

**STATUS OF THE PLATELET DERIVED GROWTH FACTOR RECEPTOR  
ALPHA IN INFLAMMATORY BREAST CANCER PATIENTS**

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

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## **ABSTRACT**

IBC is arguably the most deadly form of breast cancer due to its unique development including the lack of formed lumps, having symptoms similar to inflammation, and also due to its rapid and aggressive invasion. IBC occurrence and incidence are high at younger ages and also in some ethnic groups like African American women and North African women. Diagnosis of IBC can be made clinically if the patient shows symptoms such as swelling, orange-like skin appearance, redness, nipple inversion, breast discharges, rapid onset of the disease, young age, and the duration of the symptoms is less than 6 months. Another type of diagnosis is pathologic diagnosis where there is emboli invasion in lymphatic vessels of the skin overlying the breast and the lack of a palpable tumor. In a previous study by my laboratory we demonstrated that the IBC gene profile shows high level expression of platelet derived growth factor receptor alpha (PDGFRA), a receptor tyrosine kinase. This overexpression stimulates cell oncogenesis. My project focuses on determining the expression, localization, and phosphorylation status of PDGFRA in patient samples, as well as determining PDGFRA's role as an effective target to induce sensitivity to chemotherapy drugs and overcome chemotherapy drug resistance. A third focus of this project is to evaluate the significance that PDGFRA contributes in IBC cell line growth, emboli formation, and invasion rate.

## **Chapter 1**

### **INTRODUCTION**

#### **1.1 Breast Biology**

##### **1.1.1 Breast Tissue Anatomy**

The location of the breast is between the sternal edge and midaxillary line in the horizontal axis, and between the second and sixth ribs vertically. The breast lies on the deep pectoral fascia, and the superficial pectoral fascia covers the breast. The breast is held by Cooper suspensory ligaments which are fibrous bands connecting the pectoralis major fascial. The serratus anterior muscle provides support for the breast and allows the breast to move away from the chest wall. As illustrated in Figure1-1, the breast is composed of three major tissues: skin, subcutaneous tissue, and breast tissue. The skin contains hair follicles, sebaceous glands, and exocrine sweat glands. The nipple contains nerves ending beside the sebaceous glands, and exocrine sweat glands. The areola area is measured to be in the range of 15 to 60 mm in diameter. The Morgagni tubercles which are located around the areola are formed because of the notch of two kinds of glandular ducts: large sebaceous glandular ducts, which are in a phase in between mammary and sweat glands, and Montgomery glandular ducts. The second component of the breast is subcutaneous tissue, and the third one is the breast tissues composed of 75% stromal elements, 15% of breast tissue overall is epithelial tissue. The epithelial tissue forms ducts and almost 20 lobes separated from each other by adipose tissue. The lobes branch into 20 to 40 lobules. Ducts connect lobes and each of the



lobules to drain into the lactiferous duct. The lactiferous ducts drain into the lactiferous sinus located underneath the areola area of the breast, and then open into the nipple <sup>1, 2, 3</sup>.

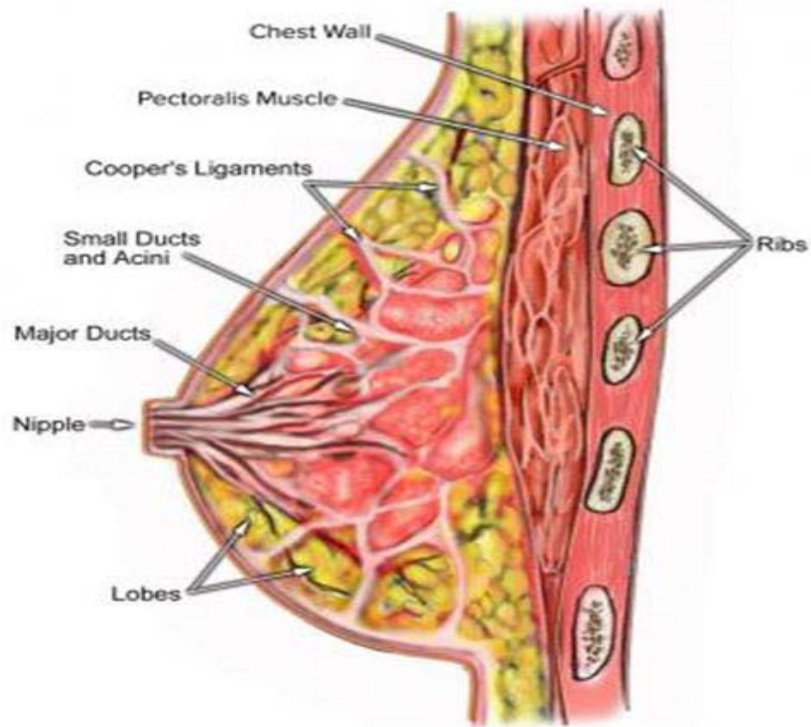


Figure 1-1 Human Breast anatomy , and different tissue that the breast composed of. Adapted from <http://reference.medscape.com/article/1273133-overview>

### 1.1.2 Blood Supply And Lymphatic System

Understanding the vascular system of the breast is important to the understanding of breast cancer metastasis and contributes to improving breast cancer treatment by choosing effective spots to inject drugs. Nutrition is supplied to the breast through a major artery called the subclavin artery that branches to the major systems of the breast blood supply systems shown in figure1-2 (A). The subclavin artery is a favorable position to inject drugs to treat breast cancer because most of the breast's

blood arteries are branching from the subclavian artery. There are three major blood systems of the breast. First is the axillary system of which one of its direct branches is the lateral thoracic artery that then branches to the lateral mammary artery found at 10 to 20 mm depth and runs the axillary tail. The lateral mammary artery branches to small nonmammary branches that supply blood to muscle and skin. The axillary system also forms other minor branches. The second system of breast blood arteries are the internal thoracic arteries that perforate rami and give direct branches to anterior medial mammary arteries and posterior medial arteries. The posterior medial arteries are found at 5 to 15 mm depth and its small branches merge with anterior cutaneous nerves. The third system of breast blood supply is called intercostals (posterior, aortic intercostals arteries). The small branches of the lateral thoracic artery, lateral mammary artery, and posterior medial arteries join together to form a plexus. There is an artery that runs between the pectorals major and minor muscle called the pectoral branch of the thoracodorsal artery, also known as the supreme thoracic artery <sup>4</sup>.

The veins of the breast illustrated in figure 1-2 (B) are classified into the superficial system, and the deep system, and the blood flows from the deep to superficial and drain into the axillary system, and thoracic and cephalic vein. The deeper system is connected with arteries. The superficial venous system includes the subareolar veins transferring blood from the nipple to the periareolar vein. The periareolar vein carries the blood from the deeper veins and the subcutaneous veins external to the areola <sup>4</sup>.

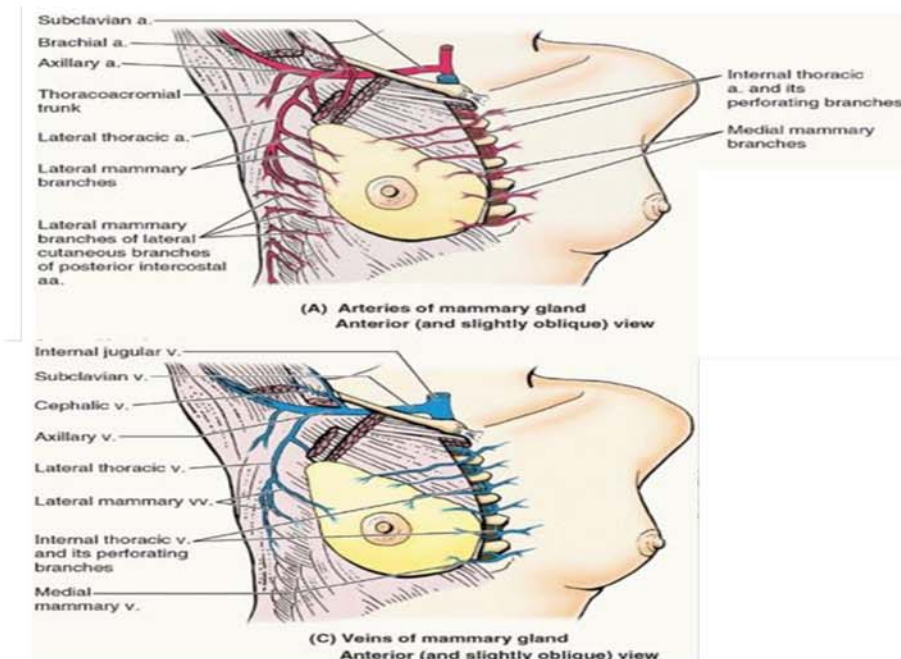


Figure 1-2: The breast vascular system including arteries , and veins modified and adapted from <http://medicoreview.blogspot.com/2012/05/blood-supply-of-female-reproductive.html>

The breast lymphatic system; figure 1-3, is important to understand because it has a significant role in breast cancer diagnostic and treatment. The doctors can predict whether the cancer cells already metastasized to the axillary lymph nodes by sentinel node biopsy (SNB). The lymphatic vessels have a smooth muscle layer. There are three lymphatic systems in the breast depending on their position of the deep fascia: the superficial lymphatic system, perforating lymphatic system, and the internal lymphatic system. The superficial lymphatic system is composed of lymphatic collectors found in the subcutaneous tissue. Lymphatic collectors branch to other lymphatic vessels in the peripheral region in close proximity to peripheral edges of the breast and lateral border of the sternum. The lymphatic collectors will unite again and become more equalized in

diameter size when reaching the lymph node. It is also observed that almost all of the lymph vessels located in the subcutaneous tissue drain into one axillary node before draining in the other node. In the dermal layer, the areola and nipple area, the lymphatic precollector vessels, and lymph capillaries become more dense and form a subareolar lymphatic capillary plexus. The second lymphatic system, perforating lymphatic system, has some similarities in morphological features with the superficial lymphatic system, and it connects the deep internal lymphatic system with thoracic blood vessels. The deepest part of the breast lymphatic system is the internal mammary lymphatic system which is found to be associated with the internal mammary artery and vein <sup>5,6,7</sup>.

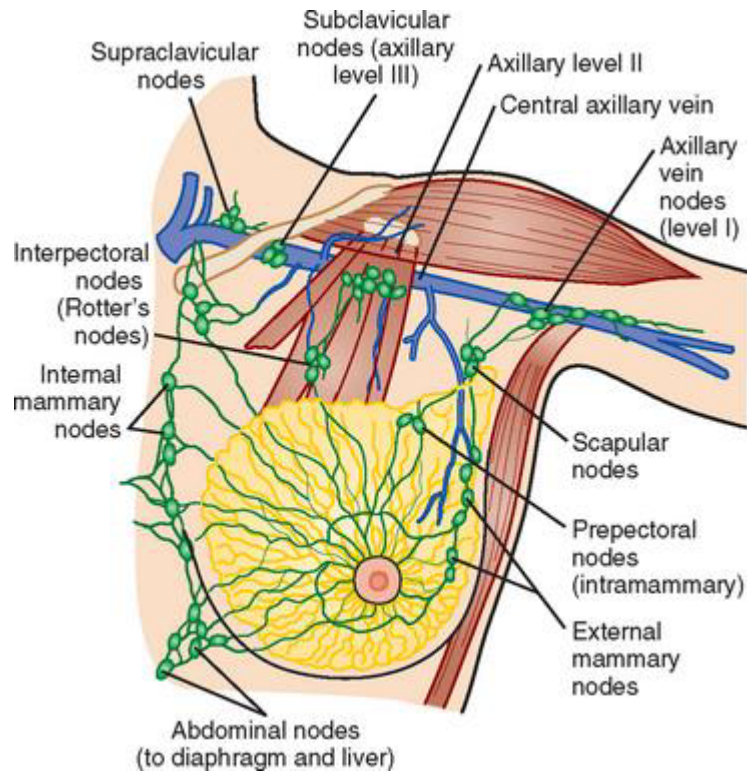


Figure 1-3: The lymphatic system of the breast adapted from <http://clinicalgate.com/local-staging-imaging-options-and-core-biopsy-strategies/>

### 1.1.3 Breast Development.

The development of the breast starts during the fifth or sixth week of fetal development where two ventral bands of ectoderm and mammary ridges exist. The mammary ridges extend from the axillary to the inguinal regions and disappear when development progresses with the exception of some pectoral regions. The breast bud is a mammary noticeable growth of the epidermis from the mammary mesenchyme layer. The formation of the primary mammary bud causes the formation of almost 20 secondary breast buds. The secondary buds will develop the lactiferous ducts and their branches. Main lactiferous ducts open into a mammary pit forming the nipple. At birth

the major lactiferous ducts will be developed, but the mammary glands will not develop until puberty. The mammary gland development will be triggered by the ovarian hormones including progesterone and estrogen. The development of the mammary glands will result in enlargement of the female breast size during puberty. In pregnancy, because of the influence of the progesterone, estrogen, placental hormones, prolactin, and growth hormones, the breast reaches complete development including enlargement in the size and density. During pregnancy, the breast is prepared for the physiological function by the veins expanding and growing at the first stage. Following that, the last stage of pregnancy includes epithelium differentiation resulting in the formation of the secretory cells that produce and secrete milk. During menopause, the breast degenerates as a result of the mammary glands and ductal regression. Aging also causes a decreased number of not only the epithelial cells forming mammary glands and ducts, but also the number of mammary glands and ducts. Aging also decreases the amount of fat the breast contains and stromal elements leading to breast reduction. The decrease in Cooper suspensory ligaments is the cause of breast ptosis<sup>2</sup>.

## **1.2 Breast Physiological Function**

The breasts go through different stages of development and based on each stage, the breast will have specific changes. During pregnancy, they go through different stages of preparation for secreting milk proteins by the time of delivery. In the first trimester of the pregnancy, the breasts become larger, veins expand, and the nipple and areola areas develop extra pigmentation. In this stage, the proliferation of the glandular epithelium will result in renewing the stromal elements. The differentiation process of epithelial cells will lead to the formation of secretory cells that formulate milk and is influenced by prolactin, growth factors, and insulin levels. The lactation initiation will

be influenced by the levels of estrogen and progesterone. The regulation of milk secretion will be linked to the neural response for sucking that causes oxytocin release <sup>8</sup>

### **1.3 Breast Cancer**

Breast cancer is the most common malignant disease affecting women. Breast cancer incidence is classified as the second cause associated with cancer deaths in women. The statistics show that 1 out of 8 women could develop breast cancer during their lifespan. Women developing breast cancer may not notice any changes in the initial stages of breast cancer. In some cases, the patient can feel a painless lump in the breast as the initial indicator of tumor growth. Other symptoms can present as breast skin changes like redness, dimpling, itching, and skin irritation. Other symptoms include breast swelling in all or some parts, pain in the breast, pain in the nipple, and nipple discharges other than milk <sup>9</sup>.

There are many causes behind breast cancer development, some of them described clinically for the individual trait, for instance, childbearing age, increasing age, and mutations in BRCA1 or BRCA2 genes. Other reasons include cultural or environmental causes with different grade of association, for instance, smoking, alcohol consumption, and diet have an association with breast cancer <sup>10</sup>. There are many procedures that could be taken in order to prevent breast cancer. Primary prevention of breast cancer includes genetic screening for the patient and chemoprophylaxis using tamoxifen which reacts with estrogen to prevent growth promotion in women who may develop breast cancer <sup>10</sup>. Secondary breast cancer prevention is based on clinical breast examination (CBE) screening, Breast self-examination (BSE) screening, and mammography <sup>11,12,13</sup>. The advantage of primary and secondary screening for the

patient is either prevention of developing cancer or starting early treatment during the early stages of breast cancer. This raises high survival rate and decreases breast cancer mortality. For instance, in primary prevention through genetic screening, women with a mutation in BRCA1 or BRCA2 can have breast conserving surgery instead of breast mastectomy in order to prevent developing breast cancer. One of the benefits to minimizing the risk is that early prevention or early detection can give the patients wider choices like having sentinel lymph node biopsy as an alternative to axillary lymph nodes removal<sup>14, 15, 16</sup>.

Breast cancer can be invasive or noninvasive depending on the tumor spread to surrounding tissues and organs. The breast cancer initiates from breast ducts or breast lobules. The noninvasive ductal cancer is called ductal carcinoma in situ (DCIS). Another form of noninvasive breast cancer is lobular carcinoma in situ (LCIS). The invasive forms of invasive breast cancer can also initiated from breast ducts called invasive ductal carcinoma (IDC) or initiated from lobules called invasive lobular carcinoma (ILC), or can be generalized as mammary carcinoma if it contains both invasive forms of breast cancer<sup>17</sup>.

Breast cancer screening is recommended for every women with average risk of developing breast cancer. This screen starts with breast self-examination (BSE) screening where the women have to examine any lump existence in the present. Also women between ages 20-39 undergo clinical breast examination (CBE) every 3 years, and every year for women at age 40 or older. It is also recommended for women at age 40 to have annual mammography. In some cases, mammography screening has a poor prognosis because it cannot provide a clear diagnosis, so magnetic resonance imaging (MRI) is highly recommended for women carrying mutations in BRCA1,2 genes which



are tested with genetic screening<sup>16</sup>. There is also a histologic diagnostic system known as the Bloom and Richardson histologic grading system<sup>17</sup>. The Bloom and Richardson system was built on evaluating three factors: frequency of hyper chromatin and mitotic figures, the degree of differentiation, and the dissimilarity of nuclei staining, cell size, and cell shape. Based on the evaluation of those factors, the tumor will be classified as low (Grade I), intermediate (grade II), or high (Grade III)<sup>18, 19</sup>.

Another system to grade the breast cancer is known as TNM system to evaluate the cancer spread when diagnosed. This is important and necessary to highlight a treatment plan and estimate prognosis. This system evaluates the tumor size, lymph node invasion, and metastasis to other tissues. The metastasis can be determined by a blood test, magnetic resonance imaging (MRI), positron emission tomography (PET), biopsy, computed tomography (CT), x-ray, bone scan, and mammograms. The TNM system (table1-1) classifies the tumor based on three categories: T describes the tumor size and invasion to the chest wall or skin. T has values from 0 to 4 and larger values indicates larger tumor and higher spreading. The second category (N) is about detecting lymph node invasion, and it is scored from 0 to 3. The letter (M) indicates if the cancer has metastasized to distant organs other than the primary tumor initiating organ. Only 0 or 1 is given to (M) grade<sup>20</sup>.

Table 1-1: TNM staging system for breast cancer modified and adapted from <https://cancerstaging.org/references-tools/quickreferences/pages/default.aspx>

Primary tumor size (T)	Lymph node invasion (N)	Distant Metastases (M)
T0: There is no evidence of tumor	N0 No regional lymph node metastases	M0:No distant metastases
T1: Tumor size ≤ 20 mm	N1 Metastases evident in type I, II axillary lymph node(s)	cM0(i+) No evidence of distant metastases
T2: Tumor size > 20 mm but ≤ 50 mm	N2 Metastases in I, II axillary lymph nodes or internal mammary nodes with no axillary lymph node metastases	clinically, but molecularly or microscopically, there are tumor cells in circulating blood, bone marrow, or other no regional nodal tissue with size > 0.2 mm without signs of metastases
T3: Tumor > 20 mm but ≤ 50mm	N3 regardless level I, II axillary lymph node involvement, there are two Metastases in ipsilateral infraclavicular (level III axillary) lymph node(s) or in metastases in ipsilateral supraclavicular lymph node(s). or regarding level	M1: Distant organ detectable metastases measured as 0.2 mm
T4 : Tumor > 50 mm in greatest dimension	I, II axillary lymph node in ipsilateral internal mammary lymph node(s) metastases; or in internal mammary lymph node metastases	

ANATOMIC STAGE/PROGNOSTIC GROUPS			
Stage 0	Tis	N0	M0
Stage IA	T1*	N0	M0
Stage IB	T0	N1mi	M0
	T1*	N1mi	M0
Stage IIA	T0	N1**	M0
	T1*	N1**	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1*	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Another accurate diagnostic which classifies breast cancer into subtypes and helps in deciding specific treatment, is evaluating the status of hormone receptors and growth factors; for instance, evaluating the expression of estrogen receptor (ER) and progesterone receptor (PR) or human epidermal growth factor (a tyrosine kinase receptor) (Her-2). This kind of classification also helps to predict the prognosis and indicate the best treatment for a positive impact <sup>21, 22</sup>. The molecular expression of hormone receptors and growth factors define the breast tumors. Based on the gene expression patterns and cell characteristics, the breast tumors are classified into two main branches: ER negative or low expressed cluster and secondly ER positive expression cluster. The first cluster of negative or low ER expression is divided into three subtypes. First is basal like which includes expression of laminin, fatty acid binding protein7, keratin 55, 7. The second subtype of ER negative cluster is called ERBB2+ which shows high expression of ERBB2 and GRB7. The third group is named normal basal like which has low expression of luminal epithelial genes and high expression of both basal epithelial genes and adipose tissue genes. The main second cluster of positive ER expression is branched into three subtypes. The first is called Luminal A which is positive for ER, x box binding protein, and GATA binding protein 3. The second subtype is luminal B which has low expression of ER as well as other luminal specific genes. The third subtype is named luminal C which has some similarities in gene expression with ERBB2+ and the basal like subtype of the negative ER cluster except that luminal C is ER positive <sup>23, 24</sup>.

### **1.3.1 Inflammatory Breast Cancer (IBC)**

Inflammatory breast cancer (IBC) is one of the deadliest and aggressive forms of breast cancer affecting women in of younger age. IBC incidence is found to be higher

in African American women, especially in Tunisia, Egypt, and North African countries, than in Caucasian women<sup>25, 26</sup>. IBC, according to the American Joint Committee on Cancer (AJCC) staging system, is classified as a TD4 tumor<sup>27</sup>. The term inflammatory carcinoma was first used by Drs. Lee and Norman in 1924 to describe a kind of breast cancer that has inflammatory symptoms that suggest the breast is infected, known as mastitis<sup>28</sup>. The name inflammatory breast cancer was chosen for this type of cancer because the symptoms look like a breast infection including inflammation, but this appearance was as result of tumor emboli blocking the lymphatics. Prior to that name, IBC was called mastitis carcinomatosa, acute mammary carcinomatosis, carcinoma mastitoides, von Volkmann's or Wokmann's syndrome in pregnant women, lactation cancer, or acute encephaloid cancer cancer<sup>29</sup>. Clinical statistics demonstrate that women with IBC have higher risk of death measured to be around 40% compared to non IBC patients<sup>30</sup>.

There are many studies being conducted to find out what could be causing IBC or what could put the patient at high risk of developing IBC. Family history, obesity or body mass index (BMI), breast density, age, and ER status may increase risk<sup>31</sup>. There is also distance organ metastases in women diagnosed with IBC for instance, brain metastasis<sup>32</sup>.

IBC diagnostic criteria is based on clinical examination, pathological examination, hormone receptors, and HER2 expression determination<sup>27,33</sup>. It is important to have these different examinations because some IBC patients have clinical symptoms of IBC, but do not show lymphatic invasion, and others have lymphatic invasion without showing any clinical symptoms of IBC (OIC), and others have both lymphatic invasion by tumor emboli and clinical symptoms<sup>34</sup>.

The clinical diagnosis of IBC is based on rapid progression of the disease, and the IBC symptoms. Shown in Figure 1-4, patients with IBC have enlargement of the affected breast with warm sensation, erythema in at least one third of the breast, edema, peau d'orange, no palpable underlying mass, nipple inversion, nipple discharge, and rapid onset occurring in less than 6 months, <sup>27, 35</sup>. Imaging is also helpful to diagnose IBC and to determine the IBC stage as well as the treatment response. The imaging systems used include mammography to show if there is any mass in the breast or structural difference like skin thickening, and stromal density. Positron emission tomography- computed tomography (PET-CT) is very helpful to determine early metastasis of IBC. Magnetic resonance imaging (MRI) can be helpful in guiding a skin punch biopsy since it can locate part of skin abnormalities. Breast ultrasound has an advantage in displaying the breast lesions <sup>33</sup>. Some of the imaging systems can help to determine which parts of the skin is favorable for a skin punch biopsy. This pathological examination is important to identify one of the important markers of IBC which is dermal lymphatic invasion (DLI) where a pathological examination can reveal tumor emboli present in dermal lymphatic vessels <sup>36, 27</sup> as shown in figure 1-5.

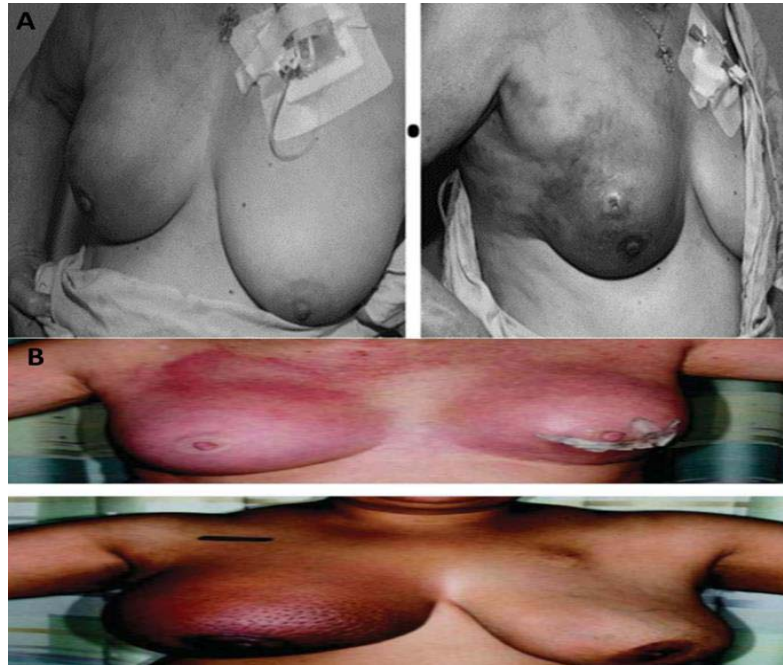


Figure 1-4: Inflammatory breast cancer symptoms: redness, skin orange peel, breast enlargement that mimicking inflammation appearance with permission from (Dawood,S.; Cristofanilli M 2015) <sup>37</sup> for panel A, and Panel B with permission from (Fredika M. Robertson et al, inflammatory breast cancer, the disease, the biology, the treatment, A cancer journal for clinicians. Wiley) <sup>38</sup>.

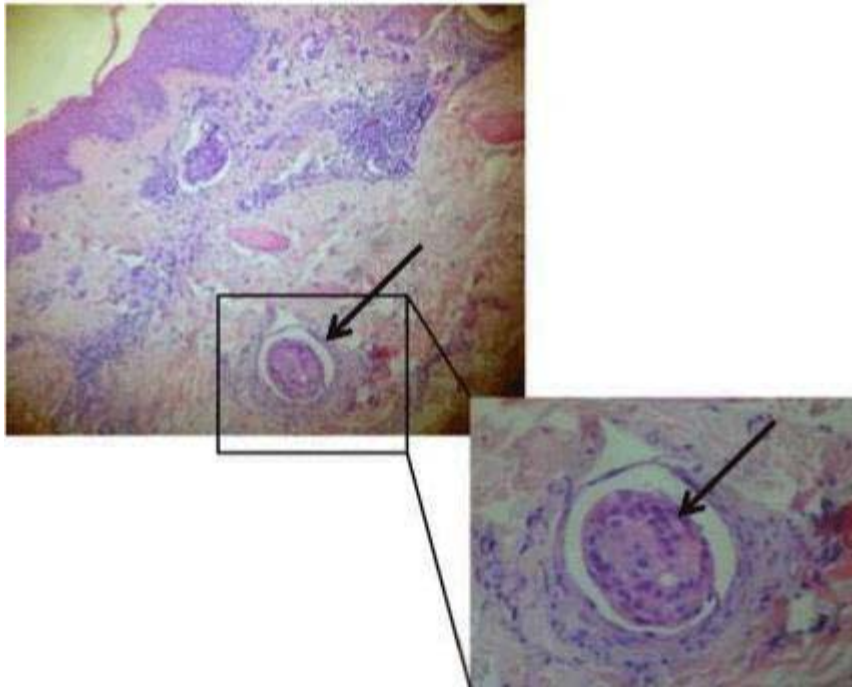


Figure 1-5: IBC on histology level showing the emboli invading the dermal lymphatics vessels adapted from (Ayman Maher et al 2012)<sup>39</sup>.

For accurate diagnosis of IBC, it is recommended to determine the status of hormone receptors and HER2 to develop an ideal treatment plan for the patient's specific IBC gene profile. IBC is one of the breast tumors with aggressive behavior and low survival rate because it lacks the expression of some the hormone receptors. Some studies show that high percentage of IBC tumors have negative expression of hormone receptors such as progesterone receptor (PR) and estrogen receptor (ER), and overexpression of Ki-67, and BAX <sup>40</sup>. As for HER2, most IBC tumors over express human epidermal growth factor- like receptor 2 (HER-2)) <sup>41, 42</sup>. Another marker for IBC which is shown to be over expressed in 90% of IBC tumors is RhoC guanosine triphosphatase (GTPase). In addition, LIBC (lost in inflammatory breast cancer) shows lack of expression <sup>43, 44</sup>.

As for IBC treatment, the international panel of IBC recommend ded that IBC patients should start chemotherapy as a first step and as a more favorable choice than surgery because there might be high chance of left behind cancer cells that can participate in recurrence with a high rate. The panel members highly recommended to use both taxane and anthracycline <sup>27, 45</sup>. In a follow-up study, Ueno et al. recommended that IBC should be treated with a chemotherapy course followed by breast mastectomy, then followed by chemotherapy then radiotherapy to improve the survival rate <sup>46</sup> . In addition to chemotherapy, surgery, and radiation, there is targeted therapy based on the gene profile of the IBC tumor. For example, a patient with Her-2 overexpression will have trastuzumab in addition to a chemotherapy course <sup>47</sup>. Other drugs used to improve the treatment and management of IBC in addition to a chemotherapy course are lapatinib to target Her-2, panitumumab targeting only epidermal growth factor receptor (EGFR), and afatinib to target Her-2 and EGFR <sup>48</sup>.

## **1.4 Platelet Derived Growth Factor Receptor Alpha (PDGFRA)**

### **1.4.1 Structure And Signaling**

Platelet growth factor receptors including platelet derived growth factor receptor alpha (PDGFRA) are considered to be members of tyrosine kinase type III family. The PDGFRA receptor is coded by gene PDGFRA on chromosome 4q12<sup>49</sup> . The receptor size is about 170 kDa<sup>50</sup>. As shown in figure1-6, the receptors of type III receptor tyrosine kinase (RTK) are commonly consisted of single spanning trans membrane domains, five extracellular domains for ligand specific binding, and split by intracellular kinase domains connected together by a polypeptide known as a linker. There are regions in the receptor responsible for sustaining the silence status of the receptor in the absence



of the ligand: the C terminal tail of the receptor, the intracellular juxtamembrane domain, and the activation loop of the kinase domain. There are two homologs of platelet derived growth factors receptor proteins, platelet derived growth factor receptor alpha (A) and beta (B), as expressed by the genes PDGFRA and PDGFRB, respectfully. There are four ligands, platelet derived growth factor A, B, C, and D that bind and activate the PDGFRs. PDGFRA is activated by the binding of PDGF A, B, or C, whereas PDGFRB is activated by ligands B and D. The PDGFRs' ligands are cell mitogens that stimulate wound healing in the adult, and play a significant role in initiating embryonic development of connective tissue<sup>51</sup>. It is also shown that the binding of the ligand motivates cell growth reforming of actin filaments, and cell motility<sup>52</sup>. The binding of the ligand to the receptor causes the two intercellular kinase domains to be dimerized, leading to transphosphorylation of tyrosine residues in the activation loop which stimulates the transphosphorylation of the juxtamembrane domain<sup>53</sup>. Ligand binding to PDGFRA leads to dimerization that causes autophosphorylation of the tyrosine residues, this will result in conformation change allowing for SH2 domain activation and starts signaling transduction. Signaling transduction is important for activating protein kinase C (PKC) and Rho family members, serine/threonine kinase p70, and s6 kinase if the signal molecule binding to PDGFRA is phosphoinositol (PI) 3 kinase. Binding of phospholipase C gamma (PLC $\gamma$ ) to PDGFRA will increase the levels of diacylglycerol and Ca<sup>+2</sup> leading to activation of some PKC family members. PDGFRA signaling is also important for some gene expression if Stat5 binds to PDGFRA, and binding of CRK causes activation of nucleotide exchange factor C3G<sup>54</sup>.

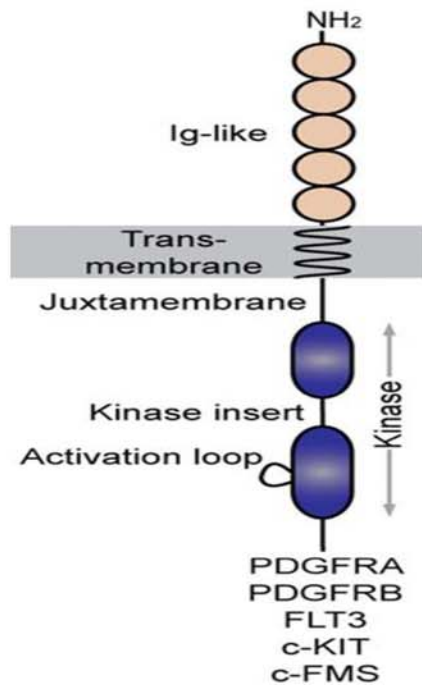


Figure 1-6: The structure of PDGFRA one of the receptor tyrosine kinase with permission from (Toffalini F1, Demoulin JB, 2010)<sup>53</sup>.

#### 1.4.2 Platelet Derived Growth Factor Receptor A Oncogenesis And Targeted Therapy

Signal transduction pathways of RTKs and some RTKs directly can contribute to oncogenesis initiation which causes uncontrolled cell growth leading to cancer<sup>55</sup>. One of the main pathways that RTKs activate is the Ras/Raf/MAPK pathway which is necessary for cell proliferation and thus continues activation by RTKs lead to increase cell growth<sup>55</sup>. Mutations of PDGFRA, one of the RTK members, such as substitution ASP842Val in exon 18 is linked to gastrointestinal stromal tumors (GISTs) and epithelial morphology. This common mutation in exon 18 affects the TK2 domain leading to continuous activation of the kinase by changing the activation loop that

regulates the ATP binding site. This causes activation of the receptor in the absence of the ligand leading to continuous downstream signal transduction. Other primary mutations of PDGFRA, TK1 (exon12, Tyr555Cys and Asp561Val) were found to contribute to gastrointestinal stromal tumor pathogenesis (GISTs). Secondary mutations occurring post a PDGFRA inhibitor treatment course were found to be responsible for drug resistance<sup>56-58</sup>.

One of the RTK inhibitors called Imatinib, “Gleevec” (or “Glivec”) was developed in the 1990s as a drug suppressing signaling pathways of PDGFRA, BCR-ABL, ABL, and c-KIT. The Imatinib mechanism prevents the protein tyrosine phosphorylation reaction in which the tyrosine kinase receptor transfers phosphate from its ATP binding site to tyrosine residues. When Imatinib binds to the ATP binding site, that bars conformational changes, and thus phosphate transfer to tyrosine, and receptor activation<sup>59,60</sup>. Imatinib is used to treat dermatofibrosarcoma protuberans (DFSP)<sup>61</sup>, The Philadelphia chromosome (Ph) disorder<sup>62</sup>, chronic myeloid leukemia (CML)<sup>63</sup>, gastrointestinal stromal tumors (GISTs)<sup>64</sup>, malignant melanoma (MM), systemic mastocytosis (SM), and hypereosinophilic syndromes (HES)<sup>65</sup>.

Crenolanib (CP-868,596), known also as 1-[2-[5-[(3-methyloxetan-3-yl) methoxy] benzimidazol-1-yl] quinolin-8-yl] piperidin-4-amine, is a very highly selective inhibitor against PDGFRs over other RTKs<sup>66</sup>. Some mutations in RTKs cause cell resistance to Imatinib, and other inhibitors such as Sunitinib<sup>57</sup>. PDGFRA (D842V), c-KIT (D816V) and FLT3 (D835V/Y) mutations linked to GIST were found to cause continuous activation of the receptor in the absence of the ligand, and since some RTKs are targeting the inactive form of RTKs receptor, patients with this

mutation are show resistance to Imatinib, and Crenolanib show significant inhibition of RTK activity<sup>67, 68, 69, 70</sup>. Due to Crenolanib binding to the active form of the receptor, Crenolanib is classified as a type I tyrosine kinase inhibitor, RTKI<sup>71</sup>.

Lenvatinib (Lenvima®) is used to improve treatment for non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), thyroid activity against platelet-derived growth factor receptor  $\alpha$  (PDGFRA), c-kit, fibroblast growth factor receptors 1–4 (FGFR-1–4), and vascular endothelial growth factor receptors 1–3 (VEGFR1–3). The inhibition of tumor behavior such as invasion, rapid growth, and migration is by inhibiting the signaling pathways of PDGFRA and FGFR1. Lenvatinib also prevents angiogenesis by inhibiting Vascular endothelial growth factor (VEGF), c-kit, and kinase insert domain receptor (KDR ) facilitating recruitment of endothelial cell circulation from bone marrow to blood flow<sup>72, 73</sup>.

In IBC patients, platelet derived growth factor receptor alpha shows higher expression compared with non-IBC patients<sup>74</sup>, with the exception of triple negative breast cancer patients. *Thus I hypothesized that Platelet Derived Growth Factor receptor alpha (PDGFRA) can play a significant role in improving IBC treatment by targeting the receptor target in Inflammatory Breast Cancer and Triple Negative Breast Cancer.* Three aims were developed to achieve answers supporting this hypothesis: 1) to determine the expression, localization, and phosphorylation status in IBC patient samples, 2) to screen for PDGFRA inhibitor doses combined with chemotherapy and evaluate cell response compared with chemotherapy alone, and 3) to evaluate IBC cell line behavior (emboli formation, cell growth, cell viability and invasion) when PDGFRA is knock down.

## **Chapter 2**

### **EXPERIMENTAL METHODS**

#### **2.1 Cell Culture**

Some of the experiments required cell culture for IBC cell lines (SUM149 and KPL-4; a gift from Dr. Junichi Kurebayashi, Kawasaki Medical School,) and triple negative breast cancer (MDA-MB-231). SUM149's were cultured in Ham's F12 base media (Mediatech, Inc., Manassas, VA) with 5% fetal bovine serum (FBS: Atlanta Biologicals, Lawrenceville, GA), 1% antibiotic/antimycotic (Mediatech, Inc.), 1% Pennicillin-Streptomycin (Mediatech, Inc.), 1% L-glutamine (Mediatech, Inc.). The final concentration of the insulin/transferrin/selenium (ITS) cocktail was 5ml of working solution (5 $\mu$ g/ml Insulin, 2.5mg/ml transferrin, 20 $\mu$ g/ml selenium: Sigma-Aldrich, St. Louis, MO). Hydrocortisone, 500  $\mu$ l of a 1mg/ml working solution was added (Sigma-Aldrich). KPL-4, and MDA-MB-231 were cultured in the same conditions as SUM149 with replacing Ham's F12 base media with DMEM-F12 supplemented with sodium pyruvate. The cultured cell lines were kept at 37°C and 5% CO<sub>2</sub>.

#### **2.2 Immunohistochemical (IHC) Staining.**

Immunohistochemical staining was performed on patient samples preserved as paraffin sections. Sections were deparaffinized through three changes of xylene, and hydrated through different ranges of ethanol. Sections were exposed to 10 mM sodium citrate buffer (pH=6) at 70 ° C for antigen retrieval then allowed for 20 minutes of

boiling at 95°C. Sections were allowed to cool on ice for 30 minutes. Sections were washed with two changes of distilled water for 10 minutes total followed by phosphate buffer saline (PBS: pH=7.4) with 0.1% Triton x-100 for 10 minutes. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 minutes. The sections were blocked using 10% horse serum in buffer containing 5% goat serum, 0.1% fish skin gelatin, 0.1% triton-x100, 1% bovine serum albumin (BSA), 0.05% Tween, 0.05% sodium azide in PBS for 1 hour. After the blocking process, sections were incubated with 1:100 ratio of rabbit anti-PDGFR $\alpha$  (c-20) polyclonal primary antibody conjugated to horseradish peroxidase (HRP), (Santa Cruz Biotechnology Inc: sc-338) overnight. The sections were washed in PBS with 0.1% Tween 20 for 10 minutes. Sections were directly incubated from 1 to 15 minutes with 3, 3'-Diaminobenzidine (DAB) (Becton-Dickinson, Franklin Lakes, New Jersey) until the brown color was observed. Sections were stained with methyl green for 10 minutes, and dehydrated. Sections finally were fixed onto the slide with permount. Axioplan system, Axiocam was used for imaging (Zeiss.)

### **2.3 Immunofluorescence Staining**

Immunofluorescence staining was performed on mouse samples containing SUM149 cells injected subcutaneously to form a tumor. After optimizing the conditions, the staining was performed on patient samples. Paraffin sections were deparaffinized in 3 changes of xylene each for 5 minutes. The sections were dehydrated in different changes of alcohol. Antigen retrieval was performed in sodium citrate (pH 6) for 20 minutes, and cooled on ice for 30 minutes. Sections were washed in 3 changes of phosphate buffer saline with 0.1% Tween. As for blocking, sections were incubated in fish skin gelatin with 5% goat serum for 60 minutes. Three changes of PBS were used

to wash the sections each for 10 minutes. Sections were incubated in primary antibodies, anti-PDGFR $\alpha$  (Santa Cruz Biotechnology, Inc: Tyr754: Sc-12911-R) or anti-E-cadherin antibody [HECD] (ab1416) the ratio of antibodies was optimized based to the tissue type. Goat anti-rabbit secondary antibody conjugated to 647 and goat anti-mouse conjugated to 488 were used at 1:2000. Sections were washed in phosphate buffer saline pH6, and co-stained with DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride). slowfade gold was used for mounting.

KPL-4 cells were grown on cover slips and allowed to reach 60% confluence. Cells were washed gently with PBS and fixed in 4% EM grade paraformaldehyde for 30 minutes then washed in PBS pH=7 and blocked with 5% goat serum. Staining was performed using the previously mentioned steps with paraffin embedded sections.

## **2.4 Cell Viability Assay**

Cell viability assays were performed on the SUM149 IBC cell line. Cells were harvested using trypsin .25% (Fisher Scientific), and seeded in triplicates in 96- well plates. The seeding ratio was (8000 cell/well). Cells were incubated for 48 hours at 37°C in 5% CO<sub>2</sub> and allowed to attach before starting the treatments. Cells were treated for 6 day time points with 10 different concentrations of Imatinib or Crenolanib (Arog Pharmaceuticals, Inc., Dallas, TX) combined with paclitaxel (LC laboratories, Woburn, MA) or doxorubicin (LC laboratories). The PDGFR $\alpha$  inhibitors were used at a 10% final concentration in a total of 200  $\mu$ l media in each well. MTT (4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide: Invitrogen, Carlsbad, CA) working solution was prepared at a concentration of 5 mg/ml in phosphate buffered saline (PBS). Depending on the time course point, 50  $\mu$ l of MTT was added to each well and incubated the at 37°C in 5% CO<sub>2</sub> for 3 hours to allow MTT conversion to formazan crystals by mitochondria

reductase. The media containing MTT was aspirated and 100  $\mu$ l of dimethyl sulfoxide (DMSO) was added to each well with mixing. The absorbance was read at 570 nm. The used concentrations of Crenolanib, Imatinib and Lenvatinib was from 1 $\mu$ m to 500 $\mu$ m. Each of the different concentrations were combined with different doses of Doxorubicin 1 nM to 10 nM or Paclitaxel 0.1 $\mu$ m to 10  $\mu$ m.

## **2.5 Transfection**

Cells were plated at 80% confluency, and allowed to adhere for 24 hours at 37°C in 5% CO<sub>2</sub>. At 24 hours post-plating, cells were transfected with siRNA against PDGFRA (Human) or scramble siRNA control (Origene Technologies, Inc) at a concentration range of 1nm, 5nm, and 10 nm. Fugene ®HD transfection reagent was used (Promega). The ratio of siRNA to Fugene was 1:1.5. Media was replaced after 24 hours post-transfection and successful knock down of mRNA and protein was determined using real time quantitative PCR (qPCR) and western blotting.

## **2.6 RNA Extraction, And Real Time Quantitative Polymerase Chain Reaction (qPCR)**

RNA extraction was performed using Purelink® RNA mini kit. Cells were washed with PBS, scraped, and collected. Centrifugation was performed to pellet the cells and aspirate the PBS. Lysis buffer, and 70% ethanol where added and samples were centrifuged to collect liquids; 700  $\mu$ l of washing buffer was added to the tubes and left for 4 minutes at room temperature. Centrifugation was performed for 15 seconds. Wash buffer II was added twice and centrifugation was performed at 10,000 rpm for two minutes. RNA was collected and solubilized in RNase free water. RNA concentrations were determined with the Nanodrop and equal amounts were loaded for the real time quantitative PCR.



As for qPCR, RNA samples were used with power SYBR® Green RNA – to – Ct™ 1-step kit (Applied Biosystems). RT-PCR reaction master mix was prepared by the manufacturer recommendations. Master mix and total reaction volume were loaded at a 20 µl final volume. Primers obtained from IDT 5'-TTGATGAAGGTGGAACTGCT-3' and 5'-ATTCCTCTGCCTGACATTGAC-3' were used as forward and reverse primers for PDGFRA, respectively. As for beta actin, 5'-ACAGAGCCTCGCCTTTG-3' was used as the forward primer and 5'-CCTTGACATGCCGGAG-3' as the reverse primer. Samples run by 7500 fast qPCR machine and the samples were kept in hold stage for 30 minutes at 48°C, then 95°C for 10 minutes to allow for reverse transcription. Then samples were incubated at 95°C for 15 seconds and 62°C was the ideal temperature for primers to anneal and extend for 1 minute. Data analysis was performed by normalizing data to the house keeping gene, beta actin, and comparing 2<sup>-dCt</sup> values. All samples were normalized to the control having 1 as its 2<sup>-dCT</sup>.

## **2.7 Western Blotting**

Protein was collected from transfected cells at different time points to determine the success of transfection. RIPA Lysis and Extraction Buffer, protease inhibitor, and phosphatase cocktail were prepared and applied on cells (Thermo Fisher Scientific, Waltham, MA, USA). Samples were kept in ice for 30 minutes and centrifuged for 20 minutes at 4°C at 12000 RPM for 20 minutes. Protein was collected and concentrations were determined with the Pierce™ BCA protein assay was performed using the manufactures recommendations (Pierce Scientific, Rockford, IL). The BCA reactions were incubated at 37°C for 30 minutes before taking the reading at 570 nm. Samples were prepared with 4X Leammli sample buffer (Bio Rad, Hercules, CA) to a final

concentration of 40  $\mu\text{g}/\mu\text{l}$  in 30  $\mu\text{l}$  final volume and incubated at 95° C for 5 minutes before loading. Denatured protein samples were loaded in a gradient 4-15% mini-protean® TGX™ (Bio rad, Hercules, CA) and run at 120 volts for 80 minutes. Protein was transferred to a nitrocellulose membrane at 80 volts for 1 hour. Nonspecific binding was avoided by blocking with 3% Bovine Serum Albumin (BSA) in Tris buffer saline (TBS) with 0.1% Tween 20 for 1hour. Membrane was incubated with anti PDGFRA to detect total receptor over night at 4° C, 3 wash changes of TBST were used before imaging the protein using Pierce™ ECL western Blotting substrate (Thermo Scientific, Rockford, IL). The membrane was re-blocked and re- probed for beta actin as a control.

## **2.8 Emboli Formation Assay**

Untreated cells, PDGFRA siRNA transfected cells, and cells transfected with siRNA scramble were collected using trypsin and counted to have 100,000 cell/ flask. Suspension culture flasks from cell star (Greiner Bio-one, NC) were used. Three flasks were prepared for each condition. Cells were suspended in 5 ml media containing 2.25% PEG 8000 (Promega, Madison, WI). Flasks were allowed to incubate in 5% CO<sub>2</sub> at 37° C for 48 hours with gentle rocking at (40 rpm). After 48 hours, emboli were counted for the three flasks and the median values were considered in statistical analysis.

## **2.9 Invasion Assay**

Emboli were collected from each condition. Centrifugation was performed to sediment emboli and aspirate the media containing PEG 8000. An equal number of emboli for each condition was suspended in serum-free media. Chambers were put in a 24 well plate, and 1ml of emboli suspension was plated in a BD bio coat invasion chambers (BD Biosciences, San Jose, CA). The lower part of the well contained media

with serum to stimulate the cell migration. The plates were incubated for 24 hours and at each time point, cells were stained with DAPI (4',6-diamidino-2-phenylindole; Thermofisher, Inc.) and fixed with 2% EM grade paraformaldehyde in PBS (pH7.4) for 5 minutes and then fixed with 2% EM grade paraformaldehyde for 20 minutes. Chambers were stored in PBS at 4° C for imaging. The chambers were imaged using a LSM 880 confocal microscope by Z-stacking at 98.5 microns.

### **2.10 Apoptosis Assay**

Cells were plated in a 6 well plate at 500,000 cell/well and allowed to adhere for 24 hours. Cells were either untreated or transfected with siRNA targeting PDGFRA transcripts or scramble plasmids for 24 hours. Three days post transfection, media containing dead cells was collected and adhered cells were harvested using trypsin and both were collected in 15 ml tubes. Centrifugation was important to acquire the cell pellet which was then re-suspended in PBS or medium. Propidium iodide (PI) was applied to probe the dead cells while avoiding exposure to light for 40 minutes at 25°C. cells were washed 3 times with PBS then suspended in PBS. A flow cytometer was used to determine the dead cell population compared to viable cell population.

## Chapter 3

### RESULTS

#### 3.1 Platelet Derived Growth Factor Receptor A Expression And Localization.

An antibody to total PDGFRA was used for immunohistochemistry and western blotting analysis. As for PDGFRA immunofluorescence staining, anti-PDGFRA that only detects the active receptor phosphorylated at tyrosine 754 was used. SUM149 tumors grown in mice were stained to optimize the conditions. 40 patient samples were stained for total PDGFRA via immunohistochemistry, and 21 of them showed over-expression of PDGFRA as shown in Figure 3.1. The remaining samples showed very low expression that was similar to the negative control tissue in the sample. For the low level expressed samples, immunofluorescence staining showed expression of the phosphorylated PDGFRA as well as co-localization with DAPI used to stain the nucleus as shown in Figure 3.2.

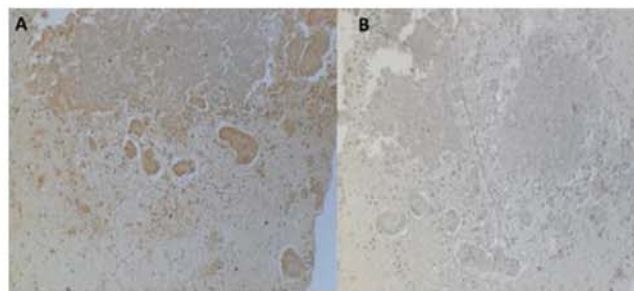


Figure 3-1 Immunohistochemical staining of PDGFRA in patient samples shown in panel A shows the over-expression of PDGFRA compared to a negative control in panel B.

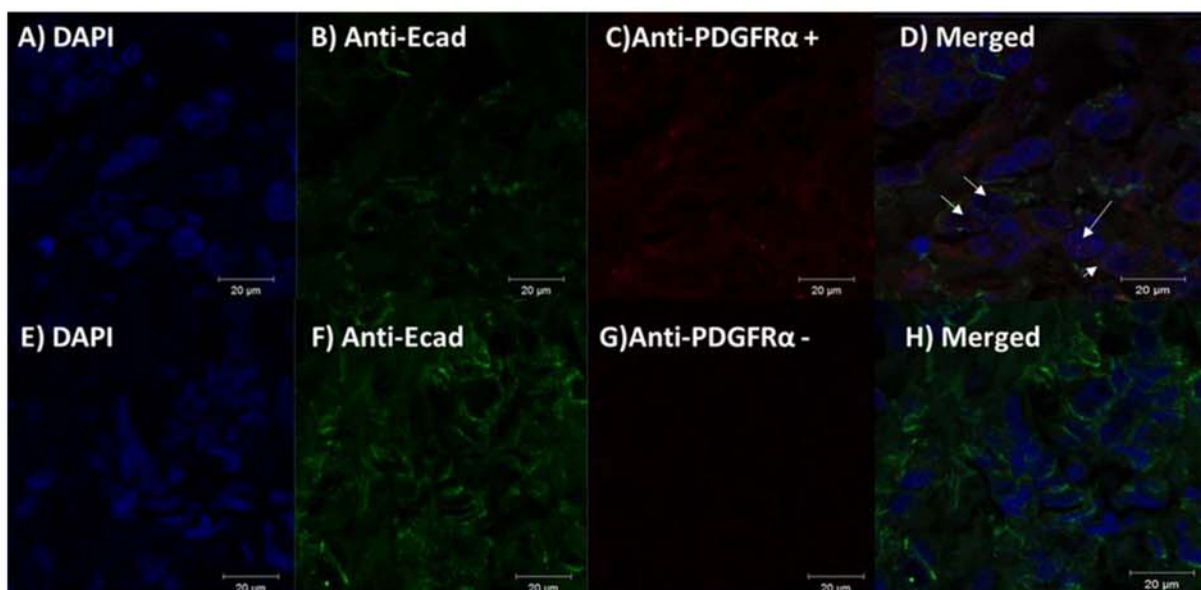


Figure 3-2: Immunofluorescent staining for detecting active PDGFRA in patient samples and co-staining with DAPI. A) positive expression and co-localization with DAPI. B) Negative control.

### 3.2 Platelet Derived Growth Factor Receptor A Targeting Therapy

The SUM149 cell line, KPL-4 cell line, and MDA-MB-231 cell lines were screened for effective doses of three PDGFRA inhibitors combined with different doses of either with Paclitaxel or Doxorubicin. SUM149 showed a significant response to Doxorubicin used at 1 nm, and 10 nM and ranges from 1 µM to 500 µM of PDGFRA inhibitors. 1 nM of Doxorubicin was significant with Crenolanib at 4, 5 µM, and with Imatinib at 6 µM. No significant response with Lenvatinib as showed in Figure 3.3 Panel A. Panel B of Figure 3.3 shows SUM149 response to paclitaxel at 10nm with the same range of inhibitors doses used with Doxorubicin. Paclitaxel was very effective with Crenolanib at the range 1 to 5 µM of doses, and with Imatinib at 3, 4 µM. Figure 3.3 panel C, D, E, and F have the results of cell viability at higher doses of Doxorubicin,

and Paclitaxel at 10 nM and 100 nM, respectfully. Doxorubicin is showing very low cell viability with 10, 300, 400, 500  $\mu$ M of Crenolanib, and 10, 50  $\mu$ M of Imatinib, and with 10, 20, 30, 40, 50, 400, 500  $\mu$ M of Lenvatinib. Paclitaxel 100  $\mu$ M is causing more cell death with 50  $\mu$ M Imatinib, 300,400,500  $\mu$ M of Crenolanib, and with 30, 40, 50, 400, 500  $\mu$ M Lenvatinib.

Figure 3.4 A, B showing the KLP-4 cell viability with range of PDGFRA inhibitors from 1 to 7  $\mu$ M with either Doxorubicin 1nM or Paclitaxel 10 nM. The cells become sensitive to chemotherapy treatments with 1 to 5  $\mu$ M of Crenolanib, and Lenvatinib combined with 1 nm Doxorubicin. When using 10  $\mu$ M Doxorubicin, a low cell viability was observed at 10 to 50  $\mu$ M, and 400 to 500 uM doses of Crenolanib, 40 to 50  $\mu$ M of Imatinib, and 400 to 500 uM Lenvatinib Figure 3.4 C,E. Paclitaxel was used at 100  $\mu$ M with very effective cell growth suppressing at 30, 40 ,50 ,400 ,500  $\mu$ M of Crenolanib, 10, 40, 50  $\mu$ M of Imatinib, and 400 and 500  $\mu$ M Lenvatinib.

MDA-MB-231 screening presented in Figure 3.5 illustrates significance at 30, 50, 400, 50  $\mu$ M Crenolanib, and 10 to 50  $\mu$ M of Imatinib, and 30, 40, 50, 200, 300, 500  $\mu$ M Lenvatinib when combined with Doxorubicn at 10 nM. A 100 nM of Paclitaxel showed lower cell viability when combined with 20, 30, 40, 50, 300, 500  $\mu$ M doses of Crenolanib. Imatinib was effective at 30 to 50  $\mu$ M. A range from 30 to 50, 500  $\mu$ M of Lenvatinib showed defeat effect in cell growth compared with chemotherapy alone.







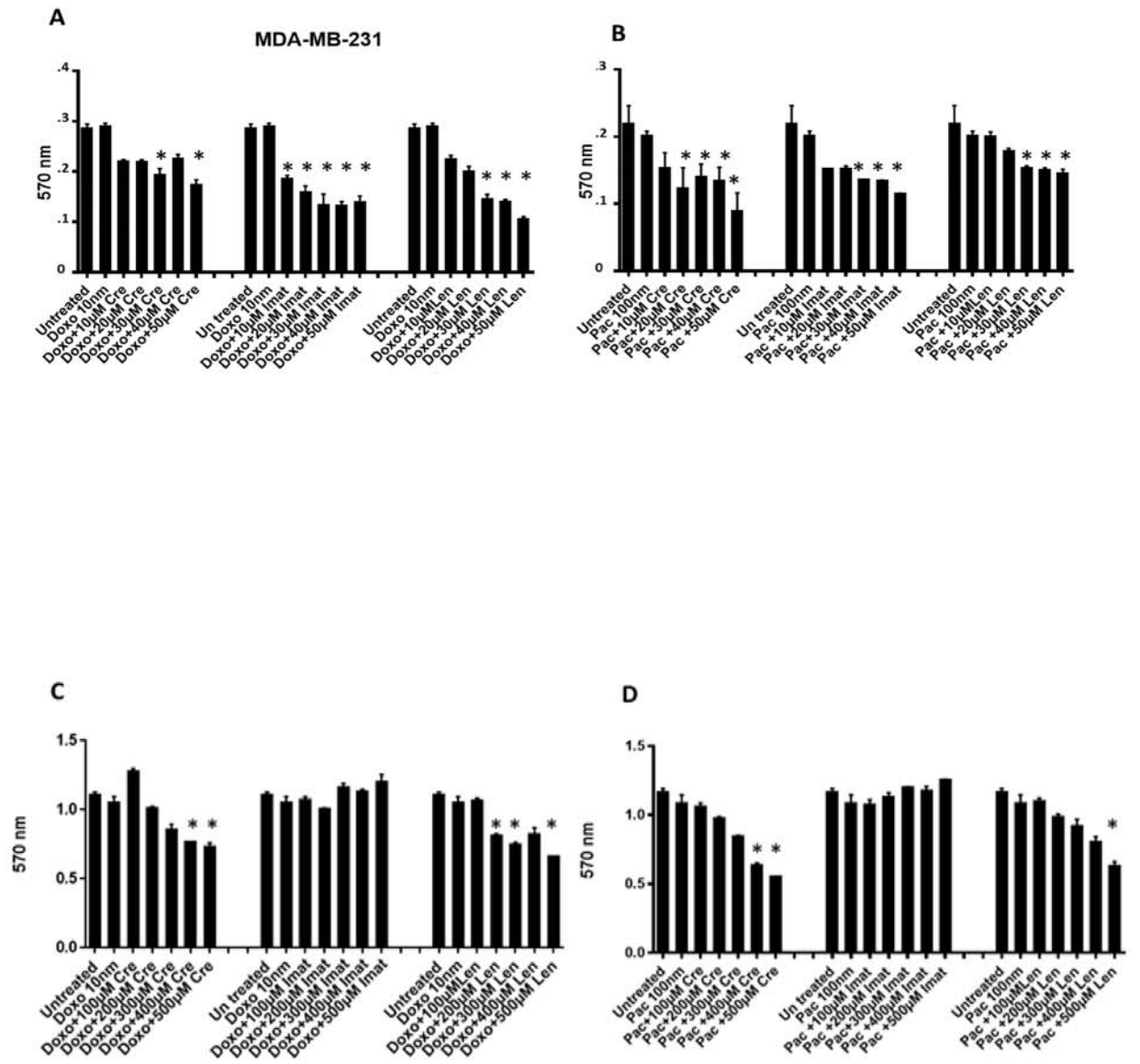


Figure 3-5: MDA-MB-231 cell line sensitivity to chemotherapy. The \* indicates p value < 0.00001.

### **3.3 Platelet Derived Growth Factor Receptor Alpha Decrease Expression**

Platelet derived growth factor receptor alpha was knock down using anti PDGFRA siRNA in two IBC cells lines, SUM149 and KPL-4. The declined expression of PDGFRA showed significant reduction in emboli formation which almost dropped to half of the number found in untreated conditions as shown in Figure 3.6. It is noticeable that the knock down condition in SUM149 tends to have less, and some of the formed emboli were smaller compared with controls Figure 3.6 A, C. Emboli size of a SUM149 control embolus was measured to be around 400  $\mu$  while some were measured to be around 200  $\mu$  as in Figure 3.6A. Whereas decreased expression of PDGFRA in KPL-4, there was more single cells in the knock down condition compared with controls as shown in Figure 3.6 B, D. As for the PDGFRA effect on cell death rate evaluation, FACS analysis showed in Figure 3.7 A, and B was used. Figure 3.7, C, and 3.7D showing the apoptosis events caused by suppressing the expression of PDGFRA in SUM149, and KLP-4 which indicates that absence of PDGFRA causes significant cell death. On the other side, cell viability was measured to determine PDGFRA knockdown affect in SUM149, AND KPL-4 as showed in Figure3, 8. SUM149 was showing significant at day 2 figure 3-8 A,C where as KPL-4 was showing a difference in day 3 Figure 3,8 B, and D compared with untreated controls. Invasion were observed since IBC is a very aggressively invaded cancer, and a tile scanned at 10x, and invasion rate were determine as ratio. Figure 3, 9 displays invasion chambers side view at high resolution in SUM149 Figure 3, 9 (A, B, C) and KPL-4 in (D, E, F). Quantifying of DAPI density were performed to determine the cell density of each side of the gel. When analyzing the derived data from DAPI quantifying, the knockdown conditions of PDGFRA did not show an affect limiting the cell invasion in both IBC cell lines as shown in Figure 3.9 (G, H).

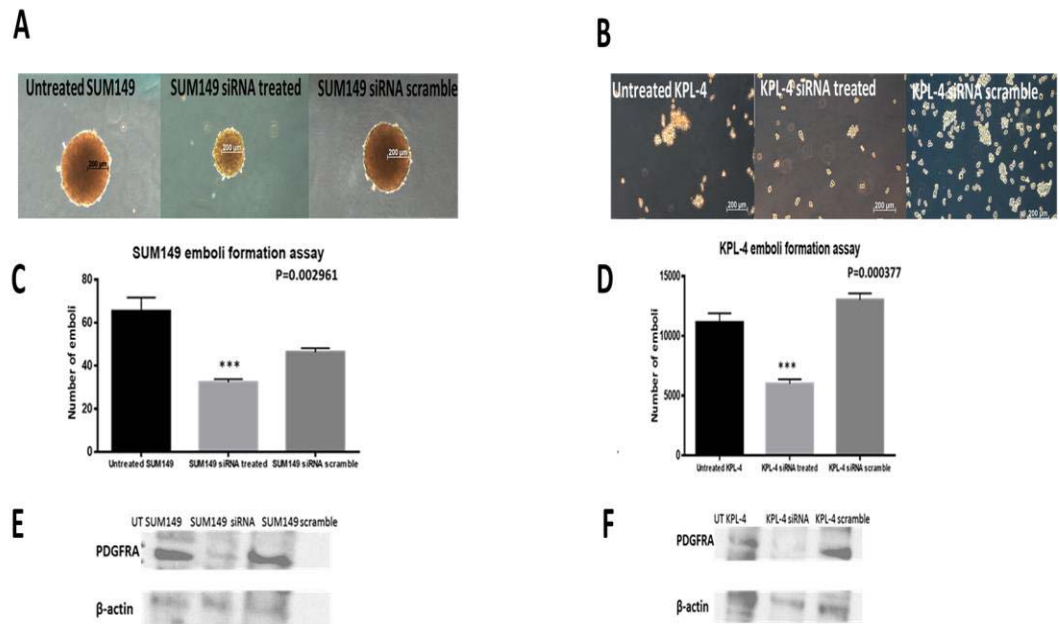


Figure 3-6: The effect of PDGFRA knock down in IBC cell lines Panel A,B showing the emboli formed in SUM149, and KLP-4 respectively. Panel C, D is the statically analysis for emboli counting, and panel E, F is showing western blot for each condition.

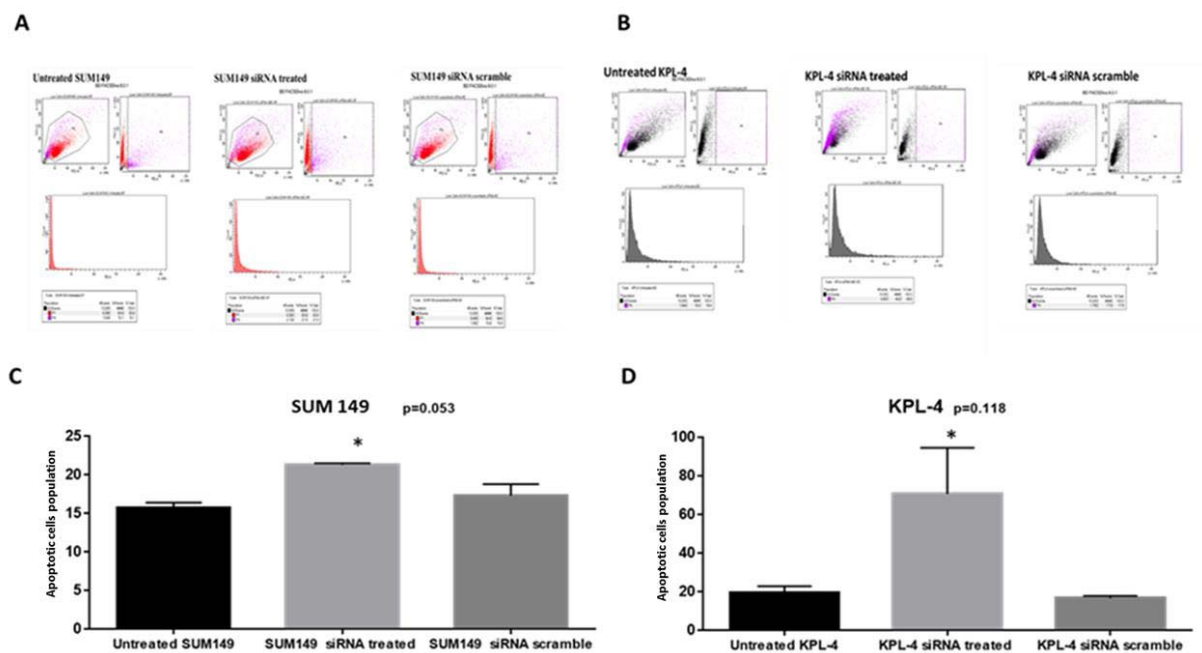


Figure 3-7: Fluorescence-activated cell sorting (FACS) for evaluating Apoptosis events in IBC cell lines (SUM149 data presented in panel A, C, and KPL-4).

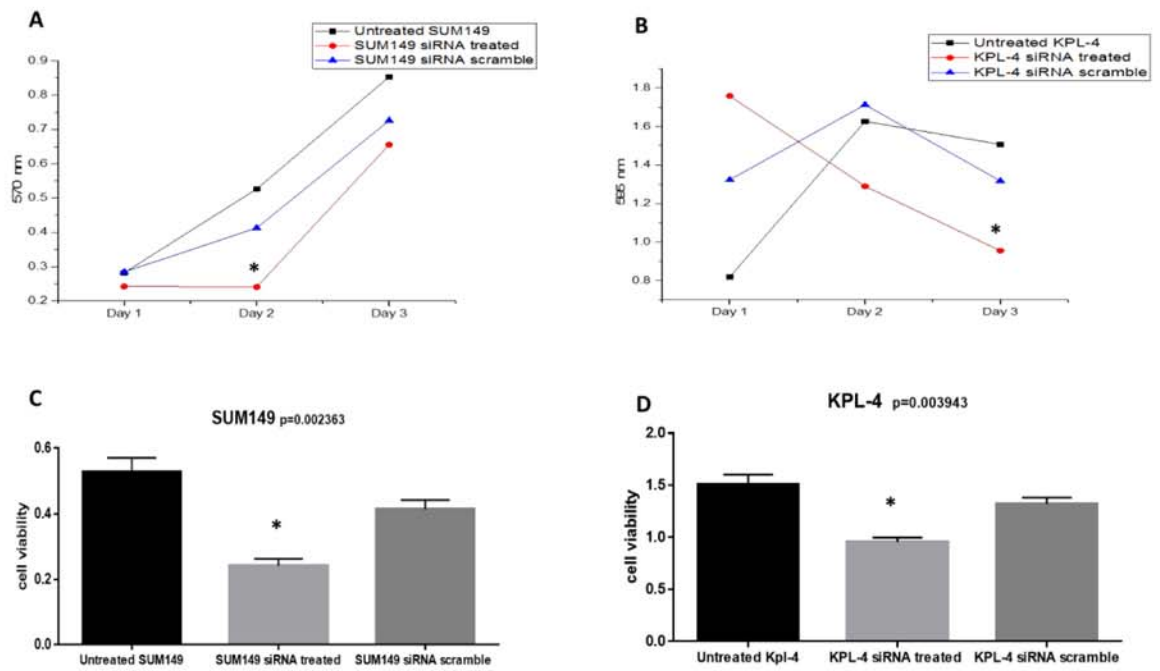


Figure 3-8: Cell viability assay showing the effect of PDGFRA on cell growth. Panel A,B showing time points for cell growth in IBC cell lines SUM149, and KPL-4 respectfully. Panel C statically analysis for SUM149 day 2, and panel D is the significance for analysis for KPL-4 day3.

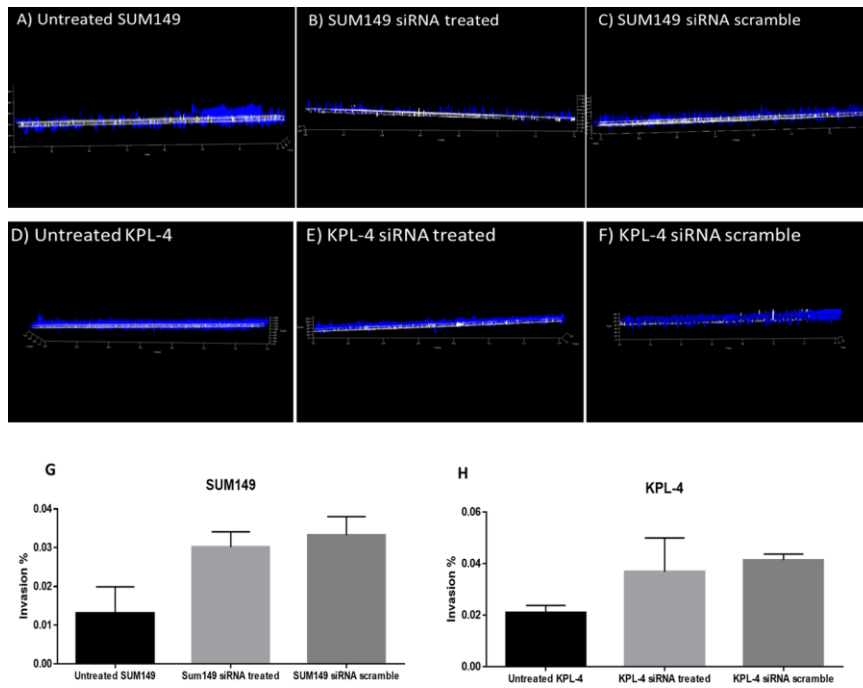


Figure 3-9: High resolution of invasion chamber showing the effect of PDGFRA decrease expression in IBC cell lines.

## **Chapter 4**

### **DISCUSSION AND FUTURE DIRECTIONS**

IBC is arguably the deadliest form of breast cancer subtypes and is always classified as T4d according to American Joint Committee on Cancer (AJCC) at the time of diagnosis. The survival rate of IBC is very low, and the incidence is increasing over time. It is uncertain if this is due an actual increase in incidence or an increase in awareness of the disease. However, it is known that IBC is more common in African American women, North African women, and Caucasian women compared with other ethnic groups. This type of breast cancer also known to be diagnosed in younger age women, typically in child bearing years, compared with other types of breast cancers. Patients can have clear symptoms, with histological evidence of emboli present in the dermal lymphatics, while other patients can have emboli present while lacking symptoms, and yet others can have emboli invasion with no symptoms. Symptoms may include breast redness, swelling, breast enlargement, nipple inversion and discharges. In addition, it is observed that patients have skin orange appearance a.k.a. ‘peau d’ orange’, skin thickening. IBC is a unique subtype of breast cancer because in the early stages of the IBC development there is no noticeable lump involvement <sup>75</sup>. For diagnostics, fine needle aspiration (FNA) will be used to screen dermal lymphatic invasion by IBC emboli <sup>76</sup>. Sonography positron is also used to determine the invasion in lymphatic nodes, to demonstrate breast lesions, and to guide skin biopsy punching <sup>77</sup>. Emission tomography/computed tomography (PET/CT), magnetic resonance imaging

(MRI), ultrasonography are used to evaluate stage, and skin thickening, and breast lesion <sup>78</sup>.

The focus of the project is very significant because the number of women diagnosed with IBC is growing, while the survival rate remains very low compared with other subtypes of breast cancers. The average survival rate for women diagnosed with IBC is almost half time period compared with the median survival rate for a women diagnosed with no inflammatory locally advanced breast cancer (LABC), , <45% compared to 90% <sup>79</sup>. Also, IBC incidence is double in African American women compared with Caucasian<sup>80</sup>. Smoot et al. reported that the survival rate is diminished from 42% for the 5 year disease-free survival rate to 21% for the 10 year disease-free survival rate in IBC patients <sup>75</sup>. It is also reported that IBC patients have very poor prognosis compared with stage-matched non-IBC patients where the median survival dropped from 13.4 years for non-IBC to 4.75 for IBC patients<sup>81</sup>.

IBC treatment includes chemotherapy followed by surgery and radiation. Doxorubicin is one of the main chemotherapy drugs used to treat many types of cancers, including IBC. Bido et al reported that Doxorubicin causes deadly metabolic effects by damaging white adipose tissue (WAT) and thus interrupting WAT function and secretions. In addition to affecting WAT, Doxorubicin also severely affects skeletal, cardiac muscles, and the liver causing very poor life quality for patients going through chemotherapy course<sup>82</sup>. Paclitaxel is another drug used to treat an enormous range of cancers, including IBC. Reported cases indicate that Paclitaxel causes cystoid macular edema (CME) which is a very rare disorder in the central retina of eye<sup>83, 84, 85</sup>. Fatigue, depression, weight loss, nausea and vomiting are some of chemotherapy side effects<sup>86</sup>. Infection because of reduction in white cell count (Myelosuppression), neurologic



toxicity, cardiac toxicity, ovarian dysfunction, development of acute myeloid leukemia or myelodysplasia, are also reported as chemotherapy side effects causing very poor life quality<sup>86</sup>. The poor quality of life in IBC patients often is without noticeable positive outcomes, and the IBC aggressive behavior leads to the rapid demise of the patient.

The serious side effect of chemotherapy, chemotherapy resistance contributing to low survival and contributing to last the side effects of chemotherapy. In addition, the survival rate is low, and the incidence is increasing, the aggressiveness of the disease, all resulting in poor life quality. Thus it is very important to find a cure, and investigating for effective target for IBC targeted therapy. This is important to make the battle for women diagnosed with IBC less painful to increase the chance of having acceptable life quality. In simple words, finding an effective target will improve the treatment of IBC, will improve the life quality, and will improve the survival rate.

A study looking at the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in breast cancer found that a number of genes are overexpressed in IBC compared with non-IBC including PDGFRA<sup>87</sup>. In addition, another study showed that Akt, PDGFRA, epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF-IR) and erbB2 have mutations causing the receptors to be constitutively active. The constitutive activity of the protein as mentioned is one of key players in chemotherapy resistance<sup>88</sup>. Targeted therapy is an important approach to overcome chemotherapy resistance but still there is drug resistance also found in targeted therapy. Receptor tyrosine kinase inhibitors are one of these approaches for targeted therapy. A study found that PDGFRA was over expressed in breast carcinoma<sup>89</sup>, and a previous study from our laboratory found it to be particularly over expressed in IBC patients compared with non-IBC patients<sup>87</sup>.

For one RTK inhibitor, Imatinib, resistance found in gastrointestinal stromal tumors (GISTs) and chronic myeloid leukemia (CML) patients leads to tumor reoccurrence<sup>90</sup>. Crenolanib is specifically targeting the active PDGFRA receptor, which is caused by mutation found to be in some types of cancers for example GIST<sup>57, 91, 92</sup>. Imatinib is targeted against inactive forms of RTKs, including PDGFRA, but does not have the same selectivity as Crenolanib<sup>59, 60</sup>. A study conducted in 2012 showed that Crenolanib selectively targeted mutated and active PDGFRA, and was successful to overcome the resistance observed when treating cells with Imatinib<sup>93</sup>. Lenvatinib is targeting varying RTKs but PDGFRA is one of its main targets. The anticancer actions of Lenvatinib is by preventing PDGFRA signaling pathway and also the inhibition of angiogenesis<sup>72, 73</sup>.

Based on these observations I hypothesized that PDGFRA is an effective target to sensitize IBC cells to chemotherapy. In SUM149, KPL-4, MDA-MB231, Crenolanib, and Lenvatinib, when combined with Doxorubicin decreased IBC cell viability, whereas Imatinib did not. A parallel pattern of cell response was observed when PDGFRA inhibitors were added individually along with Paclitaxel. Cell resistance observed with most doses of Imatinib whereas Crenolanib and Lenvatinib showed lower cell viability compared to Imatinib. These results suggest that the PDGFRA is active and the active form of the receptor is a more effective target than the inactive form, which Imatinib targets. Screening the triple negative breast cancer cell lines, and IBC cell lines is very important approach because this could lead using low level of chemotherapy drugs thus minimizing the side effect. In addition this may also serve to overcome chemotherapy resistance even with high doses of chemotherapeutic drugs. Another future potential benefit is in the identification and management of a variety of PDGFRA mutations, thus

leading to a better understating of how important inhibition of PDGFRA is in order to improve the survival rate of IBC.

Another key to understand IBC behavior, and how PDGFRA is contributing to it is to study the expression status and localization of PDGFRA receptor. Joglekar et al showed that PDGFRA is localized and active in the cytoplasm of the SUM149 IBC cell line<sup>94</sup>. The second part of my thesis screened IBC patients' samples for PDGFRA localization and expression via immunohistochemistry and immunofluorescence. It is important to know if the cell line data is representative of the patients' samples. I screened patients' samples and that approximately 52.5% overexpress while approximately 47.5% are low expressed or negative for expression of PDGFRA. When detecting the expression and localization of active PDGFRA, the IF staining showed that in the positive patients samples the receptor co-localized with DAPI, suggesting that it is localized inside the cell and not on the cell membrane. From these observations I speculate that the activity of the receptor is very important in understanding PDGFRA role in IBC behavior. As for a future direction for this aim, it would be interesting to investigate what could be causing the receptor to be mislocalized in the cell. One idea would be to determine if there is a difference in the glycosylation pattern between PDGFRA in normal breast cells compared with IBC patients. Glycosylation is required for transport of proteins to their proper cellular target. A revisit of van Golen et al's original differential display data demonstrated that IBC cells were deficient in a specific O-linked glycosylation enzyme (Joglekar and van Golen book chapter). One other approach would be to see if we knock out wild-type PDGFRA in normal breast cells and replace it with the PDGFRA gene found in IBC cells will alter the normal cells

behavior. Alternatively, we could see if re-expression of PDGFRA from normal cells reverted the IBC phenotype.

A narrower focus is the investigation of PDGFRA knockdown in IBC cell lines, and how it affects emboli formation, cell viability, invasion, and apoptosis. As demonstrated in the Results, suppressing the expression of PDGFRA by knocking down the PDGFRA resulted in few number of formed emboli in both cell lines SUM149 and KPL-4 and it is also resulted in high apoptosis rate. Each of those results explains the other. PDGFRA signaling is important for cell growth and viability, and when knocking down PDGFRA, the apoptosis rate will elevated causing less number of viable cell, and thus less number of formed emboli. It is also possible that PDGFRA signaling pathway is having a cross link with other pathways involved in initiating molecules important for emboli formation such as the adhesion molecules. This suggested to test if PDGFRA knockdown affect cell adhesion or the expression cell adhesion molecules. Another future step is see what other molecules that will be down regulated as a cause of PDGFRA down regulation, and to investigate if targeting those molecules will affect the IBC cell lines, or if targeting those molecules will sensitize the IBC cell lines for chemotherapy, and compare the outcome with the PDGFRA inhibition. One example of this is Akt3. In a previous study Lehman et al demonstrated that Akt1 was involved in IBC cell migration through phosphorylation of RhoC GTPase, while Akt3 was involved in cell viability. The link between PDGFRA expression and Akt3 has not been demonstrated however it is logical. Another example is to investigate how PDGFRA knock down is affecting the expression of Caveolin-1 facilitating IBC invasion via interacting with Akt1, and RhoC GTPase<sup>95</sup>.

Many studies reported that the high level of PDGFRA is linked to aggressive cell invasion, and when knocking down PDGFRA, the cell invasion will be controlled, and regulated. A study carried out by Ariad et al reported that women with breast cancer stage 2 had measureable level of PDGFRA, and women staged as stage 4 had higher level of PDGFRA, which indicates a linkage between metastatic levels and PDGFRA expression level<sup>96</sup>. Seyam et al showed that the level of PDGFRA expression levels, and continues activation via autocrine affect the survival rate negatively by stimulating cell growth, and thus elevating invasion, and also having a role in cell resistance for chemotherapy <sup>97</sup>. In IBC cell lines, when knocking down PDGFRA via siRNA, the expression regulation did not affect the invasion. SUM149, and KPL-4 showed higher invasion levels when treated with siRNA compared with untreated controls. This could be because of the emboli size that found to be greater than 400 micron in untreated which may play a role in slowing down the invasion process while the small size of emboli in siRNA treated where more rapidly going through the matrigel. This need to be more investigated using other types of transfection.

In conclusion, IBC patients from different locations were screened for total, and active form of PDGFRA via IHC, and IF staining. IBC cell lines were also screened for three different receptor tyrosine kinase inhibitor efficiency with two different chemotherapy drugs, Doxorubicin, and Paclitaxel at different doses. Also, PDGFRA knockdown was evaluated in IBC cell lines SUM149, and KPL-4, and how PDGFRA suppression can influence IBC cell lines to emboli formation, cell growth, cell death, and cell invasion. These findings should help in understanding the role of PDGFRA in IBC patients, and IBC cell lines behavior. Finally, it also gives insight into the influence that the level of overexpression PDGFRA has on IBC cell line behavior. It also begins

to answer the question of whether PDGFRA mislocalization has a role in the IBC phenotype. Answering these questions will lead to a better understanding of IBC, and lead to a narrower focus for targeted effective therapy.

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Date: April 5, 2016

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**Your project**

Title: "Status of the platelet derived growth factor receptor alpha in inflammatory breast cancer patients". Thesis.

Institution: University of Delaware

**Your request**

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**Anatomic Staging/Prognostic Guide tables for the following:**

Description	page #
Breast TNM & Staging	358-360

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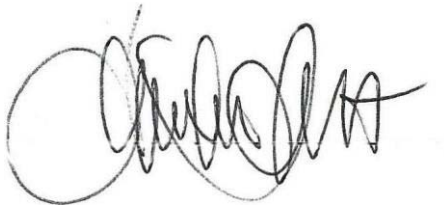
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Sincerely,

A handwritten signature in black ink, appearing to be 'Jennifer Jett', written in a cursive style.

Springer Healthcare  
Fax: +1 212 299-0423  
E-mail: [jennifer.jett@springer.com](mailto:jennifer.jett@springer.com)



**Appendix B**  
**IRBS AND CONSENT FORMS**

## IRBS AND CONSENT FORMS

Form Version 03/20/2009 GEORGE WASHINGTON UNIVERSITY & MEDICAL CENTER OFFICE OF HUMAN RESEARCH • INSTITUTIONAL REVIEW BOARD ohrb@gwumc.edu • Phone: 102.994.2715			
<b>MODIFICATION REQUEST FORM</b>			
IRB#	IRB# 230105 ER	STUDY EXPIRATION DATE:	
CLASSIFY THIS STUDY		Other (specify):	
PROJECT OR TITLE AND NUMBER: Title: The establishment of an inflammatory Breast Cancer Registry and Biospecimen Repository DEPARTMENT: Department			
PRINCIPAL INVESTIGATOR INFORMATION LAST NAME: Levine FIRST NAME: Paul Degree: MD CITI TRAINING COMPLETED: Biomedical Course SCHOOL: SPHHS DEPARTMENT: Epidemiology and Biostatistics ADDRESS: Ross Hall, 2300 I St. NW, Suite 118, Washington DC 20037 PHONE: 202.994.4582 EMAIL: sphphl@gwumc.edu			
MODIFICATION CLARIFICATION: IRB approved protocol/research consent form: <input type="checkbox"/> Change design methodology <input type="checkbox"/> Change in eligibility criteria <input type="checkbox"/> Change in number of subjects <input type="checkbox"/> Change in number of groups <input type="checkbox"/> Change in the research consent form <input checked="" type="checkbox"/> Other describe: Add Research asst. and collaborator			
IRB approved enrollment/recruitment documents: <input checked="" type="checkbox"/> Administrative/Clerical changes <input type="checkbox"/> Editorial clarification <input type="checkbox"/> New safety information for enrolled subjects <input type="checkbox"/> Other, describe:			
1. Summary of modification(s) requested (required): Addition of new staff: Michael Hagler (Research Asst) and Ken Van Golen at University of Delaware (Study Collaborator). No need to modify the Informed Consent as consents have already been signed and we are only collecting tissue samples.			
2. Verification for the modification (s): NEW STAFF			
3. Is the requested modification major or minor? Minor Modification			
4. List all attached documents. Include versions and dates: N/A			
Signature of PI:			Date:
<b>THIS BOX FOR OUR USE ONLY!</b>			
Should all subjects be re-consented: YES / NO / NA			
Date Reviewed by Full Committee or IRB Chair/Designer: ___/___/___			
Final IRB Approval:			
Chair IRB Designer:		Signature:	Date: ___/___/___

**Title of this Tissue Bank**

**Inflammatory Breast Cancer Tissue Bank**

**Description**

You are invited to take part in this tissue bank. The information in this form is meant to help you decide whether or not to take part. If you have any questions, please ask. You have a copy of the following, which is meant to help you decide whether or not to take part:

- Informed consent form
- "What Do I need to Know Before Being in a Research Study?"
- The Rights of Research Subjects

**Please read and sign this consent form, then send to:**

Dr. Paul Levine, College of Public Health  
c/o Department of Epidemiology  
984395 Nebraska Medical Center  
Omaha NE 68198-4395

**Why are you being asked to participate in this tissue bank?**

You are being asked to be a part of this tissue bank because you are an adult (19 years old or older) who has been diagnosed with inflammatory breast cancer (IBC), a rare but aggressive form of breast cancer. It often causes the breast to swell and affects the skin of the breast so that the breast looks inflamed.

**What is the reason for creating this tissue bank?**

The purpose for creating this tissue bank is to collect and store tissue and associated clinical information for use in future research studies on IBC and its possible causes.

**What will be done and what material will be collected for storage in the tissue bank?**

**Material to be collected**

With your consent, we will collect a sample of the breast cancer tissue that was collected when you were diagnosed with IBC. Additionally, we will interview you and collaborate with your physician to collect general information about you, your medical history, your breast cancer diagnosis, and possible environmental exposures.

**What will be done**

We will also analyze your tissue sample for evidence of factors that may have contributed to inflammatory breast cancer. The tissue samples will be sent to collaborating laboratories at the University of Nebraska Medical Center (and possibly Baylor, in the future) for testing.

The interview will be done by the researcher, Dr. Paul Levine, over the phone from his private office.

**What are the possible risks of participating in this tissue bank?**

There are few risks of participating in this tissue bank. There is a risk of emotional upset or discomfort in discussing your medical history. The researcher will conduct interviews when you are ready to answer questions, and you can refuse to answer questions or stop your participation in this study at any time. Since your samples will have identifying health information associated with them, there is a risk for loss of confidentiality.

**What are the possible benefits to you?**

You are not expected to get any benefit from allowing your samples to be stored in the tissue bank.

**What are the possible benefits to other people?**

Society may benefit from the research based on this tissue bank by gaining more information regarding possible exposures that can contribute to inflammatory breast cancer. This may help doctors identify inflammatory breast cancer more quickly and more precisely by knowing what can cause inflammatory breast cancer to develop.

**What are the alternatives to participating in this tissue bank?**

Instead of allowing your samples to be stored in this tissue bank you can choose not to participate.

**What will participating in this tissue bank cost you?**

There is no cost to you to participate in this tissue bank.

**Will you be paid for participating in this tissue bank?**

You will not be paid to participate in this tissue bank. In the event that any commercial products are developed from your donated tissue, there are no plans to share any revenues with you from those commercial products. It is policy that all donated tissue is the property of the Organization.

**Who is paying for this tissue bank?**

IRB Version 1

This tissue bank is being paid for by the Department of Epidemiology at the University of Nebraska Medical Center.

**What should you do if you are injured or have a medical problem as a result of participating in this tissue bank?**

Your welfare is the main concern of every member of the tissue bank team. If you are injured or have a medical problem as a result of participating in this tissue bank, you should immediately contact one of the people listed at the end of this consent form.

**How will samples and information about you be protected?**

All necessary steps will be taken to protect your privacy and the confidentiality of your samples.

You have rights regarding the privacy of your medical information collected for this tissue bank. This medical information called "protected health information" (PHI). PHI used in this tissue bank, may include, results of physical exams, blood tests, x-rays, as well as the results of other diagnostic and medical procedures, as well as your medical history. Your tissue bank data and medical records will be maintained in a secure manner.

**Who will be able to use my samples for research?**

Your samples may be studied by the Investigators listed on this protocol, such as Dr. Paul Levine, whose research proposals have obtained approval from the Institutional Review Board at the University of Nebraska Medical Center will be able to access this tissue bank for research. Your samples may also be studied in the future by other researchers here at UNMC.

**Who will have access to information about you?**

By signing this consent form, you are allowing the tissue bank team to have access to your PHI. The tissue bank team includes the individuals listed on this consent form and other personnel involved in this tissue bank at the Institution.

Your PHI will be used only for the purpose(s) described in the section "What is the reason for creating this tissue bank?"

You are also allowing the research tissue bank team to share your PHI, as necessary, with other people or groups listed below:

- The UNMC Institutional Review Board (IRB)
- Institutional officials designated by the UNMC IRB
- Federal law requires that your information may be shared with these groups:

- The HHS Office of Human Research Protections (OHRP)
- The HIPAA Privacy Rule requires the following groups to protect your PHI:
  - The Eppley Cancer Center Scientific Review Committee (SRC)

You are authorizing us to use and disclose your PHI for as long as the tissue bank is storing your sample(s).

**What will happen if you decide not participate this tissue bank?**

You can decide not to participate in this tissue bank. Deciding not to participate will not affect your medical care, if applicable, or your relationship with the tissue bank team or the Institution. Your doctor will still take care of you and you will not lose any benefits to which you are entitled.

**What will happen if you decide to stop participating once you start?**

You can stop participating in this tissue bank (withdraw) at any time. Should you wish to stop participating in the tissue bank, you must contact one of these individuals in writing requesting that your samples and associated information be removed from the tissue bank and no longer used for future research purposes.

Dr. Paul Levine, College of Public Health  
c/o Department of Epidemiology  
984395 Nebraska Medical Center  
Omaha NE 68198-4395

**Will you be given any important information during the tissue bank?**

You will be informed promptly if the tissue bank team gets any new information that may affect whether you would want to continue participating in the tissue bank.

**What should you do if you have any questions about the tissue bank?**

You have been given a copy of "What Do I Need to Know Before Being in a Research Study?" If you have any questions at any time about this tissue bank, you should contact the Principal Investigator or any of the tissue bank personnel listed on this consent form or any other documents that you have been given.

**What are your rights as a participant in this tissue bank?**

You have rights as a participant in this tissue bank. These rights have been explained in this consent form and in The Rights of Research Subjects that you have been given. If you have any questions concerning your rights or complaints about the tissue bank, you can contact any of the following:

- The investigator or other tissue bank personnel

- Institutional Review Board (IRB)
  - Telephone: (402) 559-6463.
  - Email: IRBORA@unmc.edu
  - Mail: UNMC Institutional Review Board, 987830 Nebraska Medical Center, Omaha, NE 68198-7830
- Research Subject Advocate
  - Telephone: (402) 559-8941
  - Email: unmcrsa@unmc.edu

**Documentation of informed consent**

You are freely making a decision whether to participate in the tissue bank. Signing this form means that:

- You have read and understood this consent form.
- You have had the consent form explained to you.
- You have been given a copy of The Rights of Research Subjects
- You have had your questions answered.
- You have decided to participate in the tissue bank.
- If you have any questions, you have been directed to talk to one of the individuals listed below on this consent form.
- You will be given a signed and dated copy of this consent form to keep.

Signature of Participant: \_\_\_\_\_

Date: \_\_\_\_\_

My signature certifies that all the elements of informed consent described on this consent form have been explained fully to the participant. In my judgment, the participant possesses the legal capacity to give informed consent to participate in this tissue bank and is voluntarily and knowingly giving informed consent to participate.

Signature of Person Obtaining Consent: \_\_\_\_\_

Date: \_\_\_\_\_

**Authorized Tissue Bank Personnel**

**Principal**

Levine, Paul

alt email: paullevine@earthlink.net

phone: 402-559-4248

alt #: 301-469-7394

degree: MD

**Secondary**

Paul, Ladan

alt email: Ladan.Paul@gmail.com

alt #: 703-593-7979

degree: MD



## What Do I Need To Know Before Being In A Research Study?

You have been invited to be in a **research study**. Research studies are also called "research surveys", "research questionnaires" or "scientific protocols." **Research** is an organized plan designed to get new knowledge about health, disease, behaviors, attitudes and interactions of, among and between individuals, groups and cultures. The people who are in the research are called **research subjects**. The **investigator** is the person who is running the research study. You will get information from the investigator and the research team, and then you will be asked to give your **consent** to be in the research.

**This sheet will help you think of questions to ask the investigator or his/her staff. You should know all these answers before you decide about being in the research.**

What is the **purpose** of the research? Why is the investigator doing the research?

What are the **risks** of the research? What bad things could happen?

What are the possible **benefits** of the research? How might this help me?

**How is the research different** than what will happen if I m not in the research?

Will being in the research **cost** me anything extra?

Do I have to be in this research study? How will it affect my status at the institution if I say **no**?

Can I **stop** being in the research once I ve started? How?

Who will look at my **records**?

How do I reach the investigator if I have more **questions**?

Who do I call if I have questions about being a **research subject**?

**Make sure all your questions are answered before you decide whether or not to be in this research.**

## **THE RIGHTS OF RESEARCH SUBJECTS AS A RESEARCH SUBJECT YOU HAVE THE RIGHT**

**to be told everything you need to know about the research before you are asked to decide whether or not to take part in the research study.** The research will be explained to you in a way that assures you understand enough to decide whether or not to take part.

**to freely decide whether or not to take part in the research.**

**to decide not to be in the research, or to stop participating in the research at any time.** This will not affect your medical care or your relationship with the investigator or the Nebraska Medical Center. Your doctor will still take care of you.

**to ask questions about the research at any time.** The investigator will answer your questions honestly and completely.

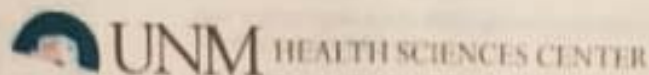
**to know that your safety and welfare will always come first.** The investigator will display the highest possible degree of skill and care throughout this research. Any risks or discomforts will be minimized as much as possible.

**to privacy and confidentiality.** The investigator will treat information about you carefully, and will respect your privacy.

**... to keep all the legal rights you have now.** You are not giving up any of your legal rights by taking part in this research study.

**to be treated with dignity and respect at all times**

**The Institutional Review Board is responsible for assuring that your rights and welfare are protected. If you have any questions about your rights, contact the Institutional Review Board at (402) 559-6463.**



Human Research Review Committee  
Human Research Protections Office

February 16, 2015

Eric Provenza, PhD  
EProvenza@salud.unm.edu

Dear Dr. Provenza:

On 2/16/2015, the HRRC reviewed the following submission:

Type of Review:	Modification and Continuing Review
Title of Study:	Molecular and Viral Characteristics of Inflammatory and Breast Cancer
Investigator:	Eric Provenza, PhD
Study ID:	11-030
Submission ID:	MODCR00001139
Funding:	None
Grant ID:	None
IND, IDE, or IDE:	None
Submission Summary:	Modification and Continuing Review #4 for Study 11-030 to add investigators Genie San Nicolas and Kaylee Deutsch.
Document Approved:	Mol Viral Protocol v02/20/2014 Study Summary of Progress v01/15/2015
Document Acknowledged:	Conflict of Interest documents and Human Subject Protection Training for added investigators.
Review Category:	Expedited: Category (1) Research involving materials that have been collected, or will be collected solely for non-research purposes and Category (1)(c) Continuing review of research previously approved by the convened IRB where the remaining research activities are limited to data analysis.
Determinations/Waivers:	Waived the requirement for informed consent. HIPAA Authorization Addendum waived. Study is closed to enrollment - no consents approved. Study is in data analysis phase only.
Submission Approval Date:	2/16/2015
Approval End Date:	3/6/2016
Effective Date:	2/16/2015

The HRRC approved the study from 2/16/2015 to 3/6/2016 inclusive. If modifications were required to secure approval, the effective date will be later than the approval date. The "Effective

Date" 3/16/2015 is the date the HRRC approved your modifications and, in all cases, represents the date study activities may begin.

Before 3/6/2016 or within 45 days of study closure, whichever is earlier, you are required to submit a continuing review. You may submit a continuing review by navigating to the active study and clicking the "Create Modification / CR" button.

Please use the consent documents that were approved and stamped by the HRRC. The stamped and approved consents are in a comment within the submission covered by this approval letter.

This determination applies only to the activities described in this submission and does not apply should you make any changes to these documents. If changes are being considered and there are questions about whether HRRC review is needed, please submit a study modification to the HRRC for a determination. A change in the research may disqualify this research from the current review category. You can create a modification by clicking Create Modification / CR within the study.

In conducting this study, you are required to follow the Investigator Manual dated July 31, 2012 (HRP-103), which can be found by navigating to the IRB Library.

Sincerely,



Mark Holdsworth, PharmD  
Executive Chair