIONS

AI : Artificial Intelligence

AJCC : American Joint Committee on Cancer

AP : Alkaline Phosphatase

ASIR : Age-Standardized Incidence Rate

BMI : Body Mass Index

BRCA : BReast CAncer gene

CAFs : Cancer Associated Fibriblasts

CCK-8 : Cell Counting Kit-8

CDH1 : Cadherin-1

CHEK : Checkpoint Kinase 2

DAB : Diaminobenzidine

EGF : Epidermal Growth Factor

ERK : Extracellular signal-Regulated Kinase

ESR1 : Estrogen Receptor 1

FFPE : Formalin Fixed Paraffin Embedded

FISH : Fluorescence In Situ Hybridization

GPR77 : G Protein coupled Receptor 77

GSVA : Gene Set Variation Analysis

GTEx : Genotype-Tissue Expression

H&E : Hematoxylin and Eosin

HER2 : Human Epidermal growth factor Receptor 2

HMDM : Human Monocyte-Derived Macrophages

HMGB1 : High Mobility Group Box 1

HRP : Horseradish Peroxidase

HRT : Hormone Replacement Therapy

IARC : International Agency for Research on Cancer

IDC-NST: Invasiva Ductal Carcinoma of No Special Type

IGF : Insulin-like Growth Factor

IHC : Immunohistochemistry

IL-6 : Interleukin-6

IRS : Immunoreactive Score

JAK/STAT3:Janus Kinase/Signal Transducers and Activators of Transcription 3

MDSC : Myeloid Derived Suppressor Cells

MMP : Matrix Metalloproteinases

MRM : Modified Radical Mastectomy

MSI : Microsatellite Instability

NFκB : Nuclear Factor kappa light chain enhancer of activated B cells

PDGF : Platelet Derived Growth Factor

SDF-1 : Stromal cell Derived Factor-1

SEER : Surveillance, Epidemiology, and End Results

STK : Serine/Threonine Kinase

TAM : Tumor Associated Macrophages

TCGA : The Cancer Genome Atlas

TDLU : Terminal Ductal Lobular Unit

TIMER2.0:Tumor IMmune Estimation Resource 2.0

TMB : Tumor Mutation Burden

TME : Tumor Microenvironment

VEGF : Vascular Endothelial Growth Factor

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Breast cancer is the most common cause of death in women all over the world and Pakistan has the highest incidence of breast cancer among Asian countries (Saeed et al.,2021). Breast cancer is a heterogeneous tumor at both morphological as well as molecular levels and needs different therapeutic regimens depending on the molecular subtype. Its complexity is intricately linked with the tumor microenvironment (TME) and is not merely dependent on the intrinsic features of tumor cells (Umar et al., 2021). Therefore, the understanding of TME in breast cancer is crucial owing to its role in tumor behavior and treatment response (Joshua et al., 2021).

1.1.1 EPIDEMIOLOGY

Breast cancer is the most common cause of death in women all over the world. Globally, a woman is diagnosed with breast cancer in every 14 seconds (Breast Cancer Research Foundation, 2021). Approximately 2.3 million cases were diagnosed and 685000 women died of breast cancer worldwide in the year 2020. Because of its increasing incidence, breast cancer has become a major burden to public health (Melina. et.al, 2022)

In Pakistan, 1.38 million new breast cancer cases are diagnosed every year while 0.458 million breast cancer patients succumb to death (Abu Bakar, 2021). One in every nine women is at risk of being diagnosed with breast cancer during their lifetime (Sohail et al,2007). According to the Annual Cancer Registry Report (2021) of Shaukat Khanum Memorial Cancer Hospital & Research Center breast cancer is the most frequently diagnosed cancer among top 10 malignancies in adult females accounting for 51.1% of cancer cases (Mahmood et al., 2022). The Karachi Cancer Registry (2017-2021) also revealed breast cancer to be the most common cancer among females in Karachi with the highest age-standardized incidence rate (ASIR) of 177.8 (Ikram et al., 2023). Diagnosis of 89% of breast cancer patients at a later stage and 59% at an advanced stage is attributed to the lack of awareness among females in Pakistan (Saeed et al., 2021). Although the mortality rate for breast cancer is gradually declining due to improvement in both early diagnosis and targeted therapy, limited success has been achieved in cases of advanced breast cancer. The inability to combat breast cancer is primarily due to its heterogeneous nature, which remains unveiled and imparts obstacles in diagnosis and resistance to treatment.

1.1.2 RISK FACTORS

Epidemiological studies have revealed a correlation of breast cancer with environmental, reproductive, and lifestyle factors, involving both modifiable and non-modifiable risk factors.

1.1.2.1 Modifiable Risk Factors

- **Marital Status**

Psychosocial factors have a strong influence on the development and prognosis of various malignant tumors, and marital status is one of the important factors (Soler-Vila et al., 2003). According to Leslie et. al, older (>65 years) unmarried women have 1.18

times increased chance of being diagnosed with breast cancer at a later stage than married women of the same age, along with an increased tendency to die of breast cancer across all AJCC stages. In married patients likely reasons for early diagnosis and better survival are financial (Osborne et al., 2005), psychosocial and personal support with better health (Bloom et al.,2001). Although older married women with breast cancer have shown improved survival, little is known about the association of marriage with breast cancer patients under 65 years of age (Hinyard et al., 2017).

Age at the time of marriage also alters the risk of breast cancer. The results of the study conducted by Hinyard et. al, state that marriage at the age of 30 or more holds a 7.0% more risk of developing breast cancer than marrying at a younger age. Early marriage but late first childbirth (>30 years) increases this risk to 1.4%. Marriage and first childbirth at a later age result in increased exposure to inherited mutations, estrogen-induced genetic mutations, and the incapability of breast tissue to differentiate (Dey et al., 2009).

- **Parity**

Parity has a profound effect on the occurrence of breast cancer, however, it differs with different breast cancer subtypes (Sun et al., 2016). During pregnancy, changes in systemic hormones particularly estrogens and progesterone, induce a defensive effect on breast cancer by differentiating the carcinogenesis target structures, the terminal end buds, and terminal ducts (Rajkumar et al., 2003). It has been found that parity has no effect on HER2 and TNBC which further augments the role of hormones in increasing the risk of breast cancer (Li et al., 2021). A 3% decrease has been shown in the risk of premenopausal breast cancer after every full-term pregnancy which increases to 12% in the case of breast cancers after menopause (Chapelon & Gerber, 2002).

- **Oral Contraceptives / HRT**

The extensive research done on the role of OC in breast cancer suggests moderate increase in breast cancer risk (Mørch et al., 2017). Risk varies with the duration of treatment and timing of use, age of the patient & type of progesterone in combined

therapy (Breast cancer and HRT, 2022). It has been found that women who have currently or recently used hormones have RR of 1.2. If OC is used for less than a year, RR is 1.09 increasing to 1.38 if continued for more than 10 years (Mørch et al., 2017)

According to the study conducted by Danielle et. al, the risk of developing breast cancer is higher in older women using oral contraceptives, while the lesser risk is associated with the use of oral contraceptives at younger ages (Fitzpatrick et al., 2023).

The Million Women Study revealed that current users of hormonal therapy are more prone not only to develop but also die of breast cancer than those who never used it. The risk decreases after stopping HRT but persists if used for more than 10 years (Breast Cancer and HRT, 2022). Hormonal therapies containing both progesterone & estrogen have a higher risk of breast cancer than therapy with estrogens alone (The Lancet-Collaborative Group, 2019).

- **Alcohol consumption**

There is evidence suggesting that drinking alcohol is a risk factor for breast cancer. Research has shown that the risk of breast cancer increases with an increase in alcohol consumption. The exact mechanism by which alcohol increases the risk of breast cancer is not fully understood. One of the proposed mechanisms is higher doses of alcohol leading to the accumulation of acetaldehyde which binds with DNA and interferes with DNA synthesis and the antioxidative defense system. Altered DNA synthesis leads to the growth of abnormal cells, which can also contribute to the development of breast cancer (McDonald et al., 2013). Additionally, alcohol also activates vascular endothelial growth factor (VEGF) which in turn stimulates mammary tumor growth (Świechowicz et al., 2022). In a study, a 46% increased risk of breast cancer was observed after a moderate amount of alcohol consumption (McDonald et al., 2013).

- **Obesity**

Obesity increases the risk of breast cancer with poor outcomes in younger as well as older women (Ruiz et al., 2017). It is thought to result from the accumulation of polyaromatic hydrocarbons in the fatty tissue of the breast, which interacts with cellular estrogen receptors leading to an increased risk of breast cancer development (Niehoff et al., 2017). In another study conducted by Ruiz et. al, it was observed that breast cancer related to obesity has worse disease-free and overall survival than underweight breast cancer females. Chances of breast cancer development are higher in females with a BMI >5.0 (Suzuki et al., 2017). It has been found that menopausal status has a profound impact on the relationship between BMI and breast cancer. According to Liu et al., (2018) an increased risk for post-menopausal breast cancer is correlated with weight gain and greater body mass index (BMI) later in life, owing to increased adipose tissue which is the major source of estrogen after menopause, however in premenopausal women, the risk decreases with higher BMI.

- **Physical Inactivity**

Studies have found that women with regular or increased physical activity have less chance of developing breast cancer compared to women with a sedentary lifestyle (Kim et al., 2013).

Exercise helps to boost the immune system, which may help to prevent the growth of abnormal cells that can lead to cancer. Physical activity can also improve insulin sensitivity and reduce inflammation, which are other factors that have been linked to an increased risk of breast cancer (Campbell & McTiernan, 2007). Hankinson et al. found that Insulin and insulin-like growth factor-I (IGF-1) play a major role in the progression of breast cancer. Exercise can help to reduce the levels of estrogen and other hormones that are associated with an increased risk of breast cancer. Additionally, physical activity can help to reduce body fat, which also decreases the production of estrogen in the body.

- **Socioeconomic status**

Although a high incidence of breast cancer is observed in individuals belonging to

higher socioeconomic groups, the clinical presentation is better than in lower socioeconomic classes (Klassen and Smith, 2011). Women with high socioeconomic status show improved overall survival. Likely reasons for this improvement are high quality care and instant treatment available for them in the health care system (Kumachev

et al., 2016).

However, this association varies among different subgroups of breast cancer. Fewer studies with inconsistent results are available in this regard and most of them favor an increased risk of lobular tumors associated with higher class by occupation (Klassen and Smith, 2011). Frequent recurrences and rapid disease progression is linked with a low socioeconomic group (Maaren et al., 2022).

- **Diet**

Among modifiable risk factors unbalanced diet intake is strongly associated with breast cancer risk. According to the study conducted by Mahdieh et. al, women on a diet rich in animal fat have more chances of developing breast cancer than women on a vegetarian diet. However, there is a varying correlation between total and subtypes of fat intake and BC. Most likely due to measurement errors, variation in fat intake among females, individual body fatness, and other dietary components e.g., fiber & antioxidants. However, several studies have shown that SFAs increases while w-3 PUFA intake reduces the risk of breast cancer development (Khodarahmi and Azadbakht, 2014).

1.1.2.2 Non-Modifiable Risk Factors

- **Age**

Age is by far the most important risk factor and older females holds the highest age-specific incidence rates of breast cancer (Cancer Research UK, 2021) but it can

occur at any age (Licia, 2021). Breast cancer usually does not develop at the age of 20s or 30s, the risk is only 5% in this age group. More recent data indicates that among young adult females between 15 -39 years of age, the most common type of cancer is breast cancer. According to a 2021 review, breast cancer is responsible for 30% of all cancers in young adult females (Selchick and Cafasso, 2023).

In developed countries, more than 1/3 of breast cancer patients are above 70 years of age and at the time of diagnosis of breast cancer not more than one in five females are below 50 years of age (32). According to the National Cancer Institute, breast cancer risk is only 0.4% at the age of 30s, which increases to 1.5% and 3.5% by the age of 40 and 60, respectively. According to the SEER database, 5.6% of invasive breast cancer were diagnosed in women younger than 40 years (Selchick and Cafasso, 2023).

Elizabeth et. al observed that not only incidence but also mortality is high in adolescent and young adult (AYAs) females than in older women. It has also been noted that AYAs are more prone to unfavourable biology, advanced disease at diagnosis, and increased chances of recurrence (Rake et al., 2021). The variation in subtypes of breast cancer has also been observed with luminal B, human epidermal growth factor receptor 2 (HER2), and aggressive resistant triple-negative breast cancer most commonly diagnosed in groups under 40 years of age, while luminal A subtype is frequently seen in older patients (Cancello et al., 2010).

- **Early menarche or Late menopause**

The study conducted by Titus et.al, revealed that women with early menarche (before 12 years) has 50% increased risk of breast cancer than those in whom menses begin after 15 years of age (Kristin and Ashley, 2016). The premenopausal breast cancer risk decreases to 9% while postmenopausal breast cancer reduces to 4% with each year delay in menarche, as indicated by a pool analysis by Clavel & Gerber (2002).

According to a worldwide pooled analysis of 118,964 breast cancer patients, with each year delay in menopause the relative risk of breast cancer increases by a factor of 1.029, as revealed by Kristin and Ashley (2016). It was also analyzed in the same pool

that premenopausal women has a higher risk of breast cancer than postmenopausal women of the similar age. The basic reason being the prolonged exposure of women to estrogen.

- **Pregnancy and Breast Feeding**

A study conducted in 2002 showed that the hypoestrogenic state during breastfeeding is associated with an overall decrease in risk of breast cancer. The premenopausal breast cancer risk decreases to 4% with each additional year of breastfeeding (Kristin et al, 2016). However, the association of breastfeeding varies with different breast cancer subtypes. There is a considerable reduction in the risk of triple-negative breast cancer but the relation with ER-positive breast cancer is inconsistent (Gaudet et al, 2011). A similar more recent population-based controlled study has revealed a reduction in the risk of triple-negative disease but not in either ER-positive or HER2-positive breast cancer (Li et al., 2013).

- **Race and ethnicity**

As far as incidence is concerned White women show the greatest incidence as compared to black women but a reverse relationship is seen in the case of breast cancer in the younger age group (<40 years). Asian and Hispanic women have revealed the lowest incidence of breast cancer. However, the mortality rate is 40% higher in black women than white women and more than two-fold increase has been observed in black women when compared with Asian breast cancer patients (Giaquinto, 2022). African Americans are subjected to more aggressive breast cancer and high recurrence risk (Iqbal et al., 2015).

- **Family History**

A family history of breast cancer has a strong association with its increased risk.

It has been noted that 13-19% of breast cancer patients have a first-degree relative with the same condition and risk increases as the number of affected family members increases. Risk is even more if the affected family member is of a younger age group. It has also been observed that breast cancer risk is higher in case of a family history of ovarian cancer due to *BRCA1* and *BRCA2* mutations (Łukasiewicz et al., 2021).

- **Genetic Factors**

In the past years, role of genetic mutations in breast cancer has been established. Among several genes linked to increased risk of breast cancer, two major genes of high penetrance are BRCA1 and BRCA2 (Shiovitz & Korde, 2015). Their inheritance is mainly autosomal but somatic mutations are also stated frequently. *TP53*, *CDH1*, *PTEN*, and *STK11* are other highly penetrant genes associated with increased chances of breast cancer (Shahbandi et al., 2020). Breast cancer has also found to be induced by interaction of DNA repair genes like *ATM, PALB2*, *BRIP1*, or *CHEK2* with BRCA genes (Shiovitz & Korde, 2015). Park et.al, has discovered a potential association between the XRCC2 gene and breast cancer (Park et al., 2012). BRCA1 and BRCA2 mutations account for approximately 5% to 10% of all breast cancer cases. BRCA1 mutation has been found in 2% of younger breast cancer patients and is frequently associated with triple-negative breast cancers (Huzarski et al., 2013). However, BRCA2 mutations are usually ER and PR positive and related cancers are frequently of higher grade (Atchley et al., 2008).

- **Hormones**

Estrogen and progestins are the hormones that play an important role in the growth and development of breast cancer. In luminal A and luminal B subtypes, the stimulating effect of estrogen on receptors is well established, accounting for two thirds of the breast cancer cases. Estrogen produces its carcinogenic effects through two pathways, either by alteration of gene expression via estrogen receptor or by oxidative

metabolism of estrogen leading to the generation of reactive oxygen species that cause oxidative damage to DNA (Lavigne et al.,2001). Therefore, the risk of breast cancer increases in women with increasing exposure to estrogen.

1.1.3 Pathological Basis of Breast Cancer

The breasts are a pair of mammary glands located on the chest wall of females and, to a lesser extent, males. Each breast contains a network of lobes made up of glandular tissue, which produces milk during lactation, and ducts, which transport the milk to the nipple. It also contains fat and connective tissue, which gives the breast its shape and support. The breast tissue is sensitive to hormonal changes and can undergo modifications throughout a woman's life. The primary hormone responsible for breast development and milk production is estrogen (Robbins, 2020)

60% of blood to the breast is supplied by the internal mammary artery. The breast also has extensive lymphatic drainage within the breast which drains into adjacent lymph nodes. Breast cancer metastasizes to the lymph nodes through these lymphatic vessels. Most of the lymph is drained into the axillary lymph nodes, while few lymphatic vessels flow to internal mammary lymph nodes located deep in the breast. The importance of this lymphatic drainage lies in breast cancer metastasis, which usually involves the first lymph node in the chain of lymph nodes. This lymph node is known as a "sentinel lymph node," and a surgeon may remove this lymph node to check for metastases in a breast cancer patient (Johns Hopkins, 2023).

The male breast structure is similar to the female breast, except for the specialized lobules being absent in male breast tissue (Johns Hopkins, 2023).

Breast cancer is a universal disease with a significant impact on all races and both sexes. It is a heterogeneous disease with a set of breast tumor subtypes having distinct molecular and cellular origins and clinical behavior (Pavani, 2023). The uncontrolled growth of abnormal cells in the breast tissue gives rise to this complex disease. Atypical growth occurs in two types of breast tissue – ductal epithelium (85-90% invasive cancer) and lobular epithelium (10-15% invasive cancer). Although ductal epithelium is

the frequent site of origin of most of breast cancers, aberrant cells can also arise within lobular (milk-producing) glands. Some proliferative and non-proliferative benign breast conditions are also involved in the development of breast cancer (ACS, 2021).

The exact pathophysiology of breast cancer is not fully understood, but it is thought to involve a combination of genetic, hormonal, and environmental factors.

Genetic factors play a significant role in the development of breast cancer. Mutations in certain genes, such as BRCA1 and BRCA2, are associated with an increased risk of breast cancer. These genes are involved in the repair of damaged DNA, and when mutated, can no longer perform this function effectively. As a result, cells may accumulate genetic mutations that can lead to the development of cancer.

Hormonal factors also contribute to the pathophysiology of breast cancer. Estrogen and progesterone are the hormones involved in the regulation of the menstrual cycle and have the ability to stimulate tumorigenesis in breast cells. Breast cancer risk increases with prolonged exposure of these hormones, especially in women who have early menarche, late menopause, or have taken hormonal therapy.

Environmental factors such as exposure to radiation, certain chemicals, and lifestyle factors such as alcohol consumption, smoking, and a high-fat diet can also increase the risk of breast cancer. These factors can cause genetic mutations, inflammation, and other cellular changes that can contribute to the development of cancer (Łukasiewicz et al., 2021).

Apart from these factors role of tumor microenvironment (TME) has also been established. It has been validated that inflammation plays a crucial rule in the development and progression of breast cancer. One of the important components of inflammation contributing in tumor development is complement receptor C5aR2.

1.1.4 Molecular Basis of Breast Cancer

The mammalian cell cycle comprises of four phases namely, G1, S, G2 and M phases. The G1 and G2 phases are followed by S and M phases respectively and during

this transition of phases, the cell cycle come across inactivation of different checkpoints. The purpose of these checkpoints is to maintain genetic integrity during the process of replication of cell. The presence of DNA damage and the integrity of mitotic spindles are assessed during these transitions. The breast cancer susceptibility gene 1 and 2 (BRCA-1 and BRCA-2) breast/ovarian cancer risk genes are both involved in DNA repair (Maser,2002 & Evan, 2001). Genome instability results in a greater potential to develop genetic changes such as gene loss, gene amplification, point mutations, and chromosomal translocations. While most of these subsequent changes may result in cell death, some can affect key genes involved in cell survival, proliferation, invasiveness, motility, drug resistance and other malignant characteristics (Waldman et al., 2000).

In tumorigenesis there is a series of progressive changes, including stimulation of oncogenes and downregulation of tumor suppressor genes. An oncogene results from a gain of function mutation of a proto-oncogene that generates a tumorigenic product. Many of the genes classified as oncogenes fall into categories of abnormally activated growth factors, growth factor receptors, intracellular signaling molecules, and nuclear transcription factors. On the other hand, mutation of a tumor suppressor causes a loss of function in the ability to restrain cell growth. Tumor suppressor genes are mostly related and known to influence the cell cycle machinery such as pRb and p53, others involved in DNA repair are BRCA1 and BRCA2 genes (Eissa et al., 1999).

The underlying mechanism of the molecular basis of breast cancer involves a complex interplay between genetic, epigenetic and environmental factors that influence the growth and division of breast cells.

Genetic alterations play a crucial role in breast cancer development and progression. Mutations in genes such as BRCA1 and BRCA2 can impair DNA repair mechanisms, leading to the accumulation of genetic mutations and the development of cancer. Other genetic alterations, such as amplification of the HER2 oncogene or mutations in TP53, can lead to uncontrolled growth and division of breast cells (Basis).

Women with mutations in the BRCA1 or BRCA2 genes have a 70% lifetime risk of developing breast cancer (Kuchenbaecker, 2017), compared to a lifetime risk of around 13% for women without these mutations (Jamie, 202161).BRCA1 interacts with protein complexes that regulate chromatin structure and has a role in transcription

(Basis). Breast cancer due to BRCA1 mutation tend to be mostly triple negative. In addition, women with BRCA1 or BRCA2 mutations tend to develop breast cancer at a younger age than women without these mutations.

HER2 is a receptor tyrosine kinase that promotes cell proliferation and opposes apoptosis by stimulating RAS and PI3-AKT signaling pathways. Amplification of HER2 gene on chromosome 17q leads to the development of HER2-positive breast cancers (Basis).

P53 is a tumor suppressor gene and it not only regulates growth and division of normal cell, but is also involved in gene transcription, DNA repair and genomic stability. The alteration in p53 function favors checkpoint defects, cellular immortalization, genomic instability, and inappropriate survival that results in continuous proliferation and evolution of damaged cells. The role of p53 in apoptosis contributes to the chemotherapy induced cell death (Al-Mansouri and Alokail, 2006).

Epigenetic alterations, such as DNA methylation and histone modifications, also play a role in breast cancer. These alterations can affect the expression of genes involved in cell growth and division, as well as DNA repair mechanisms, and can promote cancer development.

Environmental factors, such as exposure to radiation, hormonal imbalances, and obesity, can also contribute to breast cancer development.

1.1.4.1 Molecular Classification

Breast cancer is a disease of wide range of morphological features, varied immunohistochemical description and diverse histopathological subtypes. These distinct subtypes offer different clinical patterns and outcomes, specific to each pattern. This led primarily to the development of classification on the basis of histology and cytology. Breast cancer originates from the inner lining epithelium of the ducts or the lobules. The two basic morphological patterns are the tumors arising from and confined to the epithelium (carcinoma in situ) or tumors invading the stroma (invasive carcinoma), and origin of tumor whether ductal or lobular. In both patterns, the tumor evolves from the

Table 1.1: Molecular Classification of Breast Cancer

Molecular Subtype				
	Luminal A	**Luminal B**	**HER2/neu**	**Basal like**[a]
Gene expression pattern	Expression of luminal (low molecular weight) cytokeratins, high expression of hormone receptors and related genes	Expression of luminal (low molecular weight) cytokeratins, moderate-low expression of hormone receptors and related genes	High expression of HER2/neu, low expression of ER and related genes	High expression of basal epithelial genes and basal cytokeratins, low expression of ER and related genes, low expression of HER2/neu
Clinical and biologic properties	50% of invasive bresat cancer, ER/PR positive, HER2/neu negative	20% of invasive breast cancer, ER/PR positive, HER2/neu expression variable, higher proliferation than Luminal A, higher histologic grade than Luminal A	15% of invasive breast cancer, ER/PR negative, HER2/neu positive, high proliferation, diffuse TP53 mutation, high histologic grade and nodal positivity	~15% of invasive breast cancer, most ER/PR/HER2/neu negative (triple negative), high proliferation, diffuse TP53 mutation, BRCA1 dysfunction (germline, sporadic)
Histologic correlation	Tubular carcinoma, Cribriform carcinoma, Low grade invasive ductal carcinoma, NOS, Classic lobular carcinoma[b]	Invasive ductal carcinoma, NOS Micropapillary carcinoma	High grade invasive ductal carcinoma, NOS	High grade invasive ductal carcinoma, NOS Metaplastic carcinoma, Medullary carcinoma
Response to treatment	Response to endocrine therapy	Response to endocrine therapy	Response to trastuzumab (Herceptin)	No response to endocrine therapy or trastuzumab

and prognosis		(tamoxifene and aromatase inhibitors) not as good as Luminal A		
	Variable response to chemotherapy	Variable response to chemotherapy (better than Luminal A)	Response to chemotherapy with antracyclins	Sensitive to platinum group chemotherapy and PARP inhibitors
	Good prognosis	Prognosis not as good as Luminal A	Usually unfavorable prognosis	Not all, but usually worse prognosis

Reference: Eliyatkın et al., 2015.

main segment of mammary gland, Terminal ductal lobular unit (TDLU). Both ductal and lobular types can be noninvasive (DCIS/LCIS) or invasive (IDC/ILC). However, the most frequently occurring are invasive ductal carcinoma (IDC), accounting for 50%-80% of all breast cancer types **(**Makki, 2015). IDC is further categorized into "no specific type" (NST) or "special type", depending on whether the tumor presents ample morphological features along with specific cellular and molecular behavior, to limit as a particular histological subtype. The IDC-NST is the commonest subtype, accounting for about 40% to 75% of all invasive breast carcinomas (Nascimento and Otoni 2020).

Since histology alone could not reflect the complex nature of breast cancer, so gene expression profiling was considered to understand the heterogeneity of breast cancer. Perou and Sorlie, in 2000 proposed molecular classification of breast cancer on the basis of gene expression profiling. Luminal A, Luminal B, HER2 enriched, and Basal-like (Triple negative) are the molecular subtypes characterized by Perou et al.

The molecular classification of breast carcinomas allowed a better biological understanding of the disease, with impactful changes in clinical practice.

1.1.5 Pathological Staging

pTNM Classification (AJCC 8th Edition)

Reporting of pT, pN, and (when applicable) pM categories is based on information available to the pathologist at the time the report is issued. As per the AJCC (Chapter 1,8th Ed.) it is the managing physician's responsibility to establish the final pathologic stage based upon all pertinent information, including but potentially not limited to this pathology report.

Table 1.2: AJCC TNM System for Staging of Breast Carcinoma

pT		pN		pTNM-Stage	
Tis	DCIS LCIS Paget nipple	pN_{1mi}	Micrometastasis > 0,2 mm to 2 mm	0	DCIS
		pN_{1a}	1-3 axillary nodes	IA	T1N0
T1 mic	≤ 0,1 cm	pN_{1b}	Internal mammary nodes with microscopic/macroscopic metastasis by sentinel node biopsy but not clinically detected	IB	T0-1N1mi
T_{1a}	≤ 0,5 cm			IIA	T0-1N1 T2N0
T_{1b}	> 0,5 - 1 cm			IIB	T2N1 T3N0
T_{1c}	> 1 cm - 2 cm	pN_{1c}	1-3 axillary nodes and internal mammary nodes and internal mammary nodes with microscopic/ macroscopic metastasis by sentinel node biopsy but not clinically detected	IIIA	T0-2N2 T3N1-2
T2	> 2 cm - 5 cm	pN_{2a}	4-9 axillary nodes	IIIA	T4N0-2 T3N1-2
T3	> 5 cm	pN_{2b}	Internal mammary nodes, clinically detected, without axillary nodes	IIIC	anyT N3
T_{4a}	Extension to chest wall (does not include pectoralis muscle invasion only)	pN_{3a}	≥ 10 axillary nodes or infraclavicular	IV	systemic
T_{4b}	Ulceration, ipsilateral satellite skin nodules, or skin oedema - including peau d'orange.	pN_{3b}	Internal mammary nodes, clinically detected, with axillary node(s) or > 3 axillary nodes and internal axillary mammary nodes with microscopic metastasis by sentinal node biopsy but not clinically detected		
T_{4c}	a+b				
T_{4d}	Inflammatory ca	pN_{3c}	Supra-clavicular		

Reference: Robin et al., 2016. Staging and treatment of breast cancer.

1.1.6 Histologic Grade (Nottingham Histologic Score)

The grade corresponds to the largest area of invasion. If there are smaller foci of invasion of a different grade, this information should be included under "Additional Findings."

1.1.7 Tumor Microenvironment

A tumor is not merely a group of cancer cells but a heterogeneous collection of resident and infiltrating cells, extracellular matrix and secreted factors. The tumor development and progression depends upon cellular and molecular changes within the host tissues elicited by tumor cells. Different type of tumors exhibit different composition of tumor microenvironment. However, the main features common to most of the tumors are stromal cells, immune cells, blood vessels and extracellular matrix. It is now validated that tumor microenvironment actively promotes cancer advancement (Truffi et al., 2020). The heterogeneity of breast cancer is also due to the active communication between cancer cells and their environment (Baghban et al., 2020). As a result of this interaction stromal cells lose their function and acquire new phenotypes that promote development and invasion of tumor cells and also play important role in response to therapies (Joshua et al., 2021). In addition to the resident cells, various inflammatory and immune cells are also recruited to the tumor site and their anti-tumor functions are downregulated by the signals derived from tumor cells (Whiteside, 2008). Traditional grading and typing of breast cancer are based solely on the assessment of tumor cells, but now pathologic assessment of the TME is incorporated into routine breast cancer management. The components of the tumor microenvironment are categorized into cellular, physical, and soluble elements. The cellular components are further classified into local, regional, and metastatic divisions. Not only the tumor cells but also tumor-infiltrating inflammatory cells such as lymphocytes, plasma cells, dendritic cells, macrophages, and neutrophils constitute local components. However, the regional component consists of interaction between tumor cells and nearby stromal cells like fibroblasts, myoepithelial cells, and endothelial cells. The metastatic compartment involves tumor cells at lymph nodes and

Table 1.3: Histologic Grade (Nottingham Histologic Grade)

Features		Score
Tubule and gland formation	Majority of tumour (>75%)	**1**
	Moderate degree (10–75%)	**2**
	Little or none (< 10%)	**3**
Nuclear pleomorphism	Small, regular uniform cells	**1**
	Moderate increase in size and variability	**2**
	Marked variation	**3**
Mitotic count (dependent on microscopic field area, e.g. for field area 0.264 mm^2 with field diameter 0.58 mm)	0–9	**1**
	10–19	**2**
	>20	**3**

Ref: Atlas of breast cancer early detection (WHO).

distant organs. The enzymes, growth factors, and cytokines are among the various soluble and physical factors that play an important role in breast cancer progression (Joshua et al., 2021).

Stromal cells play significant role in tumor formation by secreting many factors that influence angiogenesis, proliferation, invasion, and metastasis. The composition of cells of stroma vary significantly from tumor to tumor. The foremost stromal cells that interact with tumor cells are fibroblasts, myoepithelial cells and endothelial cells. Endothelial cells are critically important in promoting cancer cell migration, invasion and metastasis, particularly by participating in new blood vessel formation. As the size of tumor increases, tumors develop their own blood supply through the secretion of proangiogenic factors such as platelet derived growth factor (PDGF), epidermal growth factor (EGF) and VEGF by endothelial cells

The most abundant cells in TME are cancer associated fibroblasts (CAFs). The normal fibroblasts in the tumor stroma are activated into cancer associated fibroblasts (CAFs) by autocrine and paracrine effects. Secretion of cytokines transforming growth factor-β (TGF-β) and stromal cell derived factor-1 (SDF-1) by normal fibroblasts, and secretion of platelet derived growth factor-α/β (PDGF- α/β), basic fibroblast growth factor (b-FGF) and interleukin-6 (IL-6) by tumor cells leads to the activation of normal stromal fibroblasts into CAFs. In breast cancer activation of CAFs is irreversible. As compared to the NF, CAFs have increased abilities of angiogenesis, proliferation, and migration of cancer and they also tend to produce large amounts of extracellular matrix (ECM) (Qiao et al., 2016). CAFs play an important role in tumor progression by regulating cancer stem cells, which act as a key population of tumor cells with high tumorigenicity and chemoresistance. Apart from initiation, development and invasion of breast cancer, CAFs also fulfils diagnostic, prognostic and therapeutic purpose. Different subsets of CAFs have been suggested in recent studies, having different phenotypes and functions. CAFs have been found to have tumorigenic as well as anti-tumor role depending upon the type of subset (Su et al., 2018). CAFs promote tumor growth by secreting chemokines and growth factor which enhances resistance to drugs by targeting tyrosine kinases and epidermal growth factor receptor (Umer et al., 2021) Therefore, therapeutic application against CAFs can only be helpful if type of subset is known.

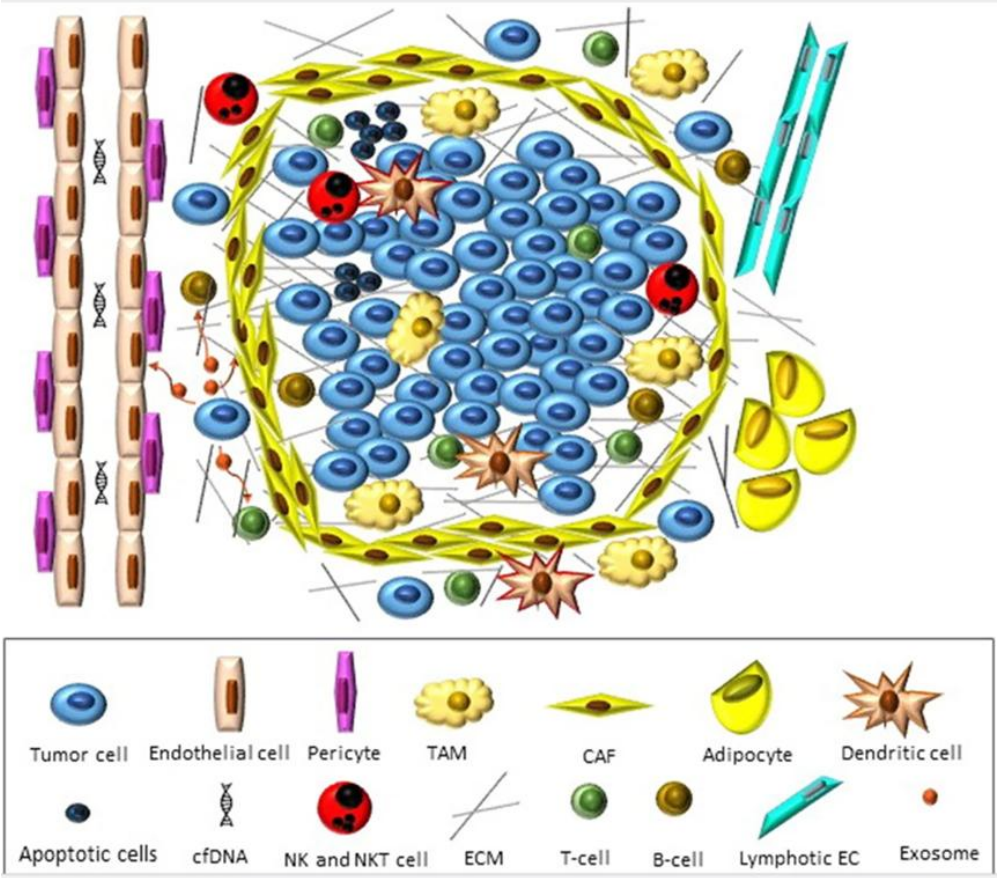


Figure 1.1: Tumor microenvironment at a glance. Tumor cells hijack different cellular and non-cellular non-malignant components of TME to promote their own growth and survival under hostile conditions. Meanwhile, the mediators for such contacts can be soluble factors (chemokines/cytokines/growth factors, etc.), or those that enable horizontal genetic/biomaterial transfer including cfDNA, apoptotic bodies, CTCs, and exosomes.

Reference: Baghban et al., 2020.

Another important component of TME are tumor associated macrophages (TAMs). It has been found that TAMs also play an important role in tumor progression, invasion, metastasis and chemoresistance. TAMs are derived not only from resident macrophages but also from tumor-infiltrating monocyte derived macrophages. M1 macrophages with antitumoral role and M2 macrophages having tumorigenic character, are the two distinct subtypes of macrophages. The functional difference of macrophages is closely associated with their plasticity while cells in TME regulate different phenotypes of macrophages (Pan et al., 2020). The tumorigenic role of TAMs was first established in breast cancer (Larionova et al., 2020).

In a nutshell, tumor development is a multifaceted process which is regulated by various cells present in tumor nest and in the stroma of TME. Immune mediated mechanisms in the tumor microenvironment play a central role in the occurrence and progression of cancer.

1.1.8 Complement System and Cancer

The complement system is an essential part of the innate immune system. Apart from its role in innate immunity, complement system is also intricated in various pathological conditions like autoimmune diseases, thrombosis, schizophrenia, graft-versus-host disease, and cancer. The compliment cascade comprises of a series of proteolytic reactions triggered by three pathways, the classical, lectin and alternative pathways. In thirty different types of cancers, gene expression of complement was analyzed and results showed increased expression of genes that code for alternative and classical pathway. The cleavage of factors C3 and C5, which are the central components, generates bioactive fragments C3a and C5a, respectively.C5a is a very potent chemoattractant and anaphylatoxin. It also acts as a strong immunomodulator. The process of complement activation involves membrane and plasma proteins and is tightly regulated. If not controlled properly, the regulatory proteins responsible for activation of complement, may result in excessive accumulation of C5a and

development of many diseases, including cancer (Kharghan, 2017). Liver is the main source of complement component production but stromal as well as tumor cells also produce complement proteins. Thus, complement components are not only infiltrated systemically but are also produced locally by different types of cells, both contributing to the enhanced levels of complement components at the tumor site. Apart from generating complement proteins, tumor cells also activate complement cascade through other proteins, produced by other host cells. It has also been observed that complement components produced by tumor cells can stimulate tumor progression directly without the activation of complement cascade Not only the different components of complement contribute to the tumor development but the membrane attack complex, formed in the last reaction of complement cascade also causes cell activation and/or the cell death by binding with the lipid bilayer of the cell membrane and altering its permeability (Revel et al., 2020).

Role of complement in tumor initiation and progression is justified by two reasons. Complement activation end products affect tumor growth by altering cancer cell behavior and modulating the immune response to the tumor Firstly, complement activation modulates adaptive immune response, and might alter T-cell response to tumors. Secondly, inflammation has been found to have a significant role in cancer development and progression, and complement system is an integral part of inflammation.

Some researches have revealed anti-tumoral role of complement in certain cancers, however many cancers have shown evidence of its pro-tumoral role. According to recent studies complement can be anti or pro-tumoral depending upon the type of cancer and may even produce opposing effects in the same type of tumor in different models. Despite its obligation to kill tumor cells it has been found to have a tumorigenic role. The first evidence was recruited from the slower tumor growth after C5aR deficiency in a mouse model of TC1 cancer. The deficiency of C5aR resulted in suppression of the anti-tumor $CD8^+$ T cell-mediated response, increased infiltration of myeloid derived suppressor cells (MDSC) into tumors and extension of their T cell-directed suppressive capabilities. The researchers have discovered that complement components have a significant role in tumor development, angiogenesis and immune surveillance. This highly suggests that the consequences of complement activation are

mostly pro-tumorigenic (Revel et al., 2020). Among different components of complement, role of C5aR2 in cancer is fairly controversial and needs further investigation to be used as a therapeutic target.

1.1.8.1 C5aR2 and Breast Cancer

One of the components of complement, C5a is a 74-aa glycoprotein and its structure comprises of a helix bundle. C5a is a very potent chemoattractant and anaphylatoxin but it also acts as a strong immunomodulator and has been shown to have both pro and anti-tumoral role.

It generates its effects by binding with two receptors C5aR1 (CD88) and C5aR2 (GPR77, C5L2) (Xaria, et al., 2019). Although C5aR2 activity has been linked to tumor progression, its role in tumorigenesis is still controversial.

C5aR2 gene is located on chromosome 19 and in humans it is expressed abundantly on bone marrow tissues, immune cells, lungs, spleen, and subsets of T-cells. Localization of C5aR2 shows great diversity in humans. In monocytes, neutrophils, natural-killer cells and macrophages it is mainly expressed intracellularly while its cell surface expression is seen in CD4+T cells. Overexpression of C5aR2 has been observed to expedite proliferation, migration and invasion of breast cancer cells (Xaria, et al., 2019).

The expression of C5aR2 has been observed not only in tumor cells but also in stromal cells of tumor microenvironment. Tumor microenvironment is rich in C5aR2 positive cancer associated fibroblasts (CAFs) which not only promote tumorigenesis by providing a supportive niche to cancer stem cells but also favors chemoresistance and modulates many components of immune system (Shicheng Su, 2018).

Differential expression of C5aR2 has been seen in various molecular subtypes of breast cancer, being higher in Luminal A & B and lower in HER2 positive and basal subtypes. The overexpression of C5aR2 is associated with poor prognosis. The Gene

Set Variation Analysis revealed a strong correlation between C5aR2 and, metastasis and relapse of breast cancer. Initial studies revealed C5aR2 as a decoy receptor but later studies demonstrated that it not only modulates C5a signaling but can also function through intracellular signaling with cytosolic β-arrestins. It has been observed that its function depends upon its expression and localization. It may act as a decoy receptor, sequestering C5a/C5a des Arg when expressed on cell surface, however possibility of C5aR2 serving as a functional receptor cannot be ruled out. When expressed in the cytoplasm it may produce its effects through intracellular networks either with C5aR1 or another proteins like β-arrestins (Zhang et al., 2017).

Its relation with malignancy is due to its involvement in several metabolic pathways including TNF α signaling via NF-kB. NF-kB pathway is an oncogenic signaling pathway which is consistently activated by C5aR2. C5aR2 upregulates ESR1 oncogene leading to endocrine therapy resistance in breast cancer. The C5aR2 mediated endocrine therapy resistance in breast tumor suggests that it can be used as a prospective biomarker in breast cancer (Yumeng et al.,2021). C5aR2.

Role of C5aR2 is still not unraveled being anti-tumoral in some cancers while propagating tumor in others. It may initiate inflammation or may act as an anti-inflammatory agent or both may co-exist in same cell type or in distinct cells, tissues or organs, counterbalancing and producing net effect as its role. Its downstream signaling and regulation is also ambiguous. Therefore, further research is required in this field to delineate exact mechanism of C5aR2 (Zhang et al., 2017).

1.1.9 Immunohistochemistry

Immunohistochemistry (IHC) is a method for detecting antigens or haptens in cells of a tissue section by using antibodies binding specifically to antigens in biological tissues.

The antigen-antibody binding can be visualized through a color-producing reaction, catalyzed by the enzymes like Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP). The distribution and localization of specific cellular components

within cells and in tissues can be detected by means of IHC. The particular IHC method used depends upon type of specimen and assay sensitivity (Boster, 2021). Immunohistochemistry has become a vital component of diagnostic modalities of breast cancer. The unfolding of newer assays and has led to the development of latest interpretation techniques. In breast cancer IHC helps to determine the source of primary or metastatic carcinomas and distinguish metastases from another source to the breast. However, none of the stains is sensitive or specific. GATA3 and Sox10 are usually negative for subtypes other than triple-negative breast carcinoma, but Sox10 along with S-100 is also positive for metastatic melanoma. For its differential diagnosis, a pan-cytokeratin immunostain must be included (Mathews, 2021).

Apart from its limited specificity and sensitivity, IHC has now become an essential part of diagnostic pathology. IHC is used not only for diagnostic purposes but it can also assess prognosis and response to therapy. It is an advanced technology and manageable for most of laboratories. Better results can be obtained with IHC if combined with traditional hematoxylin-eosin stained slides and use panels of markers (Zaha, 2014).

1.2 RESEARCH GAP/ RATIONALE OF STUDY

1.2.1 Theoretical gap

The research on role of C5aR2 in cancer is limited and controversial worldwide. In the previously reported studies, it was found that C5AR2 and IL-10 were linked to chemoresistance and poor prognosis in patients suffering from lung and breast cancer. Humans have two C5a receptors: C5aR1 (CD88) and C5aR2 (GPR77 or C5L2). The interaction between C5a and C5aR1 has proinflammatory and disease-inducing effects, whereas role of C5aR2 is not clear. Even less data is available regarding association of C5aR2 expression and breast carcinogenesis. Various studies have claimed both pro and anti-tumoral effects of C5aR2 activity mystifying the issue even more.

1.2.2 Contextual gap

Even though breast cancer is the most prevalent cancer in Pakistan, data regarding therapeutic/prognostic markers specific for our population are almost non-existing. Researches done worldwide have identified novel targets leading to a breakthrough in targeted drug development. Effective and economical techniques are the need of the time to identify these and new targets in our population so that Pakistani patients can also benefit from already available therapies and to pave way for new drug development.

1.2.3 Methodological Gap

C5aR2 has been validated using multiple different techniques including RNA analysis, DNA sequencing and flow cytometry. All these techniques are not readily available in our country and are expensive. Immunohistochemistry expression is an economical and easily available diagnostic technique and has beneficial role in diagnosing and choosing therapeutic options.

1.3 PROBLEM STATEMENT

Breast cancer is the most prevalent malignancy in females and the incidence is rising by the day. Breast cancer contributes to increased risk, morbidity and mortality in Pakistani women. Early diagnosis with the help of specific markers and prompt targeted treatment may result in better prognosis and improved outcome.

1.4 HYPOTHESIS OF STUDY/ RESEARCH QUESTION

A) Null Hypothesis

There is no correlation between C5aR2 expression and clinicopathological parameters in breast carcinoma.

B) Alternate Hypothesis

There is correlation between C5aR2 expression and clinicopathological parameters in breast carcinoma.

1.5 OBJECTIVES OF STUDY

1. To evaluate C5aR2 (GPR77) expression in stromal and epithelial cells of breast carcinoma
2. To determine correlation of C5aR2 (GPR77) expression in breast carcinoma with clinicopathological parameters

1.6 SIGNIFICANCE OF THE STUDY

Breast cancer is the most frequently diagnosed adult female malignancy worldwide. In 2020, according to global data breast cancer accounted for 684,996 deaths worldwide (WHO, 2021). Among Asian countries Pakistan has the highest rate of breast cancer. Both the risk and mortality has increased in Pakistan (Sidra, 2019). Recent cancer related research is focused on early identification of diagnostic and therapeutic targets. Novel targets have been not only found in tumor cells themselves but TME has also been shown to play crucial role in tumor survival and proliferation. Among the cells of TME, the complement activation products have been shown to maintain chronic inflammation, promote immunosuppressive microenvironment, induce angiogenesis and increase vitality and metastatic potential of cancer cells. The receptor for C5A, C5aR2 provides a good living environment by inhibiting normal immune function and aggravating the inflammatory response. This has led to the recommendation that inhibition of C5aR2 is a good therapeutic option and is an open field for drug development. This study aims to define expression of C5aR2 in breast cancer and its correlation with various clinical parameters and to evaluate its significance as a potential therapeutic target.

CHAPTER 2

LITERATURE REVIEW

According to W.H.O. 2021, statistics breast cancer is the most common cancer worldwide. Annually breast cancer contributes to approximately 12% of all new cancer cases. It is less prevalent in Asian, Native American, and Hispanic women. Increased frequency of BRCA mutations leads to a higher risk of breast cancer among Jewish women. The risk increases two-fold for a woman with a positive first-degree relative. Its occurrence is mainly (85%) due to acquired genetic mutation while inheritance accounts for 15% of breast carcinoma. Among inherited gene mutations BRCA1 and BRCA2 are the most common. BRCA2 mutations are more frequent than BRCA1 in men, increasing the risk by 6.8% (Breast Cancer Facts and Statistics). Pakistan has the highest breast cancer incidence and mortality among Asian countries. The increase in death rate is mainly because of unawareness, late diagnosis, and inappropriate treatment. Therefore, early diagnosis and prompt treatment are essential to enhance the survival rate (Sidra et al., 2019).

Breast cancer is not merely a disease, it is a collection of diseases with varying histopathologic and molecular properties. Early in the last century, all breast cancer patients were given the same treatment but later on, it was observed that breast cancer patients showed different prognoses and responses to treatment. This led to the development of traditional classification based on the morphology of tumors. Early in this century, owing to the heterogeneity of breast cancer, new molecular techniques such as gene profiling were introduced giving rise to molecular classification. The molecular classification of the new era improved the quality of life of breast cancer patients and reduced mortality due to targeted therapies and individualized treatment programs (Eliyatkın, 2015). Since then, it has undergone several modifications but

histological features remain the basis of updated classification of breast cancer. The subsequent molecular subtypes of breast cancer are

Luminal A (ER+, PR+/-, HER2−, low Ki-67),

Luminal B (ER+, PR+/-, HER2− with high Ki-67 or HER2+ with any Ki-67 status),

HER2-enriched (ER−, PR−, HER2+) and

Triple-negative (TN) breast cancer (ER−, PR−, HER2−)

For better prognosis and therapeutic guidance, it is obligatory to determine the molecular subtype precisely. These molecular subtypes also help to direct pre and postoperative systemic therapy. Although it is a breakthrough in the evolution of the classification of breast cancer but shortcomings cannot be ruled out. It only categorizes breast cancer into 4 distinct subtypes which simply do not match with the complex nature of the tumor. In fact, each subtype shows diverse features with altered prognostic and therapeutic responses (Zhang, 2023). The upgraded WHO classification shows many changes including the subtyping of lobular carcinoma in situ (LCIS) into classical, pleomorphic, and florid subtypes, replacing the histological grading of mitosis as the mitosis number per defined area (in mm^2) instead of no of mitosis in 10 HPFs, classifying solid papillary carcinomas or encapsulated papillary carcinomas as invasive carcinomas, tumors considered as a separate class are now designated as variants, etc (Lebeau & Denkert, 2021).

Currently, Immunohistochemistry (IHC) of biopsy specimens has replaced genetic testing in revealing breast cancer molecular subtypes (Zhang et al., 2023). Unfortunately, biopsy specimens can assess only a small portion of cancer, overlooking the true nature of the whole tumor. Moreover, a 2+ score of HER2 demands another genetic testing method i-e, FISH for accuracy. The worldwide availability of both IHC and FISH is uncertain leading to excessive or undertreatment of breast cancer patients. Therefore, an efficient and infallible technique is crucial to precisely diagnose the molecular subtypes of breast cancer and assist in therapeutic decisions (Zhang et al., 2023).

Recently artificial intelligence (AI) based methods are being used for the evaluation of screening mammograms, to fulfill flaws in breast cancer classification and

for further divisions of breast tumors. In this respect, a multi-modal deep learning model MDL-IIA has been established to anticipate the molecular subtypes and distinguish between Luminal and Non-Luminal breast cancer subtypes. It is a widely available, cheaper, and non-invasive technique and its efficiency can be further improved if used with other imaging modalities (Zhang et al., 2023).

In their study, Ji Li et al. (2020) demonstrated the diverse cellular composition of TME among different molecular subtypes of breast cancer, the comprehensive study of which may help in a personalized treatment strategy. Therefore, there is an indigence to develop a refined classification of breast cancer based on TME that can improve clinical utilization of new-generation sequencing assays.

The heterogeneity of breast cancer compelled researchers to emphasize its environment. A tumor along with cellular and noncellular components in its environment creates a tumor microenvironment that plays a major role in tumor behavior. The tumor microenvironment develops as a result of tumor cell interaction with host cells. The different cells taking part in the tumor microenvironment consist of proliferating tumor cells, the tumor stroma, blood vessels, infiltrating inflammatory cells, and a variety of associated tissue cells (Whiteside, 2008). The composition of TME determines its positive or negative effect on the outcome of cancer patients (Revel, et al., 2020). Despite the availability of hormonal and targeted therapies, drug resistance remains the mainstay of treatment failure. It has been observed that the interaction between tumor cells and the surrounding stromal cells is bidirectional and has a significant impact on therapeutic response. Therefore, targeting tumor-TME association may reduce tumor progression, metastasis, and chemoresistance.

Cancer associated fibroblasts (CAFs) are the most abundant stromal cells in the TME of breast cancer. Initially, CAFs were thought to play tumorigenic role by modulating extracellular matrix (ECM) and by secreting tumor supporting growth factors and chemokines. The chemoresistant role of CAFs is through the activation of different signaling pathways. Hormone resistance is mediated through activation of ER/Notch feed-forward loop by miR-221from CAFs vesicle, which in turn increases the development of CD133 stem cells, responsible for hormone resistance. CAFs derived IL-6 activates not only STAT3 which is critical for CAF-cancer stem cell niche formation but also JAK/STAT3 signaling and downregulates PTEN, both are

responsible for breast cancer stemness and resistance to trastuzumab. Other reported mechanisms of CAFs induced chemoresistance is through secretion of hepatocyte growth factor and persistent activation of NF-κB pathway. On the contrary, recent studies have found different subsets of CAFs with anti-tumoral or pro-tumoral effects.

Other important TME cells are tumor associated macrophages (TAMs). TAMs secrete survival factors and activate anti-apoptotic signaling pathways in tumor cells thereby influencing their therapeutic response. The Chemoresistance of TAMs is mediated by the activation of STAT3 signaling through the release of IL-10. In breast cancer, high M2 infiltration is associated with poor prognosis. The composition of TME is diverse and cells of chronic inflammation are a major part of this environment.

The contribution of chronic inflammation in tumorigenesis is established from the infiltration of inflammatory cells in the tumor. Rudolf Virchow was the first to describe the effect of inflammation on tumor pathogenesis (Yesim, 2019). However, the composition and number of these inflammatory cells vary from tumor to tumor.

Inflammation being a highly complex event involves several cellular and humoral factors. One of those components is complement which plays a major role in the process of inflammation.

Complement is an integral part of innate immune system acting as a first line of defense against abnormal cells like tumor cells. In cancer patients, complement activation has been demonstrated clinically. Its immunosurveillance role against cancer is inhibited by certain mechanisms, which not only allow cancer cells to get away with their elimination but also decrease the efficiency of cancer immunotherapies (Pio et al., 2014).

Recent studies have shown that the complement system has a complex relationship with human cancer (Afshar, 2017). It is generally considered that the complement system plays an important role in tumor growth and progression. On the contrary, there are researches demonstrating its anti-tumoral role (Xinye, 2022).

Many studies have found activation of the complement system in tumors and an elevation of complement activity in the sera of patients with neoplastic diseases (Pio et al., 2014). However, a study conducted by Jian et al revealed contradictory results showing increased C5aR levels in tumor tissues as compared to healthy tissues but

reduced serum C5a and C5 levels in the advanced cases of breast cancer, most likely owing to its increased activation and use up in the initial stages of the disease (Chen et al., 2020)

Tumor cells are known to activate the complement system which is evident from the presence of activation products of complement in breast and lung tumor tissue (Niculescu, F., 1994, Ajona, d., 2013). The abnormal activation of the complement system has been documented as a contributory factor for the pathogenesis of cancer and other human diseases (Reis ES, 2018). Its activation results in the generation of many components, among which C5a is one of the most powerful proinflammatory mediators (Ruben Pio, 2015). C5a acts on host immune cells such as antitumor CD8+ T cells and provides an immunosuppressive microenvironment. Suppression of antitumor CD8+ T cell by C5a is associated with the recruitment of myeloid-derived suppressor cells into tumors and augmentation of their T cell–directed suppressive abilities (Maciej, 2008).

The duration of action of C5a is short-lived and is degraded early to its much stable form, C5a des Arginine. C5a and C5a des Arginine have certain biological characteristics in common (Reis E., 2012). Various regulatory proteins monitor complement activation and failure of regulation will result in C5a overproduction which may provoke certain pathological processes including cancer (Woodruff, T. M., 2011, Markiewski M. M, 2009).

C5a generates its response by binding with two receptors C5aR1 (CD88) and C5aR2 (GPR77). The pro-inflammatory role of C5aR1 has been established but the role of C5aR2 is still uncertain. Although C5aR2 is a G-protein coupling receptor (GPCR) but still lacks coupling with Gα owing to the difference in its structure. There are changes in the amino-acid sequence of two of its motifs, DRY and NPXXY on the intracellular domains. Both are meant for recognition and binding with Gα and lacking that sequence makes it incapable of binding with Gα.

Apart from C5a, C5aR2 can also bind with other ligands. The strong affinity of C5a and C5a des Arg to bind with complement receptor C5aR2 is proved by different researchers through ligand binding assays (Ting, 2017). Cain and Monk revealed a high affinity of C5a for C5aR2 using the hC5aR2 gene expressing cell lines RBL-2H3 (rat basophilic leukemia) and I-labeled ligand, and a low affinity of C3a and C4a to bind with C5aR2 (2002). Kalant and colleagues used the same approach but different cell

lines and explained the binding of C3a and C4a, C3a-des Arg (ASP), and C4a des Arg with C5aR2 but at a different position (2003). On the contrary, Okinaga and colleagues using an I-labelled ligand binding assay showed contradictory evidence denying any interaction between C5aR2 and C3a or C4a in both permanently transfected murine pre-B lymphocytic cell line and transiently transfected HEK293T (2003).

Recently Croker et al. assayed 61 peptides resembling the C-terminal sequence of C5a and identified two functionally selective ligands for C5aR2, P32 and P59. Croker revealed that both ligands can recruit β-arrestin 2 via C5aR2, but can neither upregulate heteromer formation between C5aR1 and C5aR2 nor recruit β-arrestin 2 through C5aR1. In HMDM, C5a-induced ERK1/2 activation was inhibited by both ligands but not in a CHO-C5aR1 cell line. It was also found that P32 and P59 can inhibit the release of IL-6 from HMDMs. C5a-induced neutrophil mobilization was also inhibited by P32 in wild-type mice, but inhibition in C5aR2knockout mice was not reported.

The study conducted by Christian et.al revealed the expression of C5aR2 on several immune cells but with varying intensity. C5aR2 expression was found not only on tissue-residing immune cells but also on circulating immune cells. The expression on neutrophils was strong as well as homogenous. However, tissue macrophage expression of C5aR2 was strong but heterogeneous depending upon its site. The highest C5aR2 expression was observed in the macrophages of airways, lamina propria of the small intestine as well as microglial cells. Moderate expression was shown on pulmonary macrophages whereas only 25% of adipose tissue macrophages stained positive for C5aR2. Strong and homogenous C5aR2 expression was noted in peritoneal macrophages.

C5aR2 expression in eosinophils from the lung, lamina propria of the small intestine, bone marrow, adipose tissue of viscera, and blood was also assessed. Almost all the eosinophils in the blood were positive for C5aR2 and similar results were observed in eosinophils from lung, bone marrow, and lamina propria of the small intestine. Weaker but homogenous C5aR2 expression was noted in eosinophils from adipose tissue.

Immature dendritic cells, natural killer cells, and B-lymphocytes also showed C5aR2 expression except for T-lymphocytes.

Initially, it was thought that C5aR2 does not produce any physiological response because it does not exhibit G protein coupling due to the absence of the highly conserved DRY and NPXXY motifs that are indispensable for G protein recognition/coupling in Class A GPCRs (Xaria,2020), instead, it acts as a decoy receptor that binds and removes excess of C5a and also regulates ERK1/2 activation by C5aR1. However, according to new developments, C5aR2 functions via β-arrestins signaling and can stimulate and regulate the biological functions of C5a (Jamileh et al.,2019). It not only recruits β-arrestins but also forms heterodimers by interacting with C5aR1. The formation of heterodimers is obligatory for the internalization of C5aR1, its colocalization with C5aR2, and for C5a-driven AP2 selection. On the basis of these functions, it can be suggested that C5aR2 acts as a C5aR1 regulator (Xaria et al.,2019). Croker et al performed BRET tests and by calculating BRET ratio revealed that C5aR1-C5aR2 heterodimer formation can be induced by C5a while evaluation of ligand-induced BRET ratio showed no heterodimer formation by C5a-des Arg. It also has been found that upregulation of heteromer formation by C5a regulates the release of anti-inflammatory cytokines IL-10 and G-CSF from HMDM, supporting the anti-inflammatory role of C5aR2.

C5aR2 mediates its signaling effect through β-arrestin recruitment. Not only C5a and C5a-des Arg but selective C5aR2 ligands P32 and P59, also have the ability to recruit β-arrestins. Among the members of β-arrestin family, β-arrestin 1 and 2 have widespread distribution among various tissues and cells and accumulate in the cell cytoplasm, however, β-arrestin 1 also has a tendency to accumulate in the nucleus of the cell (Song, et al., 2017). Upon stimulation with C5a, non-canonical GPCR C5aR2 undergo phosphorylation by GRK5/6 (GPCR kinase). As a result, there is recruitment and localization of β-arrestin 2 to the plasma membrane. Localization of β-arrestin to the membrane of C5aR2 expressing cells has also been found under normal circumstances but to a lesser degree. β-arrestin 2 once recruited, colocalizes with C5aR2 on endosomal vesicles. There is not much known about the downstream signaling of β-arrestin. One of the proposed mechanisms is β-arrestin1 and p65 complex formation that induces NF-κB p65 transcriptional activity (Song et al., 2017)

It has been observed that β-arrestin regulates cell proliferation, promotes tumor cell invasion and metastases, and is also involved in drug resistance. β-arrestin 1 after recruitment into the nucleus promotes cell growth and angiogenesis by activation of

many growth factors like VEGFR2/3 and EGFR (Song et al., 2017). Scola et al denied any interaction between C5aR2 and β-arrestin 1. However, recently Pandey et al. discovered an association between C5aR2 and β-arrestin 1 (2021). After activation, C5aR2 downregulates ERK1/2 signaling through β-arrestin and also via C5aR1-C5aR2 heterodimer formation.

C5aR2 agonist P32 also downregulates ERK1/2 signaling and intracellular calcium mobilization in HMDMs by decreasing cellular response to C3a. It has been observed that not only complement receptors but other GPCRs expressed on human macrophages are also influenced by C5aR2 activation. Chemokine receptor CMKLR1 mediated ERK1/2 signaling was significantly reduced and LTB4R mediated calcium mobilization was increased by pretreating cells with C5aR2 agonist P32. In HMDM P32 induced C5aR2 activation downregulates cytokine production triggered by TLRs as well as non-TLRs like C-type lectin receptors and STING (DNA sensor stimulator of IFN genes.

Schicheng Su, et al (2018) reported that C5AR2-associated CAFs and IL-6 have a strong correlation with chemoresistance and poor prognosis in lung and breast cancer patients.

On the contrary, tumor growth restriction by C5aR2 was noted in a study of melanoma-bearing murine model (Jamileh, et al.,2019). Similar anti-tumorigenic effects of C5aR2 were seen in C5aR2 deficient, AOM/DSS-induced colorectal carcinoma showing increased tumor growth (Peipe, et al.,2020).

It has been found through bioinformatic analysis that different cancerous and non-cancerous cells and tissues show variable C5aR2 expression levels and tumors with high C5aR2 expression have a poor prognosis (Yumeng, 2021). Transwell assay using fifty thousand breast cancer cells in a serum-free medium and CCK8 assay using cancer cells and Cell Counting Kit-8 reagent were performed which explained the vital role of C5aR2 in tumor proliferation, invasion, and migration.

C5a receptors regulate the immune function of tumors. C5aR2 receptor activation results in cytokine production including IL-6 and TNF-α. Both these cytokines are essential for tumor initiation and its subsequent progression (Yilin, 2021). In a study conducted on gastric carcinoma using IHC, it was suggested that high C5aR2 expression is associated with local invasion (Yilin, 2021)

Peipei, et al., (2020) induced a colorectal carcinoma model and using immunohistochemistry, flow cytometry, and bead assay the determined role of immune cells in colorectal tumor progression. The results demonstrated that deficiency of C5aR2 augmented tumorigenesis, explaining its anti-inflammatory role in colorectal cancer.

In recent studies, Su et al. found that cancer-associated fibroblasts have different subsets that noticeably affect the prognosis of tumors (2018). CAF subset production is attributable not only to the biological behavior of the tumor but also to selective pressure due to treatment exposure. This was verified by Su et al showing an abundance of C5aR2 positive CAFs in patients treated with neoadjuvant therapy as compared to patients not treated with neoadjuvant therapy. (Alexia, 2018). These subsets show different functions having pro- and anti-tumoral effects (Ozdemir BC,2014, Rhim AD., 2014). Among different CAF subpopulations, a specific subset of cancer-associated fibroblasts with cell surface markers (C5aR2 and CD10) was determined in lung and breast tumors strongly associated with chemoresistance. The authors established the fact that these new markers can analyze the response of cancer patients to neoadjuvant therapy and its possible consequence (Su S, 2018).

The general thought of tumorigenic role of CAFs became controversial when CAFs were targeted in pancreatic cancer patients and also in pancreatic cancer mouse model resulting in disease exacerbation. This anti-tumorigenic role of C5aR2 expressing CAFs lead to the belief that CAFs have different subsets with distinct functions. Therefore, identification of CAF subset is a pre-requisite for targeting CAFs in cancer therapy.

The tumorigenic effect of C5aR2 positive CAFs is also supported by the fact that it provides a supportive niche to cancer stem cells by secreting cytokines IL-6 and IL-8. The binding of C5aR2 with C5a maintains the release of IL-6 and IL-8 through persistent transcriptional activation of NF-κB pathway (Alexia, 2018). The proposed mechanism of chemoresistance and cancer stemness by C5aR2-associated fibroblasts is through activation of the NF-κB pathway (Alexia, 2018).

Inflammation is regarded as a hallmark of cancer. NLRP3 inflammasome is a complex protein that augments inflammation through the release of HMGB1 and maturation of cytokines IL-1β and IL-18. The complement receptor C5aR2 stimulates

NLRP3 activation by increasing PKR expression. PKR expression is promoted by C5aR2 via the MAPK/ERK pathway and type I IFN signaling. The study conducted by Songlin Yu et.al demonstrated that C5aR2 deficiency attenuates NLRP3 inflammasome activation and also release of HMGB1 and IL-1β, thereby reducing inflammation.

The profound relation between disease-specific survival and C5aR2 in several types of cancer inclusive of breast cancer was analyzed by Cox proportional hazards model using the TCGA database. Similarly, high C5aR2 expression has also been strongly associated with overall survival (OS) in multiple cancers particularly those with BRCA mutations (Yumeng et al.,2021). ER-positive breast cancer cells presented with high expression of C5aR2 as compared to the neighboring normal cells, a finding consistent with the oncogenic nature of C5aR2 (Yumeng, et al., 2021). Yumeng Zhu et al using Spearman's rank correlation coefficient found a strong positive correlation of C5aR2 expression with tumor mutation burden and microsatellite instability. Transwell and CCK8 assay further illustrated that increase expression of C5aR2 in breast cancer cells is related to its progression, proliferation, invasion, and migration (Yumeng, et al., 2021)

Jian et al conducted a study verifying the role of C5a/C5aR pathway in the pathogenesis of BC development both in vivo and in vitro. He transplanted mice with BC cell line 4T-1 cells(C5aR positive), and the C5aR deficiency significantly reduced the tumor growth. Ki67 expression was also found to be downregulated in C5aR deficient mice. Treating mice with C5aR antagonist, hexapeptide AcF yielded similar results. In vitro, treatment of 4T-1 cells with C5aR antagonist led to a reduction in the growth of tumors while treating BC cell line MDA-MB-453 cells with recombinant C5a enhanced invasion and metastasis of these cell lines. The underlying mechanism of reduction in tumor growth due to C5aR blockade by C5aR antibody or its genetic removal is enhanced antitumor response or additional inhibition of growth of cancer cells by the tumor microenvironment and C5a-C5aR association in tumor cells (Chen et al, 2020).

Gene Set Enrichment Analysis discovered widespread involvement of C5aR2 in important signaling pathways such as the p53 pathway, IL6 JAK STAT3 signaling, KRAS signaling up, TNFα signaling via NFκB, IL2 STAT5 signaling, and also in pathways associated with apoptosis and generation of reactive oxygen species. According to the gene co-expression analysis high expression of C5aR2 causes

consistent activation of the NF-kB pathway by binding with its ligand C5a. C5AR2 overexpression facilitated the functions such as migration, invasion, and proliferation in breast cancer cells, which is consistent with bioinformatics analysis (Yumeng, 2021).

The correlation between C5aR2 and, the secretion and transport of hormones was established by Gene Ontology enrichment analysis. This association is of prime importance since the basic treatment modality for hormone-positive breast cancer is hormonal therapy. C5aR2 can have a profound effect on a breast cancer cure. Gene Set Variation Analysis also discovered a link between C5aR2 and, metastases, relapse, and ESR1 (Estrogen Receptor 1) upregulation. ESR1 is a proven breast cancer oncogene responsible for hormonal resistance (Yumeng, et al., 2021).

Yumeng et al. overexpressed C5aR2 in MDA-MB-231 (ER-negative) cell lines of breast cancer which augmented tumor migration, invasion, and proliferation. This is attributed to upregulation of MMP2 and MMP9 expression levels demonstrating increased metastases and invasion in many cancers, confirming their role as an oncogene. The oncogene MMP2 stimulates TGF-β to uphold epithelial-mesenchymal transformation. On the other hand, MMP9 supports tumor angiogenesis by releasing vascular endothelial growth factors. Tumor mutation burden (TMB) and microsatellite instability (MSI) were found to be negatively related to C5aR2 expression as demonstrated by Spearman's rank correlation coefficient (Yumeng, 2021).

To demonstrate the influence of C5aR2 on the immune infiltration of macrophages in breast cancer, the TIMER2.0 database was used. It was found that C5AR2 overexpression was positively correlated with immune infiltration of M2 macrophages while negatively correlated with M0 and M1 macrophages. The outcome is indicative of the active participation of C5aR2 in immune infiltration, especially the polarization of macrophages (Yumeng, 2021).

Research on C5aR2 inhibitor was conducted by Wang Yuxuan, using human gastric cancer cell lines obtained from different variants of gastric cancer such as MKN1 (from adenosquamous carcinoma), MKN7 (from tubular adenocarcinoma of well-differentiated type), NUGC3 and AGS (from poorly differentiated tubular adenocarcinoma). Scientists then tested the expression of C5aR and C5L2 (C5aR2) on gastric cancer cell lines. Stimulation of MKN1 and MKN7 cells by recombinant human

complement component C5a showed no effect on growth but invasion was markedly increased (Yuxuan, 2019).

The role of complement receptor C5aR2 in the development and progression of various cancers particularly breast cancer is quite evident. From a therapeutic perspective, C5aR2 expression can be used as a biomarker for better outcomes of target therapies.

OPERATIONAL DEFINITIONS

1.Tumor Microenvironment and Tumor Stroma

Tumor microenvironment consists of abnormally developed blood vessels, cancer associated fibroblasts (CAFs), macrophages, immune cells along with extracellular matrix surrounding the tumor cells. Tumor microenvironment (TME) and tumor cells communicate bidirectionally to promote growth, chemoresistance, and metastasis (Umar et al., 2021).

2. Cancer-Associated Fibroblasts (CAFs)

CAFs are the activated fibroblasts that form the major component of tumor stroma. CAFs have a controversial role in cancer development. Different studies have suggested its pro-tumoral and anti-tumoral effects which hamper its therapeutic use (Shicheng et al., 2018). Targeting C5aR2 associated CAFs with pro-tumoral effect will be beneficial for breast cancer patients.

3. C5a, Complement Component

C5a is a chief component of complement. It is an anaphylatoxin, chemoattractant, and a key immunomodulator. Excessive C5a production as seen in regulatory proteins dysfunction, may result in many diseases including cancer.

4.**C5aR2**

C5aR2 is the second complement receptor for C5a. It is a G-protein coupled receptor which instead of binding with G protein subunit binds with β-arrestin and induces a supplementary signaling cascade resulting in tumor invasion and metastasis. Its expression on tumor cells or tumor stroma is consistent with poor prognosis.

5.**C5aR2 Expression**

C5aR2 tumor staining will be scored using the semi-quantitative Immunoreactivity Score (IRS) according to Remmele and Stegner. We will assess the percentage of positive tumor cells in five gradations (no positive cells (0), <10% positive cells (1), 10–50% positive cells (2), 51–80% positive cells (3), and >80% positive cells (4)) and will multiply it by the staining intensity quantified in four gradations (no staining (0), mild staining (1), moderate staining (2), and strong staining (3)). Thus, IRS values ranging from 0 to 12 will be obtained. Two independent investigators will evaluate all stained slides. Final decisions on discrepant scores will be achieved by consensus.

6. **Chemoresistance:**

The inability of a tumor to respond to chemotherapy is chemoresistance and tumor microenvironment is mainly responsible for it. Components of TME when exposed to chemotherapy undergo phenotypic changes and become resistant to chemotherapy.

7. **Tumor Mutation Burden (TMB)**

It is the number of genetic changes occurring in a cell. Targeting cancer cells specifically can prevent killing body's normal and healthy cells. Cancers with low TMB have fewer mutations, so less chance for activation of immune system as compared to cancer with high TMB. Increase mutations have been associated with aggressiveness of tumor and sometimes resistance to chemotherapy.

8. **Clinicopathological Parameters**

Relating signs and symptoms of a disease with the results of laboratory examination. In case of breast cancer, the parameters involved are stage and grade of tumor, age of patient, receptor status, proliferation marker.

9. **Receptor Status**

Depending upon the presence of hormone receptor breast cancer can be estrogen positive if it has receptors for estrogen suggesting that estrogen may stimulate growth of cancer cells by sending signals. Similarly, it can be progesterone positive, her2 positive or triple negative if it has progesterone receptor, HER2 or all receptors negative respectively.

CHAPTER 3

METHODOLOGY

3.1 STUDY DESIGN

It was a Cross-sectional study. Ethical approval was obtained from the Ethical Review Committee (ERC) of Bahria University Health Sciences Campus (BUHSC) Karachi, before the study was commenced.

3.2 SUBJECTS

Formalin Fixed Paraffin embedded breast cancer tissue samples were used (cases from 2021-2023)

3.3 SETTING

Samples were collected in the Department of Histopathology, PNS Shifa Hospital, Karachi

3.4 INCLUSION CRITERIA

- Females of all age groups
- Primary breast adenocarcinoma sample

3.5 EXCLUSION CRITERIA

- Metastatic tumors
- Male breast cancer specimen
- Poorly fixed tissues
- Patients, not willing to participate

3.6 DURATION OF STUDY

3.6.1 Individual study period: 2-3 days

3.6.2 Total period of study: 06 months

3.7 SAMPLE SIZE ESTIMATION

The study was conducted in 128 FFPE Breast tissue samples

The sample size was calculated by OpenEpi, Version 3, open source calculator.

A total of 128 specimens were evaluated based on the prevalence

of breast cancer in 2020 with a 5% confidence level and 95% confidence

interval.

Sample Size for Frequency in a Population

Population size (for finite population correction factor or fpc) (*N*): 192

Hypothesized % frequency of outcome factor in the population(*p*): 56.3%+/-5

Confidence limits as % of 100 (absolute +/- %) (*d*) : 5%
Design effect (for cluster surveys – *DEFF*) : 1

Sample size n = [DEFF*Np(1-p)]/ [(d2/Z21-α/2*(N-1)+p*(1-p)]

Sample size calculated = **128**

Reference (Globocan 2021 Pakistan)

3.8 SAMPLING TECHNIQUE

Convenient sampling (Non-probability) technique was used in the study

3.9 HUMAN SUBJECTS AND CONSENT

Formalin fixed, paraffin-embedded tissue blocks of breast carcinoma patients.

In prospective cases, consent was taken prior to biopsy.

3.10 MATERIALS USED

- Formalin fixed paraffin-embedded tissue blocks of breast cancer
- Clinical, surgical (mastectomy), and biopsy records
- Hematoxylin & Eosin stained slides of all samples
- Poly-L lycine coated glass slides for Immunohistochemical markers
- Antigen Retrieval Solution
- Blocking Agent

- Primary antibodies (Rabbit polyclonal C5aR2 antibodies, IgG type). Product Identification: GPR77 Antibody (PA5-104357) in IHC (P)
- Secondary antibody Horseradish Peroxidase (HRP)
- Enzyme labeled polymer system
- Chromogen substrate
- Hematoxylin for counterstain
- Phosphate buffer solution

3.11 PARAMETERS OF STUDY

- This study was done to identify C5aR2 (GPR77) associated tumor cells and stromal cells by Immunohistochemistry on selected formalin fixed paraffin embedded blocks of breast cancer specimen

- Clinical data/history was reviewed to collect data regarding age, clinical diagnosis and stage of tumor

- Hematoxylin &Eosin slidesof the selected specimens were reviewed bytwo histopathologists, so that information regarding morphology, lymphovascular invasion, grade, stage, receptor status, Ki-67 could be assessed

- C5aR2 was expressed inmembrane/cytoplasm

- The percentage of C5aR2 positive tumor cells was assessed in five gradations:

  (no positive cells (0), <10% positive cells (1), 10–50% positive cells (2),

  51–80% positive cells (3), and >80% positive cells (4))

  and was multiplied by the staining intensity quantified in four gradations

  (no staining (0), mild staining (1), moderate staining (2), and strong staining (3)

  (Benjamin et.al., 2021)

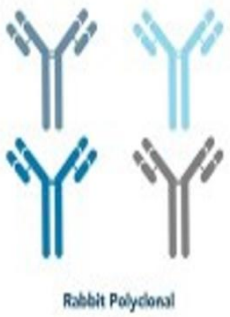
MATERIALS

MASTECTOMY SPECIMEN

TISSUE BLOCKS

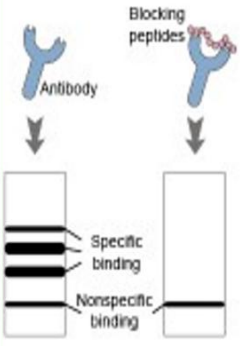
H & E SLIDES

RABBIT POLYCLONAL ANTIBODY

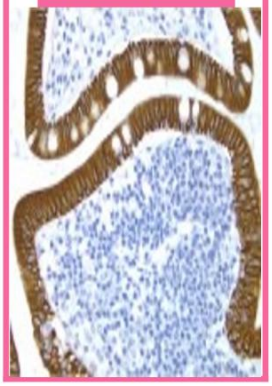


ANTIGEN RETRIEVAL SOLN

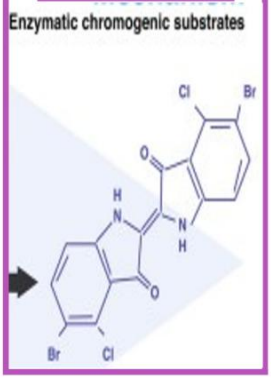
BLOCKING AGENT



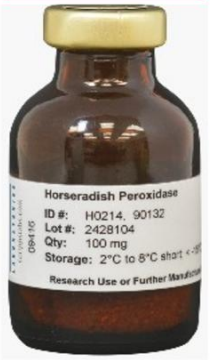
HAEMATOXYLIN



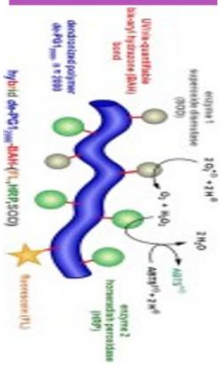
CHROMOGEN SUBSTRATE



HORSERADISH PEROXIDASE



ENZYME LABELED POLYMER SYSTEM

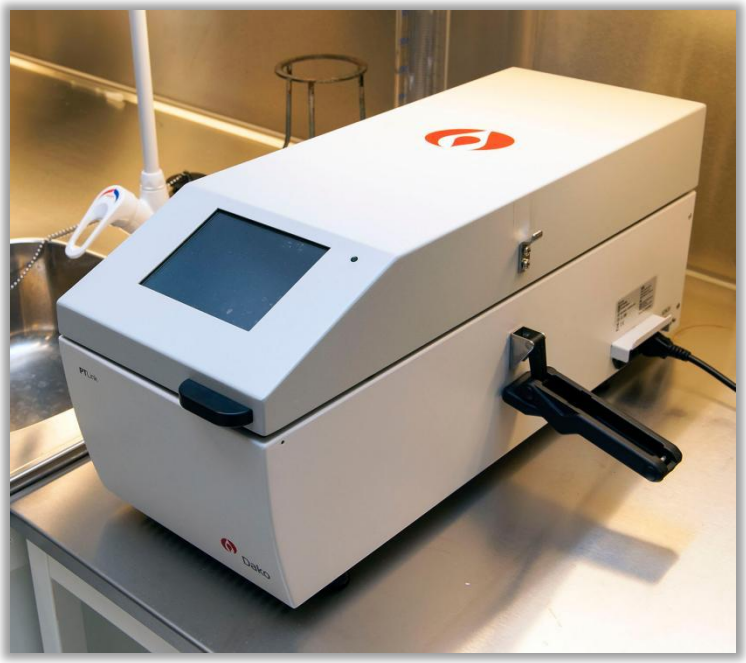


PHOSPHATE BUFFER SOLUTION

EQUIPMENTS

PROCESSOR

MICROTOME



ANTIGEN RETRIEVAL

SLIDES IN HUMIDITY CHAMBER

3.12 PROTOCOLS OF STUDY

- Biopsy reports, clinical data and all related information was reviewed and recorded
- Two histopathologists retrieved and reviewed H&E stained slides
- Immunohistochemical (IHC) staining was done on formalin fixed, paraffin-embedded tissue slides according to the protocols specified by the company
- All slides were examined under a light microscope using scanner lens (4x10 magnification), low power lens (10x10), and high power lens (40x10) and were reviewed by the supervisor.
- Parameters like staging, grading were recorded
- Statistical analysis of results was done

3.12.1 METHODS OF STAINING

3.12.1.1 Haematoxylin and Eosin (H&E) Staining

Sections proceeded through numerous solutions as follows:

a) Xylene I --- 10 min

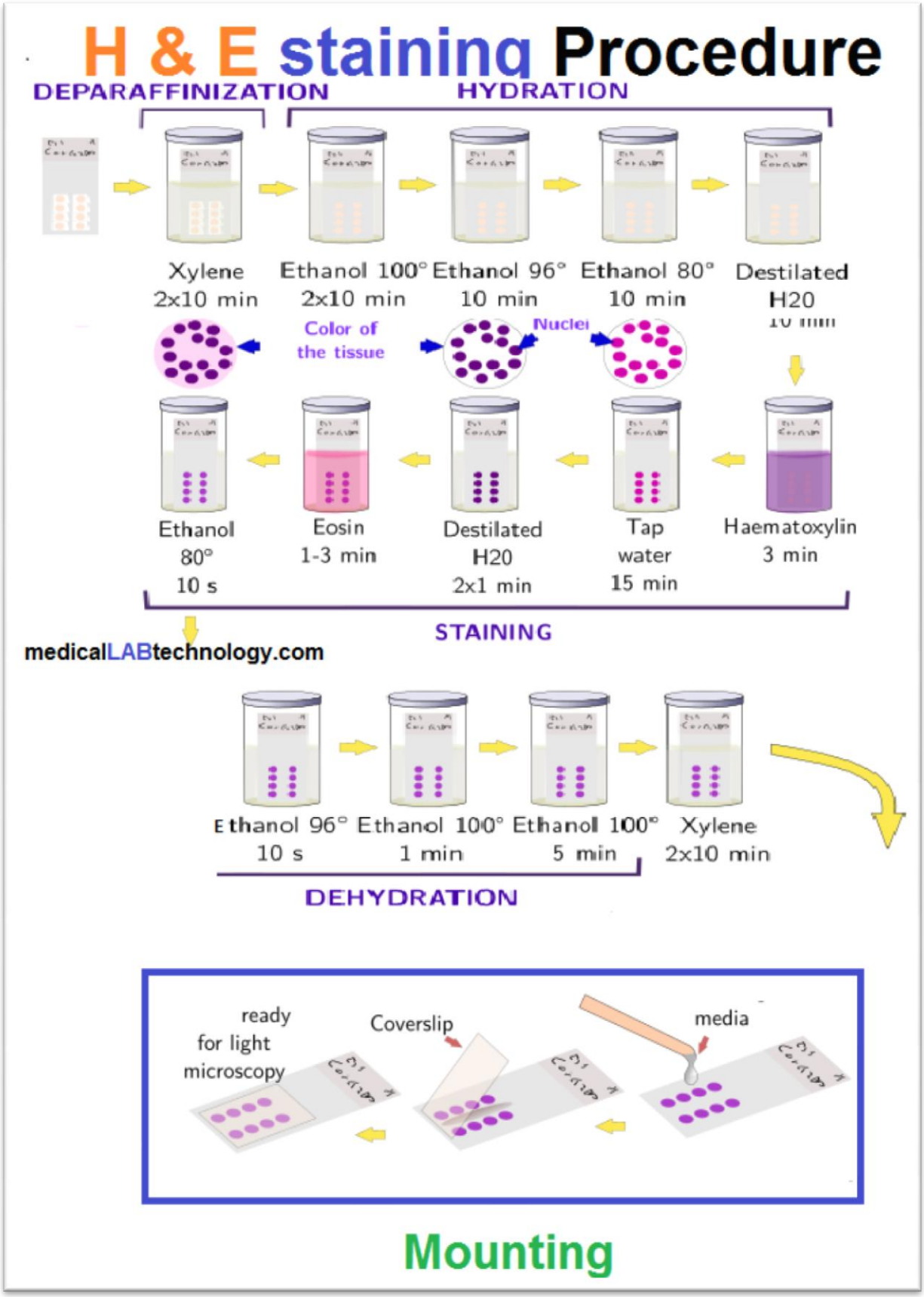
b) Xylene II--- 10 min

c) Absolute Alcohol-- 10 min

d) 95% Alcohol --- 5 min

e) 80% Alcohol --- 5 min

f) 70% Alcohol --- 5 min


H & E staining Procedure
DEPARAFFINIZATION
HYDRATION
Xylene
2x10 min
Ethanol 100°
2x10 min
Ethanol 96°
10 min
Ethanol 80°
10 min
Destilated
H20
Color of
the tissue
Nuclei
Ethanol
80°
10 s
Eosin
1-3 min
Destilated
H20
2x1 min
Tap
water
15 min
Haematoxylin
3 min
STAINING
medicalLABtechnology.com
Ethanol 96°
10 s
Ethanol 100°
1 min
Ethanol 100°
5 min
Xylene
2x10 min
DEHYDRATION
ready
for light
microscopy
Coverslip
media
Mounting

S

g) Tap water rinse -- 2 min

h) Haemtoxylin ---- 5-10 min

i) Acid Alcohol 1%, 3-5 dips then washed with tap water

j) Ammonia water, 3-5 dips then rinsed with tap water for 10 min

k) Eosin --- 2 min

l) 70% Alcohol --- 5 quick dips

m) 80% Alcohol -- 5 quick dips

n) 95% Alcohol --- 5 quick dips

o) Absolute Alcohol – 2 changes – 5 min each

p) Xylene --- 5 min

q) Mounted in Dako Toluene free mounting media

Results:

- Nuclei stained blue
- Cytoplasm stained varying shades of pink

(Reference: Kiernan,J.A (2008) Histological and Histochemical Methods: Theory and Practice. 4th ed. Bloxham, UK)

3.12.1.2 Immunohistochemical Staining

Primary Antibody

Rabbit GPR77 Polyclonal Antibody

Localization: Membrane and Cytoplasm

Positive Control Tissue : Small Intestine

Dilution : 1: 130

Procedure

- Sections were taken from FFPE tumor blocks of 2-4 micrometer thickness and were picked on poly L-lysine coated slides
- Slides were fixed in oven at 80°C for 20-25 min

Antigen Retrieval

- Deparaffinization, hydration, and antigen retrieval were done automatically through Dako PT Link pretreatment system in 40-45 minutes. Temperature started from 65°C when the slides were put in, raised to, and kept at 97°C for 40 min, then cooled down to 65°C, and then slides were taken out
- Slides were then washed with washing buffer 2 times for 5 minutes each

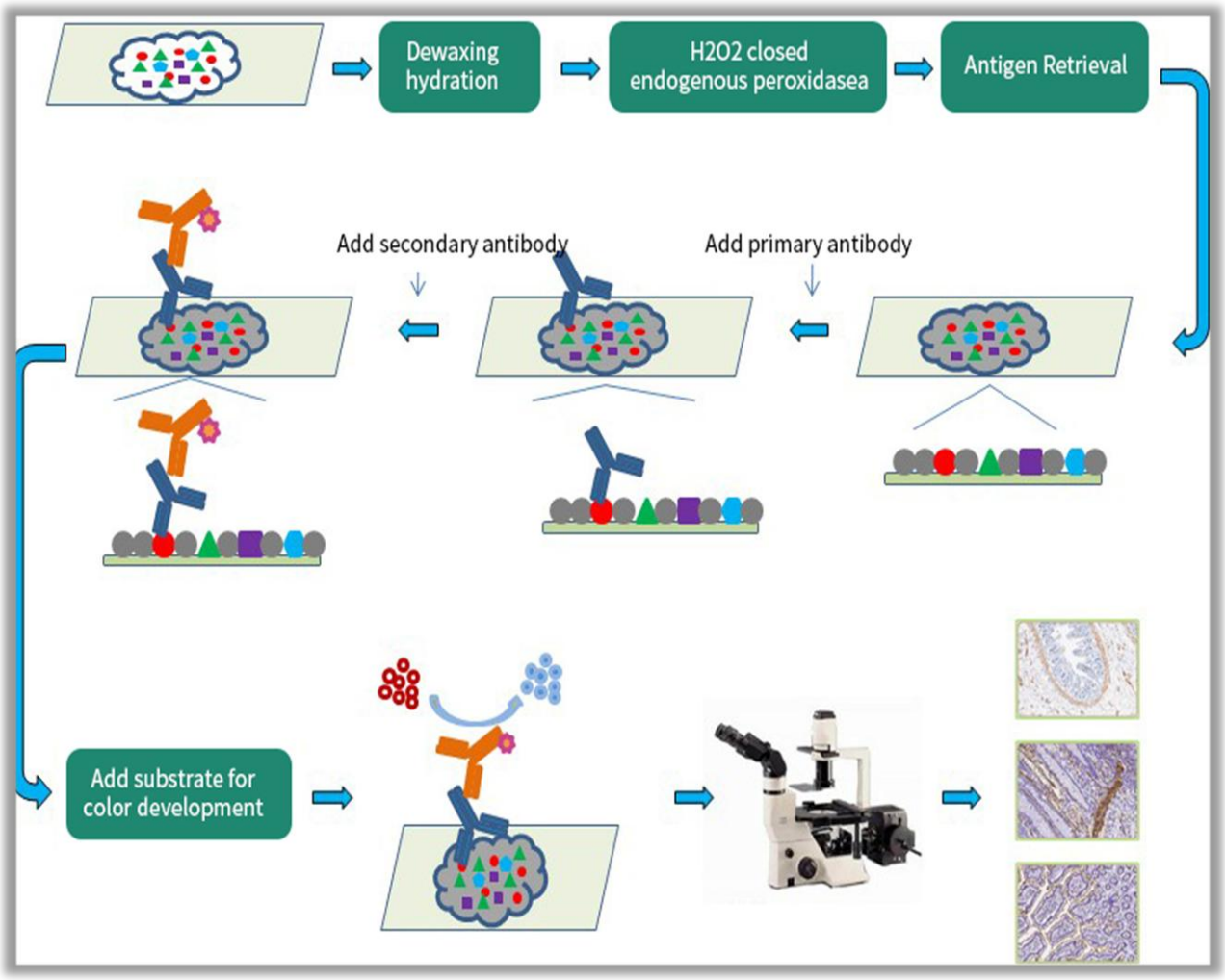
Blocking

- Endogenous peroxidase was blocked by hydrogen peroxide blocking solution
- On tissue sections 1-2 drops of blocking solution was applied to cover the section
- Slides were incubated for 20 minutes in humidity chamber at room temperature
- Slides were washed with washing buffer 2 times for 5 minutes each

Primary Antibody

- GPR77 antibody was diluted in the ratio of 1: 130 as specified in the company's protocol
  Dilution was done by antibody diluent.
- Primary antibody was applied to cover the section
- Sections were incubated overnight in humidity chamber at 4°C
- Slides were then washed with washing buffer 2 times for 5 minutes each

IMMUNOHISTOCHEMISTRY PROCEDURE



Secondary Antibody

- Sufficient Horseradish Peroxidase (HRP) was applied to cover the section
- Slides were incubated for 1 hour in a humidity chamber at room temperature
- Slides were then washed with washing buffer 2 times for 5 minutes each

DAB Substrate Chromogen

- Diaminobenzidine (DAB) substrate chromogen was prepared with 1 ml of DAB substrate and 1 drop of DAB chromogen
- Slides were wiped
- Sufficient DAB substrate chromogen solution was smeared to coat the section
- Slides were incubated for 1- 2 minutes at room temperature
- Slides were washed with distilled water

Hematoxylin Counterstain

- Slides were counterstained with Hematoxylin stain, 1-3 dips
- Slides were washed with washing buffer
- Sections were dehydrated in ascending series of Alcohol (60%, 80%, 100%) and cleared in Xylene
- Slides were mounted in Dako toluene free mounting media
- Slides were then observed under light microscope

3.12.2 INTERPRETATION

For GPR77 the observed staining was membranous and/or cytoplasmic.

The percentage of stained cells (A) was taken into account in five gradations

No positive cells (0)

<10% positive cells (1)

10–50% positive cells (2)

51–80% positive cells (3)

>80% positive cells (4)

and multiplied by the staining intensity (B) quantified in four gradations

No staining (0)

Mild staining (1)

Moderate staining (2)

Strong staining (3)

Final Immunoreactive Score (IRS) obtainedby(AxB) with range (0-12)

0-1 = Negative

2-3 = Mild

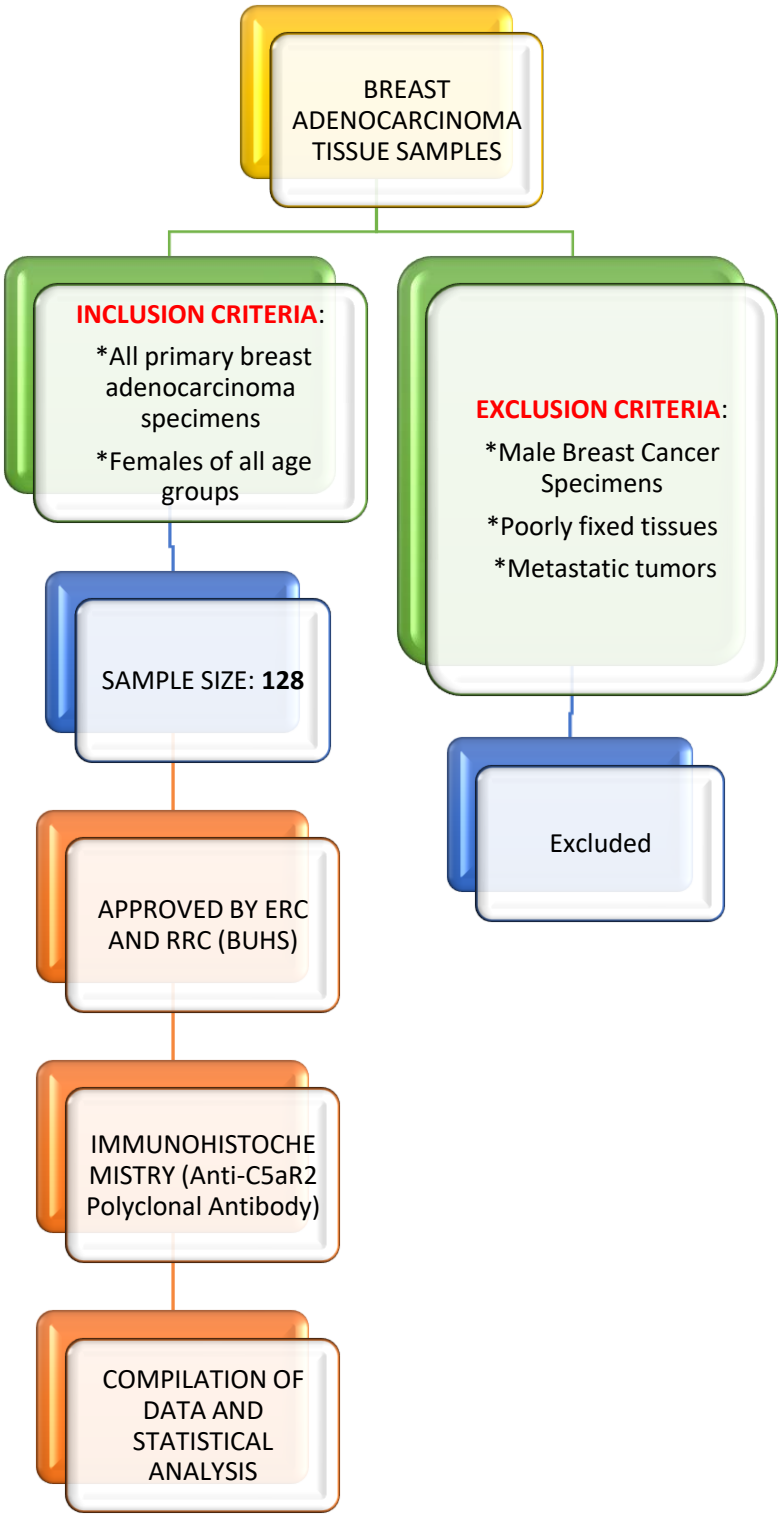
4-8 = Moderate

9-12= Strongly positive

Reference:

- Benjamin et al, February 19, 2021. Immunohistochemical identification of complement peptide C5a receptor 1 (C5aR1) in non-neoplastic and neoplastic human tissues. *PLOS ONE*.
- Fedchenko, N. and Reifenrath, J. 2014. Different approaches for interpretation and reporting of immunohistochemistry analysis results in the bone tissue – a review.*Diagnostic Pathology*.

3.13 ALGORITHM OF STUDY



3.14 STATISTICAL ANALYSIS

- Statistical analysis was done using SPSS version 23
- Continuous variables were shown as mean and standard deviation
- Categorical data was shown as frequencies and percentages
- Appropriate test statistics such as Chi-square test were used to see the association between C5aR2 density and clinicopathological variables
- Pearson correlation test was done to evaluate the relationship between C5aR2 density and continuous clinicopathological variables
- The p-value< 0.05 and confidence interval of 95% was considered statistically significant

CHAPTER 4

RESULTS

The study was conducted at PNS Shifa Hospital in the year 2021 to 2023. A total number of 128 samples were taken according to the selection criteria mentioned earlier and the sample size calculation. The related clinical data was documented in the subject evaluation form. After defining the standard features of the patients and tissue specimens, the data was analyzed and then compared with different clinicopathological parameters.

4.1 Baseline Characteristics

According to the baseline characteristics (Table 4.1) most of the specimens (68%) were obtained from modified radical mastectomy (MRM) while the rest of the cases were trucut (19%), excisional (8%), and incisional (5%) biopsies with few non-specified biopsy cases. The majority (86.7%) of cases were Invasive Breast Carcinoma of No Special Type (IBC-NST) while others included Invasive Lobular Carcinoma (ILC)(7.0%) and Ductal Carcinoma In Situ (DCIS)(3.1%).

The most frequent age group detected in breast cancer samples was 31-50 years with the age range of 24 to 90 years. The patients with grade 2 (moderately differentiated) cancer showed the highest percentage (72%) among all cases while out of 89 stage retrieved cases, 33% predominantly manifested stage II breast cancer patients and 29% were Stage III. On the basis of molecular classification, 46.1% of cases were Luminal B and the rest of the cases were Luminal A, TNBC and HER2+ve in a decreasing frequency 16%, 15.6%, and 13.3% respectively.

The laterality was almost the same with the left-sided breast tumor slightly higher (47.7%) than the right-sided tumor (44.5%). A high proliferation index (Ki67) was observed in two-thirds of cases (64.1%). Lymphovascular invasion was identified in only 35% of cases, while a small proportion of breast tumors (6%) showed perineural invasion. Lymph node involvement was seen in 41.4% of breast cancer samples.

4.2 Age

Table 4.1 shows the age-wise distribution of breast carcinoma patients. The age range of breast cancer patients was 24 to 90 years with a mean age of 57 years. To identify the relationship between age and tumor characteristics, age groups were categorized into 20-30y, 31-50y, 51-70y, and >70y, representing four stratifications. Most (48%) of the tumors fell in the age group 31-50y while 40% comprised of patients in the age group 51-70y. No statistically significant finding was observed between age and other parameters except for histologic subtype (p=0.02) with more than half of the cases (86.7%) with IBC-NST histologic type.

4.3 Molecular Classification

Table 4.1 represents the molecular classification of breast carcinoma patients. 46% of breast cancer cases were Luminal B while the rest of the 50% tumors were Luminal A, TNBC and HER2 positive tumors in a decreasing frequency. The only significant association of molecular classification was observed with grade (p=0.025). Grade 2 was the most frequently observed grade in all the molecular subtypes.

4.4 Grade

Table 4.1 shows the distribution of breast carcinoma patients according to grade. The breast cancer samples comprised mainly (72%) of Grade 2 tumors with only 27% propensity for Grade 3 and only 1% Grade 1 tumors. The molecular subtypes were found to be significantly associated with grade (p=0.04).

4.5 Stage

The grouping of breast cancer patients according to the stage is presented in Table 4.1. Most of the breast samples presented with stage II tumors (33%) followed by stage III (29%) tumors. The stage of 30% of cases was not known. Among different variables, a statistically significant association of stage was shown with histologic subtype (p-0.000) and LNI (p-0.000) mentioned in Table 4.5.

4.6 Histologic Subtype

Table 4.1 revealed IBC-NST being the most common histologic type (86.7%) found in our breast cancer samples followed by ILC and DCIS. A significant association was identified between tumor histologic subtype and DCIS (p-0.02) as shown in Table 4.6.

4.7 Receptor Status

Table 4.1 represents the receptor status of breast cancer patients. The most frequently observed receptors were ER/PR+ (40%) followed by TNBC (16%) and HER2+ (12%). The receptor status of 11 cases was not known. A significant association of receptors was observed with grade (p-0.02) and Ki-67 (p-0.01).

4.8 Additional Parameters

Figure 4.8 describes the frequency distribution of different parameters. The laterality was almost the same with the left-sided breast tumor slightly higher (47.7%) than the right-sided (44.5%) tumor. A high proliferation index (Ki67) was observed in two-thirds of cases (64.1%). Lymphovascular invasion was identified in only 35% of cases, while a small proportion of breast tumors showed perineural invasion (6%). Lymph node involvement was seen in 41.4% of breast cancer samples. The association of most of these parameters has already been stated in above mentioned Tables. DCIS was present in 35.9% of breast cancer cases.

4.9 C5aR2 Expression in Tumor and Stromal Cells

Immunohistochemistry was done to identify C5aR2 expression in tumor cells and stromal cells of the tumor microenvironment. The expression was then correlated with different clinicopathological parameters to establish an association between these parameters and C5aR2. Figures 4.9a and 4.9b shows the difference in C5aR2 expression between tumor and stromal cells. The C5aR2 expression in tumor cells was greater than in stromal cells in all parameters. The majority of tumor cells express C5aR2 either mildly or moderately while almost half of the stromal cells did not express C5aR2.

4.9.1 C5aR2 Expression and Age

Table 4.9.1 demonstrates C5aR2 expression in breast cancer patients of different age groups. C5aR2 showed an increase in expression with the increasing age both in tumor and stromal cells, and the highest strong C5aR2 expression (50%) was observed in the stromal cells of tumors in patients more than 70 years of age. The findings in Table 4.9.1, revealed a statistically significant association between age and C5aR2 expression in tumor (p-0.002) and stromal cells (p-0.000).

4.9.2 C5aR2 Expression and Histologic Subtype

The association of histologic subtype and C5aR2 expression in tumor and stromal cells is shown in Table 4.9.2. All histological types showed mild to moderate expression with only IBC-NST type displaying strongly positive expression in both tumor and stromal cells. No significant association was identified between tumor histologic subtype and C5aR2 expression in tumor and stromal cell

4.9.3 C5aR2 Expression and Grade

As evident from Table 4.9.3, Grade 2 breast cancer cells and stromal cells revealed ample C5aR2 expression with mild to strong intensities followed by Grade 3 tumors. Strong expression was predominant in stromal cells of Grade 3 tumors. A significant association (p-0.03) was observed between grade and tumor cell C5aR2 expression.

4.9.4 C5aR2 Expression and Stage

Table 4.9.4 shows tumor and stromal cell C5aR2 expression in different stages of the tumor. Maximum C5aR2 expression was observed in the tumor and stromal cells of stage II tumors. C5aR2 expression is mainly moderate in all stages of tumors. The maximum level of strong C5aR2 expression has been observed in stromal cells of stage 3 tumors.

4.9.5 C5aR2 Expression and Receptor Status

Table 4.9.5 demonstrates the association between receptors and C5aR2 expression in tumor and stromal cells. Most of the receptor types showed moderate C5aR2 expression in both tumor and stromal cells except for ER positive tumors displaying mild C5aR2 expression. Strong expression was predominantly observed in tumor cells of ER/PR/HER2 positive (33.3%) and ER/HER2 positive (16.7%) tumors and in stromal cells of ER/PR/HER2 positive tumors (11.1%) and TNBCs (10%). The association between receptor types and tumor cell C5aR2 expression was found to be significant (p-0.03). However, no significant association was observed between receptors and stromal cell C5aR2 expression.

4.9.6 C5aR2 Expression and Molecular Classification

Among different molecular variants in tumor cells, moderate to strong expression was frequently observed in Luminal B and HER2 positive tumors whereas mild

expression was commonly seen in Luminal A tumors and TNBCs. However, in stromal cells, Luminal B frequently showed moderate C5aR2 expression while strong expression was maximally displayed by TNBCs followed by HER2-positive tumors. No significant association was detected between molecular classification and tumor and stromal cell C5aR2 expression.

4.9.7 C5aR2 Expression and Tumor Remaining

In both tumor and stromal cells of breast cancer cases, definite tumor response (<30% tumor remaining) has been found to be associated with decreased or no C5aR2 expression respectively. However, breast cancer cases yielding poor treatment response (>30% tumor remaining) were associated with increased C5aR2 expression both in tumor and stromal cells. A significant association (p-0.03) was identified between C5aR2 expression in tumor cells and post treatment tumor response.

4.9.8 C5aR2 Expression and Ki-67

In Table 4.9.8 increased moderate to strong C5aR2 expression has been observed in tumor cells of breast cancer cases with a high proliferation index. On the contrary, in stromal cells, an equal distribution of C5aR2 expression was found in high and low Ki-67 profile breast cancer cases. No significant association was identified between Ki-67 and C5aR2 tumor and stromal cell expression.

4.9.9 C5aR2 Expression and LVI

Table 4.9.9 indicates increased C5aR2 expression in the tumor and stromal cells of breast cancer cases positive for lymphovascular invasion (LVI) as compared to LVI-negative breast cancer cases showing less C5aR2 expression. No significant association was identified between C5aR2 tumor and stromal cell expression and LVI.

4.9.10 C5aR2 Expression and Lymph Node Involvement (LNI)

As shown in Table 4.9.10 C5aR2 expression was enhanced in tumors with lymph node involvement compared to tumors without lymph node involvement, both in tumor and stromal cells. No significant association was identified between C5aR2 tumor and stromal cell expression and LNI.

4.9.11 C5aR2 Expression and Perineural Invasion (PNI)

Table 4.9.11 shows a preponderance of C5aR2 expression in the tumor cells of most of the breast cancer cases with absent perineural invasion (PNI). Unlike tumor cells, the stromal cells revealed greater C5aR2 expression in PNI +ve cases. PNI and C5aR2 tumor and stromal cell expression had no significant association.

4.9.12 C5aR2 Expression and Ductal Carcinoma In Situ (DCIS)

Table 4.9.12 gives the association between C5aR2 expression in tumor and stromal cells and DCIS. DCIS-negative breast cancer cases showed increased expression of C5aR2 both in tumor and stromal cells. Moderate C5aR2 expression was more pronounced in DCIS-positive tumors compared to strong C5aR2 expression being predominantly observed in DCIS-negative tumors. No significant association was identified between DCIS and C5aR2 tumor and stromal cell expression.

4.9.13 C5aR2 Correlation between Age, Ki-67 and Tumor and Stromal Cell Score

Pearson correlation test was applied to evaluate the relationship between continuous variables (Age and Ki-67) and tumor and stromal cell C5aR2 expression. A weak positive correlation was observed between age and tumor cell expression of C5aR2. However, the relation between age and stromal cell expression of C5aR2 was found to be moderately positive. A weak positive correlation was also observed between Ki-67 and stromal cell C5aR2 expression. However, the relation between Ki-67 and tumor cell C5aR2 expression was weakly negative. None of these values were statistically significant.

Table 4.1: Demographic and Tumor Characteristics

VARIABLES	CATEGORIES	FREQUENCY (PERCENTAGE)
Age	20-30y	8 (6.3%)
	31-50y	61(47.7%)
	51-70y	51 (39.8%)
	>70y	8 (6.3%)
Biopsy	MRM	87 (68%)
	Excisional Bx	8 (6.3%)
	Incisional Bx	5 (3.9%)
	Trucut Bx	19 (14.8)
	Bx NOS	9 (7%)
Histologic Subtypes	IBC-NST	111 (86.7%)
	ILC	9 (7.0%)
	DCIS	4 (3.1%)
	Others	4 (3.1%)
Pathological Stage	stage 0	2 (1.6%)
	stage I	8 (6.3%)
	stage II	42 (32.8)
	stage III	37 (28.9%)
	Not known	39 (30.5%)
Receptor	ER+	11 (8.6%)
	ER/PR+	51 (39.8%)
	ER/PR/HER2+	9 (7%)
	TNBC	20 (15.6%)
	ER/HER2+	6 (4.7%)
	PR/HER2+	2 (1.6%)
	HER2+	16 (12.5%)
	PR+	2 (1.6%)
	Not Known	11 (8.6%)
Molecular Classification	Luminal A	21 (16%)
	Luminal B	59 (46.1%)

	HER2+ve	17 (13.3%)
	TNBC	20 (15.6)
	Not known	11(8.6%)
Grade	grade 1	2 (1.6%)
	grade 2	92 (71.9%)
	grade 3	34 (26.6%)
Tumor Remaining	<30%	5 (3.9%)
	30-50%	8 (6.3%)
	51-80%	4 (3.1%)
	>80%	11 (8.6%)
	No Treatment	100 (78.1%)
Ki-67 (%)	Low (<14%)	19 (14.8%)
	High (>14%)	86 (67.2%)
	NOS	23 (18%)
LVI	Present	45 (35.2%)
	Absent	49 (38.3%)
	NOS	34 (26.5%)
DCIS	Present	46 (35.9%)
	Absent	52 (40.6%)
	NOS	30 (23.5%)
PNI	Present	8 (6.3%)
	Absent	83(64.8%)
	NOS	37(28.9%)
LNI	Present	53 (41.4%)
	Absent	35 (27.3%)
	NOS	40 (31.3%)
	Total	128(100%)

Table 4.2: Cross-tabulation of Age and Tumor Characteristics

		Age				Total	p-value
		20-30	31-50	51-70	>70		
Grade (n=128)	grade 1	0	0	2	0	2	0.25
	grade 2	5	41	41	5	92	
	grade 3	3	20	8	3	34	
	Total	8	61	51	8	128	
Histologic Subtype (n=128)	IBC-NST	8	59	38	6	111	**0.02**
	ILC	0	1	7	1	9	
	DCIS	0	1	3	0	4	
	Others	0	0	3	1	4	
	Total	8	61	51	8	128	
Pathological Stage (n=89)	stage 0	0	0	2	0	2	0.79
	stage I	1	3	3	1	8	
	stage II	2	20	16	4	42	
	stage III	1	16	18	2	37	
	Total	4	39	39	7	89	
Receptor (n=117)	ER+	1	6	4	0	11	0.83
	ER/PR+	4	19	23	5	51	
	ER/PR/HER2+	0	5	3	1	9	
	TNBC	2	10	7	1	20	
	ER/HER2+	0	3	3	0	6	
	PR/HER2+	0	1	1	0	2	
	HER2+	0	10	5	1	16	
	PR+	1	1	0	0	2	
	Total	8	55	46	8	117	
Molecular Status (n=117)	Luminal A	2	7	10	2	21	0.91
	Luminal B	4	28	23	4	59	
	HER2+ve	0	10	6	1	17	
	TNBC	2	10	7	1	20	
	Total	8	55	46	8	117	
Tumor Remaining (n=28)	<30%	1	2	2	0	5	0.25
	30-50%	1	2	5	0	8	
	51-80%	0	3	0	1	4	
	>80%	1	6	4	0	11	
	Total	3	13	11	1	28	
Ki-67 (n=105)	Low (<14%)	1	7	8	3	17	0.43
	High (>14%)	6	43	32	5	82	

	Total	7	50	40	8	105	
LVI (n=94)	Present	0	24	18	3	45	0.10
	Absent	5	18	22	4	49	
	Total	5	42	40	7	94	
PNI (n=91)	Present	0	4	3	1	8	0.81
	Absent	5	35	37	6	83	
	Total	5	39	40	7	91	
LNI (n=92)	Present	2	25	25	1	53	0.18
	Absent	3	15	13	4	35	
	Total	5	40	38	5	88	

Table 4.3: Cross-tabulation of Molecular Classification and Tumor Characteristics

Variable with categories		Molecular Status				Total	p-value
		Luminal A	Luminal B	HER2+ve	TNBC		
Age (n=117)	20-30	2	4	0	2	8	0.91
	31-50	7	28	10	10	55	
	51-70	10	23	6	7	46	
	>70	2	4	1	1	8	
Histologic subtype (n=117)	IBC-NST	15	52	16	19	102	0.06
	ILC	2	6	0	0	8	
	DCIS	2	0	0	1	3	
	Others	2	1	1	0	4	
Grade (n=117)	grade 1	0	2	0	0	2	**0.028**
	grade 2	20	44	11	10	85	
	grade 3	1	13	6	10	30	
	Total	21	59	17	20	117	
Stage (n=79)	stage 0	1	0	0	0	1	0.30
	stage I	0	3	1	2	6	
	stage II	6	21	4	7	38	
	stage III	8	11	8	7	34	
	Total	15	35	13	16	79	
Tumor Remaining (n=25)	<30%	2	2	0	0	4	0.54
	30-50%	1	5	1	1	8	
	51-80%	0	3	1	0	4	
	>80%	3	2	2	2	9	
	Total	6	12	4	3	25	
Ki-67 (n=105)	Low (<14%)	15	3	0	1	17	**0.000**
	High (>14%)	0	55	14	17	82	
	Total	15	58	14	18	105	
LVI (n=83)	Present	7	18	9	9	43	0.55
	Absent	9	18	4	9	40	
	Total	16	36	13	18	83	
PNI (n=80)	Present	0	7	0	0	7	0.16
	Absent	16	27	13	17	73	
	Total	16	34	13	17	80	
LNI (n=81)	Present	10	21	9	9	49	0.76
	Absent	5	13	3	7	28	
	Total	15	34	12	16	77	
DCIS (n=87)	Present	6	22	8	6	42	0.28
	Absent	11	17	6	11	45	
	Total	17	39	14	17	87	

Table 4.4: Cross-tabulation of Grade and Tumor Characteristics

Variable with Categories		Grade			Total	p-value
		grade 1	grade 2	grade 3		
Age (n=128)	20-30y	0	5	3	8	0.25
	31-50y	0	41	20	61	
	51-70y	2	41	8	51	
	>70y	0	5	3	8	
	Total	2	92	34	128	
Pathological Stage (n=89)	stage 0	0	1	1	2	0.06
	stage I	1	6	1	8	
	stage II	0	32	10	42	
	stage III	0	25	12	37	
	Total	1	64	24	89	
Molecular Status (n=117)	Luminal A	0	20	1	21	**0.028**
	Luminal B	2	44	13	59	
	HER2+ve	0	11	6	17	
	TNBC	0	10	10	20	
	Total	2	85	30	117	
Histologic subtype (n=128)	IBC-NST	1	78	32	111	0.21
	ILC	1	7	1	9	
	DCIS	0	3	1	4	
	Others	0	4	0	4	
Tumor Remaining (n=28)	<30%	0	5	0	5	0.07
	31-50%	0	7	1	8	
	51-80%	0	2	2	4	
	>80%	0	5	6	11	
	Total	0	19	9	28	
Ki67 (n=105)	Low (<10%)	0	17	2	17	0.15
	High (>20%)	1	58	27	82	
	Total	1	75	29	105	
LVI (n=94)	Present	0	35	10	45	0.38
	Absent	1	33	15	49	
	Total	1	68	25	94	
PNI (n=91)	Present	0	6	2	8	0.93
	Absent	1	59	23	83	
	Total	1	65	25	91	
LNI (n=92)	Present	0	37	16	53	0.47
	Absent	1	26	8	35	
	Total	1	63	24	88	
DCIS (n=98)	Present	0	32	14	46	0.47
	Absent	1	39	12	52	
	Total	1	71	26	98	

Table 4.5: Cross-tabulation of Stage and Tumor Characteristics

Variable with Categories		Stage				Total	p-value
		stage 0	stage I	stage II	stage III		
Age (n=89)	20-30y	0	1	2	1	4	0.79
	31-50y	0	3	20	16	39	
	51-70y	2	3	16	18	39	
	>70y	0	1	4	2	7	
	Total	2	8	42	37	89	
Histologic subtype (n=89)	IBC-NST	0	4	37	33	74	**0.000**
	ILC	0	1	5	3	9	
	DCIS	2	1	0	0	3	
	Others	0	2	0	1	3	
	Total	2	8	42	37	89	
Grade (n=89)	grade 1	0	1	0	0	1	0.06
	grade 2	1	6	32	25	64	
	grade 3	1	1	10	12	24	
	Total	2	8	42	37	89	
Receptor (n=79)	ER+	0	0	0	4	4	0.43
	ER/PR+	1	1	21	13	36	
	ER/PR/HER2+	0	1	4	2	7	
	TNBC	0	2	7	7	16	
	ER/HER2+	0	1	2	0	3	
	PR/HER2+	0	0	1	0	1	
	HER2+	0	1	3	8	12	
	Total	1	6	38	34	79	
Molecular Status (n=79)	Luminal A	1	0	6	8	15	0.29
	Luminal B	0	3	21	11	35	
	HER2+ve	0	1	4	8	13	
	TNBC	0	2	7	7	16	
	Total	1	6	38	34	79	
Tumor Remaining (n=28)	<30%	0	0	0	5	5	0.30
	30-50%	0	1	4	3	8	
	51-80%	0	0	1	3	4	
	>80%	0	0	3	8	11	
	Total	0	1	8	19	28	
Ki67 (n=70)	Low (<14%)	0	0	6	9	14	0.41
	High (>14%)	0	3	28	24	52	
	Total	0	3	34	33	70	
LVI (n=89)	Present	0	2	18	22	42	0.11
	Absent	2	6	24	15	47	
	Total	2	8	42	37	89	
PNI (n=88)	Present	0	1	5	2	8	0.73
	Absent	2	7	37	34	80	
	Total	2	8	42	36	88	
LNI (n=89)	Present	0	0	18	35	53	**0.000**
	Absent	1	8	26	0	35	
	Total	1	8	41	35	88	
DCIS (n=88)	Present	2	3	19	13	37	0.54
	Absent	0	5	23	23	51	
	Total	2	8	42	36	88	

Table 4.6: Cross-tabulation of Histologic subtype and Tumor Characteristics

Variable with Categories		Histologic subtype				Total	p-value
		IBC-NST	ILC	DCIS	Others		
Receptor (n=117)	ER+	10	1	0	0	11	0.96
	ER/PR+	40	6	2	3	51	
	ER/PR/HER2+	8	1	0	0	9	
	TNBC	19	0	1	0	20	
	ER/HER2+	6	0	0	0	6	
	PR/HER2+	2	0	0	0	2	
	HER2+	15	0	0	1	16	
	PR+	2	0	0	0	2	
	Total	102	8	3	4	117	
Tumor Remaining (n=28)	<30%	5	0	0	0	5	---
	30-50%	8	0	0	0	8	
	51-80%	4	0	0	0	4	
	>80%	11	0	0	0	11	
	Total	28	0	0	0	28	
Ki-67 (n=105)	Low (<14%)	15	2	0	2	17	0.06
	High (>14%)	80	5	0	1	82	
	Total	95	7	0	3	105	
LVI (n=94)	Present	39	3	0	3	45	0.18
	Absent	39	6	3	1	49	
	Total	78	9	3	4	94	
PNI (n=91)	Present	8	0	0	0	8	0.60
	Absent	67	9	3	4	83	
	Total	75	9	3	4	91	
LNI (n=92)	Present	47	5	0	1	53	0.12
	Absent	26	4	2	3	35	
	Total	76	9	3	4	88	
DCIS (n=98)	Present	39	1	4	2	46	**0.02**
	Absent	42	8	0	2	52	
	Total	81	9	4	4	98	

Table 4.7: Cross-tabulation of Receptors and Tumor Characteristics

Variable with Categories		Receptor								Total	p-value
		ER+	ER/ PR+	ER/PR/ HER2+	TNBC	ER/ HER2+	PR/ HER2+	HER2+	PR+		
Grade (n=117)	grade 1	1	0	1	0	0	0	0	0	2	**0.02**
	grade 2	6	46	6	10	5	1	10	1	85	
	grade 3	4	5	2	10	1	1	6	1	30	
	Total	11	51	9	20	6	2	16	2	117	
Tumor Remaining (n=25)	<30%	1	2	1	0	0	0	0	0	4	0.69
	30-50%	0	3	1	1	2	0	1	0	8	
	51-80%	1	1	1	0	0	0	1	0	4	
	>80%	1	4	0	2	0	0	2	0	9	
	Total	3	10	3	3	2	0	4	0	25	
Ki67 (n=105)	Low (<14%)	0	15	3	1	0	0	0	0	17	**0.01**
	High (>14%)	9	32	5	17	6	2	13	2	82	
	Total	9	47	8	18	6	2	13	2	105	
LVI (n=83)	Present	3	16	4	9	2	0	9	0	43	0.43
	Absent	1	21	4	9	1	1	3	0	40	
	Total	4	37	8	18	3	1	12	0	83	
PNI (n=80)	Present	0	5	2	0	0	0	0	0	7	0.22
	Absent	4	31	5	17	3	1	12	0	73	
	Total	4	36	7	17	3	1	12	0	80	
LNI (n=81)	Present	4	21	5	9	1	1	8	0	49	0.51
	Absent	0	14	2	7	2	0	3	0	28	
	Total	4	35	7	16	3	1	11	0	77	
DCIS (n=87)	Present	2	18	5	6	3	1	7	0	42	0.50
	Absent	3	22	2	11	1	0	6	0	45	
	Total	5	40	7	17	4	1	13	0	n=87	

Figure 4.8: Frequency/Percentage Distribution of Additional Parameters

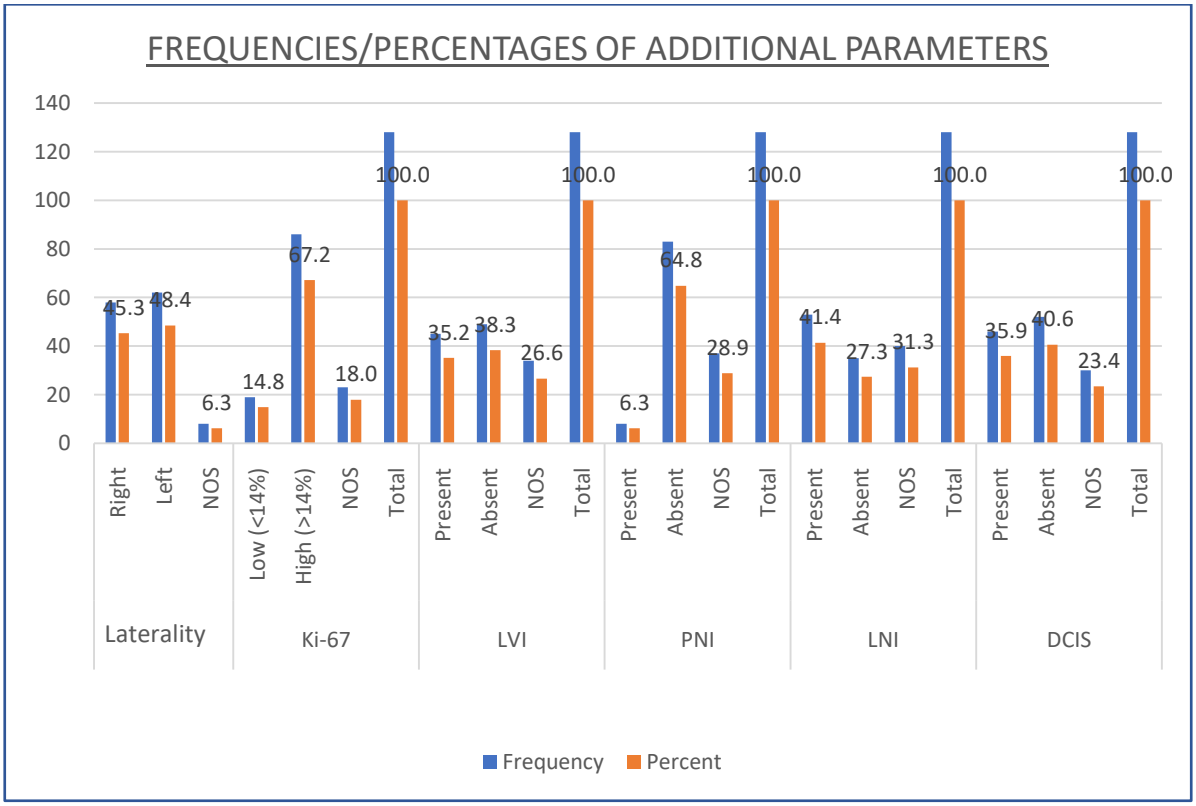
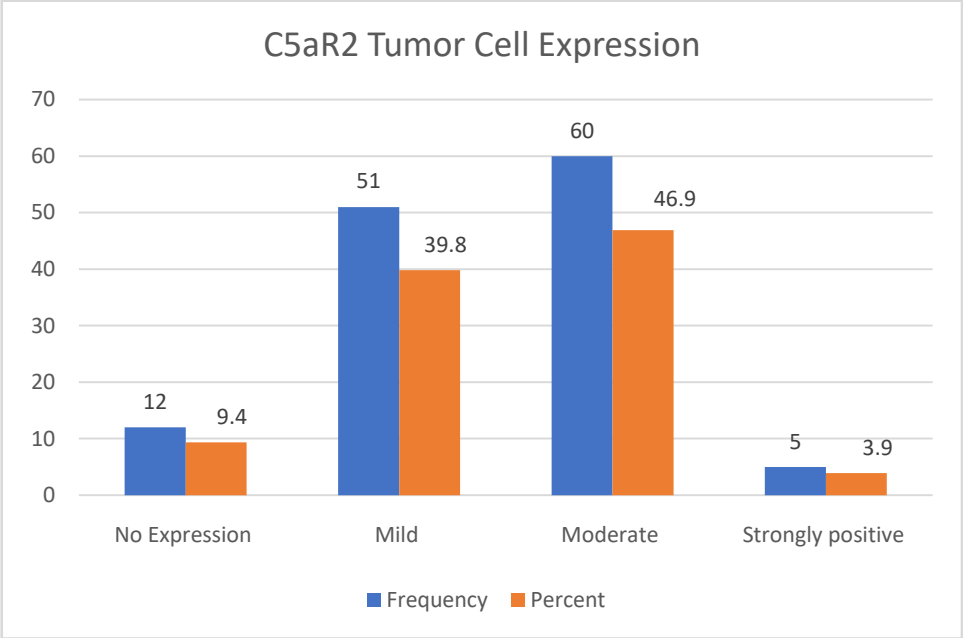
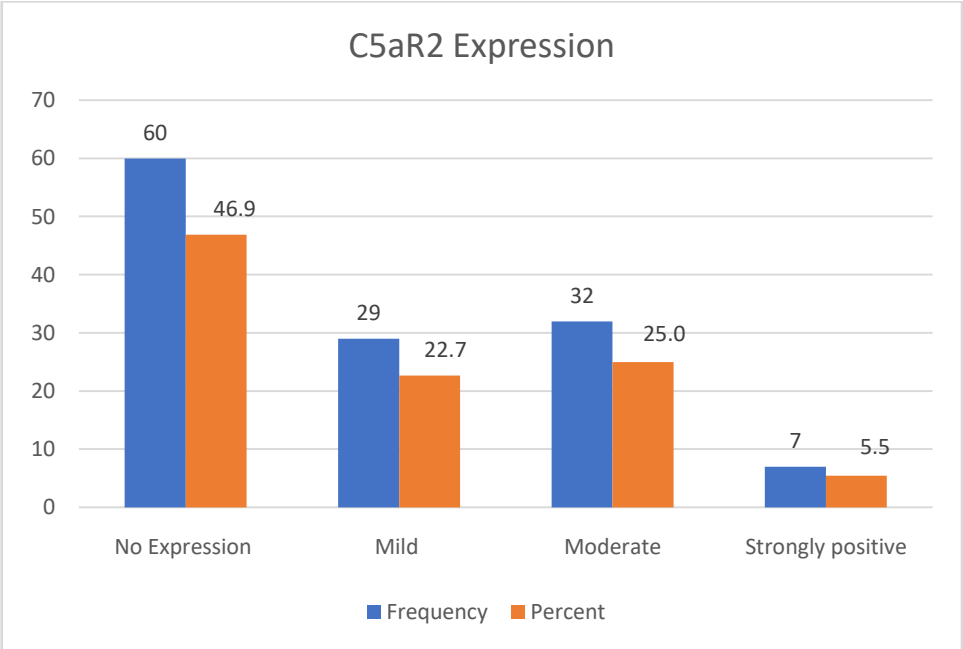


Figure 4.9a: C5aR2 Expression in Tumor Cells



(Key: 0= No expression, 1= Mild, 2= Moderate, 3= Strong)

Figure 4.9b: C5aR2 Expression in Stromal Cells



(Key:0= No expression, 1= Mild, 2= Moderate, 3= Strong)

Table 4.9.1: Association of C5aR2 Expression and Age

C5aR2 EXPRESSION		AGE				Total	p-value
		20-30	31-50	51-70	>70		
Tumor Cell Score	0	4	5	3	0	12	**0.002**
		50.0%	8.2%	5.9%	0.0%	9.4%	
	1	2	29	15	5	51	
		25.0%	47.5%	29.4%	62.5%	39.8%	
	2	2	23	32	3	60	
		25.0%	37.7%	62.7%	37.5%	46.9%	
	3	0	4	1	0	5	
		0.0%	6.6%	2.0%	0.0%	3.9%	
Stromal Cell Score	0	6	31	22	1	60	**0.000**
		75.0%	50.8%	43.1%	12.5%	46.9%	
	1	1	15	13	0	29	
		12.5%	24.6%	25.5%	0.0%	22.7%	
	2	1	14	15	3	33	
		12.5%	23.0%	29.4%	37.5%	25.8%	
	3	0	1	1	4	6	
		0.0%	1.6%	2.0%	50.0%	4.7%	
Total		8	61	51	8	128	
		100.0%	100.0%	100.0%	100.0%	100.0%	

Table 4.9.2: Association of C5aR2 Expression and Histologic Subtype

C5aR2 EXPRESSION		Histologic Subtype				Total	p-value
		IBC-NST	ILC	DCIS	Others		
Tumor Cell Score	0	12	0	0	0	12	0.41
		10.8%	0.0%	0.0%	0.0%	9.4%	
	1	45	5	1	0	51	
		40.5%	55.6%	25.0%	0.0%	39.8%	
	2	50	3	3	4	60	
		45.0%	33.3%	75.0%	100.0%	46.9%	
	3	4	1	0	0	5	
		3.6%	11.1%	0.0%	0.0%	3.9%	
Stromal Cell Score	0	51	4	2	3	60	0.96
		45.9%	44.4%	50.0%	75.0%	46.9%	
	1	25	3	1	0	29	
		22.5%	33.3%	25.0%	0.0%	22.7%	
	2	29	2	1	1	33	
		26.1%	22.2%	25.0%	25.0%	25.8%	
	3	6	0	0	0	6	
		5.4%	0.0%	0.0%	0.0%	4.7%	
Total		111	9	4	4	128	
		100.0%	100.0%	100.0%	100.0%	100.0%	

Table 4.9.3: Association of C5aR2 Expression and Grade

C5aR2 EXPRESSION		Grade			Total	p-value
		grade 1	grade 2	grade 3		
Tumor Cell Score	0	0	8	4	12	**0.03**
		0.0%	8.7%	11.8%	9.4%	
	1	1	37	13	51	
		50.0%	40.2%	38.2%	39.8%	
	2	0	43	17	60	
		0.0%	46.7%	50.0%	46.9%	
	3	1	4	0	5	
		50.0%	4.3%	0.0%	3.9%	
Stromal Cell Score	0	2	45	13	60	0.49
		100.0%	48.9%	38.2%	46.9%	
	1	0	22	7	29	
		0.0%	23.9%	20.6%	22.7%	
	2	0	22	11	33	
		0.0%	23.9%	32.4%	25.8%	
	3	0	3	3	6	
		0.0%	3.3%	8.8%	4.7%	
Total		2	92	34	128	
		100.0%	100.0%	100.0%	100.0%	

Table 4.9.4: Association of C5aR2 Expression and Stage

C5aR2 EXPRESSION		Stage				Total	p-value
		stage 0	stage I	stage II	stage III		
Tumor Cell Score	0	0	0	1	6	7	0.18
		0.0%	0.0%	2.4%	16.2%	7.9%	
	1	0	2	18	16	36	
		0.0%	25.0%	42.9%	43.2%	40.4%	
	2	2	5	21	15	43	
		100.0%	62.5%	50.0%	40.5%	48.3%	
	3	0	1	2	0	3	
		0.0%	12.5%	4.8%	0.0%	3.4%	
Stromal Cell Score	0	1	4	12	15	32	0.81
		50.0%	50.0%	28.6%	40.5%	36.0%	
	1	0	2	12	8	22	
		0.0%	25.0%	28.6%	21.6%	24.7%	
	2	1	2	16	10	29	
		50.0%	25.0%	38.1%	27.0%	32.6%	
	3	0	0	2	4	6	
		0.0%	0.0%	4.8%	10.8%	6.7%	
Total		2	8	42	37	89	
		100.0%	100.0%	100.0%	100.0%	100.0%	

Table 4.9.5: Association of C5aR2 Expression and Receptor Status

C5aR2 EXPRESSION		Receptor								Total	p-value
		ER+	ER/PR+	ER/PR/ HER2+	TNBC	ER/ HER2+	PR/ HER2+	HER2+	PR+		
Tumor Cell Score	0	2	4	0	2	0	0	2	1	11	**0.03**
		18.2%	7.8%	0.0%	10%	0.0%	0.0%	12.5%	50%	9.4%	
	1	6	22	2	9	2	1	4	0	46	
		54.5%	43.1%	22.2%	45%	33.3%	50%	25%	0.0%	39.3%	
	2	3	25	4	9	3	1	9	1	55	
		27.3%	49%	44.4%	45%	50.0%	50%	56.3%	50.0%	47.0%	
	3	0	0	3	0	1	0	1	0	5	
		0.0%	0.0%	33.3%	0.0%	16.7%	0.0%	6.3%	0.0%	4.3%	
Stromal Cell Score	0	9	25	2	8	3	1	8	0	56	0.25
		81.8%	49%	22.2%	40%	50%	50%	50%	0.0%	47.9%	
	1	2	10	1	8	0	0	4	1	26	
		18.2%	19.6%	11.1%	40%	0.0%	0.0%	25.0%	50%	22.2%	
	2	0	14	5	2	3	1	3	1	29	
		0.0%	27.5%	55.6%	10%	50%	50%	18.8%	50%	24.8%	
	3	0	2	1	2	0	0	1	0	6	
		0.0%	3.9%	11.1%	10%	0.0%	0.0%	6.3%	0.0%	5.1%	
Total		11	51	9	20	6	2	16	2	117	
		100%	100%	100%	100%	100%	100%	100%	100%	100%	

Table 4.9.6: Association of C5aR2 Expression and Molecular Classification

C5aR2 EXPRESSION		MolecularStatus				Total	p-value
		Luminal A	Luminal B	HER2+ve	TNBC		
Tumor Cell Score	0	4	3	2	2	11	0.5
		19.0%	5.1%	11.8%	10.0%	9.4%	
	1	9	24	4	9	46	
		42.9%	40.7%	23.5%	45.0%	39.3%	
	2	8	28	10	9	55	
		38.1%	47.5%	58.8%	45.0%	47.0%	
	3	0	4	1	0	5	
		0.0%	6.8%	5.9%	0.0%	4.3%	
Stromal Cell Score	0	11	29	8	8	56	0.34
		52.4%	49.2%	47.1%	40.0%	47.9%	
	1	5	9	4	8	26	
		23.8%	15.3%	23.5%	40.0%	22.2%	
	2	4	19	4	2	29	
		19.0%	32.2%	23.5%	10.0%	24.8%	
	3	1	2	1	2	6	
		4.8%	3.4%	5.9%	10.0%	5.1%	
Total		21	59	17	20	117	
		100.0%	100.0%	100.0%	100.0%	100.0%	

Table 4.9.7: Association of C5aR2 Expression and Tumor Remaining

C5aR2 EXPRESSION		Tumor Remaining				Total	p-value
		<30%	30-50%	51-80%	>80%		
Tumor Cell Score	0	4	0	0	3	7	**0.03**
		80.0%	0.0%	0.0%	27.3%	25.0%	
	1	0	3	3	6	12	
		0.0%	37.5%	75.0%	54.5%	42.9%	
	2	1	4	1	2	8	
		20.0%	50.0%	25.0%	18.2%	28.6%	
	3	0	1	0	0	1	
		0.0%	12.5%	0.0%	0.0%	3.6%	
Stromal Cell Score	0	5	2	2	4	13	0.19
		100.0%	25.0%	50.0%	36.4%	46.4%	
	1	0	2	0	4	6	
		0.0%	25.0%	0.0%	36.4%	21.4%	
	2	0	3	1	3	7	
		0.0%	37.5%	25.0%	27.3%	25.0%	
	3	0	1	1	0	2	
		0.0%	12.5%	25.0%	0.0%	7.1%	
Total		5	8	4	11	28	
		100.0%	100.0%	100.0%	100.0%	100.0%	

Table 4.9.8: Association of C5aR2 Expression and Ki-67

C5aR2 EXPRESSION		Ki67 Low (<14%)	Ki67 High (>14%)	Total	p-value
Tumor Cell Score	0	2	7	9	0.69
		10.5%	8.1%	8.6%	
	1	9	33	42	
		47.4%	38.4%	40.0%	
	2	8	42	50	
		42.1%	48.8%	47.6%	
	3	0	4	4	
		0.0%	4.7%	3.8%	
Stromal Cell Score	0	8	41	49	0.79
		42.1%	47.7%	46.7%	
	1	4	18	22	
		21.1%	20.9%	21.0%	
	2	5	23	28	
		26.3%	26.7%	26.7%	
	3	2	4	6	
		10.5%	4.7%	5.7%	
Total		19	86	105	
		100.0%	100.0%	100.0%	

Table 4.9.9: Association of C5aR2 Expression and LVI

C5aR2 EXPRESSION		LVI		Total	p-value
		Present	Absent		
Tumor Cell Score	0	4	4	8	0.61
		8.9%	8.2%	8.5%	
	1	16	22	38	
		35.6%	44.9%	40.4%	
	2	22	22	44	
		48.9%	44.9%	46.8%	
	3	3	1	4	
		6.7%	2.0%	4.3%	
Stromal Cell Score	0	18	17	35	0.27
		40.0%	34.7%	37.2%	
	1	7	16	23	
		15.6%	32.7%	24.5%	
	2	17	13	30	
		37.8%	26.5%	31.9%	
	3	3	3	6	
		6.7%	6.1%	6.4%	
Total		45	49	94	
		100.0%	100.0%	100.0%	

Table 4.9.10: Association of C5aR2 Expression and LNI

C5aR2 Expression		LNI		Total	p-value
		Present	Absent		
Tumor Cell Score	0	7	1	8	0.24
		13.2%	2.9%	9.1%	
	1	23	13	36	
		43.4%	37.1%	40.9%	
	2	21	20	41	
		39.6%	57.1%	46.6%	
	3	2	1	3	
		3.8%	2.9%	3.4%	
Stromal Cell Score	0	21	11	32	0.48
		39.6%	31.4%	36.4%	
	1	14	9	23	
		26.4%	25.7%	26.1%	
	2	14	14	28	
		26.4%	40.0%	31.8%	
	3	4	1	5	
		7.5%	2.9%	5.7%	
Total		53	35	88	
		100.0%	100.0%	100.0%	

Table 4.9.11: Association of C5aR2 Expression and PNI

C5aR2 EXPRESSION		PNI		Total	p-value
		Present	Absent		
Tumor Cell Score	0	1	7	8	0.93
		12.5%	8.4%	8.8%	
	1	3	33	36	
		37.5%	39.8%	39.6%	
	2	4	40	44	
		50.0%	48.2%	48.4%	
	3	0	3	3	
		0.0%	3.6%	3.3%	
Stromal Cell Score	0	2	32	34	0.48
		25.0%	38.6%	37.4%	
	1	1	22	23	
		12.5%	26.5%	25.3%	
	2	4	24	28	
		50.0%	28.9%	30.8%	
	3	1	5	6	
		12.5%	6.0%	6.6%	
Total		8	83	91	
		100.0%	100.0%	100.0%	

Table 4.9.12: Association of C5aR2 Expression and DCIS

C5aR2 EXPRESSION		DCIS		Total	p-value
		Present	Absent		
Tumor Cell Score	0	1	7	8	0.14
		2.2%	13.5%	8.2%	
	1	17	21	38	
		37.0%	40.4%	38.8%	
	2	27	22	49	
		58.7%	42.3%	50.0%	
	3	1	2	3	
		2.2%	3.8%	3.1%	
Stromal Cell Score	0	21	19	40	0.59
		45.7%	36.5%	40.8%	
	1	9	15	24	
		19.6%	28.8%	24.5%	
	2	14	14	28	
		30.4%	26.9%	28.6%	
	3	2	4	6	
		4.3%	7.7%	6.1%	
Total		46	52	98	
		100.0%	100.0%	100.0%	

Table 4.9.13: Correlation between Age, Ki-67 and Tumor and Stromal Cell Score

	Tumor Cell Score	Stromal Cell Score
Age		
r-value	.24	.30
p-value	.006	<.001
Ki-67		
r-value	-.03	.008
p-value	.743	.935

Table 4.9.14: Association of C5aR2 with Clinicopathological Parameters

Clinicopathological Parameters	Frequency - n (Percentage%)	Tumor Cell C5aR2 Expression				p-value	Stromal Cell C5aR2 Expression				p-value
		No	mild	mod	sev		No	mild	mod	sev	
Age	**n=128**	**12**	**51**	**60**	**5**		**60**	**29**	**33**	**6**	
20-30y	8 (6.3)	4	2	2	0		6	1	1	0	
31-50y	61 (47.7)	5	29	23	4		31	15	14	1	
51-70y	51 (39.8)	3	15	32	1	**0.002**	22	13	15	1	**0.000**
>70y	8 (6.3)	0	5	3	0		1	0	3	4	
Histologic Subtype	**n=128**	**12**	**51**	**60**	**5**		**60**	**29**	**33**	**6**	
IBC-NST	111 (86.7)	12	45	50	4		51	25	29	6	
ILC	9 (7.0)	0	5	3	1	0.41	4	3	2	0	0.96
DCIS	4 (3.1)	0	1	3	0		2	1	1	0	
Others	4 (3.1)	0	0	4	0		3	0	1	0	
Grade	**n=128**	**12**	**51**	**60**	**5**		**60**	**29**	**33**	**6**	
1	2 (1.6)	0	1	0	1		2	0	0	0	
2	92 (71.9)	8	37	43	4	**0.03**	45	22	22	3	0.49
3	34 (26.6)	4	13	17	0		13	7	11	3	
Stage	**n=89**	**7**	**36**	**43**	**3**		**32**	**22**	**29**	**6**	
0	2 (1.6)	0	0	2	0		1	0	1	0	
1	8 (6.3)	0	2	5	1	0.18	4	2	2	0	0.81
2	42 (32.8)	1	18	21	2		12	12	16	2	
3	37 (28.9)	6	16	15	0		15	8	10	4	
Molecular Subtype	**n=117**	**11**	**46**	**55**	**5**		**56**	**26**	**29**	**6**	
Luminal A	21 (16)	4	9	8	0		11	5	4	1	
Luminal B	59 (46.1)	3	24	28	4	0.5	29	9	19	2	0.34
HER2	17 (13.3)	2	4	10	1		8	4	4	1	
TNBC	20 (15.6)	2	9	9	0		8	8	2	2	
Receptor Status-	**n=117**	**11**	**46**	**55**	**5**		**56**	**26**	**29**	**6**	
ER+	11 (8.6)	2	6	3	0		9	2	0	0	
ER/PR+	51 (39.8)	4	22	25	0		25	10	14	2	
ER/PR/HER2+	9 (7)	0	2	4	3		2	1	5	1	
HER2+	16 (12.5)	2	4	9	1	**0.03**	8	4	3	1	0.25
TNBC	20 (15.6)	2	9	9	0		8	8	2	2	
ER/HER2+	6 (4.7)	0	2	3	1		3	0	3	0	
PR/HER2+	2 (1.6)	0	1	1	0		1	0	1	0	
Tumor Remaining	**n=28**	**7**	**12**	**8**	**1**		**13**	**6**	**7**	**2**	
<30%	5 (3.9)	4	0	1	0		5	0	0	0	
30-50%	8 (6.3)	0	3	4	1	**0.03**	2	2	3	1	0.19
51-80%	4 (3.1)	0	3	1	0		2	0	1	1	
>80%	11 (8.6)	3	6	2	0		4	4	3	0	
Ki-67	**n= 105**	**9**	**42**	**50**	**4**		**49**	**22**	**28**	**6**	
High (>14%)	86 (67.2)	7	33	42	4	0.69	41	18	23	4	0.79
Low (<14%)	19 (14.8)	2	9	8	0		8	4	5	2	
DCIS	**n=98**	**8**	**38**	**49**	**3**		**40**	**24**	**28**	**6**	
Present	46 (35.9)	1	17	27	1	0.14	21	9	14	2	0.59
Absent	52 (40.6)	7	21	22	2		19	15	14	4	

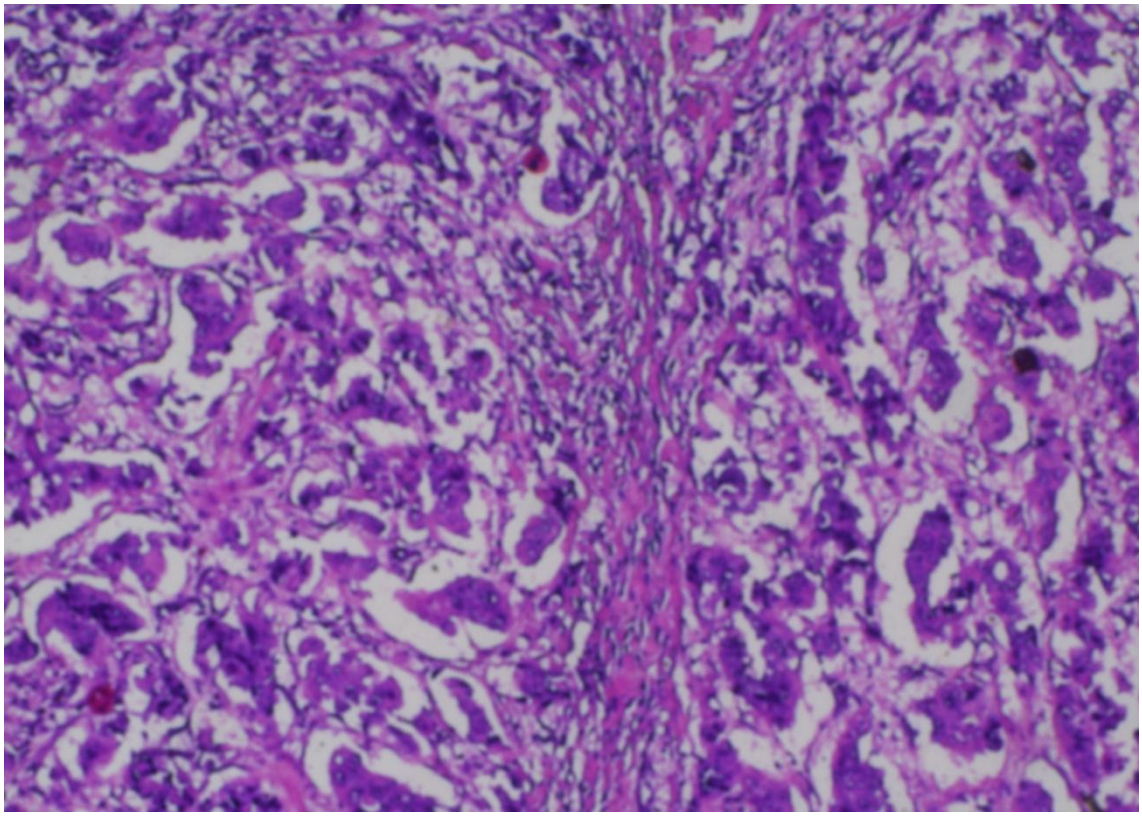


Figure 4.10: Invasive Breast Carcinoma-NST of an elderly female (H&E, x10)

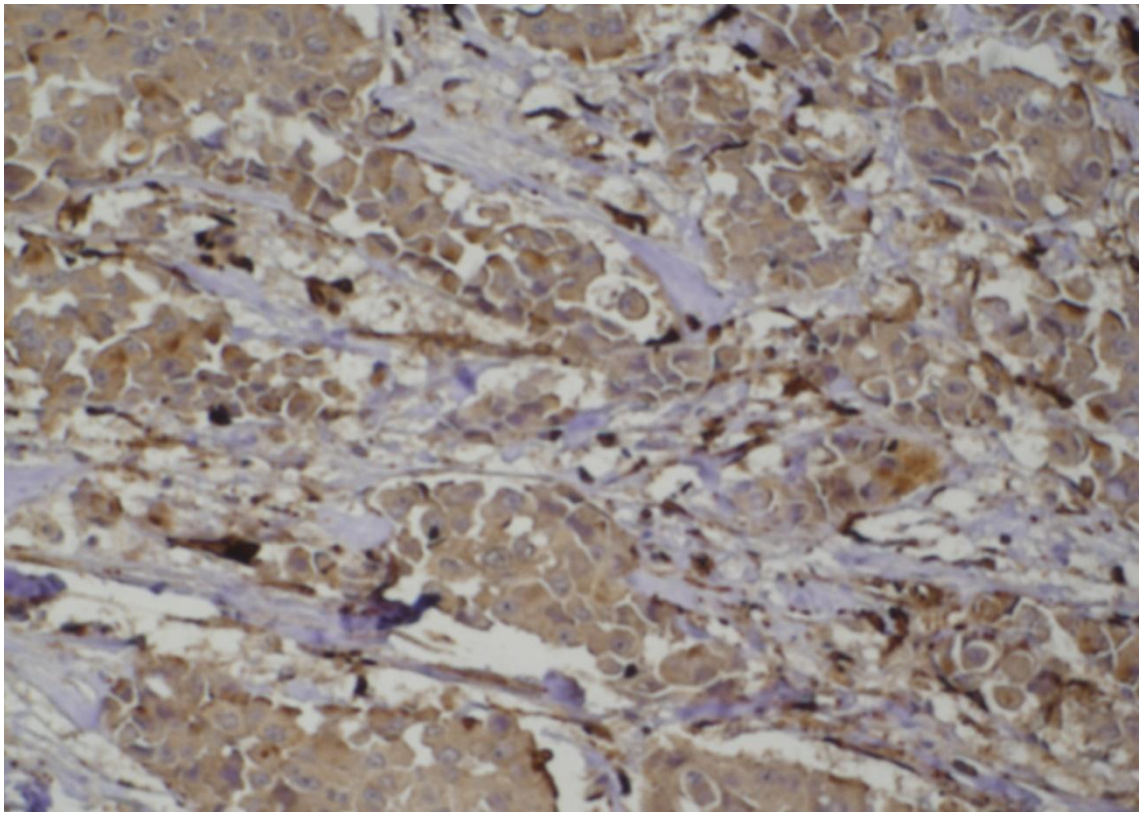


Figure 4.11: IBC-NST of an elderly female showing strong C5aR2 Expression(IHC of same case as Fig. 4.10, x20)

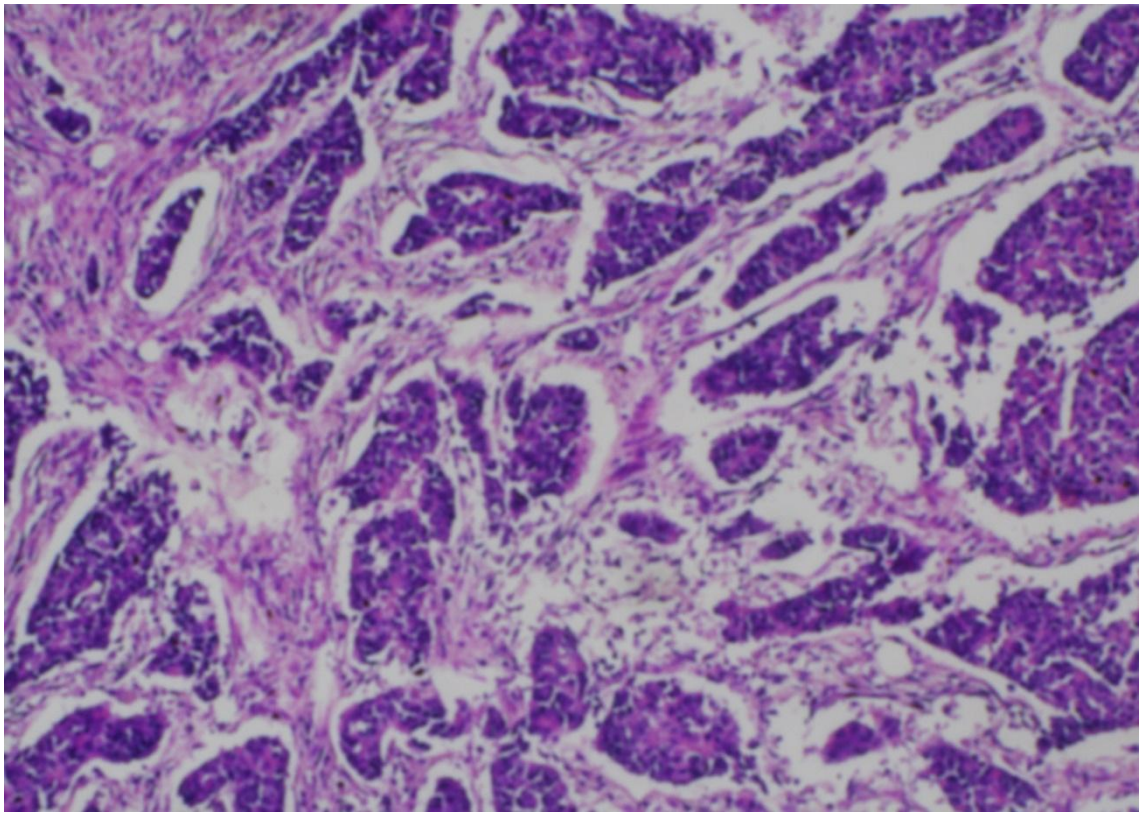


Figure 4.12:Post Treatment IBC-NST showing Poor Treatment Response (H&E, x10)

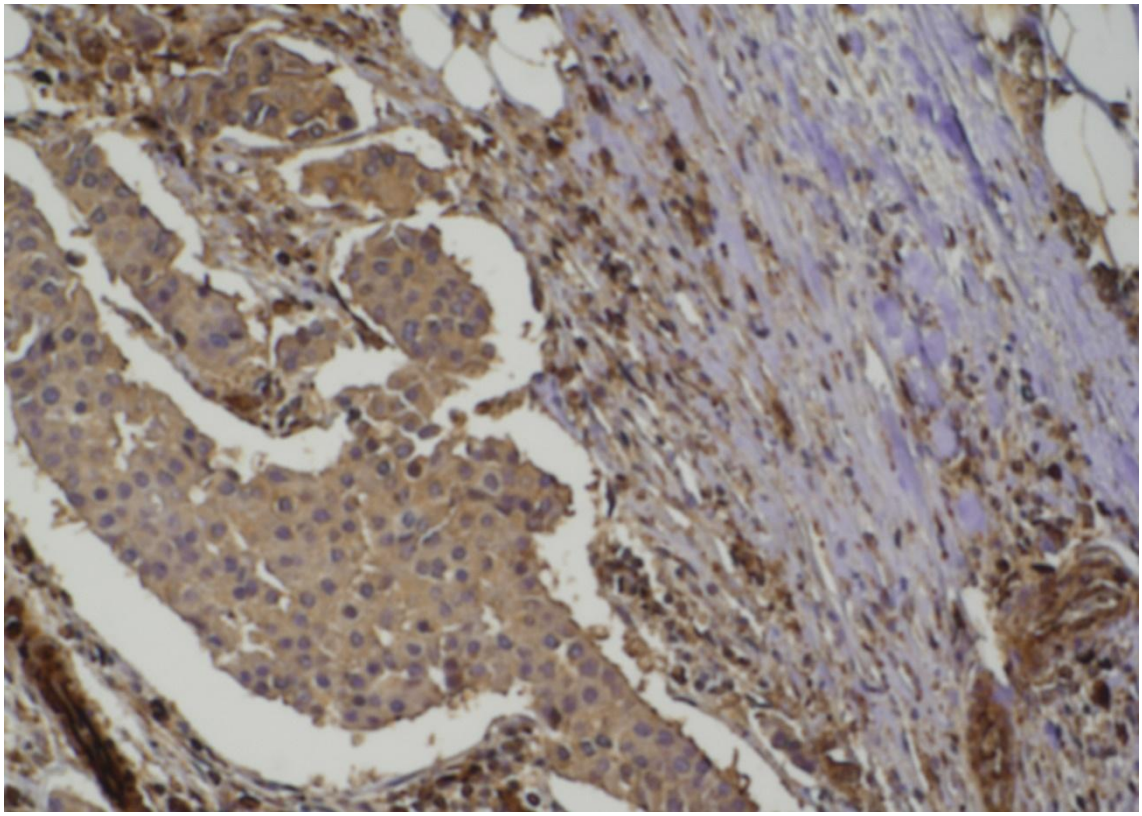


Figure 4.13: IBC-NST showing Post Treatment Strong C5aR2 Expression (IHC of same case as Fig. 4.12, x20)

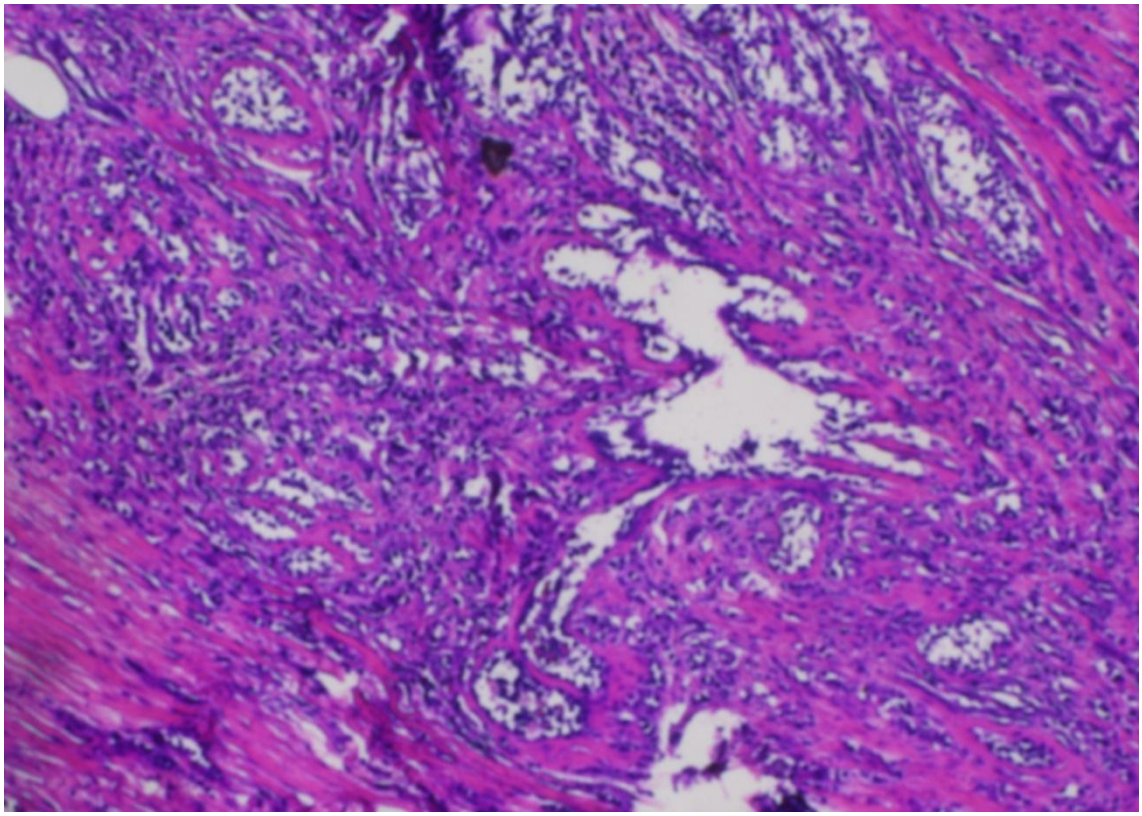


Figure 4.14: IBC-NST showing Post Treatment Definite Treatment Response (H&E, x10)

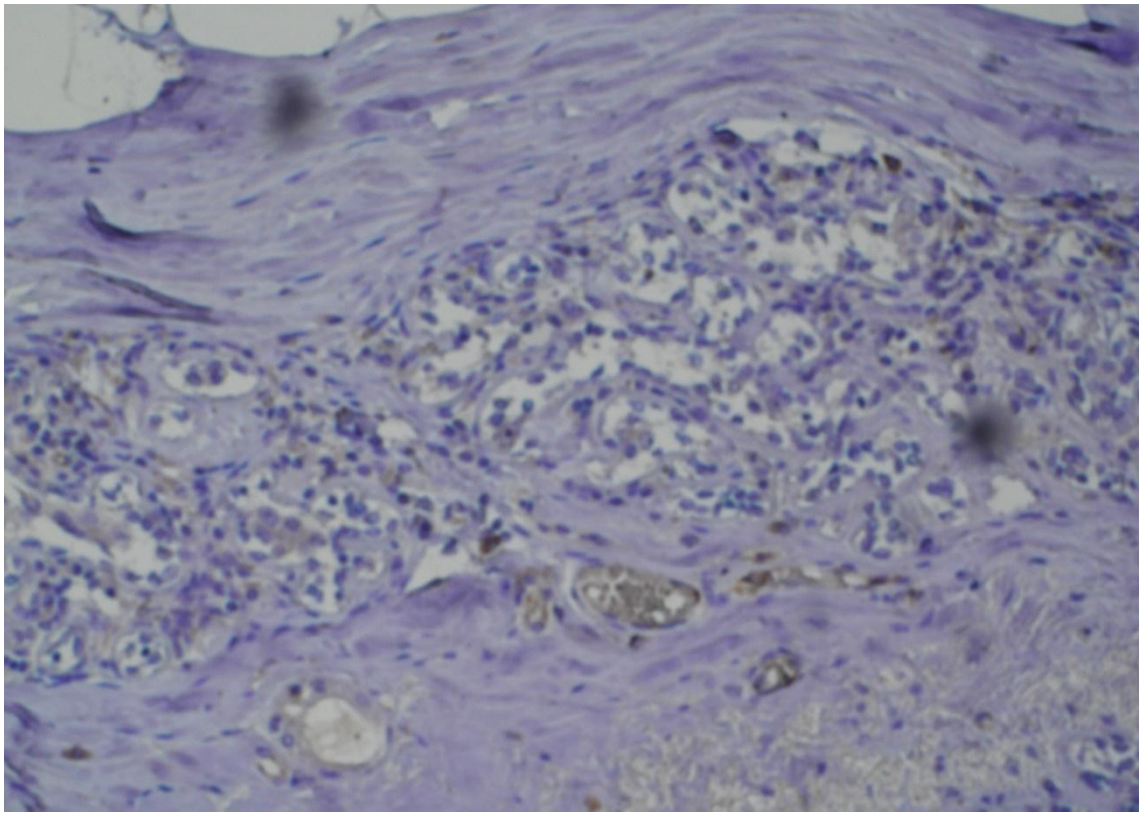


Figure 4.15: IBC-NST. Post Treatment Residual Tumor with Definite Treatment Response showing Absence of C5aR2 staining (IHC of same case as Fig.4.14, x20)

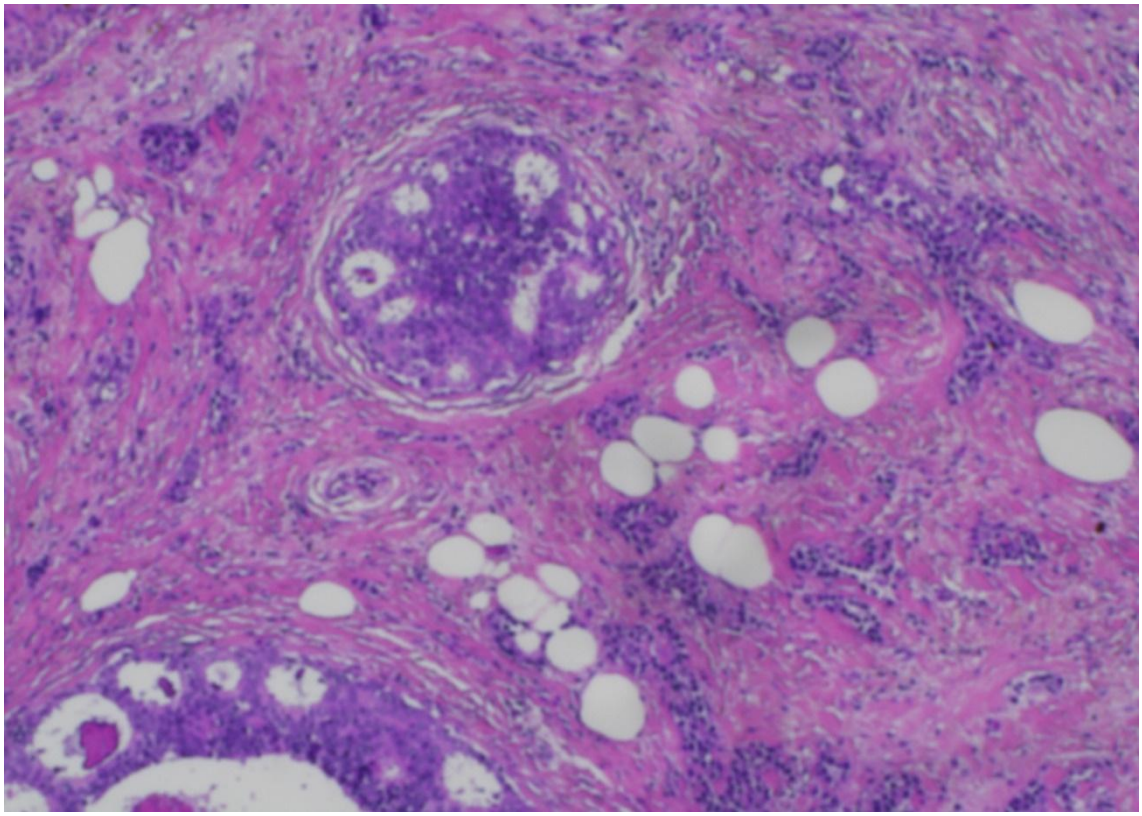


Figure 4.16: IBC-NST with DCIS (H&E, x10)

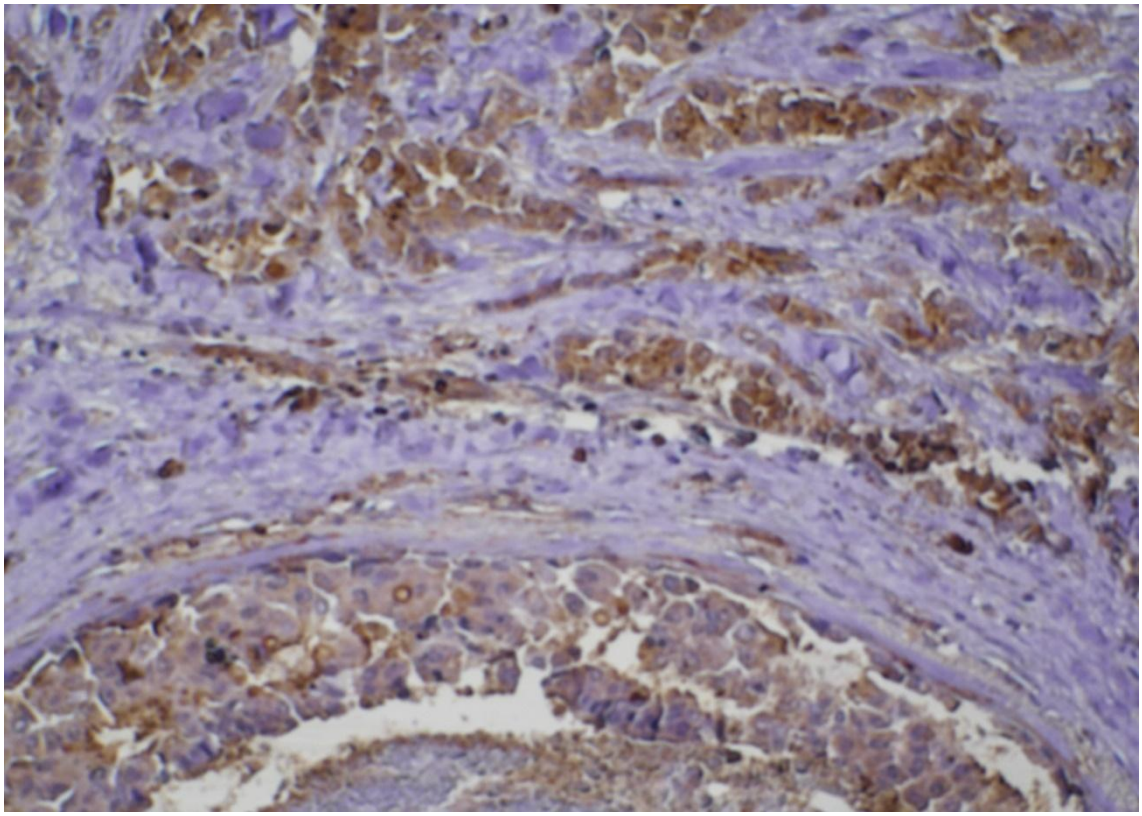


Figure 4.17: IBC-NST showing increased C5aR2 Expression in Tumor Cells (IHC of same case as Fig. 4.16, x10)

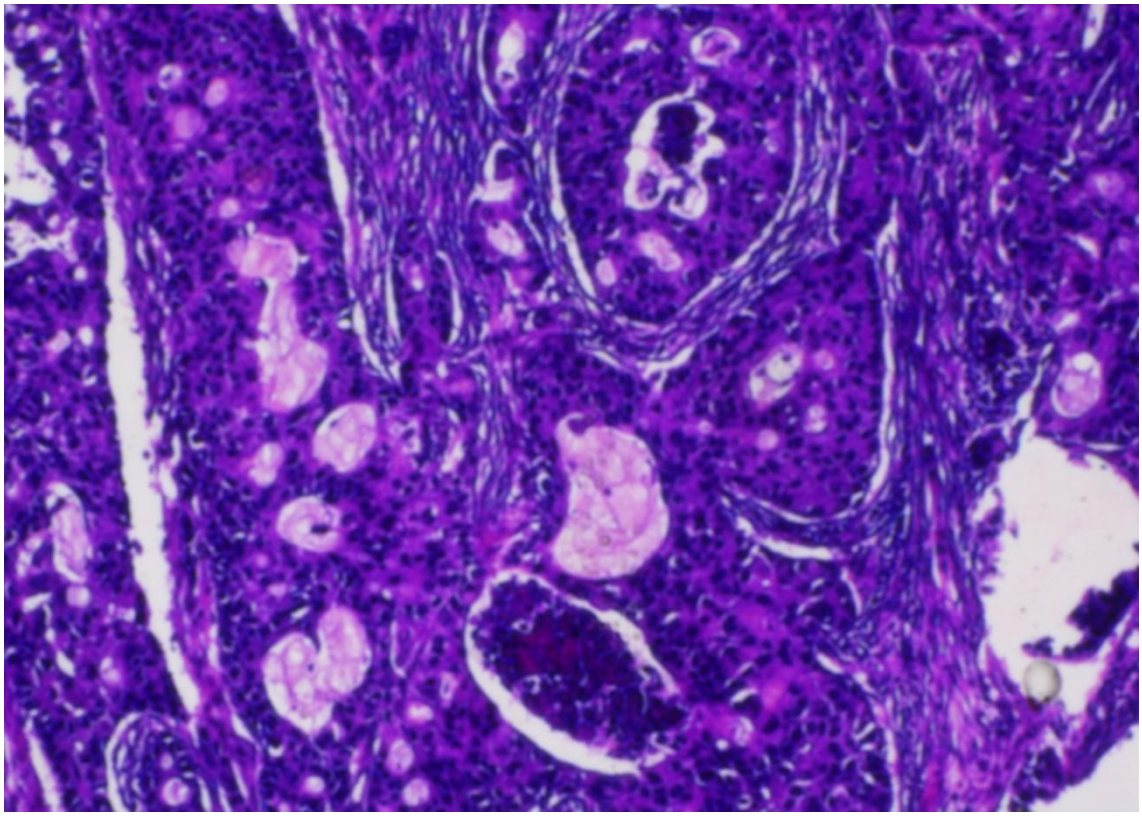


Figure 4.18: IBC-NST (TNBC) showing H&E, x10

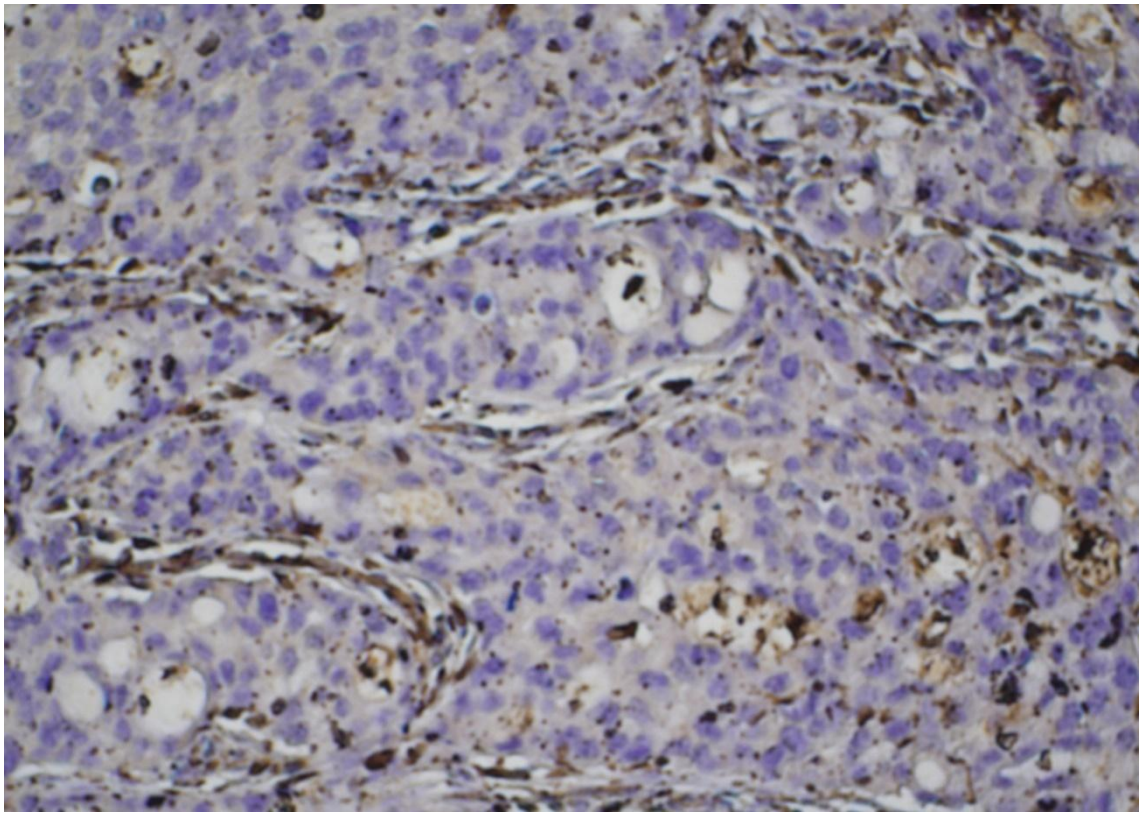


Figure 4.19: IBC-NST (TNBC) showing weak staining in Tumor Cells but Strong C5aR2 staining in Stromal Cells (IHC of same case as Fig.4.18, x20)

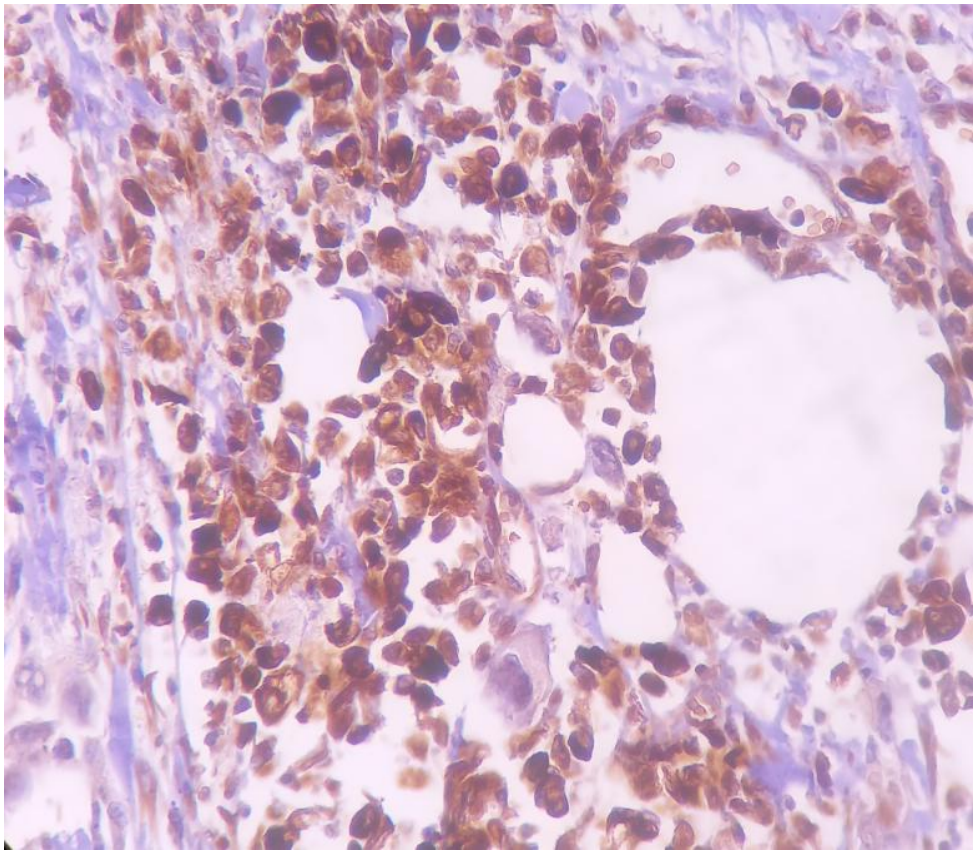


Figure 4.2: IBC-NST (Grade III) showing Strong C5aR2 Expression in Macrophages

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

In the current study, conducted from 2021-2023, all the cases were those of females, and 68% of cases were modified radical mastectomies. The vast majority of breast cancer cases in the present study were invasive breast carcinoma of no special type (IBC-NST-86.7%) followed by a small percentage of invasive lobular carcinoma (ILC) and ductal carcinoma in situ (DCIS). IBC-NST is the most common and prognostically favorable histologic subtype of breast cancer (Nascimento & Otoni, 2020, Anna et al, 2022). According to Fox et al., (2022), IBC accounted for about 75% of all breast cancer histologic subtypes.

The age range in the current study was 24-90 years with a mean age of 50 years. The highest peak in age-specific breast cancer incidence was observed in patients aged 31-50 years, followed by a second peak in the age group 51-70 years. This is contradictory to most of the previous studies observing the highest incidence of breast cancer in patients over 50 years. Lukasiewicz (2021) stated that 80% of breast cancers arise in women aged more than 50 years and the risk increases with increasing age. Different studies (Wu et al., 2014, Virani et al., 2014) conducted in Asian countries also revealed a high incidence of breast cancer in women aged 50 years or more. According to NBCC breast cancer facts and figures, older women are more likely to develop breast cancer than younger women, and 62 years is the median age at the time of breast cancer diagnosis (ACS 2022). However, Kotepui and Chupeerach (2013) and Kotepui et al., (2014) showed 40 years as the age of rising breast cancer incidence. The mean age

identified in Asian countries was 46 and 53 years by Chen et al. (2014) and Tulsyan et al. (2014) respectively. Among various variables, only histology was found to be significantly associated with age (p=0.02). Our findings uncovered that more than 50% of cases with histologic type IBC-NST were found in the 31-50 age group, which contrasts with the results of a prior study conducted by Anna et al. (2022), indicating a higher occurrence of IBC-NST in the age range of 50-70 years.

In the present study, the molecular classification of 115 cases was known while the number of unknown cases was 13. Among the known cases, the Luminal B subtype was the most frequently (46%) reported molecular subtype followed by Luminal A and TNBC cases (16% each).Khokher et al, (2016) conducted a study in Lahore and analyzed data of 261 breast cancer patients revealing similar results of increased frequency of Luminal B tumors (34.6%) while Luminal A accounted for only 26% of breast cancer cases. A contradictory trend was observed by Cortet et al. (2018), using data from three cancer registries in France from 2007-2012. Poisson models were used to analyze incidence rates by year, age class, and molecular subtype and results revealed the highest incidence of Luminal A (77.6%) followed by TNBC (10%). Another retrospective observational study with data from 2062 breast cancer patients carried out in a Tertiary Cancer Care Centre in Western India also showed conflicting results revealing a high frequency of the Luminal A subtype tumors (45.2%) among different molecular subtypes followed by TNBC (32%) (Prakash et al., 2020). In the current study, Luminal A was frequently diagnosed in the older age group (51-70y) while Luminal B, TNBC, and HER2 enriched were commonly seen in young adults (31-50y). Fatma (2020) revealed similar results in a retrospective study of 740 cases retrieved across a seven-year period, showing an increased frequency of HER2 enriched and TNBC tumors (66-70.5%) in the younger age group (<50y). The current study revealed a significant association of molecular classification with grade and Ki-67. Grade 2 was the most commonly observed grade in all molecular subtypes in this study except TNBCs showing an equal number of Grade 2 and Grade 3 cases. These findings were in accordance with a study conducted by Cheng et al (2013) on 628 patients with invasive breast cancer revealing grade 2 frequency in all molecular subtypes. However, Khokher et al (2016) revealed a frequent association of Luminal A with grade 2 while Luminal B, HER2 enriched, and TNBCs were commonly associated with grade 3. Out of 128 cases, the proliferation index of 105 was known. HER2 enriched (100%), Luminal B (94.8%),

and TNBC (94.4%) were commonly associated with high Ki-67 whereas all the Luminal A tumors showed low proliferation rates. Bustreo et al (2016) showed similar results with Luminal A breast cancer cases showing low Ki-67 and Luminal B tumors, frequently (83.9%) associated with high Ki-67 value. The contradictory results were observed in a study conducted by Lashen et al., (2021) revealing a frequent association of low Ki-67 with not only Luminal A but also with Luminal B breast tumors. However, TNBC and HER2 enriched showed high proliferation rates.

Regarding grades, most (72%) of the breast cancer cases in the present study were grade 2 tumors while 27% were grade 3 and only 1% were grade 1. The frequency of grade 2 is possibly related to Luminal B tumors which are usually grade 2 tumors and are found more often in the current study. A retrospective multicentered joint study conducted by the Western China Clinical Cooperation Group (WCCCG), including 8619 female breast cancer patients selected from the WCCCG database, showed 75% grade I/II and 25% grade III cases (Zheng et al, 2018). A significant association ($p=0.02$) between grade and molecular status was identified in the current study. Luminal A, Luminal B and HER2 enriched were frequently associated with Grade 2 tumors, whereas TNBCs showed an equal number of Grade 2 and Grade 3 cases. In Pakistan, a retrospective cross-sectional study, conducted at Liaquat National Hospital Karachi on 1951 cases of primary breast cancer yielded consistent findings indicating that both Luminal A and Luminal B subtypes were frequently associated with Grade 2 breast cancer cases (Atif et al., 2018). Contradictory results were analyzed by Fatima (2019) showing a Grade 2 predominance in Luminal A and Luminal B molecular subtypes, but HER2 enriched and TNBC were frequently grade III tumors. According to the current study findings, Grade III tumors were more commonly associated with a younger age group. The reason may be the higher occurrence of Grade III TNBC cases, which typically affect a younger age group. Similar results were identified in the study conducted by Jennifer et al. (2020).

In the current study, the pathological stage of only 89 out of 128 cases could be retrieved since the study also included trucut biopsies. The majority of the patients in this study presented with stage II tumors (47.2%) followed by stage III tumors (41.6%). The possible reasons for late presentation could be poor awareness, poverty, sociocultural behavior, and lack of screening programs. A retrospective study of 14613 Hong Kong Chinese female patients, conducted between January 2006 and February

2020 by Yik et al. (2022), also showed similar results with stage II breast cancer preponderance accounting for 37.3% of the cases. In the present study, stage III tumors (48.6%) were more frequently observed in the older age group (51-70y) compared to stage II tumors (47.6%) which were more pronounced in the relatively younger age group (31-50y). Contrariwise Jennifer et al. (2020), found a significant association ($p <$ 0.001) between age and stage and revealed an increased frequency of high pathological stage tumors (6%) in younger women (≤ 45 years) compared to only 3.3% of high pathologic stage tumors in older women. In another study, Anna et al., (2018) used Cancer Registry of Norway and identified 21,384 breast cancer women at ages 20–89 between 2005 and 2015. Anna observed a higher prevalence of advanced stages in young (<50 years) breast cancer patients compared to those older than 50 years. In the present study, stage was found to be significantly associated with histology (p=0.000) and lymph node involvement (p=0.000). In the present study, the IBC-NST and ILC subtypes were frequently associated with high stages. However, in a study conducted by Li et al. (2021), ILC was frequently diagnosed at a more advanced stage (stage III) than IBC.

Regarding histologic subtypes, the vast majority of cases were invasive breast carcinoma of no special type (IBC-NST) followed by invasive lobular carcinoma (ILC), ductal carcinoma in situ (DCIS), and 3% other histologic subtypes. Among others, 50% were micro-invasive carcinoma, 25% invasive solid papillary carcinoma, and 25% encapsulated papillary carcinoma. Most of the other studies (Waheed et al. 2019, Seung et al. 2021, Ke et al. 2018) also revealed IBC-NST as the most frequently diagnosed breast cancer followed by ILC. The preponderance of IBC in breast cancer patients followed by ILC has also been observed in several other studies (Atif et al, 2018, Samina et al,2016) conducted in Pakistan.

In the present study, out of 128 cases, 117 were known for receptor status. The majority of cases were ER/PR positive (40%) followed by TNBC (16%) and HER2-positive tumors (12%). The study conducted by Bansal et al (2017) over a period of 5 years from 2009 to 2014, including 509 cases also revealed ER/PR positive cases (42.8%) to be the most frequently diagnosed receptor status but the frequency of TNBC was less (23.6%) as compared to HER2 positive tumors (40.7%). A statistically significant association was identified between the receptor status of breast cancer and grade and Ki-67 in this study. Grade 2 was the most frequently observed grade in

almost all of the receptor statuses except for TNBC, showing an equal number of patients in both grade 2 and grade 3 tumors. Similar findings were appreciated in the study conducted by Bansal et al., except for TNBC which showed an increase in frequency with the increasing grade. Conflicting results were shown in a study conducted in Pakistan by Sohail et al., revealing increased Grade 2 frequency in ER/PR-positive cases but the majority of ER/PR-negative tumors presented with grade 3. In the present study, all the HR-positive and HR-negative receptor types exhibited elevated Ki-67 values. In Pakistan, Mushtaq et al (2021) conducted a study on 278 patients showing similar findings of high Ki-67 in all the HR-positive and HR-negative receptor types.

In the present study, the lymph node status of 88 cases was known. Since trucut biopsies were also included in the study, the lymph node status of 40 cases could not be evaluated. Out of these known cases, 60.2% were positive for lymph node involvement whereas 39.8% of cases did not show nodal involvement. Contradictory results have been observed in many studies showing increased frequency of LNI-negative cases. The study conducted by Albasri (2021) showed a higher prevalence of LNI-negative cases compared to LNI-positive breast cancer cases. In a study carried out by Waheed et al. (2019) lymph node positivity was observed in only 23.9% of breast cancer patients while majority (76.1%) of cases were negative for lymph node metastasis.

In the current study, the lymphovascular invasion status of 94 out of 128 cases could be ascertained as trucut biopsies were also included in the study. There was evidence of lymphovascular invasion in 45 (47.9%) cases while 49 (52.1%) cases did not show lymphovascular spread of cancer cells. Similar results were observed in a retrospective cohort study conducted by Houvenaeghel et al. (2021), on 17322 breast cancer patients, showing lymphovascular invasion in 24.3% of breast cancer patients while 75.7% of cases didn't show lymphovascular involvement. The presence of lymphovascular invasion is associated with poorer prognosis, as it indicates an increased likelihood of distant metastasis. In the present study, almost half of the cases showed lymphovascular invasion at the time of diagnosis possibly due to the fact that the majority of patients in our society seek medical advice at a later stage because of unawareness, illiteracy, social constraints, and inaccessible medical facilities.

The aim of this study was to determine the level of expression of complement receptor C5aR2 in breast cancer cells and stromal cells of the tumor microenvironment

and its association with clinicopathological parameters of breast cancer. Generally, the C5aR2 expression in tumor cells was more pronounced than in stromal cells. The tumor cells of 90.6% of breast cancer cases showed C5aR2 expression while stromal cells of only 53.1% of cases showed C5aR2 expression. Most of the cases (46.9% and 25.8%) showed moderate C5aR2 expression in tumor and stromal cells, respectively. However, the percentage of strong C5aR2 expression was slightly higher (4.7%) in stromal cells than in tumor cells (3.9%).

A statistically significant association was identified between C5aR2 expression in tumor (p=0.002) and stromal cells (p=0.000) and the age of the breast cancer patients. A rising trend of C5aR2 expression was noticed in both tumor and stromal cells with advancing age. The possible reason is an increased frequency of Luminal B tumors in the current study, which is frequently being observed in older age group.In tumor cells, the highest expression level was identified in patients 50 years and above, with the age group >70 years mostly displaying mild to moderate and, 31-50 years and 51-70 years age group frequently exhibiting moderate to strong expression. In case of stromal cells, an increasing trend of C5aR2 expression was observed with increasing age. The increase in moderate expression was 12.5% in the age group 20-30 years to 37.5% in the age group >70 years, whereas strong expression was intensified from 1.6% in the age group 31-50 years to 50% in the age group >70 years. This data suggests the role of C5aR2 in the progression of breast cancer in older patients. The comparable data showing the association of age and C5aR2 expression is not available yet. The animal models or cell lines were mostly used to study C5aR2 expression. A study conducted by Yumeng et al. (2021) demonstrated differential expression of C5aR2 in tumor and normal tissues using The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx). Yumeng et al. also used clinical breast cancer samples to display C5aR2 expression through immunohistochemistry but demographic data was not available.

Regarding the histologic subtypes of breast cancer, C5aR2 expression in ILC is mostly mild while in IBC and DCIS, the expression level is frequently moderate in both tumor and stromal cells. However, strong expression was frequently observed in tumor cells in ILC (11.1%) and in stromal cells in IBC-NST (5.4%). Comparable data of C5aR2 expression is not available in relation to histology.

The tumor grade and C5aR2 expression in tumor cells displayed a significant association in the current study. In tumor cells, the C5aR2 expression increased with increasing grade with 46.7% of grade II and 50% of grade III tumors showing moderate intensity. Although no significant association was identified between stromal cells and tumor grade but an escalating trend of expression was likewise noted in stromal cells, particularly at moderate and strong levels of expression as the grade increased from 23.9% to 32.4% and 3.3% to 8.8% respectively. The strong C5aR2 expression was predominantly seen in grade 2 tumor cells and grade 3 stromal cells signifying the role of C5aR2 in the progression of breast cancer. A cohort study conducted by Shicheng et al. (2018) revealed that grade 3 breast cancer patients with CAFs overexpressing C5aR2 showed a significantly shorter disease-free survival (DFS) while DFS in grade 1 and grade 2 patients were independent of tumor infiltration of C5aR2 positive CAFs. The possible cause of decreased DFS is chemoresistance induced by C5aR2 expressed on tumor and stromal cells. The positive correlation between C5aR2 expression and higher-grade breast cancers may have clinical significance. The higher-grade tumors are associated with poor prognosis thus, C5aR2 may act as a prognostic marker. The association of C5aR2 positive CAFs with poor disease-free survival highlights the importance of considering not only the tumor cells but also cells in the TME in breast cancer research and treatment approaches.

The trend of C5aR2 expression in the stage was similar to grade. The intensity of C5aR2 expression was higher in stromal cells as compared to tumor cells. Many of the cases showed an increasing trend of C5aR2 expression with increasing stage. The maximum strong expression was observed in the tumor cells of stage I tumors (12.5%) whereas in case of stromal cells, stage III tumors (10.8%) frequently exhibited strong C5aR2 expression. However, this association was not statistically significant. The results obtained in the transwell assay performed by Yumeng et al using 10% fetal bovine serum and fifty thousand C5aR2 overexpressing breast cancer cells, signified that C5aR2 overexpression enhanced the migratory and invasive capacity of breast cancer cells. Yumeng et al. also conducted Western Blot analysis and determined that C5aR2 promotes epithelial-to-mesenchymal transition (EMT) by increasing the expression of matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9). Regarding stromal cells, a cohort study conducted by Shicheng et al revealed an association between abundant C5aR2-positive CAFs and poor patient survival in

stages I, II, and III of breast cancer. The findings in the current study suggest a role of C5aR2 in the aggressiveness of breast cancer which is supported by Yumeng et al. study showing C5aR2 directly influencing the invasive potential of tumor cells.

In this study, the significance of C5aR2 expression in hormone receptor-positive and HER2-positive tumors has come into focus, illuminating its crucial role in breast cancer. A significant association (p=0.03) was observed between hormone receptor-positive tumors and tumor cell C5aR2 expression. In both tumor and stromal cells, most of the receptor-positive tumors expressed C5aR2 moderately except for ER-positive tumors which frequently showed mild expression. However, in case of stromal cells, TNBCs and HER2-positive tumors displayed mild C5aR2 expression. The strong C5aR2 expression was frequently noticed in both tumor and stromal cells of ER/PR/HER2 positive and HER2 positive tumors and also in stromal cells of TNBCs. On the contrary, Yumeng et al observed an association between high C5aR2 expression and ER-positive tumors. Yumeng et al used the Kaplan-Meier plotter database and PrognoScan database to conduct a cohort study including only ER-positive breast cancer samples that displayed a significant association between high C5aR2 expression and poor disease-free interval (DFI) in ER-positive breast cancer. Evaluation of C5aR2 expression in multiple breast cancer cell lines was done and then compared with T47D and MCF7 cells (ER-positive). C5aR2 expression was relatively low in MDA-MB-231 cells (ER-negative). A strong association was identified between C5aR2 expression, and hormone receptor-positive and HER2-positive tumors. As HER2 positivity is related to the aggressiveness of breast tumors, it is plausible that C5aR2 may contribute to the heightened aggressiveness observed in HER2-positive tumors.

The current study highlighted the correlation between elevated C5aR2 expression in tumor and stromal cells and molecular subtypes of breast cancer. Most of the breast cancer cells and stromal cells with Luminal B and HER2 positive molecular subtypes have shown moderate to strong C5aR2 expression, while C5aR2 expression was mild to moderate in tumor cells of TNBC and mild in Luminal A cases. However, in stromal cells, strong C5aR2 expression was relatively higher in TNBC (10%). No significant association was detected between molecular classification and C5aR2 expression in tumor and stromal cells. The results in the current study appeared partially similar to the Yumeng et al. study based on the TIMER 2.0 database revealing much higher C5aR2 expression in tumor cells of Luminal A and Luminal B breast cancer cases than in

HER2 positive and TNBC. However, in a cohort study conducted by Shicheng et al., the abundance of C5aR2-expressed stromal cells (CAFs) in HR-positive and HR-negative tumors was found to be associated with decreased patient survival. Luminal B and HER2 enriched are both aggressive tumors and increased C5aR2 expression in these molecular subtypes might be linked to their aggressive behavior.

In the present study, a significantly (p-0.03) positive correlation between C5aR2 expression in tumor cells and the post-treatment residual tumor was observed. C5aR2 expression was not observed in the stromal cells and was only detected in 20% of tumor cells of the breast cancer cases showing a definite treatment response (with less than 30% of the tumor remaining). On the other hand, the majority of breast cancer cases with moderate to poor treatment response showed higher C5aR2 expression. These findings are consistent with the role of C5aR2 in chemoresistance. Although a statistically significant association was not noted between C5aR2 expression in stromal cells and treatment response in this study but the increasing trend of C5aR2 expression was observed in stromal cells of most of the cases with poor treatment response. Cancer-associated fibroblasts (CAFs), the activated fibroblasts, are the most abundant and heterogeneous stromal cells in the tumor microenvironment. Studies have been conducted revealing its association with chemoresistance. Similar findings were observed by Shicheng et al. who conducted an investigation on 578 breast cancer patients to assess the role of activated fibroblasts, in paired primary tumor samples before and after neoadjuvant chemotherapy. The findings revealed increased survival of tumor cells and C5aR2 positive CAFs after neoadjuvant therapy in chemoresistant tumors as compared to chemosensitive tumors. The consistently higher proportions of both tumor cells and C5aR2 positive CAFs in resistant tumors indicated that C5aR2 positive CAFs not only induce chemoresistance in tumor cells but are also resistant to chemotherapy themselves. The possible mechanism of chemoresistance is the cancer stemness provided by C5aR2 overexpressed CAFs which causes sustained release of IL-6 and IL-8 through persistent activation of the NFκB pathway.

Another retrospective study was conducted by Yilin et al. who analyzed samples from 171 patients with locally advanced gastric cancer to assess C5aR2 expression in cancer-associated fibroblasts (CAFs) before and after neoadjuvant chemotherapy. The study also revealed a significant association between C5aR2 expression in CAFs, chemoresistance, and poor survival. Through Gene Set Variation Analysis (GSVA)

Yumeng et al. also noticed a significant association between C5aR2 expression and upregulation of ESR1. ESR1 is a well-established oncogene implicated in breast cancer and associated with endocrine resistance.

In the present study, the cut-off value taken between low and high Ki-67 was 14 (Mushtaq et al, 2021). In the current study, increased moderate to strong C5aR2 expression has been observed in tumor cells of breast cancer cases with a high proliferation index. On the contrary, in stromal cells, an equal distribution of C5aR2 expression was found in high and low Ki-67 profile breast cancer cases. Although C5aR2 tumor and stromal cell expression did not show any significant association in the present study but studies in the past have shown the relation of high proliferation index with C5aR2 expression in tumor cells. A similar finding was observed by Yumeng et al., who performed Cell Counting Kit 8 (CCK8) assay on C5aR2 overexpressed MDA-MB-231 cells (ER-negative) in 96-well plates. After measuring cell proliferation by cell counting kit-8 reagent for seven days it was concluded that proliferation rates of MDA-MB-231 cells were significantly increased after C5aR2 overexpression. The findings of the present study demonstrated the involvement of C5aR2 in breast cancer proliferation possibly because C5aR2 expression is significantly and positively associated with many proliferative pathways like MAPK3, MTOR, and STAT3.

In the present study, the lymphovascular invasion status could be determined in 94 out of 128 cases, since trucut biopsies were small and the surrounding vessels could not be observed, if any. Out of known cases, 47.8% were positive for lymphovascular invasion while 52.2% did not show lymphovascular invasion. The findings associated with C5aR2 expression in tumor and stromal cells and lymphovascular invasion were not significant. However, increased C5aR2 expression was observed in the tumor and stromal cells of breast cancer cases positive for lymphovascular invasion (LVI) as compared to LVI-negative breast cancer cases showing less C5aR2 expression. Our findings are consistent with the outcomes reported by Yumeng et al., who conducted Transwell assay and Western Blot analysis. Yumeng et al. demonstrated that C5aR2 overexpression augmented the migratory and invasive capacity of breast cancer cells.

The lymph node involvement (LNI) was identified in 88 out of 128 cases because of biopsies involved in the study apart from Modified Radical Mastectomies. Out of 88 known cases, the majority of the LNI-positive cases exhibited higher levels of C5aR2 expression both in tumor and stromal cells compared to tumors with no lymph node

involvement. Although no significant association was identified between C5aR2 tumor and stromal cell expression and LNI but findings in the current study augment the results of Yumen et al. study revealing the increased migratory capacity of breast cancer cells expressing C5aR2.

Out of 128 breast cancer cases, the status of perineural invasion (PNI) was not specified in 37 cases. Out of the 91 specified cases, perineural invasion was identified in only 8.8% of breast cancer cases while 91.2% of cases did not show perineural invasion. Most of the PNI-positive cases showed an increased frequency of C5aR2 expression compared to breast cancer cases negative for perineural invasion. Among the PNI-positive breast cancer cases, C5aR2 expression was more pronounced in stromal cells than tumor cells. In this study, no significant association was identified between PNI and C5aR2 tumor and stromal cell expression.

The ductal carcinoma in situ (DCIS) was specified in 98 breast cancer cases, out of 128 breast cancer samples. DCIS was present in 46.9% of cases and 53.1% of cases did not show DCIS. The expression of C5aR2 was higher in tumor cells of DCIS-positive tumors (97.8%), On the contrary, in stromal cells increased C5aR2 expression was observed in breast cancer cases not having DCIS. C5aR2 expression was also identified in DCIS, referring to the possible role of C5aR2 not only in tumor progression but also in tumorigenesis. No significant association was identified between DCIS and C5aR2 tumor and stromal cell expression.

To evaluate the correlation between continuous variables (Age and Ki-67) and tumor and stromal cell C5aR2 expression, Pearson Correlation test was done. A weak positive correlation was observed between age and tumor cell expression of C5aR2 but the relation was not statistically significant. The results indicated that although C5aR2 expression both in tumor and stromal cells increases with age but this linear relationship is not strong enough to be significant. However, the relation between age and stromal cell expression of C5aR2 was moderately positive. A statistically insignificant, weak positive correlation was also observed between Ki-67 and stromal cell C5aR2 expression. However, the weak negative correlation between Ki-67 and tumor cell C5aR2 expression indicated that tumor cell proliferation is not affected by C5aR2 expression, rather it decreases with increasing tumor cell C5aR2 expression. This finding is contrary to the results of Yumeng et al., showing a significant association between tumor cell proliferation and high C5aR2 expression in tumor cells.

The significance of macrophages expressing C5aR2 has emerged in breast cancer cells. Almost half of the breast cancer cases were positive for C5aR2 expressing

macrophages in varying proportions. Most of the tumors exhibiting C5aR2-expressed macrophages were Luminal B followed by TNBC. The majority of tumors showing C5aR2 expressing macrophages were grade 2 and Stage 3 tumors. Lymphovascular invasion was present in almost all of the C5aR2-expressing macrophages. The presence of C5aR2 expressing macrophages in most of breast cancer cases suggests the role of macrophages in breast cancer progression. Similar findings were observed in the study conducted by Yumeng et al (2021). In the study, the TIMER2.0 database was used to explore the role of C5aR2 in the infiltration of immune cells, especially macrophages in the tumor microenvironment. The study revealed that C5aR2 expression levels were positively correlated with M2 macrophages, however, its negative correlation was observed with infiltrating M0 and M1 macrophages. These findings showed the possible role of C5aR2 in the progression and invasion of breast cancer.

An interesting aspect that emerged in this study was the manifestation of strong C5aR2 expression in the blood vessel endothelium in all breast cancer cases, with slight variation in intensity. A study conducted by Yoshishige et al (2019) explored the role of C5aR2 expression on endothelial cells. The study revealed that neutrophil arrest and its adhesion to endothelium depend on the presence of the atypical complement C5a receptor 2 (C5aR2) in the endothelial cells. This receptor facilitates the transport of C5a into the vessel lumen, which, in turn, is necessary to initiate the process of C5aR1-driven neutrophil arrest. Neutrophils play a significant role in triggering an inflammatory response, and the role of inflammation in tumorigenesis and its response to therapy is clearly discernible.

To summarize, a significant role of C5aR2 in breast cancer has emerged in our study. A strong correlation between C5aR2 and tumor grade, stage, and high cell proliferation has been identified revealing its tumorigenic role in breast cancer. Its prognostic value is discernible from its strong association with hormone receptors positive and HER2-positive tumors, and TNBC. In addition, its significant role in chemoresistance increases the need to utilize C5aR2 not only as a prognostic but also as a therapeutic target to combat tumor progression and reverse chemoresistance thereby improving survival and reducing mortality rate in breast cancer patients. A study

conducted by Shicheng et al highlighted the therapeutic potential of a neutralizing monoclonal antibody against C5aR2 due to its efficacy in eradicating C5aR2-positive

stromal cells (CAFs) and cancer stem cells (CSCs). Well-designed clinical trials and new combinational therapies including biological as well as immunological agents are necessary to recognize the best treatment options for breast cancer patients with adverse prognoses.

5.2 Implications of the Study

5.2.1 Theoretical Implications

N/A

5.2.2 Practical Implications

- It is a prognostic marker for breast cancer progression, invasion, and chemoresistance.
- It might prove to be a therapeutic target for breast cancer especially for those not responding to conventional chemotherapy.
- The findings in our study are associated with higher grade, stage and chemoresistance which might prove to be a basis for the development of targeted therapy against this receptor

5.2.3 Policy Implications

C5aR2 may be used as a marker of the diagnostic panel as it is imperative to identify cases especially chemoresistant cases before devising any treatment plan.

5.3 Limitations and strengths of study

(A) Limitations

- It was a single-centered study
- The sample size was small, although based on Open-source calculation, thereby limiting the generalization of results
- A good comparable data was not available
- The retrospective data obtained was limited to initial assessment and post-operative histopathology results so survival analysis was not possible and the pathological stage was considered as an outcome variable
- Since trucut biopsies were also involved, the cases showing pathologic stage were further narrowed down

(B) Strengths

- This is theinauguralresearch study done to evaluate the expression of C5aR2 and its correlation with multiple clinicopathological parameters of breast in Karachi, Pakistan.
- This analysis is among a limited number of research investigations done on human breast cancer tissue

5.4 Recommendations

- Multi-centered studies with large sample sizes and follow-ups are required to assess disease progress and overall survival
- IHC evaluation of C5aR2 is subjective so there is a need to standardize the detection methods and techniques for C5aR2 cell count to obtain consistent results
- It is reported that C5aR2 expression varies across species and most of the C5aR2 analysis was done on animal models. Therefore, more studies on human tissues are required to comprehensively evaluate their role in cancer.

5.5 Conclusion

As evident from previous studies, C5aR2 plays an important role in promoting tumor growth and resistance to therapy. Our study demonstrates that elevated C5aR2 expression is associated with high tumor grade and proliferation rate, corroborating its role in tumorigenesis. Thus, it may be used as a biomarker for disease progression and prognosis. The differential expression of C5aR2 in tumor and stromal cells of breast cancer emphasizes its complex role in the tumor microenvironment. Furthermore, its upregulation in chemoresistant cases suggests its potential as a therapeutic target which may prove helpful in hampering tumor formation and reversing chemoresistance.

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A

Bahria University
Discovering Knowledge
Health Sciences Campus, Karachi

FACULTY RESEARCH COMMITTEE
BAHRIA UNIVERSITY HEALTH SCIENCES - KARACHI

LETTER OF APPROVAL

Date: 15-09-2022

To,
Dr. Erum Khaliq
MPhill - Student
Department of Pathology
BUHS - Karachi

Subject: **Faculty Research Committee**
FRC-BUHS Approval of Research Study

Title of Study: Correlation of Clinicopathological findings and C5aR2 Expression in Breast Cancer.

Name of Student: **Dr. Erum Khaliq**

Reference No: **FRC-BUHS -50/2022-518**

Dear: **Dr. Erum Khaliq**

Thank you for submitting research proposal to FRC-BUHS. The committee has approved your project.

This letter is referred to ERC for approval.

Regards

FOR Lateef

Dr. Mehreen Lateef,
Associate Professor,
CO- CHAIRPERSON FRC-BUHS

Cc:
DG-BUHS
Principal Medical
Principal Dental
Vice Principal BUHS
Co-chairperson FRC
Secretary

B

Bahria University
Discovering Knowledge
Health Sciences Campus Karachi

ETHICAL REVIEW COMMITTEE

LETTER OF APPROVAL

Date: 19-Dec-22

Reference:
FRC-BUHS-50/2022-518

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Prof. Ambreen Usmani
Principal & Dean
Health Sciences(BU)

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Ms NajmusSahar Ilyas

Dr. Erum Khaliq
MPhil Candidate
Department of Pathology
BUHS-Karachi

Subject: Institutional approval of research study

Title of Study: The correlation of Clinicopathological findings and C5aR2 expression in Breast Cancer

Principal Investigator: Dr. Erum Khaliq

Reference No: ERC 111/2022

Dear Dr. Erum Khaliq,

Thank you for submitting the above mentioned study proposal. ERC Bahria University Health Sciences Campus has reviewed this project in the meeting held on 16-Dec-2022 and gives approval. Kindly notify us when the research is complete.

Regards,

19/12/2022

DR. AMBREEN SURTI
Secretary, ERC
BUHS

19/12/2022

DR. QURATULAIN JAVAID
Chairperson, ERC
BUHS

Cc:
Principal BUHS

C

INFORMED CONSENT FORM FOR PATIENT

You are giving consent to participate voluntarily and at your own will in this research project which aim todetermine effective tumor markers of breast carcinoma.

You have been explained that tissue specimen will be taken for the purpose of research.

You have been told that findings of your disease and your data will be kept strictly confidential and will be used only for the benefit of community, publications and paper presentations.

You have been explained that tissue specimens will be taken for the purpose of research.You fully agreed to give your samples (tissue) at the beginning and end of study and when required in between. You also agree to give all relevant information when needed, in full and to the best of your knowledge to the researcher. It is clarified to you that no incentive will be provided to you for participating in the study, whereas you do have the right to withdraw from the study at any time.

You are advised to contact Dr. Erum Khaliq, on mobile number: 03423145600 or visit PNS Shifa Hospital in case of any query/ emergency related to your disease.

Name of Patient:_____ Sex (Male, Female) _____

D/O, W/O P

Signature / Thumb impression of Patient: _____

Name of Researcher: _____

Signature of Researcher: _____

Date: _____

C

مریض کی تحریری رضامندی کا فارم

آپ اس تحقیقی پروجیکٹ میں رضاکارانہ طور پر اور اپنی مرضی سے حصہ لینے کے لیے رضامندی دے رہے ہیں جس کا مقصد بریسٹ کینسر کے مؤثر نشانات کا تعین کرنا ہے۔

آپ کو بتایا گیا ہے کہ تحقیق کے مقصد سے بریسٹ ٹشو کا نمونہ لیا جائے گا۔

آپ کو بتایا گیا ہے کہ آپ کی بیماری کے نتائج اور آپ کے ڈیٹا کو سختی سے خفیہ رکھا جائے گا اور صرف کمیونٹی، اشاعتوں اور کاغذی پیشکشوں کے فائدے کے لیے استعمال کیا جائے گا۔

آپ نے اپنے نمونے (ٹشو) کو مطالعہ کے آغاز میں اور اس کے درمیان ضرورت پڑنے پر دینے پر مکمل اتفاق کیا۔ آپ نے ضرورت پڑنے پر تمام متعلقہ معلومات مکمل طور پر اور اپنی بہترین معلومات کے مطابق محقق کو دینے پر بھی اتفاق کیا۔ آپ کو واضح کیا جاتا ہے کہ مطالعہ میں حصہ لینے کے لیے آپ کو کوئی ترغیب نہیں دی جائے گی، جبکہ آپ کو کسی بھی وقت مطالعہ سے دستبردار ہونے کا حق حاصل ہے۔

آپ کو مشورہ دیا جاتا ہے کہ ڈاکٹر ارم خلیق سے موبائل نمبر 0342-3145600 پر رابطہ کریں۔ یا اپنی بیماری سے متعلق استفسار / ایمرجنسی کی صورت میں پی این ایس شفا ہسپتال تشریف لائیں۔

مریض کا نام: _____ والد / شوہر کا نام _____

مریض کے دستخط: _____

محقق کا نام: _____

محقق کے دستخط: _____

تاریخ: _____

D

SUBJECT EVALUATION FROFORMA

Department of Pathology PNS Shifa Hospital, Karachi

Epidemiological Data:

S.No _____ Case No_____ Date _____

Patient's Name _____ W/O, D/O _____

Age _____

Address _____

Clinical Diagnosis _____

Laboratory Investigations

Nature of Specimen

1) Modified Radical Mastectomy ☐
2) Excisional Biopsy ☐
3) Trucut Biopsy ☐

Gross Findings:

_____

_____

_____

Histopathological Findings:

_____

_____

_____

Tubular formation _____

Nuclear Pleomorphism _____

Mitotic Count _____

Lymphovascular Invasion _____

DCIS/LCIS _____

Receptor Status _____

Ki-67 _____

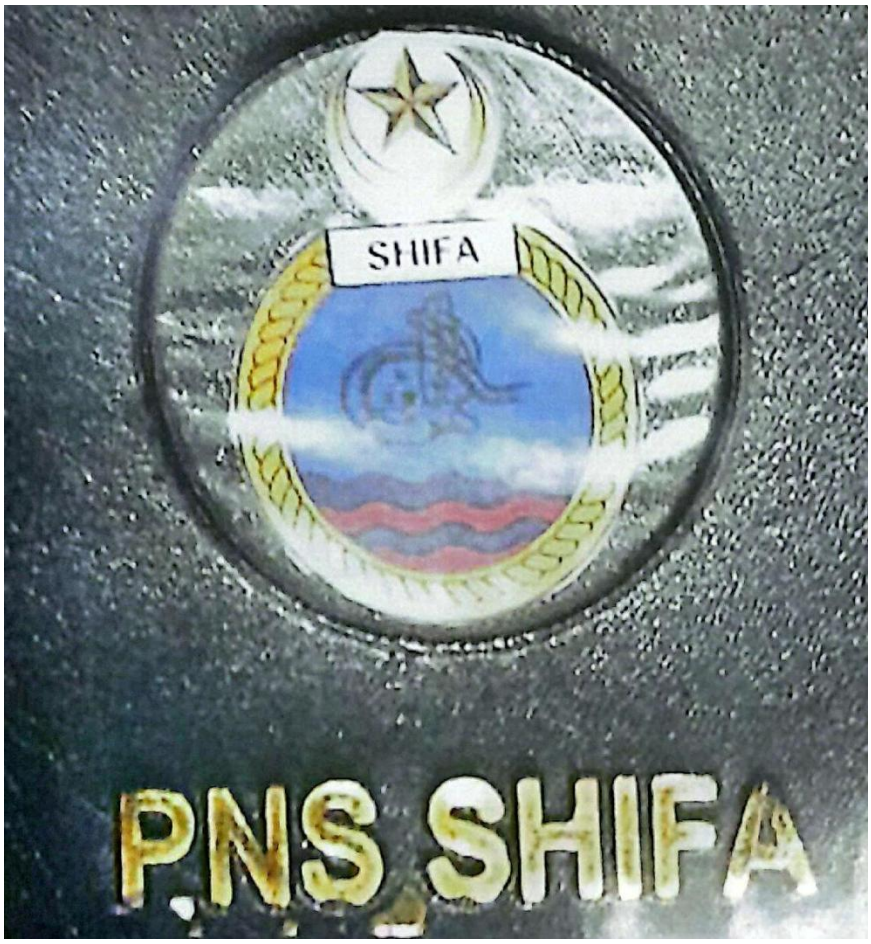
Tumor Grade_____

Tumor Stage _____

Localization of tumour_____

LOCATION	**C5aR2**
Cytoplasmic	
Nuclear	
Cell Membrane	
Intensityof Staining	
No staining (0)	
Mild staining (1)	
Moderate staining (2)	
Strong staining (3)	
Percentage of Positive cells	
no positive cells (0)	
<10% positive cells (1)	
10–50% positive cells (2)	
51–80% positive cells (3)	
>80% positive cells (4)	

E



F

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