Analysis of Expression and Activity of Indoleamine 2,3-dioxygenase in Breast Cancer Patients from Pakistan

By

Asim Farooq

0000119335

Department of Biomedical Engineering and Sciences
School of Mechanical and Manufacturing Engineering (SMME)
National University of Sciences and Technology (NUST)

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Thesis Supervisor:

Dr. Kashif Asghar

Thesis Supervisor's Signature: _____

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Declaration

I certify that this research work titled *"Analysis of Expression and Activity of Indoleamine 2,3-dioxygenase in Breast Cancer Patients from Pakistan"* is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

Asim Farooq
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Copyright Statement

Dedicated To

My Beloved Mother,

Father and Sister

and to all the curious minds solving the mysteries of universe

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Praise to the Almighty, Lord of the worlds, for blessing humans with frontal cortex and the ability to use it for peace and welfare of living beings.

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Abbreviations

APCs	Antigen presenting cells
CAFs	Cancer associated fibroblasts
CI	Confidence interval
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
COX-2	Cyclooxygenase 2
CSCs	Cancer stem cells
CTLs	Cytotoxic T-lymphocytes
DCs	Dendritic cells
DCIS	Ductal carcinoma in situ
DNA	Deoxyribonucleic acid
ECs	Endothelial cells
EP2/4	Prostaglandin E2/4 receptor
ER	Estrogen receptor
Foxp3	Forkhead box P3
GM-CSF	Granulocyte macrophage colony stimulating factor
$HER2^{+}$	Human epithelial growth factor receptor 2 positive
HLA	Human leukocyte antigen
HO-1	Heme oxygenase-1

IBC	Inflammatory breast cancer
ICs	Immune inflammatory cells
IDC	Invasive/ infiltrating ductal carcinoma
IDO	Indoleamine 2,3-dioxygenase
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IL	Interleukin
ILC	Invasive/ infiltrating lobular carcinoma
ITIM	Immunoreceptor tyrosine-based inhibitory motif
Kyn	Kynurenine
LCIS	Lobular carcinoma in situ
LOH	Loss of heterozygosity
L-Trp	L-tryptophan
MCP	Monocyte chemoattractant protein
MDM2	Mouse double minute 2
MDSCs	Myeloid derived suppressor cells
MHC	Major histocompatibility complex
1-MT	1-Methyl Tryptophan
NK-cells	Natural killer cells
NO	Nitric Oxide
OR	Odds ratio
PCs	Pericytes

PGE2	Prostaglandin E2
PI-9	Proteinase inhibitor-9
PR	Progesterone receptor
ROS	Reactive oxygen species
SCF	Stem cell factor
SDF-1	Stromal cell derived factor 1
SHP	Src homology region 2 domain-containing phosphatase-1
SKMCH&RC	Shaukat Khanum Memorial Cancer Hospital and Research Centre
TCR	T-cell receptor
TGF-β	Transforming growth factor beta
TNBC	Triple negative breast cancer
TNF-α	Tumor necrosis factor alpha
T-regs	Regulatory T-cells
VEGF	Vascular endothelial growth factor

Abstract

Modulation of immune system is associated with development and progression of cancers. Owing to the complexity of tumor formation, involving various genetic, epigenetic and environmental factors; immune system generates varied responses under different circumstances. A shift in balance from pro-inflammatory factors and towards immune tolerance promoting factors leads a patient towards poor prognosis and reduces the chances of cancer-free survival. Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme which is involved in suppression of effector T-cell responses by catabolizing essential amino acid tryptophan. Involvement of IDO in breast cancer is critical to understand the significance of immune system in breast cancer development and progression. Here, we conducted the first study to analyze IDO expression and its enzymatic activity in breast cancer cases from Pakistan

Paraffin fixed formalin embedded (PFFE) breast cancer biopsies were obtained from one hundred (100) patients from Pakistan. Expression of IDO in breast cancer biopsies was examined through immunohistochemistry (IHC). Clinicopathological characteristics of patients like age at onset of breast cancer, tumor size, histology of tumor, tumor grade, and metastasis of cancer, expression of estrogen, progesterone and human epithelial growth factor receptor were compared with IDO expression. Activity of IDO was analyzed through colorimetric assay in plasma samples of fifteen (15) breast cancer patients. It was compared with fifteen (15) healthy controls.

IDO was over-expressed in majority of breast cancer patients. Triple negative breast cancers had significantly higher expression of IDO as compared to non- triple negative

breast cancers ($p<0.01$). Overall survival of breast cancer patients with higher expression of IDO was decreased ($p=0.04$). IDO enzymatic activity was also higher in breast cancer patients as compared to controls ($p<0.0001$). IDO might be associated with prognosis of triple negative breast cancer patients and its role in modulation of immune system in breast cancer needs to be explored.

Chapter 1

Introduction

Complexity of tumors has been a part of rigorous investigation for over a decade now and it has been established that, to comprehend the biology of tumor, it is important to understand a detailed structure of tumor and its surroundings (Hanahan and Weinberg, 2011). Tumor microenvironment which is constituted along the complex process of tumorigenesis, contains multiple cells of diverse origins. These include cancer-associated fibroblasts (CAFs), endothelial cells (ECs), cancer stem cells (CSCs), pericytes (PCs), and immune inflammatory cells (ICs) etc. (Figure 1.1).

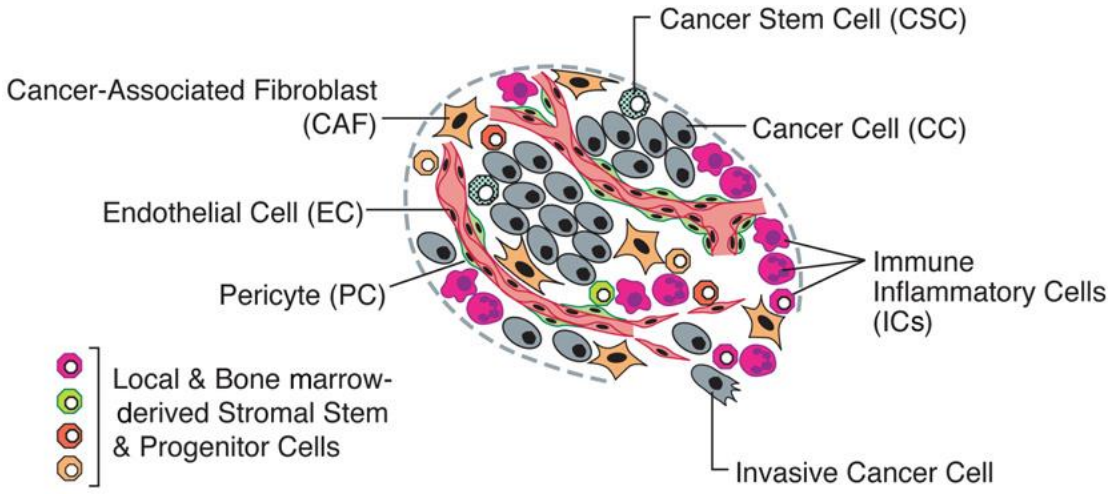

Figure 1.1 Cells in the tumor microenvironment. A collection of different cells is present in most of the tumors. Collective effects of cells from stroma and parenchyma enable tumor growth and progression. (Adapted from (Hanahan and Weinberg, 2011))

Cellular and chemical mediators of inflammation play a critical role in development and progression of tumors (Mantovani et al., 2008). Inflammatory

conditions are either present before the onset of cancer or a genetic mutation leads to induction of inflammatory environment which stimulates the development of cancer (Mantovani et al., 2008). Owing to the complex nature of immune responses, the type of immune response provoked by the immune system against a cancer can either be anti-tumorigenic or pro-tumorigenic (DeNardo and Coussens, 2007). Humoral immune responses and pro-tumor inflammatory cells tend to promote tumor development while cytolytic T cellular responses inhibit tumor growth and development (DeNardo and Coussens, 2007).

The intracellular enzyme Indoleamine 2,3 –dioxygenase (IDO) participates in catabolism of essential amino acid tryptophan. This metabolic pathway yields multiple metabolites including the immunosuppressive metabolites like kynurenines (Prendergast, 2008). Munn et al. initially discovered its immunosuppressive role in mice where IDO inhibition leads to rejection of fetus (Munn et al., 1998). Interferon gamma (IFN-γ) is a potent inducer of IDO (Di Pucchio et al., 2010) and macrophages and dendritic cells express IDO in various tissues upon activation (Munn et al., 1999, Fallarino et al., 2002b). IDO has been reported to play diversified roles in different diseases. IDO is associated with poor prognosis of cancer (Okamoto et al., 2005). Higher IDO expression promotes T-cell suppression in HIV infected patients which leads to poor prognosis (Boasso et al., 2007) but on other hand, in autoimmune diseases like rheumatoid arthritis IDO might be able to reverse the effects of disease (Bianco et al., 2009)

IDO expression in breast cancer has been analyzed by multiple researchers through animal models, in silico analyses and human samples. Kim et al. recently established that expression of IDO might reflect high mutational loads in TNBCs (Kim et al., 2017). Li et al found out that IDO and IL-6 expression are linked with advanced stages of the disease and are also involved in poor response of the tumor towards chemotherapy (Li et al., 2017). On the other hand, Jacquemier et al observed favorable prognosis in patients expressing higher levels of IDO (Jacquemier et al., 2012). A study by Soliman et al in 2013 associated favorable outcomes with increased IDO expression in estrogen receptor positive breast cancers as compared to estrogen receptor negative breast cancers (Soliman et al., 2013).

Due to the complexity of subject matter and contradictory results of various studies, we sought out to establish the expression and activity of IDO in breast cancer patients from Pakistan. In this study, we took breast cancer biopsies from one hundred to examine the expression of IDO through immunohistochemistry. Plasma samples to examine enzymatic activity of IDO were only available from fifteen breast cancer patients. Enzymatic activity of IDO was also examined in healthy controls to establish relative levels between the two groups. We explored the relationships between IDO expression and age of patient at onset of breast cancer, size of tumor, \and grade of tumor. Expression of molecular factors like estrogen receptor (ER), progesterone receptor (PR), human endothelial growth receptor 2 (HER2) and triple negative breast cancers (TNBCs) were also examined and compared with IDO expression.

Objectives

- To establish the expression of IDO in Pakistani Breast Cancer patients.
- To analyze the relationship between IDO expression and clinico-pathologic characteristics of breast cancer patients.
- To examine enzymatic activity of IDO in breast cancer patients in comparison with healthy controls.

Chapter 2

Literature Review

2.1 The Breast

The breasts (mammary glands) lie in the superficial fascia covering the anterior chest wall. They are specialized accessory glands of skin which produce milk (Snell, 2012). In children and males, they are rudimentary but in females after puberty, they increase in size and assume a spherical shape (Snell, 2012).

2.1.1 Anatomy of the Breast

Each breast consists of lobes, milk ducts, areolas, nipples, fat tissue, suspensory ligaments and retromammary space (Figure 2.1). Each human female breast consists of 15-20 lobes radiating out of the nipple (Snell, 2012). Each lobe has a

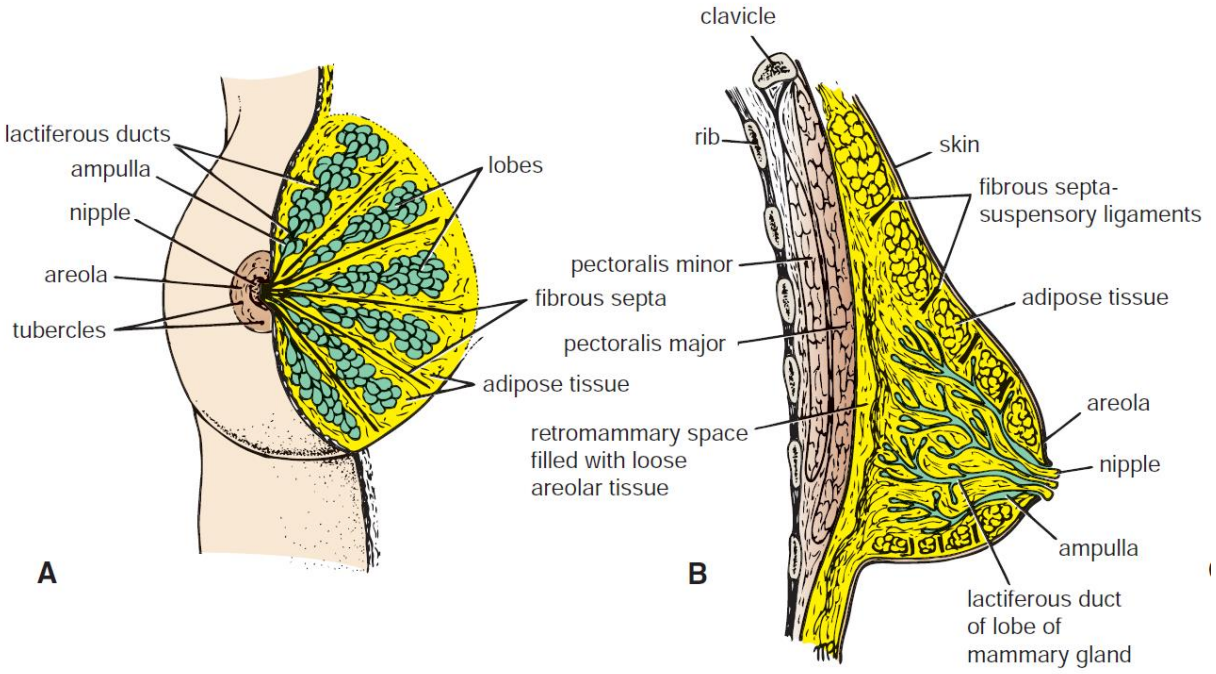

Figure 2.1 Mature breasts in the female. A. Anterior view with moderately detached skin to show inner organization of breast. B. Sagittal section. (Adapted from (Snell, 2012)).

main duct which opens discretely on the top of the nipple and contains a dilated ampulla just before its termination (Snell, 2012). Basal region of each nipple is surrounded by areolas which have minute tubercles produced by areolar glands. Fibrous septa separate the lobes of gland and also serve as suspensory glands (Snell, 2012). Retro-mammary space is present behind each breast and is filled with loose connective tissue (Snell, 2012).

2.2 Breast Cancer

Breast cancer is a mass of uncontrolled dividing cells that develops in breast tissue. It is one of the leading causes of cancer associated deaths world-wide (NCI, 2017). It can develop in ducts as well as lobes of the breasts.

2.2.1 Epidemiology

Cancer is one of the leading causes of mortality world-wide. In past decades, increased rates of cancer incidence have been reported. According to statistics generated by international agency for research on cancer (IARC), in 2012, 32.6 million people were diagnosed with cancer, 8.2 million of these patients died and nearly 14.1 million new cancer cases were diagnosed (IARC, 2012). Highest incidence of male cancers was in Australia/ New Zealand and Northern America had highest incidence of female cancer (GLOBOCAN, 2012). Central and eastern Europe was ranked top in cancer associated mortality while western Africa was among lowest in cancer incidence as well as mortality (GLOBOCAN, 2012).

In 2012, breast cancer had highest incidence (23%), mortality (16%) and 5-year prevalence (35%) among all cancers in both sexes in Pakistan (GLOBOCAN, 2012). Approximately 0.15 million new cancer cases were reported and 0.10 million cancer associated deaths were reported till 2012 (GLOBOCAN, 2012). Risk of getting cancer before 75 years of age was 10.5% in males and 13.1% in females (GLOBOCAN, 2012). Lip/ oral cavity cancer, lung cancer, non-Hodgkin's lymphoma, colorectal cancer and prostate cancer were five most frequent cancers in males, while breast cancer, lip/ oral cavity cancer, cervical and uterine cancers, ovarian cancer and esophageal cancer were five most frequent cancers in females (GLOBOCAN, 2012). There was an overall 8.8% risk of dying before 75 years of age due to cancer with 9.2% risk in females and 8.5% risk in males (GLOBOCAN, 2012).

2.2.2 Types of Breast Cancer

Breast cancer has been classified into various types. These include:

2.2.2a Ductal Carcinoma

As the name suggests, it begins in the milk carrying ducts of breasts (Hudis et al., 2005). **Ductal carcinoma in situ (DCIS)** is non-invasive cancer. When the cancer starts to spread to surrounding tissues it becomes **invasive/ infiltrating ductal carcinoma (IDC) (**Hudis et al., 2005). About 80% of all breast cancers are IDC. IDC has been further categorized into subtypes.

i) Tubular carcinoma is a type of IDC in which the tumor cells assume a tubular shape.

ii) Medullary carcinoma is a soft tumor tissue. It has a fleshy mass resembling the ‘medulla of brain’.

iii) Mucinous carcinoma is a form of IDC in which tumor cells hang in pools of mucin. Mucin is a high molecular weight glycosylated protein which plays an imperative role in formation of mucus. Breast cancer cells generate mucus which surrounds the tissue and becomes a part of it (Hudis et al., 2005).

iv) Papillary carcinoma is very rare subtype of IDC and its tumor cells appear as finger like projections with well-defined borders.

v) Cribriform carcinoma consists of cancer cells in nest-like formations, invading the stroma.

2.2.2b Lobular Carcinoma

Lobular carcinoma originates in the milk producing lobules of the breasts. It is second most common type of cancer. At initial stages, when tumor cells are localized within lobules, it is called **lobular carcinoma in situ (LCIS)**. Upon metastasis of tumor cells to surrounding tissues, it becomes **invasive/ infiltrating lobular carcinoma (ILC)** (Hudis et al., 2005).

2.2.2c Inflammatory Breast Cancer

Inflammatory breast cancer (IBC) is bellicose form of breast cancer which originates with the swelling and redness of breasts without any distinct lump.

2.2.2d Molecular Subtypes of Breast cancer

Molecular subtypes of breast cancer are classified as follows:

i) Estrogen receptor positive (ER+) breast cancers have elevated expression of ERs on tumor cells (Hudis et al., 2005). Estrogen is a female sex hormone involved in maintenance and regulation of female secondary sex characters and reproductive organs (Ryan, 1982).

ii) Progesterone receptor positive (PR+) breast cancers over-express PRs on tumor cells (Hudis et al., 2005). Progesterone is a steroidal hormone playing critical roles in human menstrual cycle, embryogenesis and pregnancy (Fritz and Speroff, 2012).

iii) Human epidermal growth factor receptor 2 positive (HER2+) breast cancers have increased expression of HER2 on tumor cells (Hudis et al., 2005). HER2 is a proto-oncogene which plays important role in development of various cancers (Mitri et al., 2012).

iv) Luminal A is ER+ and/ or PR+ with HER2- and low levels of Ki-67 protein (Inic et al., 2014).

v) Luminal B is ER+ and/ or PR+ with HER2+/- and high levels of Ki-67 protein (Inic et al., 2014).

vi) Triple negative breast cancer (TNBC) is a type of cancer that expresses none of the above mentioned receptors on its cells.

2.2.3 Risk Factors

Breast cancer is a multi-factorial disorder. Some of the major risk factors are as follows (Madigan et al., 1995):

i) Older age

ii) Family history

iii) Genetics

iv) Obesity

v) Hormone therapy

vi) Old-age pregnancy

vii) Early onset and late stoppage of menstrual cycle

viii) Alcohol usage

ix) Smoking

x) Lack of exercise

xi) Breast density

xii) Carcinogen exposure in food, water and cosmetics

xiii) Oral contraceptives

2.2.4 Cancer Staging

The "American Joint Committee on Cancer" (AJCC) in collaboration with "TNM Committee of the International Union Against Cancer" (UICC) established a system for staging cancer in clinically important sites. This system has now been adopted globally and is known as the TNM system. 'T' represents the size of tumor, 'N' represents lymph node metastasis and 'M' represents distant metastasis. Different stage of cancer requires different treatment approaches. A detailed system of TNM staging is given at (Annexure A).

2.2.5 Tumor Grading

To establish the aggressiveness of tumor cells, Nottingham grading system developed by Ellston and Ellis is used (Elston and Ellis, 2002, Galea et al., 1992). This system is based on 3 factors:

i) Glandular/ tubular differentiation which establishes a difference between tumor cells and normal cells

ii) Nuclear Pleomorphism which describes the size and shape of tumor cells

iii) Mitotic count which characterizes the number of divisions tumor cells are going through

Each category gets a score of 1-3; ‘1’ representing tumor cells close to normal cells and ‘3’ representing tumor cells away from normal. Score in each category is added to give a final score which has been divided into 3 categories

i) Grade 1 with a score of 3-5

ii) Grade 2 with a score of 6-7

iii) Grade 3 with a score of 8-9

A detailed system of tumor grading is given at (Annexure B)

2.2.6 Breast Cancer Immunology

Development of breast cancer is generally initiated by genetic and epigenetic modifications in mammary epithelial cells (Jiang and Shapiro, 2014). Diverse intrinsic tumor-suppressor mechanisms initiate apoptosis and senescence of these neoplastic cells to prevent tumor progression. Simultaneously, immune system acts as an extrinsic tumor suppressor which eliminates transformed mammary cells and obstructs their progression after they have succeeded in escaping the intrinsic tumor suppressing mechanisms (Jiang and Shapiro, 2014). Three types of inflammation are involved in cancer development and progression. Chronic inflammation that initiates tumor development, tumor associated inflammation and therapy-induced inflammation (Jiang and Shapiro, 2014). The involvement of immune system in breast cancer progression and inhibition has been summarized in Figure 2.2. The idea that immune system has capacity to regulate tumors has been a topic of debate for over a century. It

has recently been accepted that immune system not only has the ability to hinder tumor growth, but also has the power to promote tumors by a process called immune-editing (Jiang and Shapiro, 2014). Evading the destruction of immune system has recently been acknowledged as a hallmark of cancer. Previously, increased risk of breast cancer has been associated with pre-existing inflammation. A recent study by Kristensen et al. suggested that critical information on patient prognosis and treatment can be obtained from the immune response profile and inflammatory signature of breast cancer (Kristensen et al., 2012). The research associated with immune system

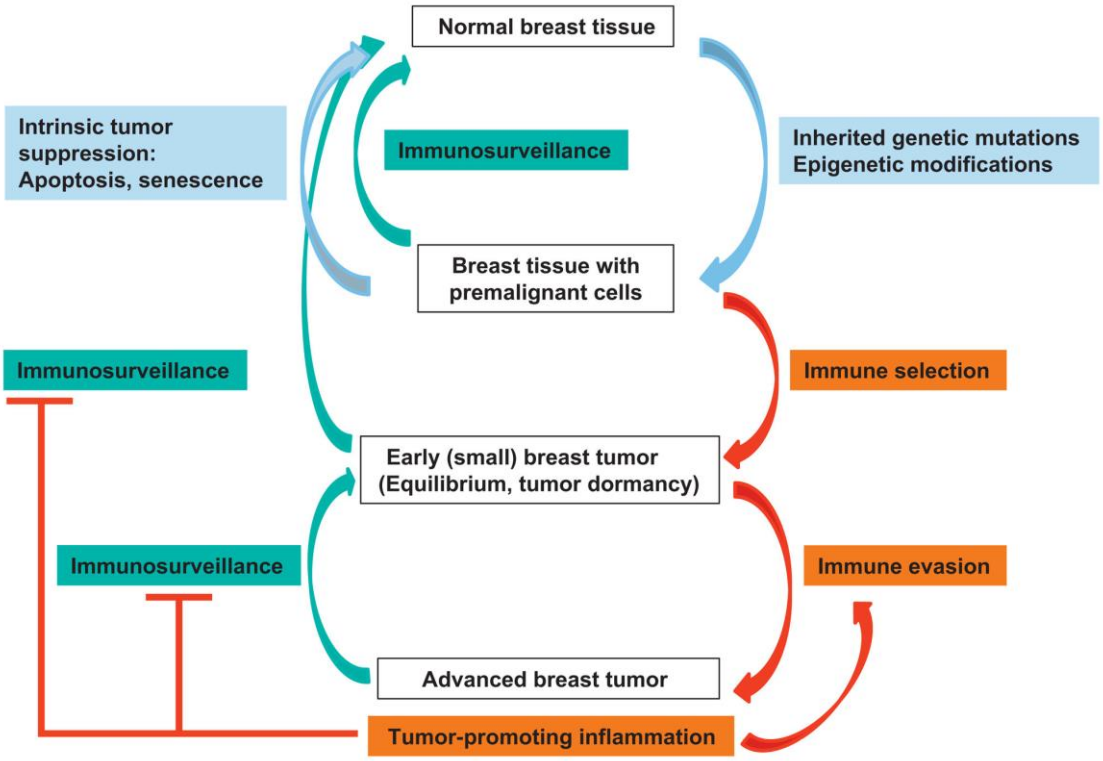

Figure 2.2 Involvement of immune system in breast cancer. Genetic and epigenetic mutations cause premalignant transformation of mammary cells. These cells can be eliminated by various tumor suppressor mechanisms. Immunediting (immune selection) and evasion can lead breast cancer to advanced stages. Tumor promoting inflammation boosts immune evasion and restrains immune surveillance. (Adapted from (Jiang and Shapiro, 2014))

and its manipulation may augment the possibilities of finding better therapeutics for breast cancer.

2.2.6a Pre-tumor inflammation in breast cancer

The ephemeral induction of IL-6 by monocyte-derived monocyte chemoattractant protein (MCP) initiates a signaling pathway that leads to constitutive production of IL-6 revealing a link between IL-6 and breast cancer development (Rokavec et al., 2012). IL-6 enhances the recruitment of mesenchymal stem cells (MSCs) and derives a pro-inflammatory loop leading to progression of breast cancer cells (Liu et al., 2011) and resistance against trastuzumab in $HER2^+$ breast cancer (Korkaya et al., 2012). Tumor necrosis factor alpha (TNF-α) induces epithelial-mesenchymal transition (EMT) in breast cancer by activating Twist1 protein through NF-kβ pathway, thereby promoting metastasis (Li et al., 2012).

2.2.6b Cellular immunity in breast cancer

$CD8^+$ cytotoxic T-lymphocytes (CTLs) and natural killer cells (NK-cells) carry out the most effective response against breast cancer cells (Jiang and Shapiro, 2014). Various researchers have concluded that CTLs can be induced to target breast cancer cells expressing specific antigens (Kontani et al., 2001, Neidhardt-Berard et al., 2004, Treilleux et al., 2004, Wang et al., 2006, Mine et al., 2009, Mittendorf et al., 2012). Patients with increased numbers of CTLs have shown better disease outcomes, independent of various other prognostic factors like tumor grade, lymph node stage, size and vascular invasion (Mahmoud et al., 2011). CTL infiltration has also been

associated with better cancer-specific survival (Liu et al., 2012). Vaccines against breast cancer achieve at least partial efficacy through CTL mediated immune responses (Wang et al., 2012, Rech et al., 2012, Schlom, 2012). Combinational therapies involving chemotherapy and immunotherapy enhance cytolytic effects of CTLs leading to an amplified antitumor effect (Ramakrishnan et al., 2010).

NK-cells achieve their cytotoxic effects by eliminating tumor cells without MHC constraint (Waldhauer and Steinle, 2008). Deterioration of NK-cell function has been linked to progression of human breast cancer (Mamessier et al., 2011). Patients with advanced stage (stage IV) familial breast cancer have shown suppressed numbers of NK-cells (Konjevic and Spuzic, 1993). Mouse breast cancer xenograft model elicited robust antitumor immunity through memory $CD4^+$ T-cell dependent NK-cell mediated immunity (Tkach et al., 2012). NK-cells play a dual role by directly suppressing tumor cells as well as improving antitumor effects of chemotherapy or immunotherapy (Jiang and Shapiro, 2014).

2.2.6c Immunosuppressive environment of breast cancer

Breast cancer cells promote an immunosuppressive environment comprising of cellular as well as molecular factors. Major immunosuppressive cells associated with immunosuppression in breast cancer are forkhead box P3 (Foxp3) regulatory T cells (T-regs) and myeloid-derived suppressor cells (MDSCs) (Jiang and Shapiro, 2014).

Peripheral immune tolerance is strongly mediated by $Foxp3^+$ T-regs. They can suppress majority of immune cells including $CD4^+$ T-cells, $CD8^+$ T-cells, NK-cells, B-

cells and antigen presenting cells (APCs) (Shevach, 2009, Sakaguchi et al., 2010). Breast cancer cells recruit T-regs through CCL22/CCR4, CXCL12 (SDF-1)/CXCR4, CCL5/CCR1 axes and also through EP2 or EP4 by up-regulating prostaglandin E2 (PGE2) (Gobert et al., 2009, Karavitis et al., 2012, Tan et al., 2011, Yan et al., 2011). Diminished expression of interferon alpha (IFN-α) by dendritic cells (DCs) also supports enhancement of T-regs in breast cancer tissue (Sisirak et al., 2012). T-regs mediate metastasis by producing large amounts of nuclear factor kappa B (NF-kB) receptor activator (Tan et al., 2011). Researchers have shown that blocking $Foxp3^+$ T-regs through anti-CD25 antibody supported stronger anti-tumor response and better disease outcomes (Rech et al., 2012, Weiss et al., 2012).

MDSCs are $CD14^-$ $CD11b^+$ immune cells of myeloid origin with a strong T-cell suppressing function in humans (Gabrilovich and Nagaraj, 2009). MDSCs are activated by interferon gamma (IFN-γ), interleukins 13 and 4 (IL-13 and IL-4) and transforming growth factor β (TGF-β) (Gabrilovich and Nagaraj, 2009). Expansion of MDSCs is carried out by granulocyte macrophage colony stimulating factor (GM-CSF), PGE2, IL-6, stem cell factor (SCF) and vascular endothelial growth factor (VEGF) (Gabrilovich and Nagaraj, 2009). MDSCs suppress T-cell growth and function by producing reactive oxygen species (ROS), nitric oxide (NO) and arginase (Gabrilovich and Nagaraj, 2009). MDSCs also carry out nitration of T-cell receptor (TCR) complexes leading to disruption of binding capacity of peptide with major 16istocompatibility complex (MHC) dimers to $CD8^+$ T-cells (Nagaraj et al., 2007). NK-cell activity is also hindered by MDSCs (Mauti et al., 2011). Modifying

immunosuppressive function of MDSCs is important for IL-12 mediated anti-breast cancer immune response (Steding et al., 2011).

2.2.6d Immune evasion in breast cancer

Breast cancer cells have evolved various mechanisms to avoid destruction through the immune system. One of the mechanisms involves bypassing immune system by avoiding immune recognition. In a study on 212 breast cancer patients, more than 30% of breast cancer patients were shown to have a reduced expression of human leukocyte antigen (HLA) class I (Kaneko et al., 2011). Patients with maintained HLA class I expression had better disease outcomes as compared to those with reduced HLA class I expression (Kaneko et al., 2011).

CTLs and NK-cells majorly utilize granzyme B to induce apoptosis of target cells (Kaiserman and Bird, 2010). Proteinase inhibitor 9 (PI-9) is an estrogen regulated gene that is intracellular inhibitor of granzyme B (Kaiserman and Bird, 2010). PI-9 was vigorously up-regulated in breast cancer cells leading to inhibition of CTLs and NK-cells (Jiang et al., 2006, Jiang et al., 2007). Increased expression of an anti-apoptotic molecule, Survivin, has been established in breast cancer (Ryan et al., 2006). This molecule has also been associated with poor prognosis in breast cancer patients (Ryan et al., 2006). On the other hand, apoptosis promoting molecule BAX-α is expressed at extremely low levels in breast cancer cells (Jiang and Shapiro, 2014). This suggests the interplay of breast cancer cells with cellular molecules to successfully evade immune system recognition (Jiang and Shapiro, 2014).

2.3 Indoleamine 2, 3-dioxygenase

Initially discovered in rabbit intestine in 1967, Indoleamine 2, 3-dioxygenase (IDO) is a heme containing, non-secretory, intra-cellular enzyme transcribed from chromosome 8 in Homo sapiens (Mellor and Munn, 2004). It has a single gene consisting of 10 exons (Mellor and Munn, 2004). IDO protein contains 403 amino acids with a weight of ~45 kDa (Lancellotti et al., 2011). X-ray diffractometry shows that IDO has two α-helical distinct domains (large and small) and the heme ring is situated amongst them (Sugimoto et al., 2006). Heme ring contains ferric ion (Fe^{3+}) whose reduction leads to activation of IDO enzyme (Thomas and Stocker, 1999).

IDO is involved in catabolizing the rate-limiting step of conversion of least-abundant, essential amino acid L-tryptophan (L-Trp) into kynurenines (Kyn) (Lancellotti et al., 2011). Kynurenine metabolites exert their effects by inhibiting cytotoxic T-cells and proliferating T-regs (Fallarino et al., 2002a). In humans, IDO is majorly expressed by antigen presenting cells of the immune system like macrophages and dendritic cells. High expression of IDO has been detected in epididymis, prostate, spleen, thymus, lungs, digestive tract and placenta (Dai and Zhu, 2010). Immunomodulatory cytokines such as IFN-γ, PGE-2, cyclooxygenase 2, (COX-2) (Sayama et al., 1981) and hepatocyte growth factor (HGF) (Galimi et al., 2001) enhance enzymatic activity of IDO, while nitric oxide (NO) and TGF-β may hinder enzymatic activity of IDO (Thomas et al., 1994, Yuan et al., 1998).

2.3.1 IDO and the Immune System

Two ways in which IDO regulates immune responses are:

1- Formation of Kyn metabolites which are natural ligands for aryl hydrocarbon receptor (AhR) (Mezrich et al., 2010). AhR is a transcription factor activated by various exogenous and endogenous ligands (Stockinger et al., 2011). Depending on affinity and duration of signaling; ligands of AhR have diverse effects on subsets of T-cells (Stockinger et al., 2011). Nevertheless, binding of Kyn pathway metabolites to AhR leads to immunosuppressive effects (Figure 2.3) including, enhanced proliferation of Foxp3+ T-cells (Mezrich et al., 2010, Nguyen et al., 2010), reduction in immunogenicity of DCs (Nguyen et al., 2010) and suppression of anti-tumor immune responses (Opitz et al., 2011, Pilotte et al., 2012).

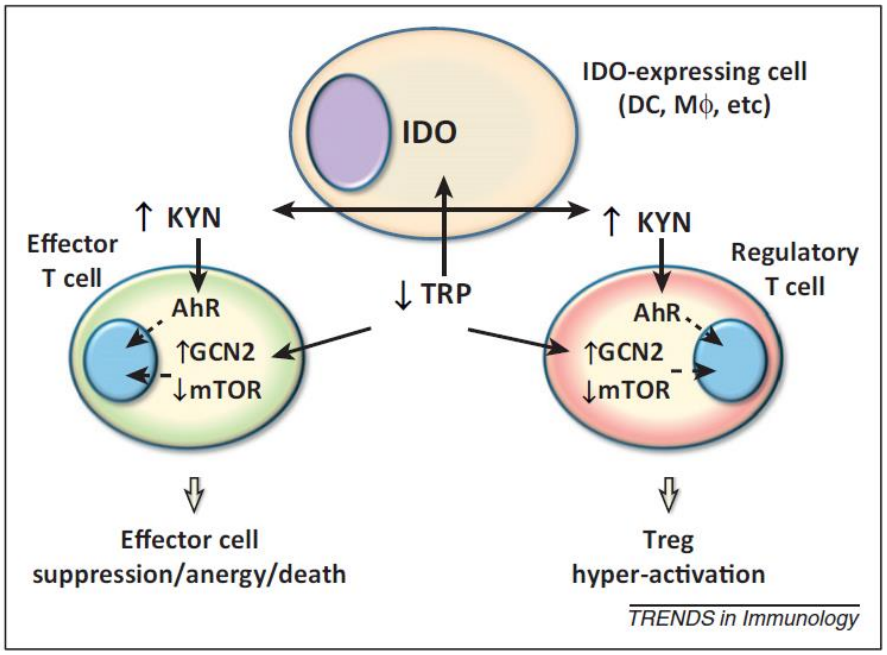

Figure 2.3 Involvement of IDO in regulating T cell and T-reg responses. Tryptophan consumption and kynurenine release generates signals via GCN2, mTOR and AhR respectively. It results in suppression of effector T cell responses and promotion of T-regs. (Adapted from (Munn and Mellor, 2013))

2- Depletion of Trp which initiates amino acid sensing regulatory pathways such as general control nonderepressible 2 (GCN2) kinase pathway and mammalian targets of rapamycin (mTOR) (Figure 2.3) (Munn and Mellor, 2013). The GCN2 molecule contains an allosteric regulatory domain in addition to a kinase domain which is involved in regulation of amino acids (Wek et al., 2006). Allosteric domain senses absence of tRNA and activates kinase domain which phosphorylates eukaryotic initiation factor 2 alpha (eIF2α) (Munn and Mellor, 2013). Activated eIF2α blocks translation of various mRNAs (Munn and Mellor, 2013)

Intracellular activity of IDO in DCs involves binding of SHP-1/SHP-2 phosphatases to ITIM motifs located inside IDO (Pallotta et al., 2011). In CD8+ T-cells, activation of GCN2 kinase through IDO leads to cell cycle arrest (Munn et al., 2005). In CD4+ T-cells, TH-17 differentiation is blocked (Sundrud et al., 2009, Keller et al., 2012) while T-reg proliferation is enhanced by IDO-induced GCN2 (Fallarino et al., 2006, Sharma et al., 2007). Thus, TH-17/ T-reg balance is shifted towards T-regs leading to a controlled inflammation and immunopathology (Favre et al., 2010).

As IDO gene expression is primarily induced by IFNs, the IDO pathway is induced in multiple pro-inflammatory mechanisms (Munn and Mellor, 2013). It can be induced in epithelial/ endothelial cells, fibroblasts, myeloid and non-lymphoid cells in inflamed tissue as well as surrounding lymphoid tissues (Munn and Mellor, 2013). In professional APCs, expression of IDO leads to tolerogenicity and in surrounding stromal cells, IDO expression can block immune responses which are triggering tissue inflammation (Desvignes and Ernst, 2009, Liu et al., 2009, Jasperson et al., 2008).

IDO is a part of complex network of signals (cellular and microenvironment) regulating immune responses (Figure 2.4).

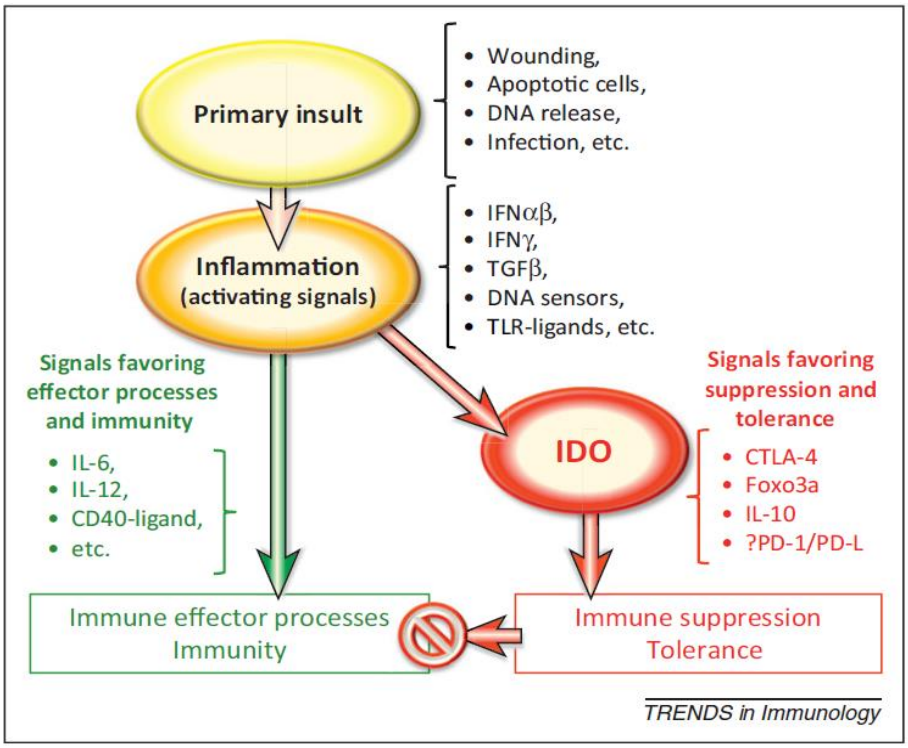

Figure 2.4 Role of IDO in immune suppression and tolerance. Inflammation activates immune cells but overall outcome depends on balance of signals that either support effective immunity or tolerance. Signals supporting regulatory outcomes might trigger IDO activity. (Adapted from (Munn and Mellor, 2013))

2.3.2 IDO and Breast Cancer

In 2004, Travers et al. established that MDA-MB-231 (ER- cell line) cells expressed IDO in presence of IFN-γ while 1-MT inhibited IDO expression in the cell line (Travers et al., 2004). In 2005, Muller et al. conducted a study on MMTV-Neu mice and submitted that loss of Bin1 results in deregulation of IDO, and IDO

inhibitors might improve responses to cancer therapy (Muller et al., 2005). Another study by Hill et al. in rat and human breast cancer cell lines concluded that the crosstalk between IDO and heme oxygenase-1 (HO-1) hinders cancer cell proliferation (Hill et al., 2005).

In 2009, Mansfield and coworkers demonstrated that IDO induced FoxP3+ T-regs are linked with advanced stages of breast cancer (Mansfield et al., 2009). In 2011, Lyon et al. observed higher levels of tryptophan degradation in patients with early stage breast cancer (Lyon et al., 2011). Yu et al. found out that IDO might promote metastasis through intrusion of FoxP3+ T-cells in the tumor microenvironment (Yu et al., 2011). In 2012, Levina et al. concluded that functional activity of IDO in tumor immunomodulation makes it an appealing target for therapy (Levina et al., 2012). In 2013, Soliman et al. established that IDO expression was higher in patients with ER+ tumors as compared to patients with ER- tumors (Soliman et al., 2013). In 2014, Larrain et al. conducted an in silico analysis and demonstrated that IDO has a role in tumor immune escape (Isla Larrain et al., 2014). In 2015, Salvadori et al. observed decreased expression of IDO through a combination of 1-MT with paclitaxel in cancer cell cultures (Salvadori et al., 2015).

Researchers from South Korea recently established that expression of IDO might reflect high mutational loads in TNBCs (Kim et al., 2017). Li et al found out that IDO and IL-6 expression are linked with advanced stages of the disease and are also involved in poor response of the tumor towards chemotherapy (Li et al., 2017). Noonepalle et al conducted a study on breast cancer cell lines and concluded that

methylation of IDO1 promoter is involved in regulation of anti-immune responses in breast tumors and can be employed as a predictive biomarker for IDO based immunotherapy (Noonepalle et al., 2017).

Chapter 3

Materials and Methods

Expression and activity of IDO breast cancer was examined through immunohistochemistry and colorimetric assay respectively (Figure 3.1). For statistical analysis, chi-square, T-test and linear regression were performed.

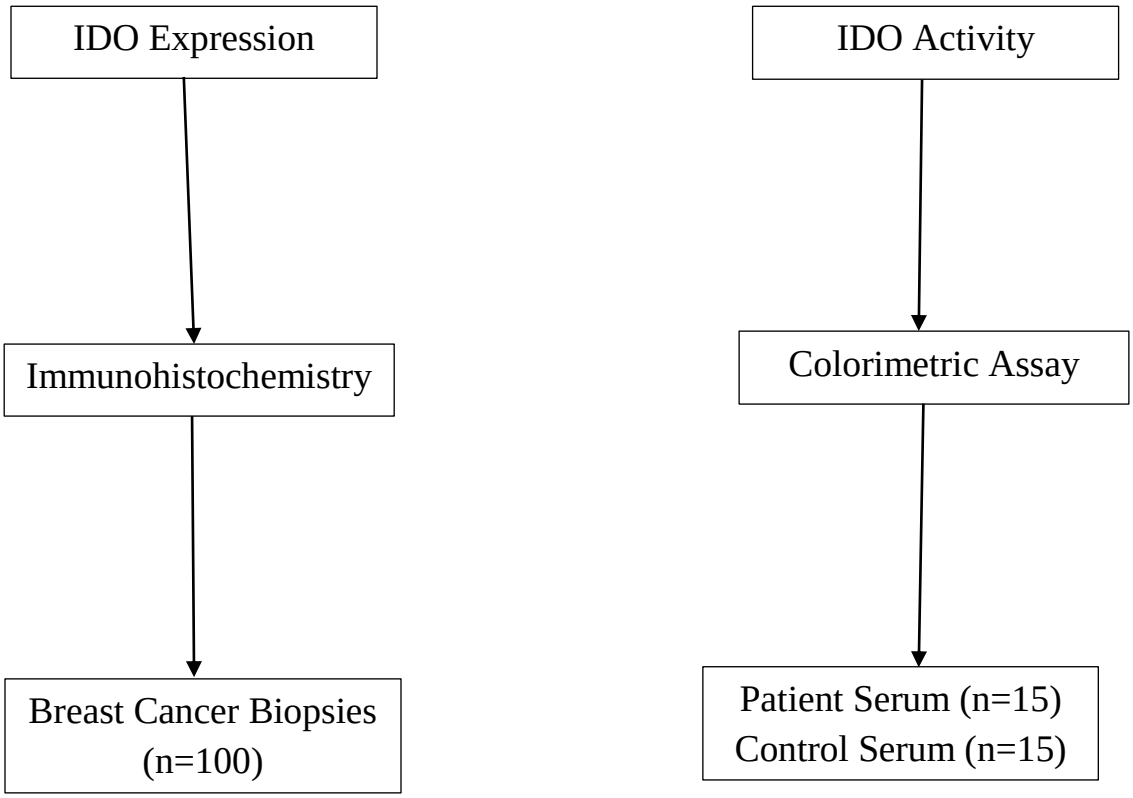

Figure 3.1 Experimental Methodologies. Expression of IDO was examined in cancer biopsies by immunohistochemistry. Activity of IDO was analyzed by colorimetric assay in patients and healthy controls.

3.1 Patients

One hundred breast cancer biopsies were retrieved from Pathology Department at Shaukat Khanum Memorial Cancer Hospital and Research Centre (SKMCH&RC), Lahore from 2007 to 2008 were randomly selected from clinical research database. Patients were at least 18 years of age, had no history of breast cancer, hepatitis B virus infection, hepatitis C virus infection, human immune deficiency virus infection and were treatment naïve.

Mean age of patients was 48.2 ± 11.8 years and the range was 23 – 75 years. 88% percent patients were from Punjab, 7% patients were from Khyber Pakhtoon Khwa, 3% patients were from Kashmir and 2% patients were from Sindh. All of the patients were female. Patients exhibited ductal carcinoma (91%), mammary carcinoma (6%), lobular carcinoma (2%) and metaplastic carcinoma (1%). 61.5% patients had grade 3 breast cancer and 38.5% patients had grade 2 breast cancer. 7 (11%) patients had T1 tumors and 57 (89%) patients had T2/T3 tumors. Tumor was metastized to lymph nodes in 56.3% patients. Estrogen receptors, progesterone receptors and human epithelial growth factor receptors were negative in 69%, 74% and 74% patients respectively. 49% patients had triple negative breast cancer. The data of clinico-pathologic characteristics of breast cancer patients is summarized in table 3.1.

Characteristic	Division	Value
Age at onset of breast cancer (Years)	Mean ± S.D.	48.2 ± 11.8
	Range	23-75
Region	Punjab	88 (88%)
	KPK	07 (7%)
	Kashmir	03 (3%)
	Sindh	02 (2%)
	Total	100 (100 %)
Gender	Female	100 (100%)
	Total	100 (100%)
Histology	Ductal	91 (91%)
	Mammary	06 (6%)
	Lobular	02 (2%)
	Metaplastic	01 (1%)
	Total	100 (100%)
Tumor Grade	3	56 (61.5%)
	2	35 (38.5%)
	Total	91 (100%)
Tumor Size	T1	07 (11%)
	T2/T3	57 (89%)
	Total	64 (100%)
Metastasis	Positive	49 (56.3%)
	Negative	38 (43.7%)
	Total	87 (100%)
ER Status	Positive	31 (31%)
	Negative	69 (69%)
	Total	100 (100%)
PR Status	Positive	26 (26%)
	Negative	74 (74%)
	Total	100 (100%)
HER2 Status	Positive	26 (26%)
	Negative	74 (74%)
	Total	100 (100%)
TNBC	Positive	49 (49%)
	Negative	51 (51%)
	Total	100 (100%)

Table 3.1 Clinico-pathologic characteristics of breast cancer patients.

3.2 Immunohistochemistry

Formalin fixed paraffin embedded (FFPE) sections of breast cancer patients were obtained from Pathology department of SKMCH&RC. Tonsils biopsies were taken as standard positive controls. Immunohistochemistry (IHC) was performed on automated Leica systems (Leica, Biosystems Melbourne, Australia). Primary antibody was mouse anti human antibody obtained from Abcam (ab55305). The dilution was 1:200. IDO labeling was visualized by Bond polymer kit and counterstained by hematoxylin. Slides were washed at each step with Bond wash buffer (AR 9590). The major steps are as follows:

1. Brief deparaffinization with Bond dewax solution (AR922) (20 min)
2. Primary antibody incubation (5 min)
3. Incubation with peroxidase block (5 min)
4. Post primary antibody (rabbit anti-mouse IgG) incubation (8 min)
5. Application of DAB (10 min)
6. Hematoxylin and eosin stain

Slides were visualized under microscope at x40 magnification. Numeric scoring of slides was performed by pathologist. Slides were scored for intensity of staining (0, 1, 2 and 3) and percentage of cells stained positive (1= 1 – 33%, 2= 34 – 66% and 3= >66%). The product of two scores was the final staining score as follows:

1. Negative = 0
2. Low = 1 – 3

3. Medium = 4 – 6
4. High = 7 – 9

3.3 Colorimetric Assay

Colorimetric assay was performed to analyze the activity of IDO in plasma of breast cancer patients and healthy controls. Three solutions were made as follows:

i. Solution A: 30% trichloroacetic acid (3g in 10ml glacial acetic acid)
ii. Solution B: Serum from patients and healthy controls
iii. Solution C: 200mg echlerich reagent in 10ml glacial acetic acid

100µl of solution A was added in 200µl of solution B. The mixture was centrifuged at 14000 revolutions per minute (rpm) for 3 minutes at room temperature. 125µl of supernatant was taken afterwards and poured in a 96 well micro-plate. Same procedure was repeated for all the samples. 125µl of solution C was added in every well containing the supernatant. The plate was read at 492nm through ELISA reader.

3.4 Statistical Analysis

Statistical analysis to compare IDO expression was performed on SPPS. (version 20.0, USA). Chi-square (bivariate analysis) was used to determine significant differences between different categorical variables. Independent t-test was used for continuous variables. Significant variables were analyzed through multivariate analysis to determine the associations. Kaplan-Meier curve was used to analyze survival of patients and the differences were analyzed by Breslow test. Unpaired t-test

was performed on GraphPad Prism (version 6) to calculate difference of IDO activity in breast cancer patients and healthy controls. p-value less than 0.05 was considered significant for all analyses.

Chapter 4

Results

4.1 Immunohistochemistry for IDO expression

All of the patients were stained positive for IDO expression. Out of 100 patients, 24 patients had low IDO expression, 27 had medium IDO expression and 49 had high IDO expression (Figure 4.1). The staining was generally cytoplasmic and detected in malignant cells (Figure 4.2).

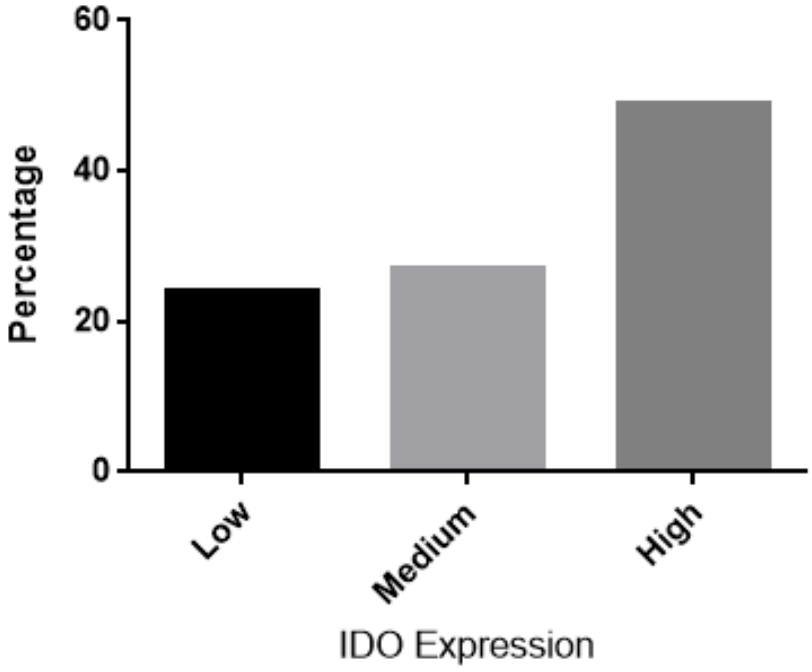

Figure 4.1 Histogram of IDO expression. 24% breast cancer patients had low IDO expression, 27% breast cancer patients had medium IDO expression and 49% breast cancer patients had high IDO expression. None of the patients was negative for IDO

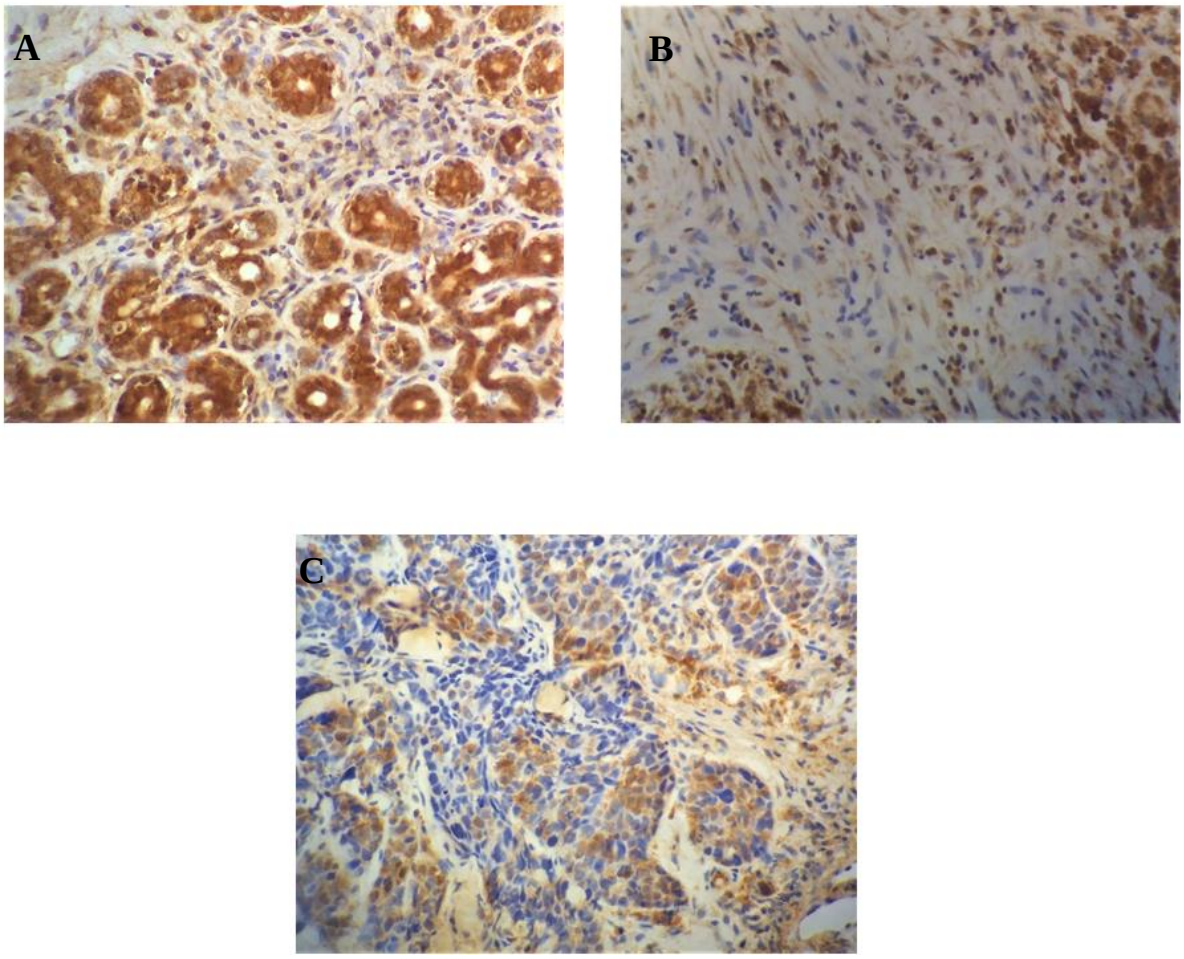

Figure 4.2 Expression of IDO in breast cancer biopsies. The IDO expression was observed in breast cancer tissues taken from patients (n=100) using anti-IDO antibody. **A** represents high IDO expression. **B** reveals medium IDO expression. C represents low IDO expresion. Images were captured at x40 magnification.

4.2 Clinicopathologic Characteristics of Patients

Several clinical and pathologic characteristics of breast cancer patients were analyzed to discover their relationship with IDO expression. Univariate statistical analyses were performed. No relationship was observed between age at onset of breast cancer, demographic region of patients, tumor size, tumor grade, metastasis, ER, PR and HER2 (Table 4.1).

Variable	**Level**	**N (%)**	**IDO Scores**			**p-value**
		Total	Low	Medium	High	
Tumor Size	T1 T2/T3 Total	7 (11%) 57 (89%) 64 (100%)	03 (42.9%) 15 (26.3%) 18 (28.1%)	01 (14.3%) 17 (29.8%) 18 (28.1%)	03 (42.9%) 25 (43.9%) 28 (43.8%)	0.565
Metastasis	Negative Positive Total	38 (43.7%) 49 (56.3%) 87 (100)	10 (26.3%) 12 (24.5%) 22 (25.3%)	7 (18.4%) 16 (32.6%) 23 (26.4%)	21 (55.3%) 21 (42.8%) 42 (48.3%)	0.309
Tumor Grade	2 3 Total	35 (38.5%) 56 (61.5%) 91 (100%)	10 (28.6%) 12 (21.4%) 22 (24.2%)	10 (28.6%) 11 (19.6%) 21 (23.1%)	15 (42.6%) 33 (58.9%) 48 (52.7%)	0.324
Estrogen Receptor	Negative Positive Total	69 (69%) 31 (31%) 100 (100%)	15 (21.7%) 09 (29%) 24 (24%)	06 (8.7%) 11 (35.5%) 27 (27%)	38 (55.1%) 11 (35.5%) 49 (49%)	0.188
Progesterone Receptor	Negative Positive Total	74 (74%) 26 (26%) 100 (100 %)	18 (24.3%) 06 (23.1%) 24 (24%)	17 (23%) 10 (38.5%) 27 (27%)	39 (52.7%) 10 (38.5%) 49 (49%)	0.284
HER2 Receptor	Negative Positive Total	74 (74%) 26 (26%) 100 (100%)	17 (23%) 7 (26.9%) 24 (24%)	19 (25.7%) 08 (30.8%) 27 (27%)	38 (51.3%) 11 (42.3%) 49 (49%)	0.729
Triple Negative Breast Cancer	No Yes Total	51 (51%) 49 (49%) 100 (100%)	14 (27.4%) 10 (20.4%) 24 (24%)	20 (39.2%) 07 (20.4%) 27 (27%)	17 (33.3%) 32 (65.3%) 49 (49%)	**0.003**

Table 4.1 Univariate analysis of patients' characteristics. Bold values represent statistical significance

Triple negative breast cancer was associated with higher IDO expression (OR= 8.5, 98% CI= 2.07 – 34.8, p-value= 0.003) (Table 4.2).

Effect	OR (95% CI)	p value
TNBC – Yes versus No	8.04 (1.7 – 39.02)	**0.01**

Table 4.2 Multivariate logistic regression for IDO scores. Bold p-values represent statistical significance.

4.3 Survival Analysis

Survival analysis was performed to analyze its relationship with IDO expression. The comparison was done between IDO low, medium and high groups. There was a significant difference between the three groups (p= 0.04) (Figure 4.3). Patients with higher IDO expression had lower mean survival time as compared to patients with lower and medium IDO expression (Table 4.3).

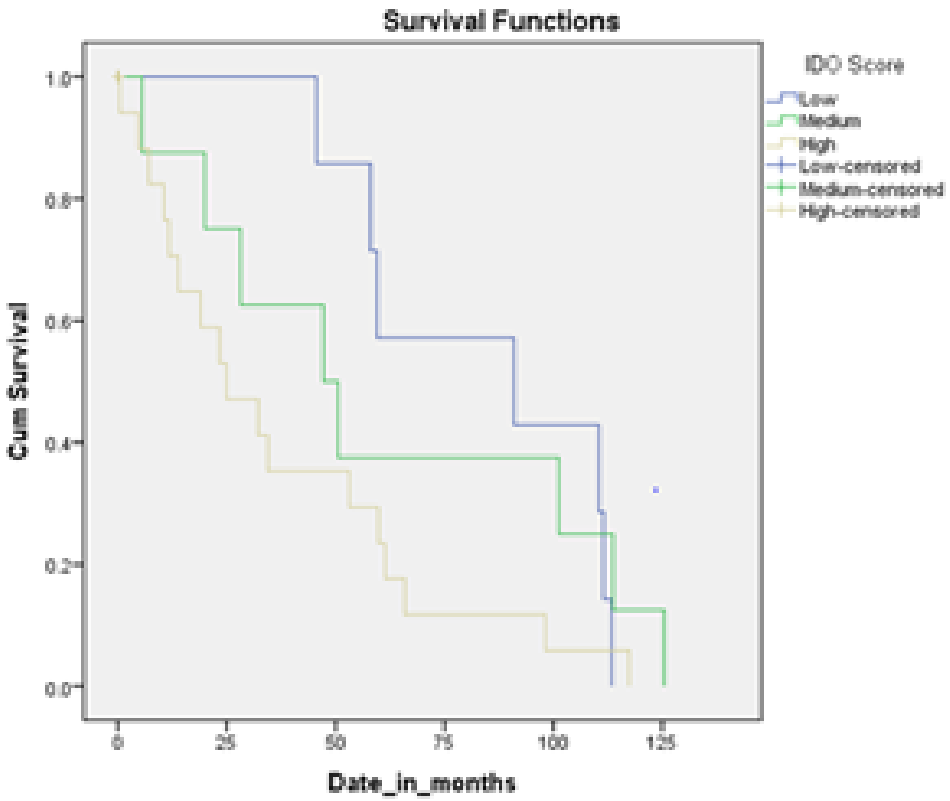

Figure 4.3 Overall survival stratified by IDO score.
p = 0.04

IDO Score	Mean Time (Months)	
	Estimate	**Std. Error**
Low	84.262	11.033
Medium	61.521	16.189
High	37.577	8.191
Overall	53.765	7.064

Table 4.3 Mean survival time (months) with low, medium and high IDO expresison

4.4 IDO activity in patients versus healthy controls through colorimetric assay.

Enzymatic activity of IDO was compared between breast cancer patients (n=15) and healthy controls (n=15). A significant difference ($p < 0.0001$) was observed between the two groups (Figure 4.4).

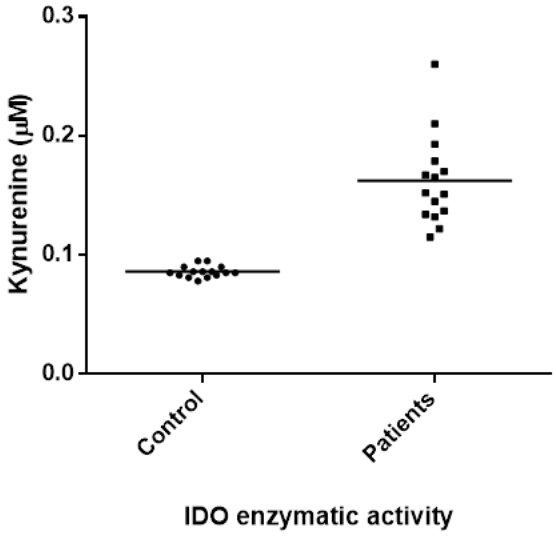

Figure 4.4 IDO enzymatic activity in breast cancer patients and healthy controls. A significant difference ($p<0.0001$) is present among the two groups.

Chapter 5

Discussion

Complexity of tumors has been a part of rigorous investigation for over a decade now and it has been established that, to comprehend the biology of tumor, it is important to understand a detailed structure of tumor and its surroundings (Hanahan and Weinberg, 2011). Tumor microenvironment, which is constituted along the complex process of tumorigenesis, contains multiple cells of diverse origins (Pilotte et al., 2012). Cellular and chemical mediators of inflammation play a critical role in development and progression of tumors (Mantovani et al., 2008). Inflammatory conditions are either present before the onset of cancer or a genetic mutation leads to induction of inflammatory environment which stimulates the development of cancer (Mantovani et al., 2008). Owing to the complex nature of immune responses, the type of immune response provoked by the immune system against a cancer can either be anti-tumorigenic or pro-tumorigenic (DeNardo and Coussens, 2007).

IDO expression in breast cancer has been analyzed by multiple researchers through animal models, in silico analyses and human samples. Kim et al. recently established that expression of IDO might reflect high mutational loads in TNBCs (Kim et al., 2017). Li et al found out that IDO and IL-6 expression are linked with advanced stages of the disease and are also involved in poor response of the tumor towards chemotherapy (Li et al., 2017). On the other hand, Jacquemier et al observed favorable prognosis in patients expressing higher levels of IDO (Jacquemier et al., 2012). A study by Soliman et al in 2013 associated favorable outcomes with increased

IDO expression in estrogen receptor positive breast cancers as compared to estrogen receptor negative breast cancers (Soliman et al., 2013). Due to the complexity of subject matter and contradictory results of various studies, we sought out to establish the expression and activity of IDO in breast cancer patients from Pakistan.

The major finding of this pilot study from Pakistan is that breast cancer patients overexpress IDO and the activity of IDO is higher in breast cancer patients as compared to healthy people ($p<0.0001$). Overall survival of breast cancer patients might decrease with increased expression of IDO ($p=0.04$). This study could not establish any significant difference between IDO expression and clinical and pathological factors of breast cancer patients. The results of this study support the fact that IDO expression in TNBC might play a role in reducing the survival rate of TNBC patients. This study shows that TNBCs expressed higher concentrations of IDO as compared to Non-TNBCs (0.01). A similar study was conducted by Kim et al. where they proposed that hindering IDO expression in TNBC patients might be a better treatment option (Kim et al., 2017).

Why IDO expression is enhanced in TNBC patient is unknown. It might be the result of binding of Kyn with AhR leading to enhanced proliferation of Foxp3+ T-regs (Mezrich et al., 2010), reduction in immunogenicity of DCs (Nguyen et al., 2010) and suppression of anti-tumoral immune responses (Opitz et al., 2011). IDO expression in cancer is a part of multifaceted network of immune regulatory pathways which can both enable and obstruct tumor development and progression (Soliman et al., 2013).

Factors such as type of cancer, stage of malignancy and host environment decide whether IDO would facilitate or hinder tumor progression (Soliman et al., 2013).

Therefore, identifying role of IDO at individual steps of development and progression of various tumors is imperative to determine ideal targeting of this pathway. This study suggests that IDO inhibition in breast cancer patients might lead to a better disease prognosis.

Chapter 6

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Annexure A

TNM Staging System for Breast Cancer*	
Primary Tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
Tis (DCIS)	Ductal carcinoma in situ
Tis (LCIS)	Lobular carcinoma in situ
Tis (Paget)	Paget's disease of the nipple with no tumor
T1	Tumor ≤ 2 cm in greatest dimension
T1 mic	Micro-invasion ≤ 0.1 cm in greatest dimension
T1a	Tumor > 0.1 cm but not > 0.5 cm in greatest dimension
T1b	Tumor > 0.5 cm but not > 1 cm in greatest dimension
T1c	Tumor > 1 cm but not > 2 cm in greatest dimension
T2	Tumor > 2 cm but not > 5 cm in greatest dimension
T3	Tumor > 5 cm in greatest dimension
T4	Tumor of any size with direct extension to (a) chest wall or (b) skin
T4a	Extension to chest wall, not including pectoralis muscle
T4b	Edema (including peau d'orange) or ulceration of the skin of the breast, or satellite skin nodules confined to the same breast
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed (e.g., previously removed)
N0	No regional lymph node metastasis

N1	Metastasis in movable ipsilateral axillary lymph node(s)
N2	Metastases in ipsilateral axillary lymph nodes fixed or matted, or in clinically apparent ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastasis
N2a	Metastasis in ipsilateral axillary lymph nodes fixed to one another (matted) or to other structures
N2b	Metastasis only in clinically apparent ipsilateral internal mammary nodes and in the absence of clinically evident axillary lymph node metastasis
N3	Metastasis in ipsilateral infraclavicular lymph node(s), or in clinically apparent ipsilateral internal mammary lymph node(s) and in the presence of clinically evident axillary lymph node metastasis; or metastasis in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement
N3a	Metastasis in ipsilateral infraclavicular lymph node(s) and axillary lymph node(s)
N3b	Metastasis in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
N3c	N3c Metastasis in ipsilateral supraclavicular lymph node(s)
Regional lymph nodes (pN)	
pNX	Regional lymph nodes cannot be assessed (e.g., previously removed or not removed for pathologic study)
pN0	No regional lymph node metastasis histologically, no additional examination for isolated tumor cells
pN0(i-)	No regional lymph node metastasis histologically, negative IHC
pN0(i+)	No regional lymph node metastasis histologically, positive IHC, no IHC cluster > 0.2 mm
pN0(mol-)	No regional lymph node metastasis histologically, negative molecular findings (RT-PCR)

pN0(mol+)	No regional lymph node metastasis histologically, positive molecular findings (RT-PCR)
pN1mi	Micrometastasis (> 0.2 mm, none > 2.0 mm)
pN1	Metastasis in one to three axillary lymph nodes and/or in internal mammary nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent
pN1a	Metastasis in one to three axillary lymph nodes
pN1b	Metastasis in internal mammary nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent
pN1c	Metastasis in one to three axillary lymph nodes and in internal mammary lymph nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent
pN2	Metastasis in four to nine axillary lymph nodes, or in clinically apparent internal mammary lymph nodes in the absence of axillary lymph node metastasis
pN2a	Metastasis in four to nine axillary lymph nodes (at least one tumor deposit > 2.0 mm)
pN2b	Metastasis in clinically apparent internal mammary lymph nodes in the absence of axillary lymph node metastasis
pN3	Metastasis in 10 or more axillary lymph nodes, or in infraclavicular lymph nodes, or in clinically apparent ipsilateral internal mammary lymph nodes in the presence of one or more positive axillary lymph nodes; or in more than three axillary lymph nodes with clinically negative microscopic metastasis in internal mammary lymph nodes;

pN3a	or in ipsilateral supraclavicular lymph nodes
pN3b	Metastasis in 10 or more axillary lymph nodes (at least one tumor deposit > 2.0 mm), or metastasis to the infraclavicular lymph nodes Metastasis in clinically apparent ipsilateral internal mammary lymph nodes in the presence of one or more positive axillary lymph nodes; or in more than three axillary lymph nodes and in internal
pN3c	mammary lymph nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent Metastasis in ipsilateral supraclavicular lymph nodes
Distant Metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

*Adapted from American Joint Committee on Cancer

Annexure B

Tumor Grading System*	
Glandular/ Tubular Differentiation	
Score 1	>75% of tumor area forming glandular/tubular structures
Score 2	10% to 75% of tumor area forming glandular/tubular structures
Score 3	<10% of tumor area forming glandular/tubular structures
Nuclear Pleomorphism	
Score 1	Nuclei small with little increase in size in comparison with normal breast epithelial cells, regular outlines, uniform nuclear chromatin, little variation in size
Score 2	Cells larger than normal with open vesicular nuclei, visible nucleoli, and moderate variability in both size and shape
Score 3	Vesicular nuclei, often with prominent nucleoli, exhibiting marked variation in size and shape, occasionally with very large and bizarre forms
Mitotic Count	
Score 1	≤ 7 mitoses per 10 high power fields
Score 2	8-14 mitoses per 10 high power fields
Score 3	≥ 15 mitoses per 10 high power fields

*Adapted from (Elston and Ellis, 2002)