# Elucidating the role of STAT3 and VEGFA in Breast Cancer and Diabetes Mellitus Type 2 patients


**BY**

**Aiman Fatima**

**00000277875**

**Atta-Ur-Rahman School of Applied Biosciences (ASAB)**

**National University of Sciences & Technology**

**Islamabad, Pakistan**

**2018-2021**

# Elucidating the role of STAT3 and VEGFA in Breast Cancer and Diabetes Mellitus Type 2 patients

A thesis submitted as a final year project in partial fulfillment of the requirement for the degree of Master of Science


**BY**

**Aiman Fatima**

**00000277875**

**Supervised By: Dr. Peter John**

**Atta-Ur-Rahman School of Applied Biosciences (ASAB)**

**National University of Sciences & Technology**

**Islamabad, Pakistan**

**2018-2021**

*I would like to bestow this*

*thesis*

*to My*

**BELOVED PARENTS and HUSBAND**

**For their unconditional love and**

**support.**

FORM TH-4

# National University of Sciences & Technology

## MS THESIS WORK

We hereby recommend that the dissertation prepared under our supervision by:

Aiman Fatima & Regn No. 00000277875

**Titled**: Elucidating the role of STAT3 and VEGFA in Breast Cancer and Type 2 Diabetes Mellitus patients be accepted in partial fulfillment of the requirements for the award of MS in Healthcare biotechnology degree with ( A grade).

### Examination Committee Members

1. Name: Dr. Iram Murtaza Signature:

2. Name: Dr. Attya Bhatti Signature:

3. Name: Dr. Saira Justin Signature:

Dr. Saira Justin
Assistant Professor
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Supervisor's name: Dr. Peter John Signature:

Dr. Peter John
Tenured Associate Professor
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Date: 15th Feb 2021.

Dr. Tauqeer Ahmed
Head of Department (HoD)
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Date: _____ Head of Department

### COUNTERSINGED

Date: _____ Dean/Principal

## DECLARATION

I certify that this research work titled **"Elucidating the role of STAT3 and VEGFA in Breast Cancer and Diabetes Mellitus Type 2 patients"** is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources has been properly acknowledged/referenced.

Signature of Student

Aiman Fatima

00000277875

**THESIS ACCEPTANCE CERTIFICATE**

Certified that the thesis entitled **"Elucidating the role of STAT3 and VEGFA in Breast Cancer and Diabetes Mellitus Type 2 patients"** submitted by Aiman Fatima has been found satisfactory for the requirement of the degree.

Supervisor: _____

Dr. Peter John
Tenured Associate Professor
Deptt of Healthcare Biotechnology
Atta-ur- Rahman School of Applied
Biosciences (ASAB), NUST Islamabad

Dr Peter John

ASAB, NUST

Head of the Department: _____

Dr. Touqeer Ahmed
Head of Department (HoD)
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied
Biosciences (ASAB), NUST Islamabad

Dr Touqeer Ahmed

ASAB, NUST

Principal: _____

Dr Hussnain A. Janjua

ASAB, NUST

Dated: _____

# CERTIFICATE FOR PLAGIARISM

It is certified that M. Phil/MS Thesis Titled **"Elucidating the role of STAT3 and VEGFA in Breast Cancer and Diabetes Mellitus Type 2 patients"** of Ms. Aiman Fatima Reg No. 00000277875 has been examined by us. We undertake the follows:

a. Thesis has significant new work/knowledge as compared already published or are under consideration to be published elsewhere. No sentence, equation, diagram, table, paragraph, or section has been copied verbatim from previous work unless it is placed under quotation marks and duly referenced.
b. The work presented is original and own work of the author (i.e., there is no plagiarism). No ideas, processes, results, or words of others have been presented as Author own work.
c. There is no fabrication of data or results which have been compiled / analysed.
d. There is no falsification by manipulating research materials, equipment, or processes, or changing or omitting data or results such that the research is not accurately represented in the research record.
e. The thesis has been checked using TURNITIN (copy of originality report attached) and found within limits as per HEC plagiarism Policy and instructions issued from time to time.

Supervisor: _____

Dr Peter John

ASAB, NUST

Dr. Peter John
Tenured Associate Professor
Deptt of Healthcare Biotechnology
Atta-ur- Rahman School of Applied
Biosciences (ASAB), NUST Islamabad

# Acknowledgements

All ultimate praises to ***Almighty Allah***, for the wisdom HE provided to me, the peace of mind HE gave and the good health, by which I was able to complete this research.

Afterwards, foremost, I would like to express sincere gratitude to my supervisor, **Dr. Peter John**, for the continuous support on the MS study and research. His guidance helped me in all the time of research and writing of this thesis. He has been supportive since the day I began working on this project. I am grateful to him from the core of my heart for his expert guidance, skilled advice, generous criticism, and sympathetic attitude without which it could not have been possible to complete this research work. My sincere gratitude to my GEC members **Dr Saira Justin** and specially **Dr Attya Bhatti** for their immense guidance and support. They have always welcomed the queries and assisted me throughout my research period. Also, I would like to praise **Dr. Irum Murtaza**, the external supervisor for the thesis, for her patience, motivation, enthusiasm, and immense knowledge, which she poured into me and my project to make it meaningful and worthy. Besides my supervisors, I thank my fellow lab mates in ASAB; Nida Fatima, Ilhaam Ayaz Durrani, Saria Shafique Bhutta, Ayesha Imran and from SMME, Maria Qubtia Malghani for their company and moral support in the ups and downs of lab work. I would thank them for their continuous help and encouragement, stimulating discussions, for the sleepless nights we were working together before deadlines. I would also like to thank Nida Ali Syed, Shanzay, Zuhra Qayyum for their self-less guidance and **Dr Jahangir** and **Dr Maryam** from Holy Family Hospital Rawalpindi for helping in sampling. Nobody has been more important to me in pursuit of my MS studies than the members of my family. I would like to thank my husband **Muhammad Faisal Munir Virk** for his support during my studies. I also want to thank my sister and brothers Saman Fatima, Abdullah and Amanullah for providing me all the support in times when I needed the most. My sincerest regards to my in-law parents **Tahira Yasmeen** and **Munir Ahmed Virk** for the trust and love they bestowed me with. And last but not the least, I would like to thank my **Mama (Afshan Jabeen)** and **Papa; Muhammad Qasim Ali** (late), for bringing me to this world, for giving the leap of faith and supporting me spiritually, physically, mentally, and financially throughout my life. Without them nothing would have been possible. ***Aiman Fatima.***

# Table of Contents

# LIST OF TABLES

# LIST OF FIGURES

# List of Graphs

# List of Acronyms

| | |
|---|---|
| AGE | Advanced glycation end product |
| AKT | Protein kinase b |
| AMPK | AMP activated protein kinase |
| AR | Androgen receptor |
| BCL2 | B cell lymphoma 2 |
| BMI | Body mass index |
| CCLE | Cancer cell line encyclopedia |
| EDTA | Ethylene-diamine-tetra-acetic-acid |
| EGF | Epidermal growth factor |
| ER | Estrogen receptor |
| GAPDH | Glyceraldehyde3-phosphate dehydrogenase |
| GLUT4 | Glucose transporter type 4 |
| GSK3β | Glycogen synthase kinase 3 beta |
| HDL | High density lipid |
| HER | Human epidermal growth factor receptor |
| IGFR | Insulin growth factor receptor |
| IL-6 | Interleukin-6 |

| | |
|---|---|
| IR | Insulin resistance |
| JAK/STAT | Janus kinase |
| MAPK | Mitogen activated protein kinase |
| NEFAs | Non-esterified fatty acids |
| NEU | N-ethyl-N-nitrosourea Oncology |
| NF-KB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NOTCH | Notch homolog 1, translocation-associated (Drosophila) |
| P13K | Phosphatidinositol-3 kinases |
| PARP | Poly-ADP ribose polymerase |
| PR | Progesterone receptor |
| ROS | Reactive oxygen specie |
| SDS | Sodium dodecyl sulphate |
| SHBG | Sex hormone binding globulin |
| SRC | sarcoma |
| STAT3 | Signal Transducer and transcription 3 |
| TNF-α | Tumor necrosis factor alpha |
| VEGFA | Vascular endothelial growth factor A |
| VLDL | Very low-density lipids |
| WNT | Wingless and int |

# ABSTRACT

Breast Cancer and Diabetes Mellitus type 2 are complex and leading diseases worldwide resulting in huge morbities. These are characterized with some common risk factors including oxidative stress, sedentary lifestyle, obesity, family history, poor diet, hyperlipidemia, hyperglycemia, hyperinsulinemia, stress, elevated blood pressure, smoking, and high cholesterol. Also, these diseases share complicated pathophysiology, and various etiologies. There are certain genetic factors influencing the severity of the diseases. There are many people who suffer from both diseases because of the uncontrolled and unpredictable signaling process. The identification of crucial biomarkers is inevitable for designing of drug that can be targeted to the desired area lessening the pathogenesis. The present medications available are either solely for the Diabetes or Breast cancer because the etiology or mode of other disease development is still not well studied.

The present study aims to find more effective biomarker to be targeted by drugs that can lessen the severity of disease by lowering the expression of these biomarkers. Two genes namely STAT3 and VEGFA were selected from JAK/STAT signaling pathway that had roles in carcinogenesis, high blood pressure induction, hyperinsulinemia, and hyperglycemia. The blood of patients was obtained to get RNA extracted by Trizol method which was later quantified to check the purity of RNA. cDNA was then synthesized. RT PCR of STAT3 and VEGFA showed higher expression of these genes in disease individuals as compared to healthy ones. The higher expression of STAT3 causes the high expression of VEGFA as well. The statistical analysis of RT-PCR results proved these genes to be possible effective biomarkers for Breast cancer and Diabetes Mellitus Type 2 patients.

# INTRODUCTION

## 1.1. Diabetes Mellitus Type 2 and Breast Cancer

T2DM and Breast cancer are complex diseases characterized by insulin resistance, hyperinsulinemia, changes in steroid hormones, mutated genetic makeup and abrupt signaling of certain growth hormones. These diseases share multiple etiology, common risk factors and complex pathophysiology (Samuel *et al*., 2018).

Research studies had already shown sedentary lifestyle, more consumption of carbohydrates combined with saturated fats in more developed countries see high prevalence of these diseases as compared to developing countries. The developing countries are however, now increasingly adopting the trend of lifestyle established by more rich societies which has tremendously enhanced the Breast cancer and diabetic patients (Devesa *et al.,* 1995). Diabetes is most prevalent chronic illness affecting millions of lives which includes women, youth, children, and adults. Diabetes is caused when body becomes unable to use insulin consisting of 90% of Diabetic subjects around the globe (Wolf *et al*., 2005).

Pre-Diabetes is often defined by the occurrence blood glucose in amount more than normal leading to insulin resistance and glucose intolerance but is not enough to be labelled as Diabetes. When Pre-diabetic stage progresses the insulin resistance in the body causes metabolic reprogramming. The process of reprogramming causes three main changes in metabolism namely, hyperinsulinemia, hyperglycemia, and dyslipidemia. The series of fluctuations proceeds to spread inflammation. The metabolic changes are responsible to subsequently lead towards a state of cell signaling dysregulation. The pathways getting affected include NF-KB, P13K/AKT/mTOR, MAPK, Notch, WNT and glucotoxicity related oxidative stress. This helps to generate a suitable microenvironment for proliferation of cancerous cells (Michels *et al*., 2003).

Breast cancer is most common cancer type with almost 28% and stands 2nd in cancer reported deaths exactly 15% among women. Breast cancer and Diabetes often occur simultaneously with almost 18% of Breast cancer suffering individuals have Diabetes. It is also reported from a meta-analytic study investigated in MEDLINE showing a positive link between Breast cancer and

T2DM. In a diabetic woman there is 20% elevated chances of Breast cancer development (Cohut, 2017).

Breast cancer incidence increases in aged people and is found in association with several hormonal triggers, family history, benign stage of Breast cancer and genetic factors. One of the critical risk factors shared by both the diseases is Obesity which is very common in developed countries. Obesity is caused by chronic inflammation that can ignite the cancer onset and result in cancer progression. The establishment of molecular association in adipose tissue, obesity and Breast cancer go behind inflammatory details where it is found that the neoplastic transformation is initiated by reactive oxygen species that may act indirectly or directly to influence the components of the cell. The free radical is also accountable for the induction of secondary amplification of inflammation process thus generating bundle of other reactive species (Baron *et al*., 2001).

## 1.2. Symptoms

Although symptoms of Breast cancer and Diabetes Mellitus type 2 are completely different but that will help distinguish when the disease begin. Following symptoms are of Diabetes which are Polydipsia often called (increased thirst), Polyuria in other words (increased urination), Polyphagia that is increased hunger, weight loss and sudden blurry vision. You often have asymptomatic hyperglycemia that would be enough to cause functional and pathological alterations in various tissues of body. These changes could be present from a extended time before diabetes could be noticed. While on the other hand Breast cancer symptoms are mostly region specific which are thickened tissues in either of the breast absent before the cancer, A change in the shape or size of any of the breasts or both, discharge of fluid from the nipple, presence of lumps in either of the armpits, presence of rash on the breasts. Breast pain is generally not a major symptom of Breast cancer.

## 1.3. Role of Oxidative Stress, Inflammation and Obesity in T2DM and Breast Cancer Microenvironment

A loss of balance state between oxidants and antioxidants is called Oxidative stress, which is essential for cells to work properly. The increased levels of free radical species because of hyperglycemia causes peroxidation of lipids which is symbolic of cell damage. For a healthy cellular function maintenance, normal redox reaction and proper signaling pathways, low levels of

reactive species are required. But the diseased state in body causes disruptions to produce excessive amount of these free radicals, causing damage to carbohydrates, proteins, DNA, and lipid particles that are essential in the maintenance of cell function resulting in the complete development of disease. DNA damage have link with variety of diseases which include T2DM and Cancer as DNA is the target of oxidative stress. Few examples of DNA damage due to oxidative stress include excised ds or ss DNA, apurinic DNA and oxidized nitrogenous bases (Vigneri *et al*., 2009). The action of free radicals is on chromatin and nitrogenous bases causing changes in the gene expression. High chances of activation of events that could induce tumor suppressing genes to become expressed and trigger cancer. This situation justifies the proposed link between diabetes and cancer showing a risk in diabetic patients of developing different type of cancers effecting major organs such as pancreas, lung, breast, sex organs and stomach. The varied reactive species involved in disturbing normal bodily metabolic functions are ROS, reactive chlorine species RCS and reactive nitrogen species RNS. Though body have counteracting forces like repair enzyme for oxygen damage like glutathione peroxidase, superoxide dismutase, catalase, vitamin C and E, zinc, Cu, glutathione reductase that help delay the oxidative attack and help body to regulate its homeostasis but the imbalance between the ROS and antioxidant defense leads to loss in intricated network of cellular function (Shikata *et al*., 2013).

The DNA damage is usually induced by inflammation caused by ROS initiating genomic alterations, instability in epigenetic events, cellular proliferation, metastasis, neovascularization, apoptotic resistance, and invasion. The series of such irregular events lead towards the initiation of cancer through mechanism in which oxidative stress affects the breast cancerous cells is unknown. Several evidence and studies support research finding that the reactive oxygen species involved in oxidative stress produces products like DNA adducts, malondialdehyde, isoprostanes and lipid peroxidation and these products are identified frequently in breast cancer diagnosed patients (Boyle *et al*., 2012).

In addition to oxidative stress, second most common trigger for the disease development is inflammation. Several factors are responsible for chronic inflammation such as sustained cellular injuries, physical and chemical factors leading towards carcinogenesis. There are signaling and metabolic pathways derived by genomic events and inflammation cause predisposition to cancer and neoplasia. Tumor development and inflammatory cells go hand in hand as inflammation acts

as promoter of tumor development. Tumor microenvironment involves activity of powerful cytokines that along with inflammatory cells is involved in cancer development. The entire tumor organ is maintained by them regulating growth, differentiation, and migration of all cell types inside the tumor state. The essential cytokines could also influence suppression of immune system and remodeling of tissues, that is important constituent of all types of tumors (Rivenbark *et al*., 2013). Chronic activation of immune system and chronic inflammation may occur in cancer population when there is imbalance between anti-inflammatory mechanism and pro-inflammatory process. Inflammation is most of the time viewed as causative of cancer, but recent studies suggest that cancer also cause inflammation. The inflammation induced by cancer can be due to induced activity of intrinsic pathways because of genomic events leading to production of new abnormal tissues. It is also concerning fact that malignancies become severe in the presence of inflammatory cells while on the other hand cancer could also produce oncogenic alterations causing tumor-enhancing inflammatory climate. Inflammation also participates in disturbing body's response to cancer treatments and chemotherapeutic drugs. Inflammation also aids in growth, angiogenesis, and metastasis (Vigneri *et al*., 2009).

Obesity has also concerning effect on the insulin resistance as it decreases body's response to insulin action. The increased abdominal obesity is associated with inflammation of adipose tissues resulting in the production of cytokines causing changes in the circulation of adipokines concentration. The alterations in body's BMI may contribute to increase insulin resistance causing release of more insulin from the pancreatic beta cells to sustain normal glucose levels, leading to hyperinsulinemia. Insulin like growth factor (IGF-I) synthesis occurs creating response for action of endogenous insulin on liver by a growth hormone resulting in fluctuation of IGF binding proteins. In type 2 Diabetes mellitus beta cells of pancreas decompensate and hyperglycemia develop. The increase in insulin resistance is linked with lipid abnormalities, causing elevation of VLDL and triglycerides, decrease in production of sex hormones which bind to globulin leading to high number of free hormones like estrogen and testosterone. Moreover, more adiposity leads to increase in local aromatization of estrogen and androgens that affect growth of tumor (Smith *et al*., 2010).

## 1.4. Deciphering Dysregulation of Epigenetic Machinery

Heritable changes in the expression of genes mitotically or meiotically which occurs without changing/altering DNA sequence, is referred as Epigenetics. Cellular regulation is achieved by epigenetic machinery like histone modification, non-coding RNAs, positioning of nucleosome and DNA methylation. Among all the mechanisms, common marker known is definitely DNA methylation. It occurs in CPG islands to regulate the integrity and conformation of chromosomes to prevent plausible damage from mobile genomic elements. DNA modification as a result of inflammation and oxidative stress occurs not only by direct mutations in DNA but also by epigenetic factors. ROS and inflammation contribute to carcinogenesis by deregulating the epigenetic elements in body (Font-Burgada *et al*., 2016). It impacts on the activity of enzymes and gene expression implicated in epigenetic control causing hypo/hyper methylation. Changes in the expression of noncoding RNAs and posttranslational modification is also influenced by oxidative stress. This results in modification of tumor suppressor gene activity and oncogenes sparking carcinogenesis process. The main participants of the epigenetic process are DNA methyltransferases, histone methyltransferases, histone acetyl transferases, histone demethylases and histone deacetylases (Hoy *et al*., 2017).

Non-coding RNAs have important role to control gene expression. Human genome though produces varied number of non-coding RNAs where miRNA is most studied. The microRNA has role to bind at specific regions on target mRNAs and mediating posttranslational silencing of gene by degrading or blocking the transcripts. By this mechanism it plays impression on the cell differentiation, proliferation of cells, regulation of cell cycle, apoptosis, and cell development. Irregular changes in epigenetic regulation have extensive range of effects on the health and has been defined in many disorders like T2DM, overweightness, autoimmune diseases, cancer, and inflammatory bowel disorder (Vona-Davis *et al*., 2009). Variety of factors influence the regulation of epigenetics like environmental exposure, oxidative stress, inflammation and diet and it clearly show link between disease risk and lifestyle. In cancer oxidative stress causes hypomethylation at CpG island by producing ROS and carcinogens, also induces hypermethylation at tumor suppressing genes such as E-cadherin and p16INK4a. For miRNA expression and histone modification one of the elements implicated are damage by oxidative stress. This shows an indicator that by regulating the epigenetic profile of certain genes oxidative stress plays pivotal role in beginning of tumor and its progression (Delort *et al*., 2015).

## 1.5. Metabolic Complications and RISK FACTORS

T2DM causes abnormal body mass (BMI), hyperactive adipocytokines and is also implicated with pathophysiology of cancer. There are three metabolic instabilities in diabetes including dyslipidemia, hyperglycemia, and hyperinsulinemia.

### 1.5.1. Dyslipidemia

The state of increased triglycerides, non-esterified fatty acids and cholesterol is dyslipidemia. High level of circulating triglycerides, decreased HDL, increased level of total cholesterol has been found to be associated with 18%, 15% and 20% of more chances of cancer. The presence of Diabetes mellitus type 2 causes enhancement in levels of non-esterified fatty acids that have crucial part in PKCθ initiation which in return causes activation of AKT which initiates series of events leading to the oncogenic phenotype by the expression of certain genes. The pathway shown below gives us insight how breast cancer cells confer multidrug resistance (Baumann *et al*., 2013).

### 1.5.2. Non esterified fatty acids

Non esterified fatty acids play crucial role as mentioned early in the tumor promotion, aggressiveness, migration, and metastasis by activation of atypical protein kinases. PKCθ induces Akt and translational activity to influence the expression of genes like STAT3, Bcl-xl and Rel B (increase invasiveness). In adipose tissues and skeletal muscle of diabetic patients NEFA trigger PKCθ which phosphorylate and activate IκB-kinase which in return phosphorylate (IRS1). The IRS1 capability to join SH2 domain of p85 (P13K) gets inhibited. This cause damage of insulin signal transduction and ultimately results in insulin resistance (Santos *et al*., 2012).

### 1.5.3. Cholesterol

Cholesterol's levels in elevated state along with LDL-C (low-density lipoprotein cholesterol) and very LDL-C could be autonomous risk factor in causing Breast cancer. For lipid raft development, Akt signaling and steroid hormone synthesis cholesterol play important role and in dividing cells of breast membranes it increases incidence of angiogenesis. Cholesterol metabolite that is 27-hydroxycholesterol (27-HC) plays role in ER-positive Breast cancer by acting as agonist. In ER-positive Breast cancer, cholesterol gets metabolized by CYP27A1 to make 27-hydroxycholesterol to activate signaling pathways. The expression increases of gene CYP27A1 in breast tumor cells which is relevant with high cancer grades. The 27-HC is chosen estrogen receptor modulator called as (SERM) bind to ER intracellularly in ER-positive cancerous cells in Breast and excites

proliferation of cells by activating Akt/GSK3β/ β-catenin and Akt/mTOR pathways. So, to maintain cholesterol homeostasis, 27-HC activate receptor of nucleus and liver-x-receptor to promote cholesterol abolition. It is main precursor for variety of sex hormones too like estrogens, androgens, progesterone, and other derivatives (Monaco et al., 2017).

### 1.5.4. Adipokines

*Adipokines* are cytokines secreted by adipose tissue and adipocyte are lipocyte or fat cells present in adipose tissues. It is widely known that both diabetes and Breast cancer have shared risk factors one of them is obesity. The altered BMI and excessive body weight contribute to 90% incidence of diabetes, also hypercholesterolemia, insulin deficiency and insulin resistance are important pathophysiological reasons behind diabetes and diabetes derived Breast cancer. Most predominant population of cells in the breast are of adipocytes. The dysregulation of normal stroma adipocyte has crosstalk between Breast cancer cell is related with improved cellular propagation and incursion of cancer. High levels of adipocyte induced inflammatory cytokines in tumor area like TNFα, IL-6 and IFNs are indicative of their association with amplified development of cancer. Leptin, the first discovered adipokine have a known role in the risk of Breast cancer. The high level of leptin in postmenopausal women indicate elevated risk of Breast cancer while low plasma leptin level in women with pre-menopause show high risk of Breast cancer. Moreover, the high leptin level is known to promote cell growth and survival. The activation of JAK2/STAT3, PKC, JNK/p38 MAPK, MAPK/ERK and P13K/Akt pathways causes proliferation of cells, angiogenesis, invasion, migration, and Breast cancer cell metastasis. *Adiponectin* has a significant role in inhibition of leptin induced inflammation, angiogenesis by instigation of AMPK pathway causing inhibiting of mTOR and p42/p44/MAPK pathways thereby reducing tumorigenesis. But the decreased levels or abrogated adiponectin has negative role carried by inhibition of AMPK pathway and ultimately activating cancer promoting pathways (Luo *et al*.,2017).

### 1.5.5. Altered lipid metabolism

Transformed/altered lipid breakdown is hallmark features of Diabetes and its prevalent and consistent state creates appropriate tumor microenvironment meeting anomalous demands of cell division. The immediate availability of diacylglycerol and ceramide courtesy of lipid accumulations in liver and muscle affects insulin signaling. Thus, increased insulin secretion and

compensatory enhanced IGF accompanied by lack of IGF-BP (Insulin growth factor binding protein) stimulate anti-apoptotic process and cell proliferation (Maruthur *et al*., 2009).

### 1.5.6. Hyperinsulinemia

Insulin released from the β-cells of pancreas as a result of elevated glucose concentration in blood. There are two components of insulin pathophysiology one is pathological derived from increased apoptotic activity and other is functional that comes from lessened insulin sensitivity and abnormal kinetics of insulin secretion. Though insulin has basic role in lipid, protein, and carbohydrate breakdown but it is also important as growth factor. Insulin stimulates mitosis and migration of cells by inhibiting cell apoptosis. Glucose transport, one of the metabolic effects of insulin is mediated via P13K whereas the mitogenic effects like Ras gene activation is done by MAPK pathway (Kim *et al*., 2015).

Insulin works by binding to IR in skeletal muscle which is a tyrosine kinase receptor of insulin, liver and adipose tissue that promote glucose uptake. It is also seen that IR is expressed abruptly in Breast cancer cell and its high occurrence causes rise in malignancy of Breast cancer epithelial cell line. IR binding to insulin causes phosphorylation of the tyrosine residue present in the cytoplasmic region of IR leading to high tyrosine kinase activity. This consequences in phosphorylation of IRS and SHC adaptor of protein 1. It also causes initiation of P13K/Akt pathway which thereby primes to phosphorylation of GSK3β. The phosphorylation of glycogen synthase kinase β causes β-catenin secretion of death complex that consists of axin, GSK3β, cancer suppressors and casein kinase. GSK3β initiates the expression of marked genes ultimately causing proteins translation that control migration, differentiation, cell cycle and anti-apoptosis. High insulin level induced p13k activation induces VEGF to promote and sustain growth of tumor and angiogenesis (Carracedo *et al*., 2013).

### 1.5.7. Insulin like growth factor and the receptors (IGFR)

IGFR are associated with harmful effects in cancer cells. It is clear though that insulin attaches to its receptor IR but in hyperinsulinemia condition insulin attaches and activates IGFR mechanism for signaling because of identical homology of receptor. It is also significant as mentioned early that increased insulin levels cause decrease of IGF-BP. Decreased levels of IGF-BP leads to growth in of plasma bioactive IGFs level. There are multiple pathways of signaling that are modulated by the response of IGFR signaling and are involved in the defense of Breast cells from

the process of apoptosis developing anti-apoptotic strength. This also induces increase in drug resistance of these Breast cancer bodies by regulation of IGF system (Samuel *et al*., 2012).

### 1.5.8. Hyperglycemia

Systematic increase in insulin resistance causes hyperglycemia, a state with elevated circulating glucose levels in blood. This causes more cell proliferation, anti-apoptosis, and metastasis of cancer cells. Especially in post-menopausal women improper glucose tolerance has direct link with Breast cancer commencement and invasiveness because of free glucose circulation in blood. The enhanced uptake of glucose in these cells is well-known hallmark of cancer. There are four main ways by which hyperglycemia can affect the proliferation and cell division of Breast cancer cell. First, the presence of insulin growth factor or insulin in excessive amounts due to lack of IGF-BP. Second, increased level of cytokines playing role in inflammation such as TNF-α or IL-6 can provide possible tumor sustainable conditions. Third, by the help of free reactive oxygen species creating an oxidative stress. Fourth, by the help of platelet activation in tumor cells (Dalamaga *et al*., 2012).

Hyperglycemia is associated with active aerobic glycolysis contributing to increased glucose oxidation and production of superoxide radicals. Elevated level of free radicals induces damage to DNA and subsequent activation of polymerase enzyme (PARP) and other reparative enzymes. But GAPDH ribosylation via PARP directs glucose to enter biochemical pathway instead of glycolytic pathway. This results in increase amount of production of hexosamine and polyol which thereby increases the levels of advanced glycation products (AGE) that are linked to hyperglycemia derived increased risk Breast cancer. AGEs produced by glucose intolerance interact with its receptors (RAGE) causing ROS production leading to activated signaling of NF-KB and damage to cells (Chen and Wang, 2011).

## 1.6. Possible Biomarkers

### 1.6.1. Role of Signal Transducer and Activator of Transcription 3 (STAT3)

STAT3 causes proliferation of cells induces cytokines and other growth factor. The activation of STAT3 transmits signaling from various receptor to nucleus so as to regulate the down-stream gene expression like those involved in uncontrolled cell division. Stat3 also positively modulates other growth factors like proangiogenic VEGF. The process of programmed cell death called as apoptosis is crucial for carcinogenesis and tumor formation. Stat3 induces Bcl-2 and Bcl-xL to limit apoptosis thus worsening the situation for breast cancer patients.

STAT3 plays as pivotal modulator of cancerous cell apoptosis and proliferation. The constitutive expression of STAT3 involves in conversion of normal cells to variety of cancers. *G-protein coupled receptor signaling pathway, receptor tyrosine kinase signaling pathway, cytokine/JAK/STAT3 pathway* are three most vital pathways in the activation of STAT3 and malignant transformation. STAT3 has vital part in advancement of insulin resistance in T2DM and skeletal muscle. The beta cell islets get damaged by abnormally activated STAT3, thus reducing insulin synthesis (Kujawski et al., 2008).

### 1.6.2. Vascular Growth Endothelial Factor A

The angiogenic switch in multiple cancers has been found to be related with vascular endothelial growth factor A (VEGFA) up-regulation stimulated by STAT3 binding to the nucleus and initiating transcription process. The aberrant growth of blood vessels facilitates various disease processes. VEGF cancer cell signaling is liable for resistance to apoptosis stimuli and its relocation and invasion. VEGF is up regulated in breast cancer. About 72–98% of breast cancer cases are positive for Vascular endothelial growth factor confirmed by immunohistochemistry. The VEGF amount in the bloodstream is raised in people with elevated blood pressure and diabetes (Mantovani et al., 2008).

## 1.7. Complications associated with T2DM and Breast Cancer

Chronic hyperglycemia when proceeds unattended for long time it may be accompanied with growth impairment and susceptibility to certain infections. Uncontrolled diabetes could invite several other complications like it can result in acute life-menacing episodes of ketoacidosis or non-ketonic hyperosmolar syndrome. Peripheral neuropathy, retinopathy with the danger of foot

ulcer and amputation leading to kidney failure, autonomic neuropathy producing gastrointestinal, genitourinary, and cardiovascular symptoms, and sexual dysfunction are the common long-term complications of T2DM and Breast cancer. Hypertension and irregularities of lipoprotein breakdown are common in Diabetes and Breast cancer (Wolf et al., 2005).

## 1.8. Epidemiology

Breast cancer and Diabetes are most lethal diseases affecting millions of people across the globe. Breast cancer is responsible for affecting almost 12% of women inside Europe and North America while 11% of these cases happen in women who are younger than that of 35 Years. Therefore, Breast cancer becomes the foremost reason of deaths amongst young women in developed world. While the comparative risk causing T2DM development for an overweight person comes 7.19 (95% Cl: 5.74-9.00) and 2.99 (95 % Cl: 2.42-3.72) for obese individual in comparison to normal weight person, the Figure.1 shows the WHO health statistics of T2DM and Breast cancer below.

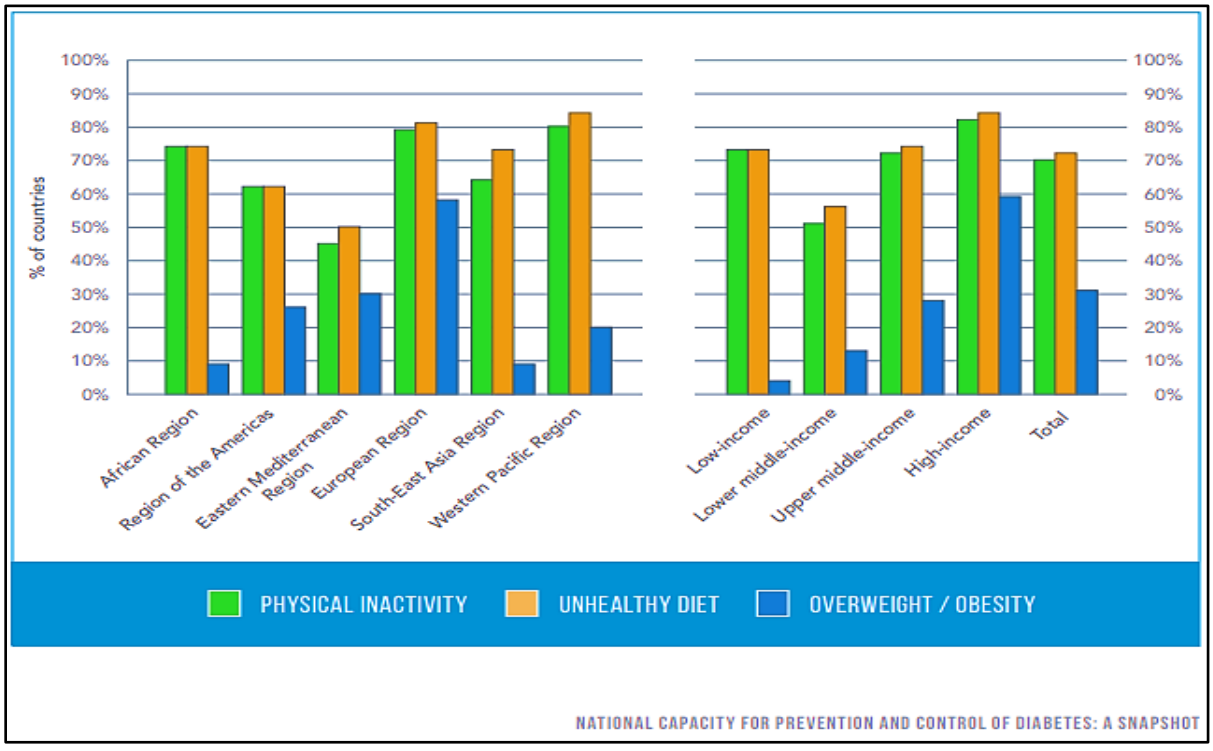



**Figure.1.1. Percentage of countries reporting operational policies for selected Risk factors, by WHO region and country income group. (WHO report, 2016).**

Meta-analytical report shows 20-28% enhanced danger of Breast cancer in women already have history of T2DM. Also, the subjects with Breast cancer and diabetes have 50% increased chance of mortality than alone any of these diseases. Though Breast cancer is genetically diverse and complex disease with multiple clinical characteristics but 16% of Breast cancer subjects also show signs of Diabetes indicating chances of 10-12% of high risk of developing the cancer amongst the diabetic women. It is also reported that the impaired glucose worsens the prognosis of Breast cancer (Shera *et al*., 2007).

## 1.9. Treatments

There are multiple treatments are use separately for each disease. Modern technology has been enabling the mankind to undergo surgeries and early detection of the both the diseases, despite of that the mortality rate is alarmingly high. The lack of research on determining the common prognostic biomarkers is hailing the route to determine the exact pathways being affected.

Following are some of the treatments used for Diabetes Mellitus Type 2:

### 1.9.1. Oral Anti-diabetic drugs

Several of the anti- diabetic drugs has been used to stop the mechanism of action of T2DM. Some of these agents aim to improve the insulin secretion this include sulphonylureas, glinides and rapid acting secretagogues, while on the other hand some of the agents act to target the insulin resistance these include metformin and thiazolidinediones (Krentz and Bailey, 2005).

### 1.9.2. Insulin

Exogenous insulin is used with other oral agents to stop the severe diabetes mellitus state as it progresses to the exhaustion of beta-cells in pancreas (Krentz and Bailey, 2005).

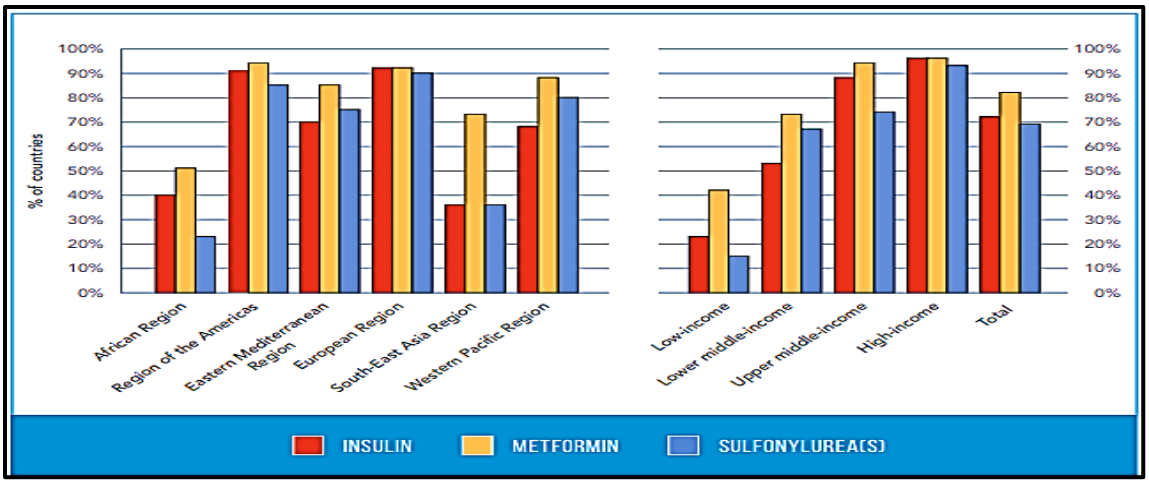



**Figure.1.2. Percentage of countries reporting essential medicines which are generally available in publicly funded pharmacies in Primary Healthcare facilities (WHO report, 2016).**

Following are the treatments used for Breast cancer:

### 1.9.3. Surgery

Breast cancer cannot be cured by just medicines it requires urgent surgery so to stop the tumor from spreading which include surgery for breast conserving called as lumpectomy, mastectomy, and dissection of the lymph node.

### 1.9.4. Chemotherapy

Radiations are used to cure the cancer, but it does have some side effects that's why gene therapy is been studied to go for as a substitute of the chemotherapy.

### 1.9.5. Hormonal Therapy

It happens to block the cancer cells from getting the required hormones that will help them proliferate.

### 1.9.6. Drugs to reduce the risk

A list of reference drugs is used to diminish the danger of Breast cancer and these are given according to the stage of the cancer.

## 1.10. Aims and Objectives

This study aims to reveal the possible biomarkers that will help in crosstalk between two major ailments Breast cancer and Diabetes Mellitus Type 2. I tend to achieve my objectives by

- Investigating the critical role of STAT3 and VEGF expression in individual's with T2DM and breast cancer in comparison to healthy controls through RTPCR.
- Conducting gene expression profiling of candidate genes that may play a role in linking both the diseases.

It will ease in finding the possible drug target and will also give an insight for drug designing. This will ultimately help in the treatment and diagnosis of breast cancer early in diabetic patients.

# Review of Literature

## 2.1. Background

Diabetes mellitus type 2 and Breast cancer are most common health diseases. Both the diseases are closely linked. Despite of the great epidemiological data present, the mechanism of association is still undetermined. The proposed mechanism through which obesity cause Breast cancer is same as the diabetes affect Breast cancer prognosis and risk. The adipose tissue dysfunction induces inflammation which is assumed to be vital association between cancer and obesity. It causes production of free radicals and ultimately result in Oxidative stress that establishes a microenvironment suitable for the tumor spread. The epidemiology suggests that Diabetes Mellitus affects 15% of older people more than 60 years and 7% of adults. Both these diseases occur frequently together about 18% of time. Most common risk factor for both the diseases is overweightness. The adipose tissue dysfunction and different adipocyte-derived hormone secretion or cytokine production are detected to cause release of adipokines resulting in inflammation and large number of fatty acids. The resultant fat deposition and lipotoxicity accumulates in pancreas, liver, and muscle cells. The obese condition also promotes hyperinsulinemia, insulin resistance and T2DM (Lorincz and Sukumar, 2006).

## 2.2. Possible Associations: Signaling Pathways

There are certain metabolic changes in the body which affect the mechanism of signaling pathways that are contributing to associate Type 2 Diabetes Mellitus and Breast cancer which includes the insulin pathway activation, Endogenous Sex hormone's impaired regulation and abrupt activation of IGF pathway.

### 2.2.1. Insulin Pathway

Insulin pathway is implicated in severity of Diabetes Mellitus type 2 caused by insulin resistance which increases insulin amount in cell. This free insulin roams and binds to the insulin receptor IR which is present in the transmembrane region of cell. Insulin receptor is a tyrosine kinase and upon insulin binding it leads to autophosphorylation. Once the tyrosine residues are phosphorylated it cause the activation of tyrosine kinase. We are already aware that the targets of the insulin are

liver, skeletal and muscle tissue but many other tissues are also under attack like Breast cancer cells and healthy Breast tissue which are expressing the insulin receptor (Pollan, 2010).

Once the insulin receptor is activated the receptor activates many other intracellular proteins which includes insulin receptor substrate family and SHC adaptor. When the Insulin Receptor Substrate joins to Insulin Receptor, it causes instigation of phosphatidylinositol 3-kinase followed by AKT activation. The binding of the IR also activates extracellular-signal-regulated-kinase. Though insulin signaling pathway has metabolic function, but both of the ERK and AKT pathways play crucial role in tumorigenesis.

Insulin receptor causes stimulation of insulin pathway in Breast cancer. It could also get stimulated via free insulin in the Breast tumor cell lines. The overexpression however, of IR could cause induction of malignant tumor transformation in Breast cancer cell lines. The overexpression of IR can be stimulated by *WNT1*, *RET* and *ERBB2* in breast cell of cancerous patients. It is also reported by scientists that the value of IR in 159 sample was six-fold greater obtained from the Breast cancer patients as compared to 33 samples obtained from healthy individuals. The higher concentration of IR can be correlated to the size of tumor, grade, and estrogen-receptor concentration. A future study involving 512 number of patients with very early staged Breast cancer had shown that there is straight link between concentration of insulin, cancer reappearance and death (Zou and Shao, 2008).

### 2.2.2. Regulation of Sex Hormones

Sex hormones impart its pathological functions and physiologic effects via steroid receptors like estrogen receptors (ERs), androgen receptors (ARs) and progesterone receptors. ER has two subunits ERα and ERβ having role in cancer development, where ERα is predominant in uterus, ovaries, and breasts. While ERβ have role in cardiovascular systems, bone, and kidneys. ER, PR, HER/NEU are essential biomarkers for the detection of breast cancer amongst patients having risk factors. These markers are usually confirmed by immunohistochemistry assay. As it is confirmed by animal studies as well that estrogens encourage mammary tumors production and the elimination of animal ovaries/ insertion of anti-estrogenic medicine have opposite response. Estrogen has direct role in the cancer identification.2 out of 3 women suffering from breast cancer are positive for hormone receptor, either estrogen positive or progesterone positive. The details are illustrated in fig below. The cancer cells which are estrogen receptor-positive are stimulated by

estrogen. The hormonal therapies used for cancer treatment such as selective estrogen receptor modulator causes decrease in estrogen level or restricts estrogen from its action on breast cancer tissues. Progesterone contributes to proliferation of cells and breast cancer development by acting in collaboration with mitogenic kinases and mediators of cell cycle. Progesterone stimulates regulation of progesterone target genes and proliferation responses. Both these hormones play role in determination of cancer and helps as a biomarker for the treatment of the disease (Cusi K, 2011).

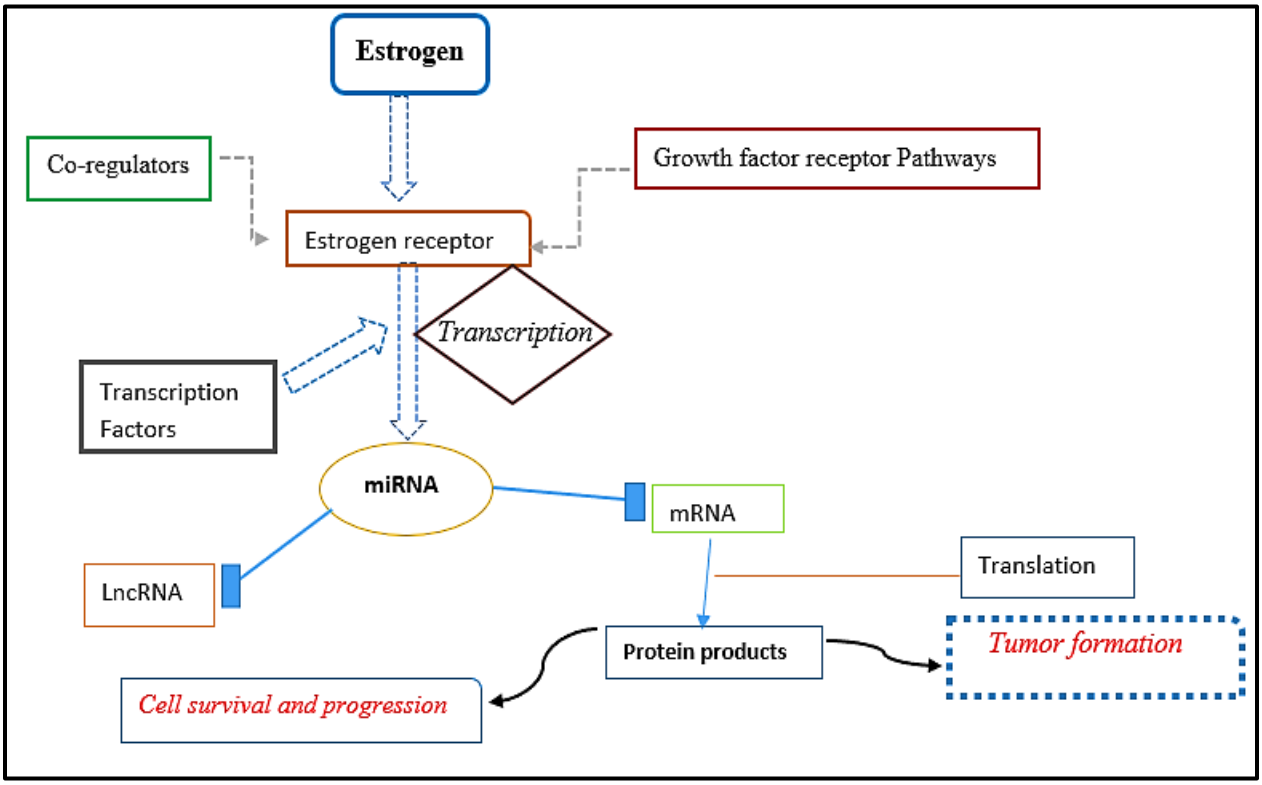



**Fig.2.1. Illustrates the mechanism of Tumor formation by the action of Sex Hormones**

### 2.2.3. Insulin like Growth factor (IGF) Pathway

Insulin like growth factor pathway contains ligands like IGF1 and IGF2, also proteins binding to insulin-like growth factor (IGFBP) and insulin-like growth factor receptor (IGFR). The insulin like growth factor namely (IGF1, IGF2) is very homologous to insulin and IGFR share almost 55% homology with insulin receptor. There are more chances of IGF1 to bind with IGFR hybrid with IR because of the little affinity with insulin. The initiation of IGFR via IGF1 causes the activation of similar set of proteins as IR and insulin.

This system of IGF-1 acts a pivotal figure in Breast cancer regulatory pathway. If there is high concentration of circulatory IGF1 and IGF-BP3 then they are related with high peril of causing Premenopausal Breast cancer. The high level of IGF1 is also an important link in associating obesity and Breast cancer. Insulin-like growth factor pathway could also be stimulated by elevated concentrations of insulin in diseases like T2DM via the non-specific activation of hybrid IGF1R/IR and IGF1 (Anderson and Neuhouser, 2012).

## 2.3. Inflammatory Mediators

Inflammation has role in mediating the host defense but sometimes the aberrant inflammatory responses cause spread of various diseases which includes cancer onset. There are variable factors responsible like the biological, chemical, physical, and genetic factors that result in neoplasia relating cancer and inflammation (Mantovani *et al*., 2008). The genetic alterations lead to initiation of STAT3 and NF-KB which coordinate the proinflammatory cytokines and other inflammatory mediators. This connection builds an atmosphere for carcinogenesis inducing all stages that are initiation, promotion, and progression. There are several studies showing the cytokines that are highly expressed such as IL-6, TNFα, that are causing unduly activation of VEGF and BCL-XL that are resulting unexpected differentiation leading to Breast cancer. Apart from that TNFα promote key enzymes and genes which participate in estrogen metabolism process that have DNA adduct products which implicate various mechanisms causing inflammation-associated Breast cancer (Halliwell and Gutteridge, 2007).

## 2.4. Oxidative Stress Domain

Oxidative stress is one of the critical aspects in the progression of certain diseases. Chronic hyperglycemia that is caused by the overload of glucose result in devastating events one of which is oxidative stress that connects the onset and succession of T2DM. Lipid Peroxidation is a oxidative degradation of lipids that causes cellular damage due to free radicals and reactive oxygen species. ROS under normal circumstances is kept within limits by using body's system of oxidant and antioxidant enzymes (Reuter et al.,2010). But when the hyperglycemic condition occurs then biomolecules get damaged. These biomolecules include DNA, peptides, and lipids. The DNA damage is found has been found to be associated with multiple diseases like cancer.

Alterations in the gene expression is found to be done by attack of free radical on chromatin and nitrogenous bases, it can affect tumor suppressor genes triggering cancer. Therefore, it is well

proposed that diabetic patients are at risk of developing cancer affecting many organs like liver, pancreas, stomach, colorectal, and breast (Panis et al., 2012).

ROS production due to inflammation initiate cancer by increasing damage of DNA, instability of genome, modifications in gene expression and epigenetic events, apoptosis resistance, metastasis, invasion, and neovascularization. The mechanism through which oxidative stress progresses in breast cancerous cells is not known but large amount of literature report that ROS are implicated in progression and etiology of Breast cancer and Diabetes. Several of oxidative stress markers are found in abundance in breast cancer cells like DNA adducts, lipid peroxidation remains, 8-isoprostanes, and malondialdehyde (Kundu and Surh, 2012).

There are several cancer inflammatory factors that are found to cause growth of an advanced stage of breast cancer where elevated level of oxidative stress, lessened catalase activity has been observed followed by serious lipid peroxidation and elevated level of nitric oxide. One of the significant ways to initiate tumorigenesis via inflammation and oxidative stress is by the modifications of DNA which silences oncogenes or inactivate the tumor suppressor genes. The changes implicated could be done by direct mutations in DNA or via the epigenetic changes (Portela and Esteller, 2010).

The free radicals play their role in the activation of apoptotic pathway which happens when cells are damaged due to elevated level of oxidative stress. The elevated levels of oxidative stress cause cellular damage such that slight increase in free radical concentration would change the phenotype. Also, it is well demonstrated that cell responses depend on the ROS baseline and duration of cell exposure to the ROS. It is also seen that association of senescence-related oxidative stress and tumorigenesis proves that the accumulation of ROS during senescence triggers cancer progession (Rodriguez-Paredes and Esteller, 2011).

## 2.5. Adiponectin

Adiponectin normally plays important part as vasoprotective adipokine by working on the lipid metabolism. The plasma level of adiponectin are correlated negatively with triglyceride and correlated positively with cholesterol (HDL). The alterations in adiponectin disrupt normal pathway of its working and may cause risk of cardiovascular disease and dyslipidemia. Also, minute level of adiponectin in patients with obesity increase risk of breast cancer. It plays major

role in the glucose regulation, insulin sensitization which affects the regular uptake of glucose, in homeostasis of lipid and atherosclerosis pathophysiology. There are numerous studies which support the fact that inhibition of adiponectin can cause proliferation of multiple cancer types and endothelial cells along with smooth cells (Matsuura et al., 2007).

### 2.5.1. Leptin

Leptin is secreted by adipocytes and proadipocytes activates P13K/AKT and MAPK pathways. These signaling processes caused by leptin (pleiotropic protein) are also influenced by high fat mass and obesity. Leptin concentration in plasma is positively associated to Body mass index and its amount is extreme in obese female subjects (Rose *et al*., 2004). Obesity is known to be one of the most critical risk factors for establishment of an association between post-menopausal Breast cancer and hyperleptinemia. High levels of leptin in stromal cells of adipocytes that surround Breast tumor bodies mediate Breast cancer development through paracrine mechanism (Celis *et al*., 2005).

Leptin level increases in pancreatic cells in diabetic condition and shows positive relation with insulin resistance. Availability of leptin is regulated by insulin which acts to release leptin form the pre-existing pool or from new leptin synthesis. The increased levels of leptin indicate association between glucocorticoids and insulin, and it is dependent upon the JAK/STAT pathway (Bradley and Cheatham, 1999).

Breast cancer cell have leptin receptor which induces anti-apoptotic process and mitogenesis. As a result, tumor interacted adipocyte produce endocrine stimulus that causes leptin generation in hyperinsulinemia microenvironment. The elements required for the production of insulin-derived growth enhancing paracrine loop are in place between the leptin secreted by adipocyte and leptin receptor expressed by the Breast cancer cells (Perera *et al*., 2008). Also, insulin acts by the mode of MAPK and P13K pathway and stimulates the leptin overexpression in Breast cancer cells of human (Bartella et al., 2008). Aromatase activity is also increased by hyperleptinemia that results in elevated estrogen synthesis, backing cross talk between ER and receptor of leptin through JAK/STAT, MAPK/P13K pathway (Cirillo et al., 2008). Leptin supports angiogenesis, like insulin, induces VEGF to cause cellular proliferation and progression of cancerous cells along with capillary tube formation in animal research models (Vona-Davis and Rose, 2009). Though leptin produced by adipocytes, could also be produced via fibroblasts which is value constituent of stroma that is adjacent to tumor. Fibroblasts which are linked by cancer helps in secretion of leptin

which then binds to its particular receptor present on distorted ER expressed tumor cells thus developing a milieu of association between tumor and stroma (Barone *et al*., 2012).

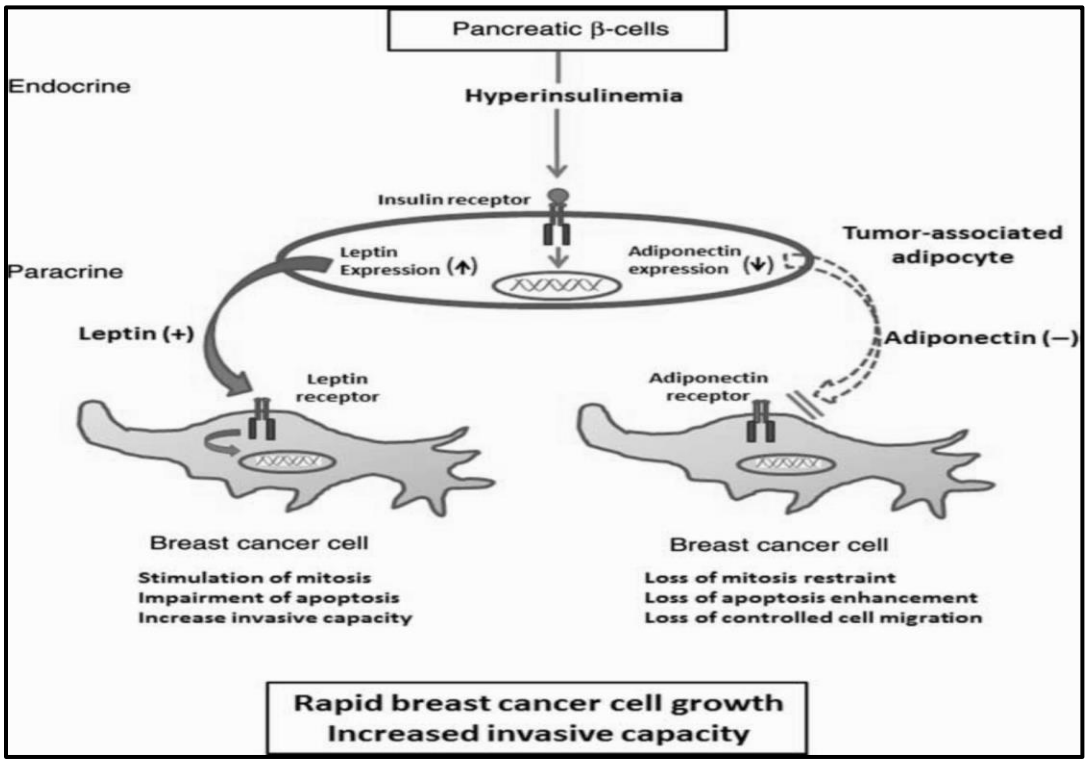



**Fig.2.2. Explains crosstalk between Obesity, Diabetes Mellitus Type II and Breast cancer.**

## 2.6. Obesity and Adipose tissues:

There is always an increase in energy intake in the obesity condition and decrease in the energy expenditure that results in inflammation and insulin resistance leading to development of diseases such as hypertension, non-alcoholic fatty liver disease, T2DM, prothrombic state, dyslipidemia and atherosclerotic heart disease (Lumeng and Saltiel, 2011). The adipocyte dysfunction and excess of adiposity causes dysregulation in variety of secretory factors derived from adipose tissues via glucose alteration and defect in lipid homeostasis (Kershaw and Flier, 2004). Insulin sensitivity is modulated by certain critical factors such as the excess fat. The fat from the intake gets stored and its buildup encourages release of fatty acid inside circulation from adjacent adipocytes (Hauner, 2005).

Adipose tissues are responsible for storing energy in triglyceride form but during positive balance of energy it enhances its storage via the hyperplasia (surge in adipocyte quantity) and hypertrophy (upsurge in magnitude of adipocytes). The amount of these adipocytes is known usually in adolescence and childhood and it remain same in obese and lean people. Thus, the increase in the

fatty acids and fat mass could be attributed to hypertrophy (Halberg and Wernstedt-Asterholm, 2008). The dysfunction of adipose tissues has a role in establishment of insulin resistance and several other metabolic diseases including Breast cancer. Free fatty acid openly arrives in liver through the movement of blood in obese condition and it is reported that the increase in free fatty acids of liver causes more synthesis of lipid and gluconeogenesis along with insulin resistance in liver. The extraordinary amount of free fatty acid circulating in liver could also affect the peripheral resistance in insulin in both humans and animals. Free fatty acids can aid as a ligand for TLR4 complex stimulating production of macrophages and certain cytokines enhancing inflammation process which leads to many obesity- associated metabolic syndromes including Breast cancer (Parseghian et al.,1997).

## 2.7. Estrogen Signaling

The chances of Breast cancer development are higher in post-menopausal women as compared to young diabetic Pre-menopausal women. An increase in the estrogen levels is observed in Post-menopausal women. The conditions such as hyperinsulinemia and insulin resistance cause reduction in the level of SHBG (Sex hormone binding globulin) that causes an increase in bioavailability of estrogen resulting in an upsurge in the risk of cancer (Acharya *et al*., 2016).

Intracellular ER which is a transcription factor interacts and binds with Estrogen and gets translocated in nucleus. ER binds and modulates gene expression that is relevant to survival, proliferation, and cellular apoptosis. Moreover, considerable crosstalk is observed between IGF and estrogen signaling pathway that can possibly increase chances of Breast cancer. The establishment of a relation between estrogen and insulin like growth factor develops a resistance to endocrine therapy among Breast cancer. Glucose acceptance and usage of aerobic glycolysis is caused via ER-activated P13K/AKT signaling that increases movement of GLUT4 to plasma membrane (Ricciardiello *et al*., 2018).

## 2.8. Vascular Endothelial Growth Factor

Vascular endothelial growth factor has potential to exert effects on the vascular cells of endothelium for that it acts as strong mitogen and anti-apoptotic feature (Veikkola and Alitalo, 1999). VEGF is synthesized in adipocytes and preadipocytes playing its role in adipose tissue development and vascularization. Amount of VEGF in serum depicts a positive association with BMI. It also acts in synergistic manner with leptin to promote angiogenic process (Cao et al., 2001).

One of the important regulators of VEGF is Hypoxia inducible factor HIF1α which causes hypoxia. The expression of VEGF is coordinated by several other hypoxia factors like epidermal growth factor (EGF), RAS, VHL, WNT-KRAS genes and platelet-derived growth factors PDGF (Semenza, 2000). The role of VEGF has been identified with its discovery a century ago detailed below in the figure.

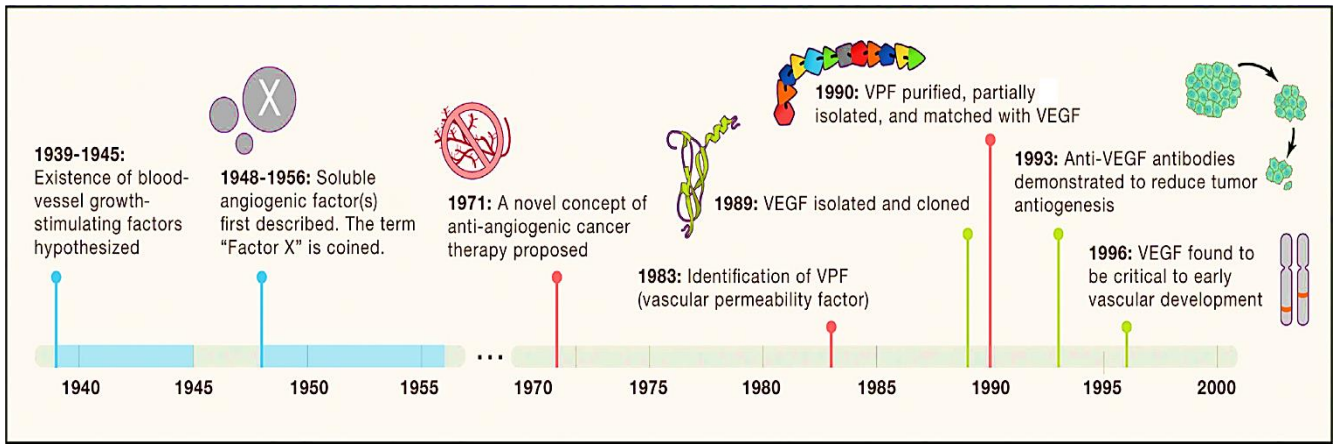



**Fig.2.3. The Discovery Timeline of VEGF.**

VEGF signaling is carried out through VEGFR1/R2 that regulates activities of other kinases which causes migration, survival, cellular proliferation during angiogenesis and vascularization. There are endothelial cells which are composed of stalk and tip present at the edge of the vascular propagation. VEGF induces these tip cells to creation of filopodias. The molecular control and regulation of this process is through the stimulation of notch signaling via elevated expression of the notch ligand present on the endothelial cells (Wacker and Gerhardt, 2011).

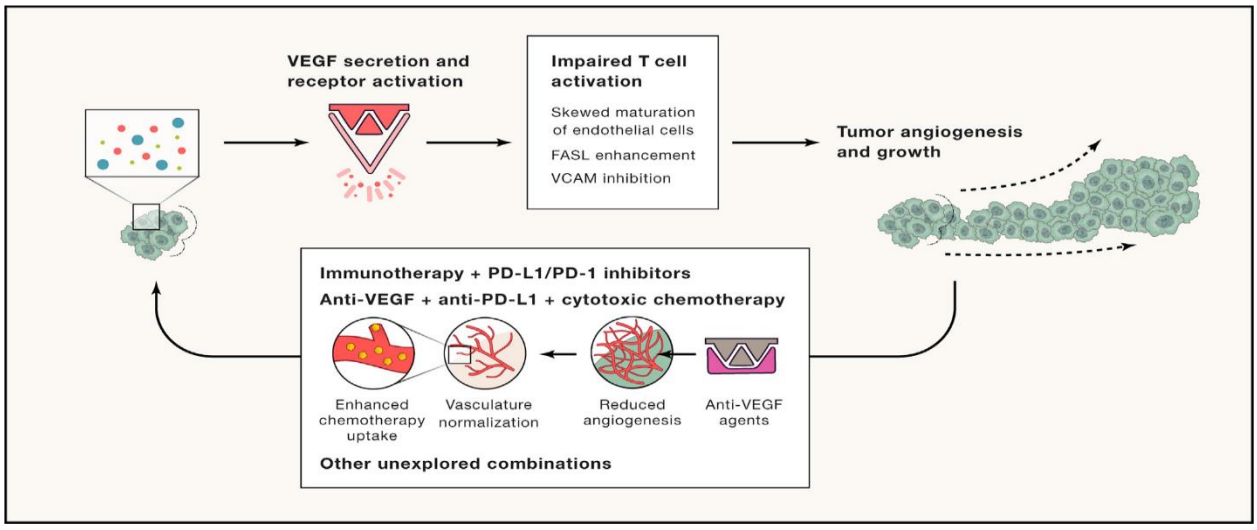



**Fig.2.4. VEGF and Tumor Angiogenesis.**

The VEGF mRNA is overexpressed in most of the tumor cases in humans that correlates to vascular density, invasiveness, recurrence, prognosis, and metastasis. VEGF-A has very profound role in abnormal growth of blood vessels. Likewise, other VEGFs also play its part in development of lymphatic vessels and disease-related angiogenesis. Angiogenesis driven by VEGF-A causes pathogenesis of human diseases such as Cancer, Eye disorders and Rheumatoid arthritis. The significant role of VEGF in the development of several diseases has motivated the discovery of Avastin, which is a humanized monoclonal antibody to VEGF-A, required for the cure of colorectal cancer. It has also been interesting to note that VEGF-A has occurred to play role in the treatment of Ischemic heart disease. But despite of several studies supporting effective VEGF-A gene therapy, clinical trials has not been much successful (Kerbel, 2008).

Although literature has recommended that the expression of VEGFR-1 in the human Breast cancer tumor tissues are amplified and connected with prognostic factor but exact mechanism, the biological effects and therapeutic influence of VEGF/VEGFR-1 signaling is unidentified.

### 2.8.1. Combination and Multitarget Therapies

Anticancer therapies never solely depend on a single drug and it requires combination of other approaches. Because simultaneously attacking more targets help in achieving better effectiveness. Preclinical trials had exposed additive and collaborative benefits from cytotoxic agents and VEGF inhibitors. The normalization of cancer vasculature by effects of anti-VEGF factors promotes combinatorial benefits. Another hypothesis has stated that VEGF inhibition will cause cropping of several endothelial cell that are not enclosed by pericytes and result in reduced contortion and tumor hyperpermeability in cancer vessels. These actions are predictable to lessen cancer interstitial pressure and enhance proliferation of cytotoxic agents and antibodies by tumor (Jain, 2014). The general mechanism of action is shown in figure below.

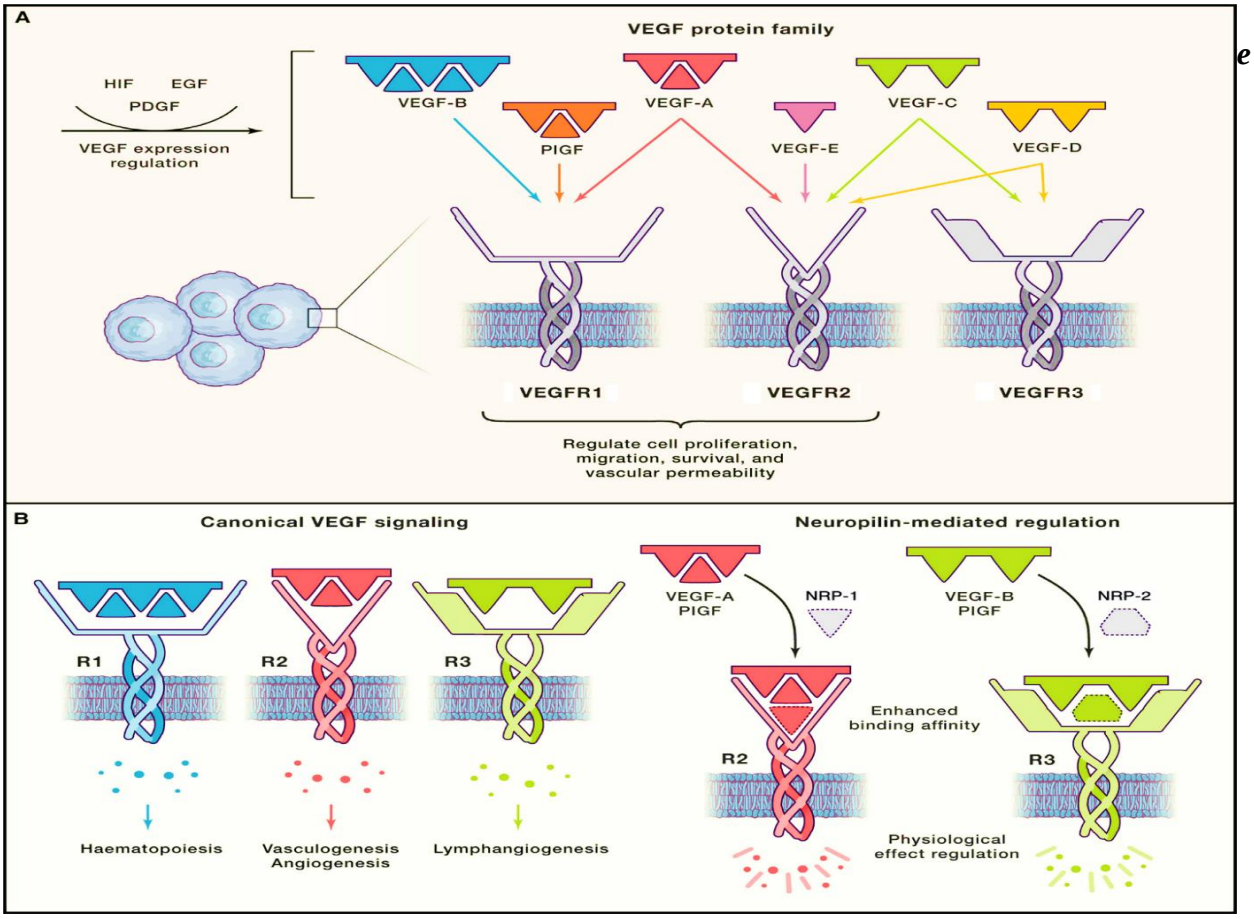



**Fig.2.5. VEGF activation and Signaling Pathway.**

## 2.9. Signal Transducer and Activator of Transcription STAT3

The role of STAT3 is to control gene regulation. The abnormal stimulation of STAT3 have been involved in process of oncogenesis but also in many other diseases like diabetic nephropathy pathogenesis. It has been found that increase activation of STAT3 signaling leads to develop skeletal muscle resistance of insulin causing Diabetes type 2. STAT3 plays its role in causing Breast cancer pathology by overactivation of JAK/STAT pathway. It enhances expression of cytokines such as IL-6 and IL-10. These activating cytokines are frequently found in cancers. The stimulation of STAT-3 by V-SRC leading to initiation of NF-KB that in turn induces IL-6 and IL-10. The inappropriate activation of STAT3 is mostly due to determined tyrosine 705 phosphorylation signaling that contribute to cancer development and establishment of malignant phenotype. The constant expression of STAT3 transfer signal from cytokine and growth factor stimulating precise target genes like cyclin-D, VEGF, c-Myc that play central part in cellular proliferation, suppression of apoptotic genes. The figure below is displaying role of STAT3 in several other diseases apart from cancer.

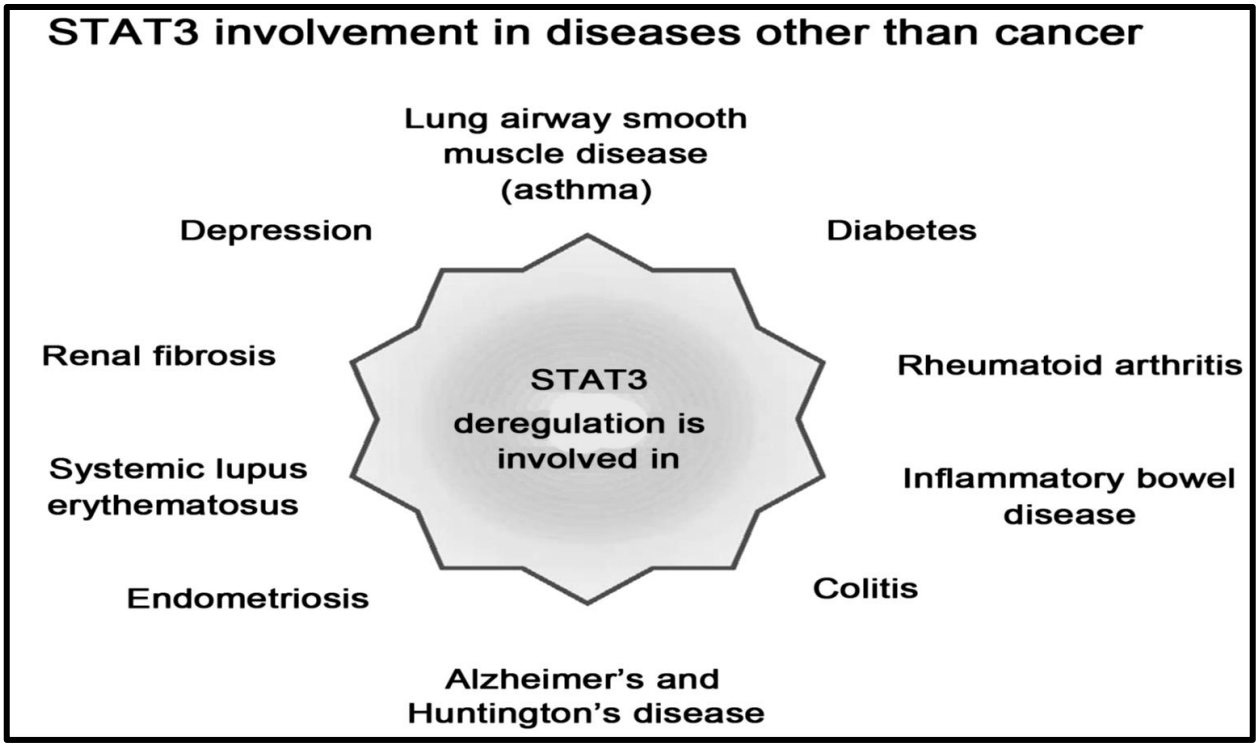



**Fig.2.6. Describes the pathologies related to STAT3**

### 2.9.1. Role of STAT3 signaling in Cancer and Diabetes Mellitus type 2

In cancer, IL-6 is released by macrophages and T-cell that are involved in inflammatory response and immune function. It activates when ligand binds to its particular receptor IL-6R and a mutual receptor gp130. Due to the formation of an interaction a complex is resulted comprised of IL-6, IL-6R, and gp130 heterotrimers. This type of signaling is called classical signaling of IL-6 activated through early immune responses, thus exciting expression of numerous acute -phase proteins. The IL-6 signaling when reaches to plasma membrane of cell it activates the Janus kinase JAK kinases. JAKs are tyrosine kinases that are present in cytoplasm generally constitute a family of only 4 members namely JAK1, TYK2 and JAK2 that are expressed inside many cells. Though, JAK3 is only present in hematopoietic system. JAK1 activates STAT3 through phosphorylation of specific residues of tyrosine. When STAT3 gets phosphorylated it form dimer and transfers to cytoplasm and then to nucleus activating STAT3 marked genes such as cyclin D, Bcl-xl, c-Myc, and VEGF (Xiong et al., 2014).

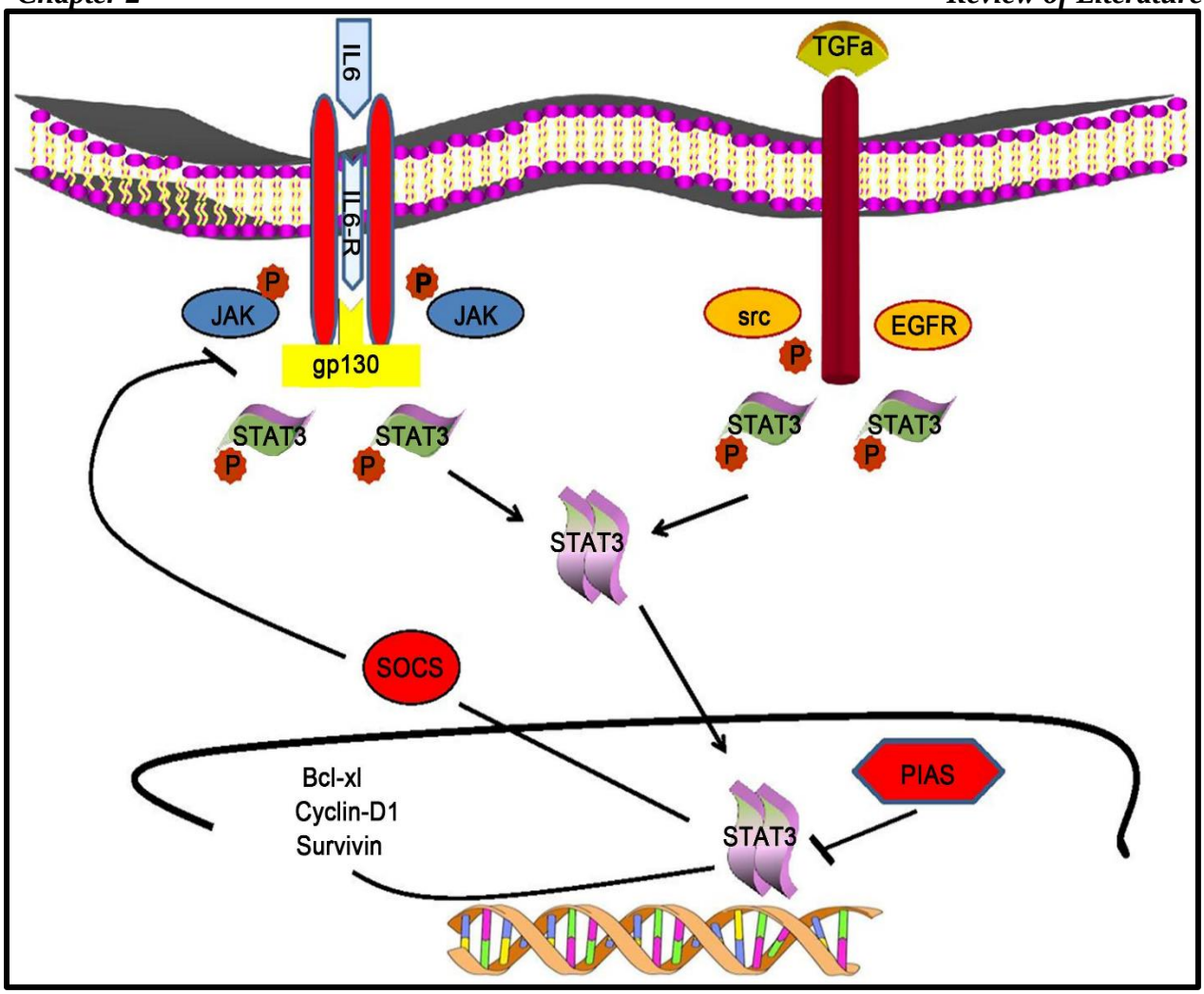



**Fig.2.7. The JAK/STAT pathway Activation**

It has been noted that constitutive activation of STAT3 contributes to the tumor progression and IL-6/JAK pathway play vital role in abnormal STAT3 signaling cascades. While in diabetes Mellitus type 2 STAT-3 sensitizes the Insulin signaling pathway by down regulating GSK-3β which is negative regulator of Insulin.

# Materials and Methods

## 3.1. Real Time Polymerase Chain Reaction Analysis

The association of genes namely Signal Transducer and activators of Transcription (STAT3) and Vascular endothelial growth factor (VEGF) level of expression in blood of study subjects is compared with the control (healthy) group by the help of RTPCR. Real time PCR utilizes specific primers of the gene for expression determination. The CDNA amplified by the semi-quantitative PCR is used against which the primers of desired gene are amplified to determine their expression. The expression level obtained as a result will be helpful in associating the genes to crosslink the Breast cancer and Diabetic patients against the healthy controls.

### 3.1.1. Study Subjects

Study was permitted by Ethics group of Holy Family Hospital as well as ASAB, NUST. Informed consent form was taken from unrelated individuals with diabetes and breast cancer diagnosis. This study was outlined as a case control study which included 80 human subjects. The sample type used is blood. For the study 7 samples were taken of diabetic patients. 26 samples were obtained from Breast Cancer patients. Almost 6 samples were taken from patients who had both Diabetes Mellitus and Breast cancer. 40 controls were taken from healthy individuals who do not have any symptoms of diabetes or breast cancer from blood.

**Inclusion Criteria:**

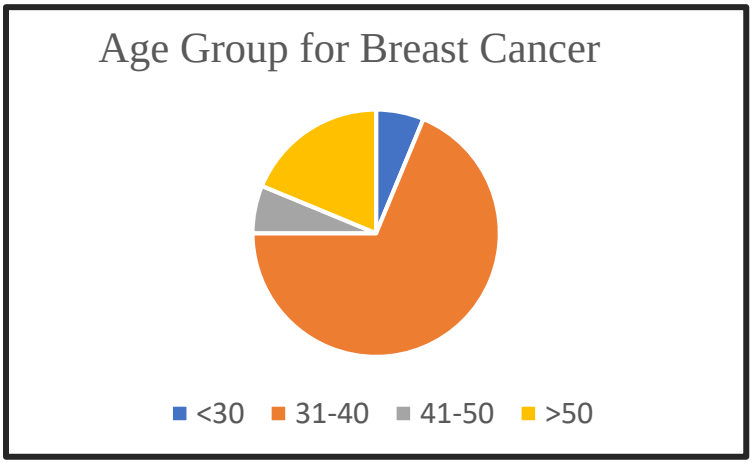
Patients detected with Diabetes Mellitus type 2 and Breast cancer were included in case control study. Age factor measured for all study groups and the human subject's ≥40s was incorporated in the study.

**Exclusion Criteria:**

Patients detected with chronic disease condition linked to renal liver and mental illnesses were omitted from study.

**Table 3.1: depicts the categories made for sample collection and distribution**

| | Sample | Group A | Group B | Group C | Group D | Group E | Total no. of Samples |
|---|---|---|---|---|---|---|---|
| | Type | (Diabetic) | (Cancer) | (Diabetic>>cancer) | (Cancer>>Diabetes) | (Control) | |
| 1 | Blood | 7 | 26 | 3 | 3 | 40 | 80 |




**Graph.3.1. Shows Age Distribution Statistics in Sample Collection**

## 3.2. Sample Collection and Storage

The chosen patients were required to provide blood sample, withdrawn using 5 ml syringe by a skillful phlebotomist in the purple capped Lavender EDTA tubes. Written permission was taken before taking blood. And for handicapped contributors, their permission was gained from their custodians. The blood sample taken was cared of in a storage ice box during whole sample collection period to prevent hemolysis, degradation, and clotting. After being properly labeled with identification number of samples, date of collection and name, age, and gender of individual, blood samples were stored on ice and taken to Immunogenetics Laboratory (IGL), Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad. They were then kept at 4°C before further treating. Adipose tissue and cancer biopsy surgeries were performed under general anesthesia. Subcutaneous fat tissue and tissue or fluid were removed respectively and up to 5-10 grams was taken on average. Biopsy was stored

immediately in liquid nitrogen and were shifted to minus 80 degrees Celsius in ultra-low freezer for long term storage.

| SR.NO | BREAST CANCER | DIABETIC | BC+DM | B-Control | CONTROL |
|---|---|---|---|---|---|
| QUANTITY | 26 | 7 | 5 | 1 | 40 |
| GENDER | F-26, M-0 | M-2, F-5 | F-5, M-0 | F-1, M-0 | F-21, M-19 |
| AGE | 23-52 | <65 | 45-50 | 36 | 23-65 |

**Table 3.2: The number of blood samples collected, distributed in table according to age and gender**.

## 3.3. STAT3 And VEGF Expression Profiling

### 3.3.1. RNA Extraction

For cryopreserved adipose and breast biopsy tissues, around 50-100 mg of tissue was homogenized using liquid nitrogen. Homogenized tissue powder was added to 750 ul of Trizol in a micro centrifuged tube. Now, vortex for further homogenization. To clean out fat and tissue debris, the mixture was centrifuged at 12000rpm for 10 minutes at 4ºC.Transfered the red/pink intermediate layer to a fresh, labelled micro centrifuge tube. Incubated it at room temperature for 5 minutes. Freshly prepared 5N glacial acetic acid. Added 20 ul of glacial acetic acid to the tube. The solution was mixed vigorously for 15 seconds. It was incubated at room temperature for 5 minutes. Chloroform 200 ul was added and solution was vigorously mixed manually for 15 seconds (shaking results in better emulsification and higher volume of aqueous phase later). Again, the mixture was left at room temperature for 10 minutes. After which it was spun at 12000 rpm for 15 minutes at 4ºC to obtain two layers. Upper layer was transferred to a new micro centrifuge tube and 500 ul of chilled isopropanol was added and vortexed to precipitate RNA. Incubated it at room temperature for 5-10 minutes. Centrifuged it at 12000 rpm for 10 minutes at 4ºC. Supernatant was removed and pellet was washed by 1ml of 75% ethanol in DEPC-treated water. Vortexed for couple of seconds. Centrifuged at 14000 rpm for 10 mins at 4ºC. Decant supernatant. Air dried the pellet. Then resuspended pellet in 25 ul of DEPC-treated water. Pellet was incubated at 55-60 degree Celsius for 10-15 minutes.

### 3.3.2. Quantitative and Qualitative Analysis

The RNA was taken from -80ºC and was immediately be transferred to ice and was quantified using Thermo Scientific Nano Drop ND-2000 spectrophotometer. The absorbance ratio A260/280 was used to determine the purity of the RNA sample.

- 1ul of DEPC treated water was used as a blank.
- 1ul of RNA in DEPC water was used for the measurement.

### 3.3.3. Complementary DNA Synthesis

Complementary DNA synthesis (cDNA) was done using Thermo Scientific Revert Aid Reverse Transcriptase. 1 ul of purified RNA sample was used as template in complementary DNA synthesis. Added 1 ul of oligo DT primers to a fresh tube. And raised the volume to 12.5 using DEPC- treated water. Gently mixed it. Centrifuged briefly. The mixture was then given heat shock at 65ºC for 5 minutes followed by quick chilling on ice. Centrifuged briefly. Transferred back to ice. Added 4 ul of reaction buffer, 20 UNITS i.e. 0.5 ul of RNase inhibitor, 2ul of 10mM dNTP mix, 200U of Reverse Transcriptase to the tube of volume 1 ul. Added DEPC-treated water to raise the reaction volume to 20 ul. Incubated at 42 degree Celsius for 60 minutes. Heated at 70 degree Celsius for 10 minutes to terminate the reaction. Stored the cDNA at -20 degree Celsius.

### 3.3.4. Semi-quantitative PCR

For the confirmation of cDNA synthesis 0.25 ul of cDNA, 2ul Taq buffer, 2 ul $Mgcl_2$, 2 ul of 2mM dNTP mix, 2 ul forward primer for GAPDH, 2ul reverse primer for GAPDH and 0.25 ul of Thermo Scientific Taq DNA Polymerase was added to PCR tube. Final volume was raised to 20 ul by adding nuclease free water. The reaction mixture was slightly spin loaded to thermal cycler. The reaction conditions for GAPDH were set to 94ºC for 3 minutes followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 52ºC for 30 seconds and extension at 72ºC for 30 seconds. Final extension was given at 72ºC for 10 minutes and the reaction product was kept at 4ºC.

### 3.3.5. Gel Electrophoresis

To confirm the amplification of CDNA, PCR product was run in 2% agarose gel. For this preparation 1.2g of agarose was dissolved in 60 ml of water and stained with 3 ul of ethidium bromide (0.5ug/ml). 5 ul of PCR product was loaded with 3 ul of loading dye. The gel was made by 1X TAE buffer made from 50X stock solution. The gel was allowed to solidify and after that

comb was removed. The sample was loaded, and gel was analyzed on Dolphin-Doc plus gel documentation system. Length of the product was determined by comparing the product size with Thermo Scientific Gene Ruler 50 bp DNA ladder.

### 3.3.6. Tris-Acetic Acid-EDTA (TAE) Buffer

The 1x TAE solution is 40mM Tris, 20mM Acetate and 1mM EDTA and typically has a pH around 8.6. TAE buffer used in gel electrophoresis was originally prepared as a 50X stock by adding 121 g Tris base, 28.55 mL of glacial acetic acid and 50 mL of 500mM/ 9.3 g EDTA to a final volume of 500 mL with pH adjusted to 8.0. The stock was diluted to 1X concentration by adding required volume of 50X to distilled water. M1V1= M 2V2 formula was used for making calculations.

## 3.4. Designing Primers for Quantitative analysis

Primers for B-actin gene has already been reported in literature. They were checked for self-complementarity and other properties by Oligo Calc (http://biotools.nubic.northwestern.edu/OligoCalc.html).

Primers for STAT3 and VEGF has been designed using Primer3web (version 4.0.0) software. There mRNA sequence was taken from NCBI. Primers has been tested for their self-complementarity and other properties by Oligo Calc: Oligonucleotide properties calculator. Primers has also been checked for non-specific binding using UCSC In-silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr). Primers was ordered from Gene Link.

## 3.5. In-Silico Analysis

For In-silico analysis three software's were used (Panther analysis, Enrichr analysis and STRING analysis). To use these software's methodology is quite similar but their outcomes are different. By STRING analysis protein-protein interaction is determined in pathways, while by Enrichr analysis gene set libraries are searched to find the role of specific genes and Panther analysis gives us the function of gene products. For the methodology first selected the multiple protein option because I was interested to find out the role of more than one gene. Secondly, mentioned the ID

of the gene retrieved from the NCBI and selected specie *Homo sapiens*. In this way by multiple analysis options would be given from which your interested analysis could be retrieved.

## 3.6. Real Time PCR

The quantity of cDNA was measured using real time PCR with the Applied Bios systems 7300 Real time PCR system and fluorescence based SYBER green technology. Quantitative expression of STAT3 and VEGF along was measured by real-time PCR. GAPDH was used as internal control for normalization of the data. PCR was done in final volume of 20 ul and it will contain 0.8 ul of each forward and reverse primer, 10 ul of Thermo Scientific Maxima SYBER green/ ROX qPCR Master mix(2X) and 25 ng of cDNA and Nuclease free water. Cycling conditions included initial denaturation at 95ºC for 10 min, 40 cycles of 95ºC for 30 sec and 60ºC for 1 min, followed by a single hold of 1 min at 72ºC and dissociation stage consisting of 95ºC for 15 sec, 60ºC for 30 sec and 95ºC for 15 sec. reaction of every sample was performed in triplicates. Non template control was run in each real time PCR reaction. The PCR product was further be proceeded on 2% agarose gel along with Thermo scientific Gene Ruler 50 bp ladder to ensure amplification of correct sized fragment.

## 3.7. Gel Electrophoresis

To check the amplification results, 2% agarose gel was made by dissolving 2 g agarose in 100 ml 1X Tris-acetate-EDTA (TAE) buffer stained with 6 µL of ethidium bromide. Five microliters of PCR product were loaded in each well. To predict and verify the size of product, 100 bp ladder was also run on the gel along with the samples. The gel image was taken using Dolphin Doc Gel Documentation System.

## 3.8. Statistical Analysis

The statistical analysis of genes can be performed using delta CT values obtained from the real-time PCR. Moreover, SSPS analysis can also be performed that will help us compare the results

# Results

## 4.1. STRING Analysis

When protein interaction of STAT3 with VEGFA, INS, Glut4 and Hif1a was analyzed, the functional enrichment inside network was observed in Biological processes. Gene ontology analysis presented that these proteins are involved in gene expression, regulation, regulation of autophagy, MAPK cascade and cell differentiation. Molecular function (Gene ontology) showed E-box binding, DNA-binding, transcription factor binding and protein dimerization activity. KEGG pathway analysis showed link of these proteins found in Type 2 diabetes mellitus, Hif1a signaling pathway, autophagy, insulin resistance, Jak-STAT signaling, MAPK signaling, signaling pathways in cancer and PI3K-AKT signaling pathway. Interlink between STAT3, VEGFA and HIF1a is experimentally determined. Overall, when these proteins were observed collectively in STRING the text mining showed co-occurrence of these proteins. In STRING analysis, the link between STAT3 and VEGFA was shown by collecting data from curated databases.

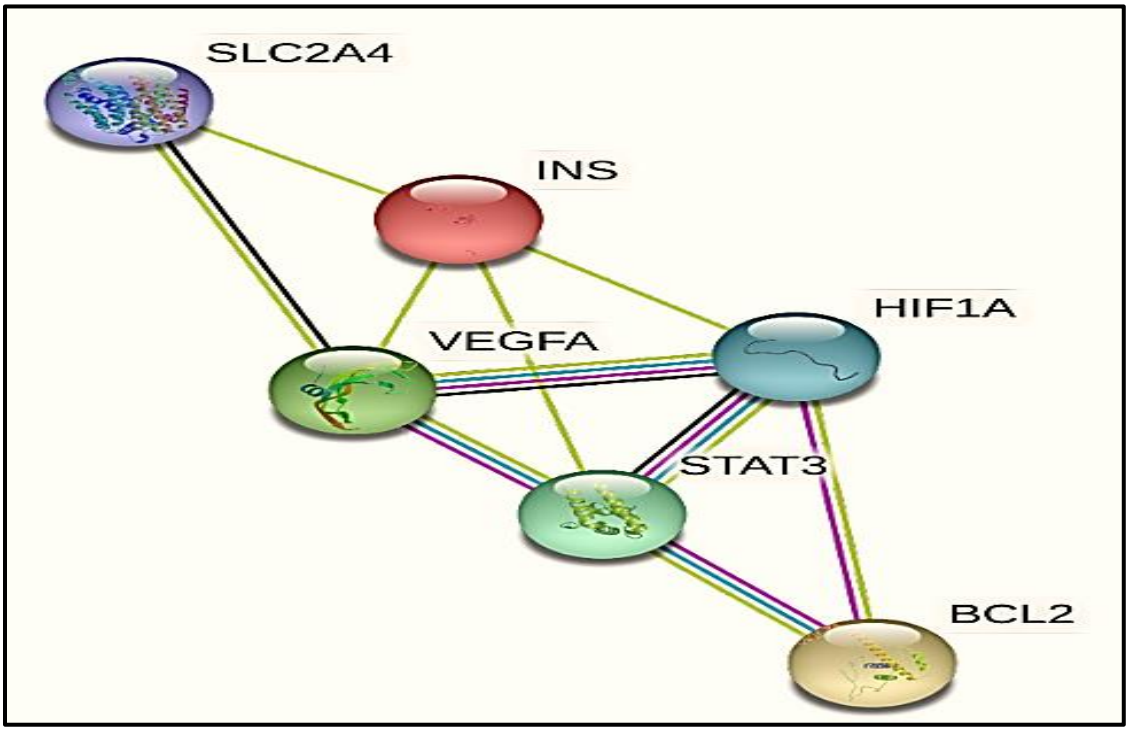



**Fig.4.1. The STRING analysis of multiple protein suggest complex interaction during various activities in metabolic and genetic pathways.**

## 4.2. Enrichr-Computational System Biology

The Enrichr computational system biology is web-based gene list enrichment analysis tool that helped in showcasing the gene set libraries and our desired genes interactive relation to other genes. The result obtained after giving input of 5 genes namely STAT3, VEGFA, GLUT4, BCL2, HIF1a, and INS observed the enrichment of them in Hif1a signaling, AGE-RAGE signaling pathway, cancer-cell pathways. The result obtained is filtered according to the significant P-value of the research analysis. The clustergram showed below displays the involvement of gene of interests in particular signaling pathway.

**i) KEGG 2019 Human**

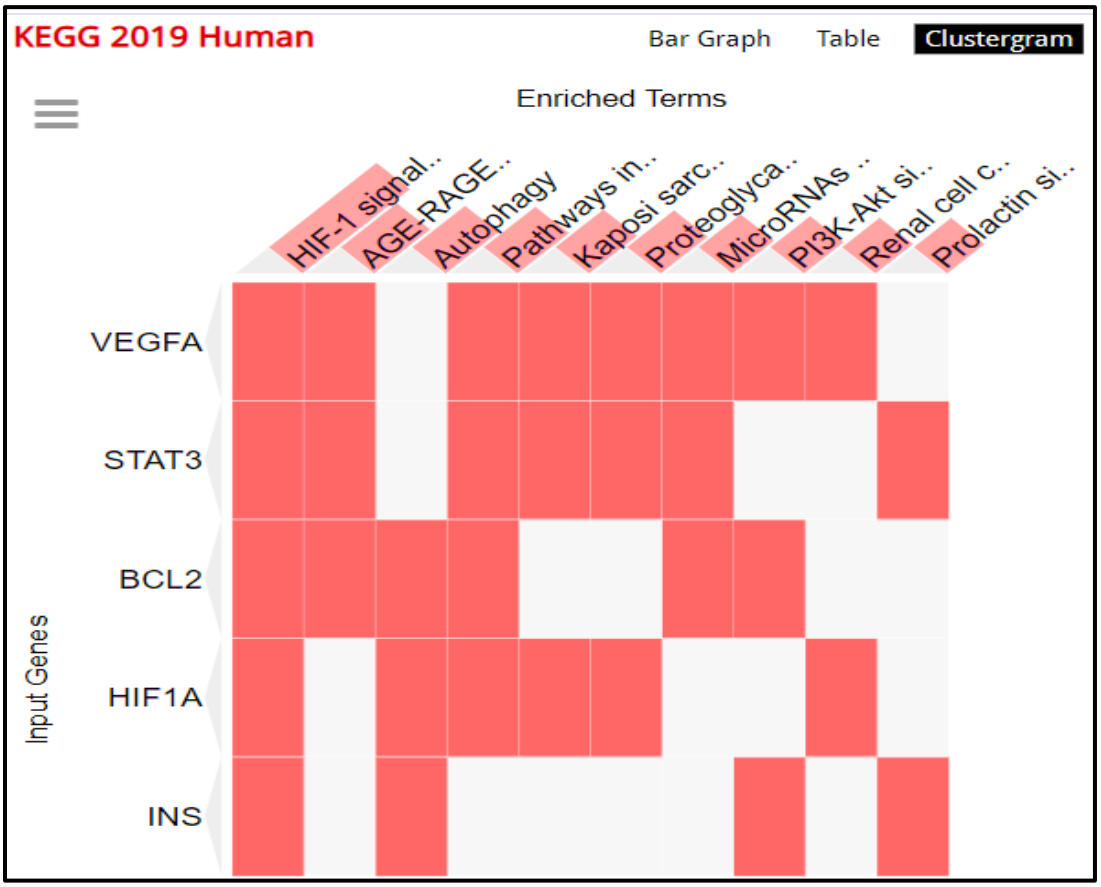



**Fig.4.2. The KEGG 2019 Human gene library shows the 5 gene enrichment analysis aligned according to significant P-value.**

### ii) Cancer Cell Line Encyclopedia

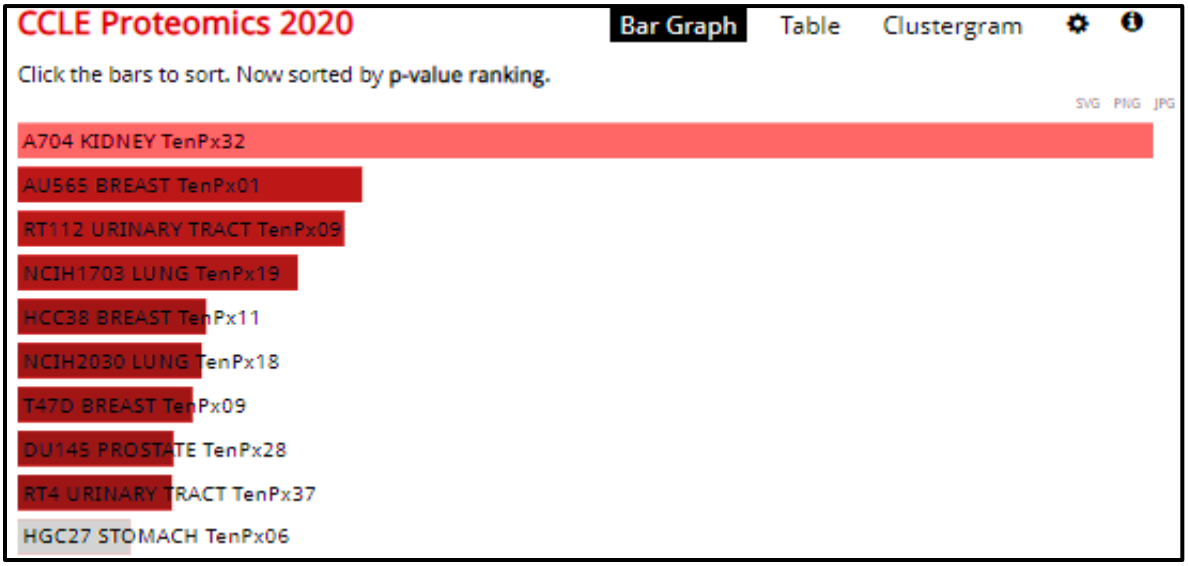



**Fig.4.3. The CCLE library showing the STAT3 and VEGFA involvement in mentioned cell lines.**

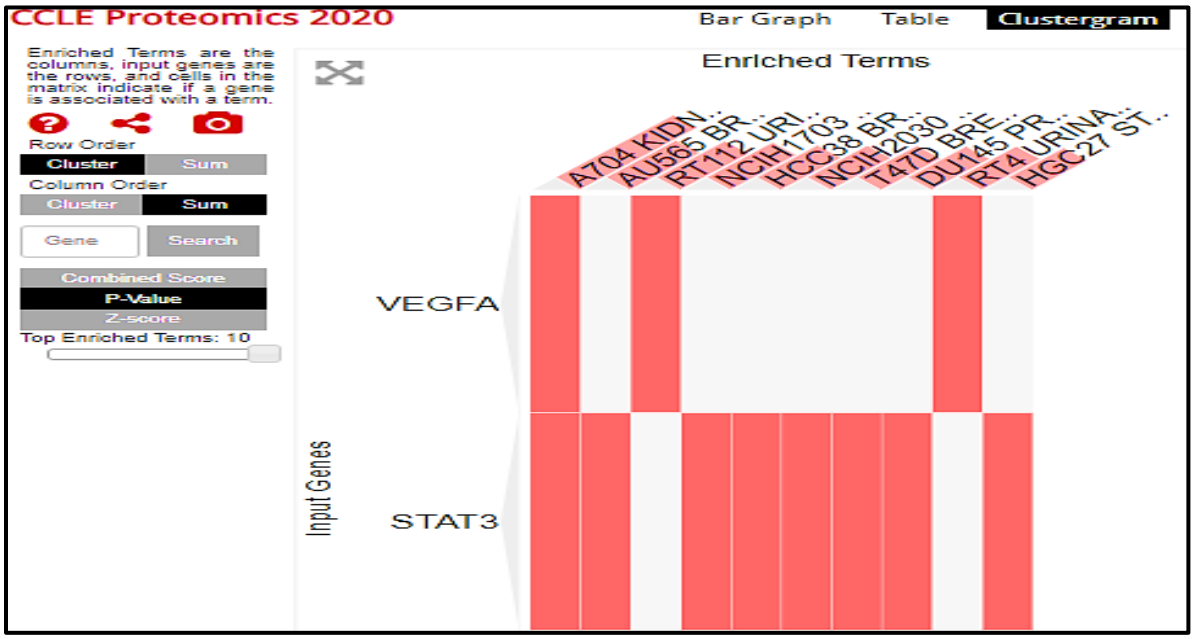



**Fig.4.4. The clustergram of the CCLE proteomics 2020 library showing the enrichment of input genes.**

CCLE Proteomics 2020 Bar Graph **Table** Clustergram

Hover each row to see the overlapping genes.

10 entries per page Search:

| Index | Name | P-value | Adjusted p-value | Odds Ratio | Combined score |
|---|---|---|---|---|---|
| 1 | AU565 BREAST TenPx01 | 0.02484 | 1.000 | 40.00 | 147.81 |
| 2 | A704 KIDNEY TenPx32 | 0.001943 | 0.7343 | 22.68 | 141.58 |
| 3 | RT112 URINARY TRACT TenPx09 | 0.02632 | 1.000 | 37.74 | 137.25 |
| 4 | NCIH1703 LUNG TenPx19 | 0.03056 | 1.000 | 32.47 | 113.25 |
| 5 | HCC38 BREAST TenPx11 | 0.04107 | 1.000 | 24.10 | 76.93 |
| 6 | NCIH2030 LUNG TenPx18 | 0.04166 | 1.000 | 23.75 | 75.49 |
| 7 | T47D BREAST TenPx09 | 0.04293 | 1.000 | 23.04 | 72.54 |
| 8 | DU145 PROSTATE TenPx28 | 0.04567 | 1.000 | 21.65 | 66.80 |
| 9 | RT4 URINARY TRACT TenPx37 | 0.04586 | 1.000 | 21.55 | 66.42 |
| 10 | HGC27 STOMACH TenPx06 | 0.05249 | 1.000 | 18.80 | 55.40 |

Showing 1 to 10 of 37 entries | Export entries to table Previous Next

**Fig.4.5. The Table of CCLE Proteomics 2020 showing the enrichment of STAT3 & VEGFA in Breast cancer lines with significant P-value, Odd ratio, and combined score.**

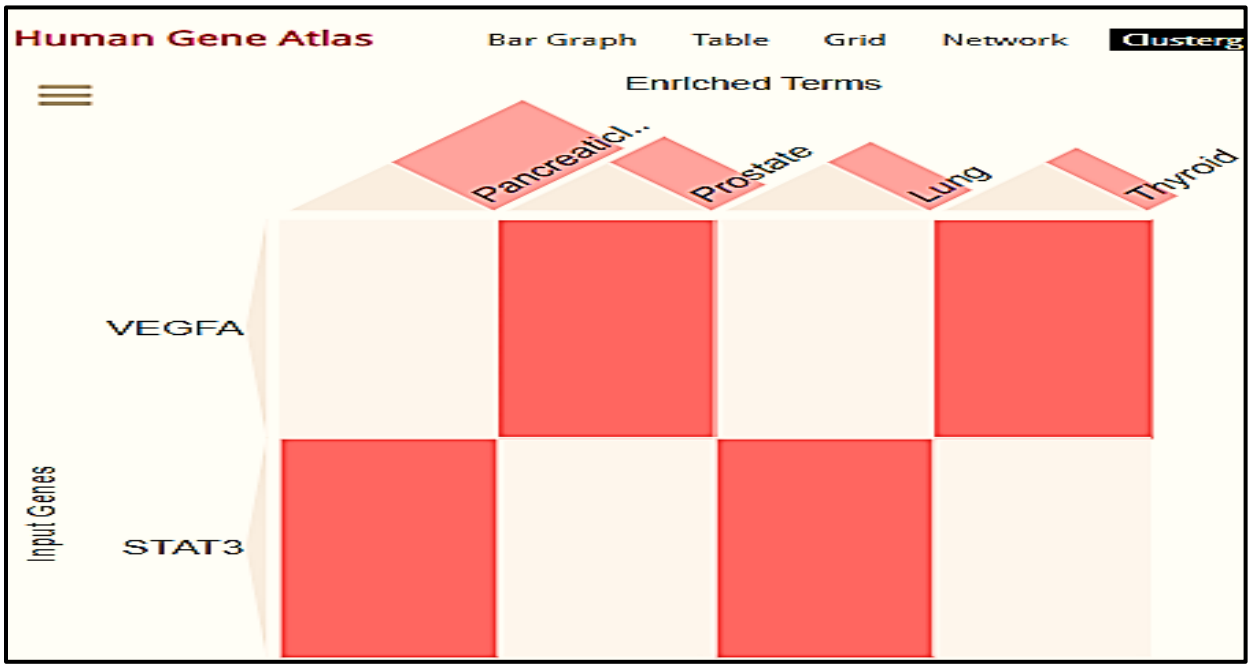



**Fig.4.6. The human gene atlas shows the involvement of STAT3 &VEGF in particular organs.**

## 4.3. PANTHER Analysis

In bioinformatics studies, PANTHER (protein scrutiny via evolutionary relationship) the classification structure is huge curated biological catalogue of gene/protein families and the functionally linked subfamilies which could be implied to categorize and recognize the purpose of gene products.

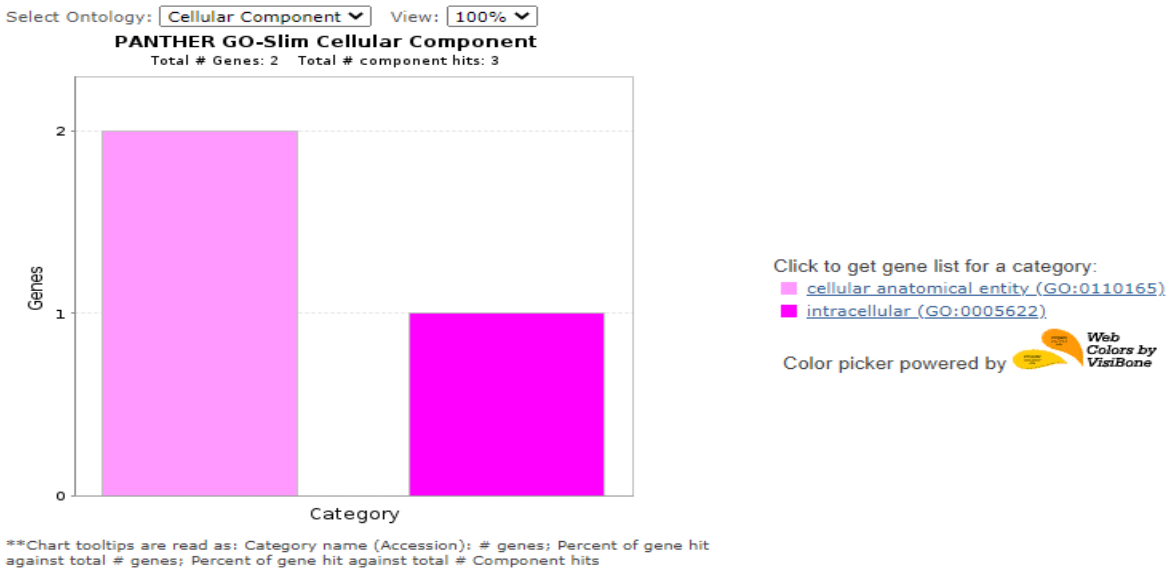



**Fig.4.7. Cellular components show the role of STAT3 and VEGFA in Breast cancer and T2DM pathologies.**

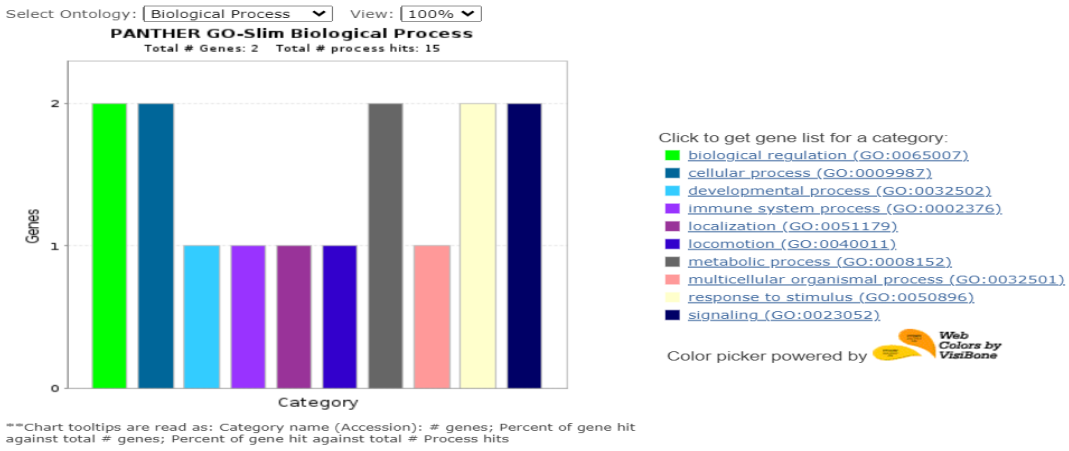



**Fig.4.8. Biological Processes show the role of STAT3 and VEGFA in Breast cancer and T2DM pathologies.**

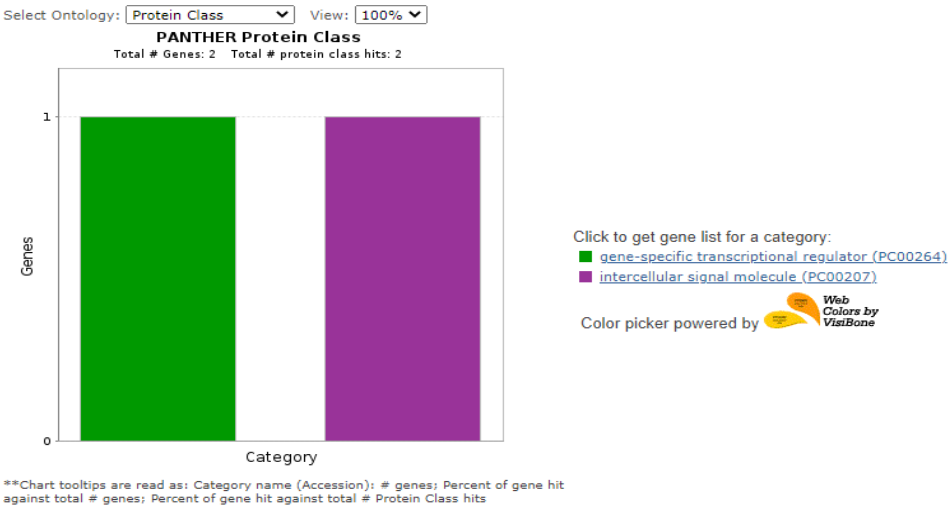



**Fig.4.9. Protein class show the role of STAT3 and VEGFA in Breast cancer and T2DM pathologies.**

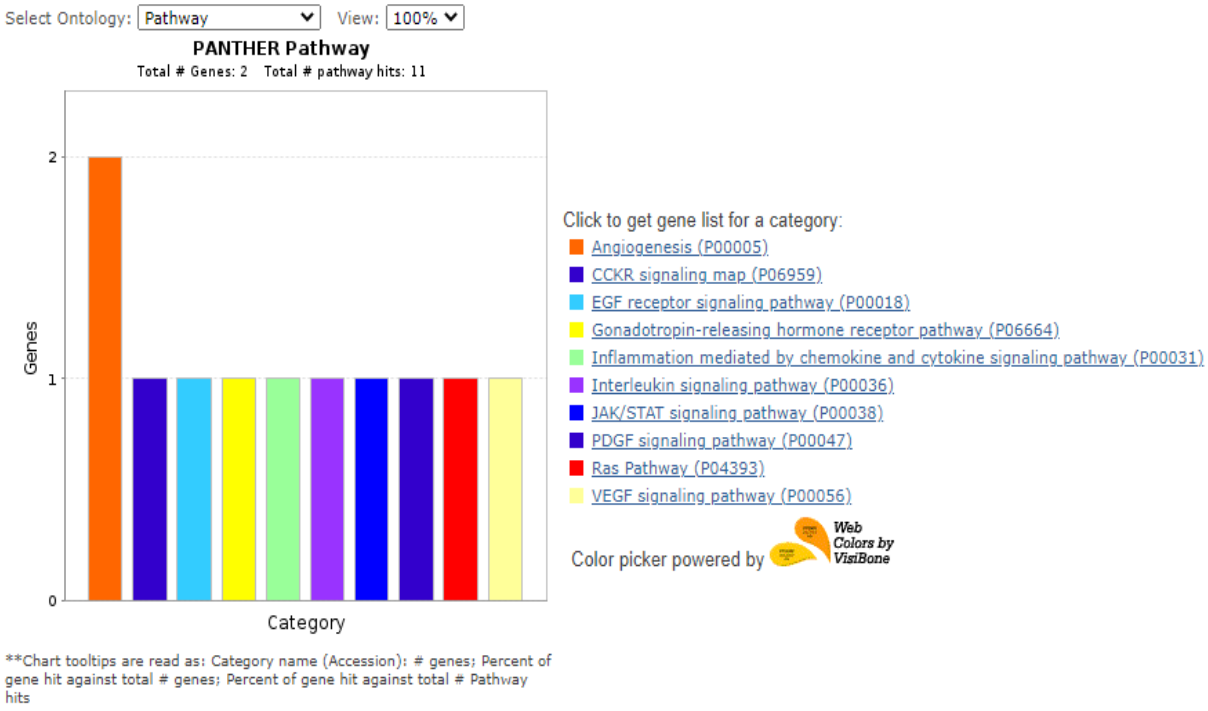



**Fig.4.10. Pathway analyses show the role of STAT3 and VEGFA in Breast cancer and T2DM pathologies.**

## 4.4. Primer Designing

The primer for STAT3 and VEGFA was designed through Primer3 Plus. The sequence homology of all primers was confirmed using primer BLAST and In-Silico software, genome browser.

**Table 4.1. Primer Sequence and Properties**

| GENE | Primer Sequence | Product size | Tm ºC | GC content | Self - Complementarity |
|---|---|---|---|---|---|
| STAT3 | (F)5' **GAAGAAGAGGGGGAGAGAGTT** 3' | 138bp | 58 | 52.4 | 0.00 |
| | (R) 5' **GAAGACGCCATTACAAGTGC** 3' | | 58.4 | 52 | 2.00 |
| VEGFA | (F)5' **ACACACCCACCCACATACAT** 3' | 178bp | 58.93 | 50.00 | 2.00 |
| | (R)5' **ATTCCCCTCCCAACTCAAG** 3' | | 56.28 | 52.63 | 2.00 |
| B-ACTIN | (F)5' **GGACTTCGAGCAAGAGATGG** 3' | 200bp | 58.07 | 55 | 4.00 |
| | (R) **TGTGTTGGCGTACAGGTCTTTG** 3' | | 61.32 | 50 | 4.00 |

### 4.3.1. Primer3 Plus Result

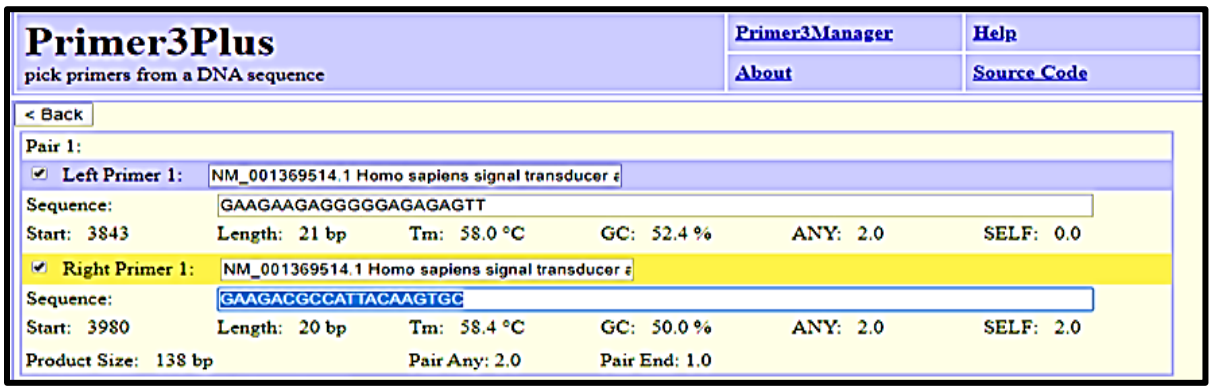



**Fig.4.11. The Primer 3 Plus result of STAT3 showing the product size of 138bp.**

### 4.3.2. UCSC In-Silico PCR Results

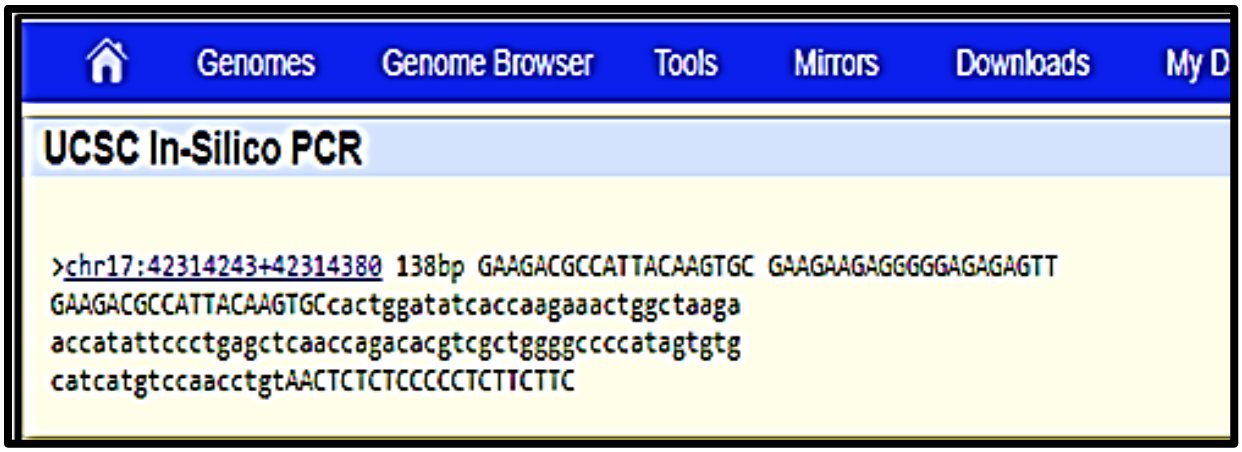



**Fig. 4.12. UCSC result confirms product size of STAT3 to be 138bp.**

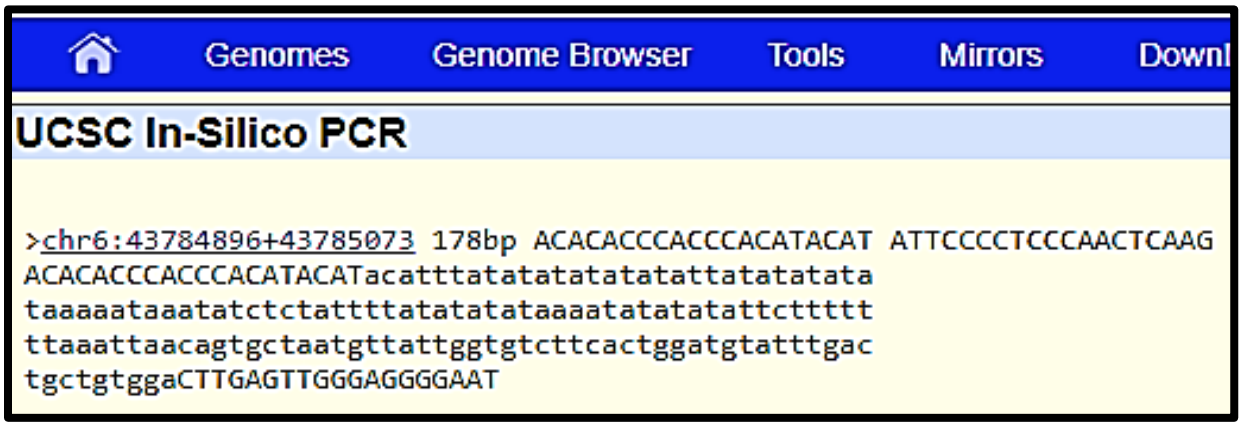



**Fig.4.13. UCSC result of VEGFA indicates size of 178bp.**

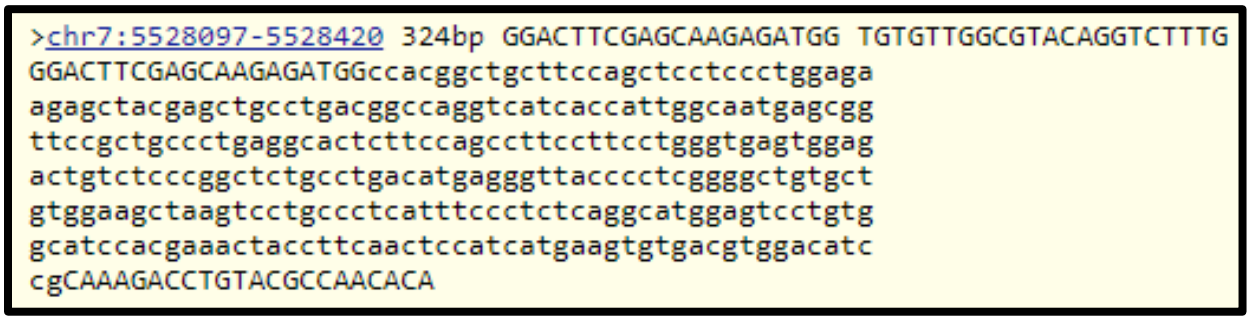



**Fig.4.14 The UCSC result of B-actin indicate size of 324bp.**

### 4.3.3. Primer Blast Results

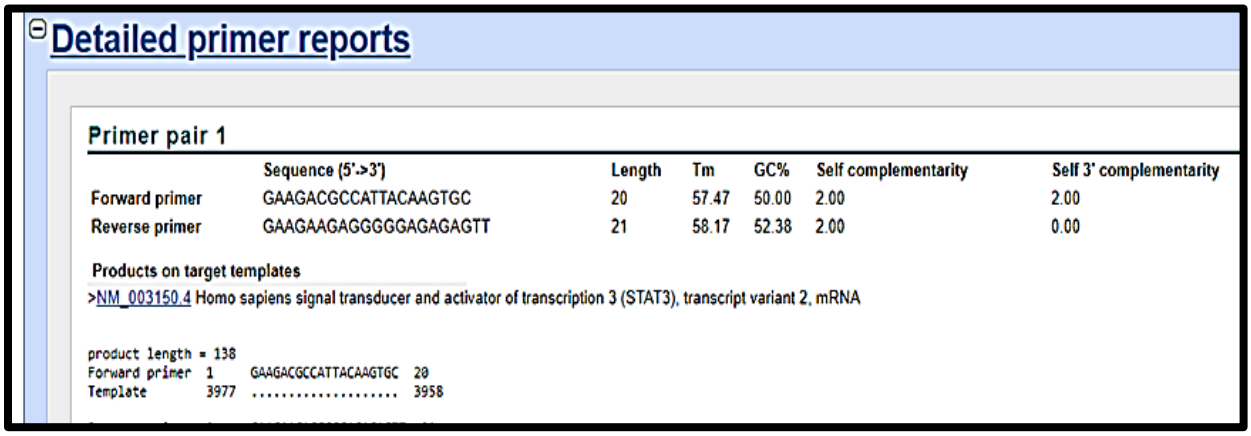



**Fig.4.15. Primer Blast result of STAT3 shows product size of 138**

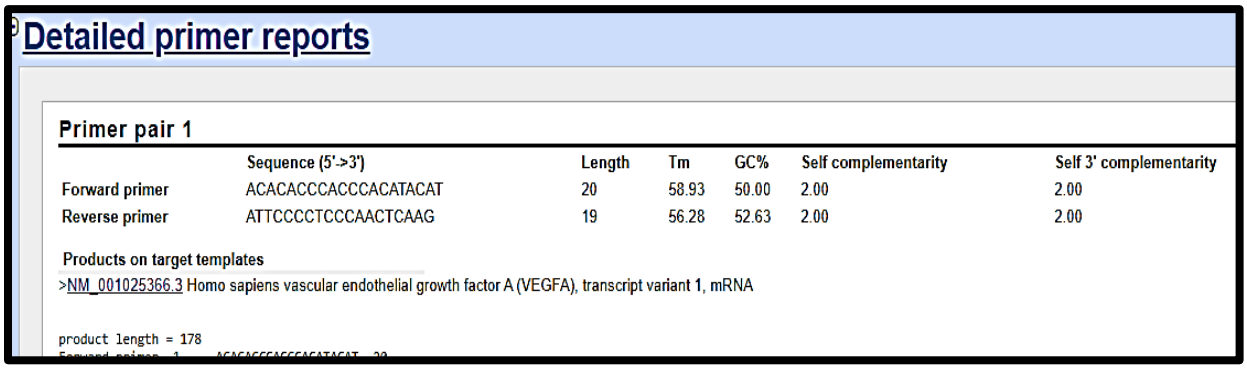



**Fig.4.16. Primer Blast result of VEGFA shows product size of 178bp.**

## 4.4. Gel Electrophoresis

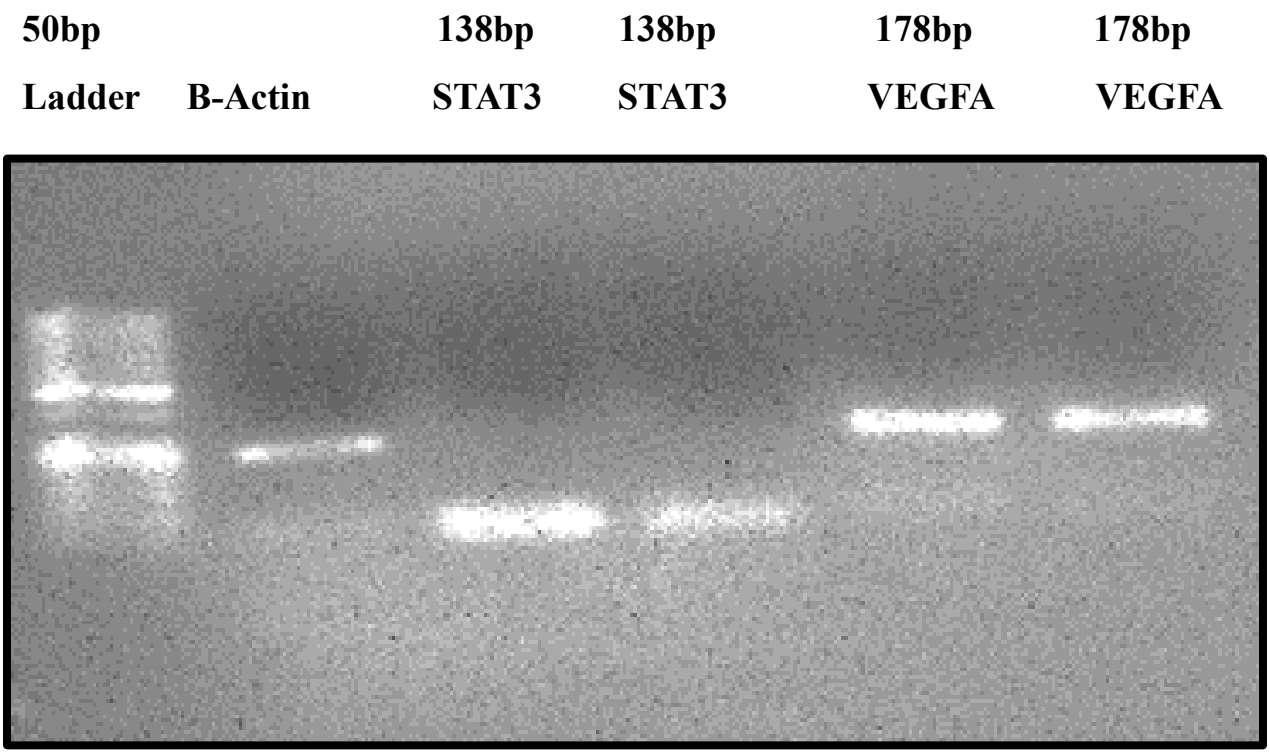
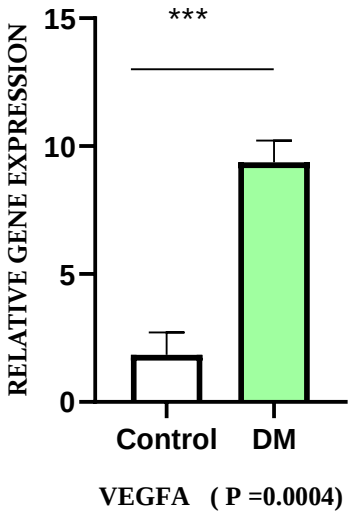
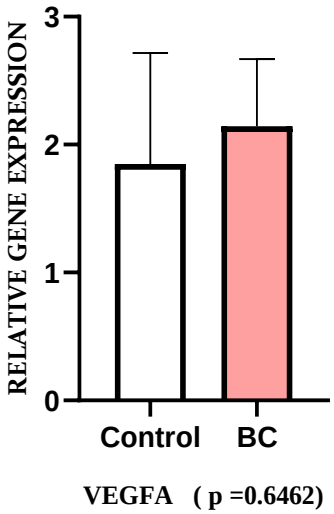



**Fig.4.17. The Gel electrophoresis result of Graded PCR optimized STAT3 and VEGFA gene.**

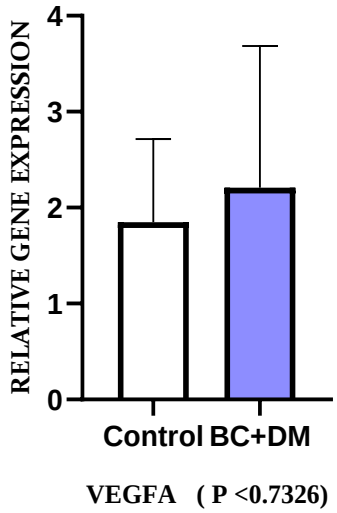
## 4.5. Real Time PCR Result



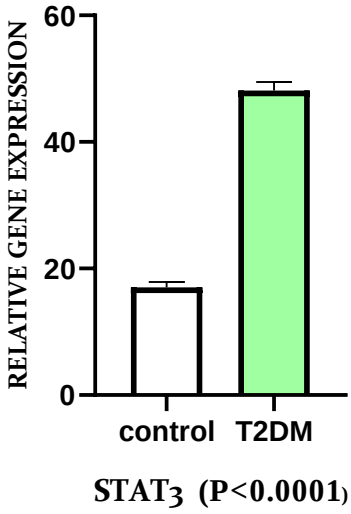

**Graph 4.1. Indicates the expression of VEGFA in Diabetes patients is higher as compared to control samples with significant P=0.0004 value and n=4, each group.**



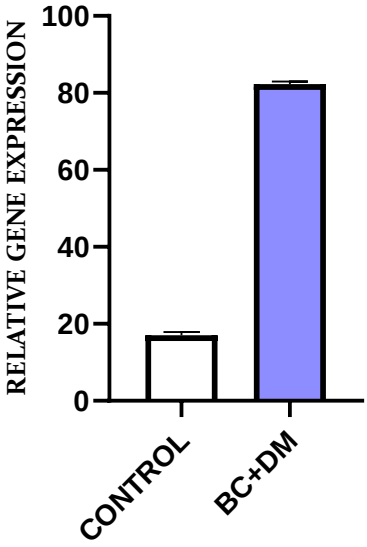

**Graph 4.2. Indicates the expression of VEGFA in Breast cancer patients is insignificantly higher than controls with p=0.6462 value and n=3.**



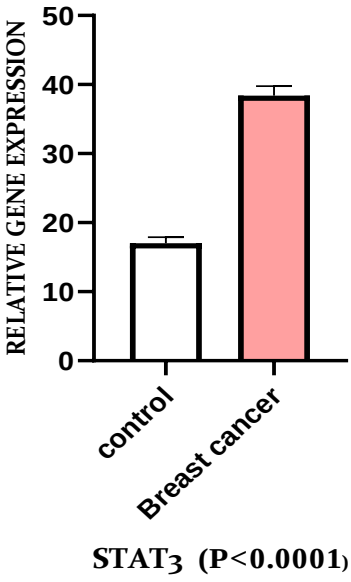

**Graph 4.3. Indicates the higher expression of VEGFA in BC+DM compared to control with p value <0.7326 and n=2, each group.**



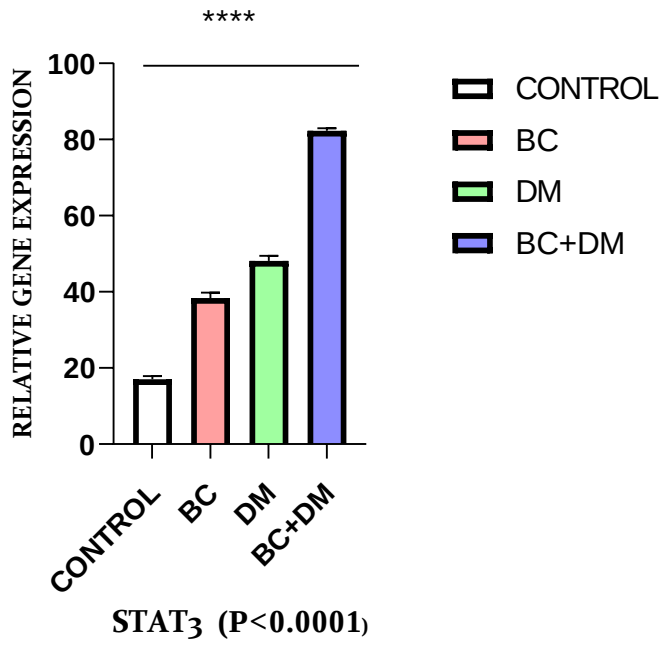

**Graph 4.4. Indicate the higher expression of STAT3 in DM as compared to control with P value<0.0001 and n=7.**



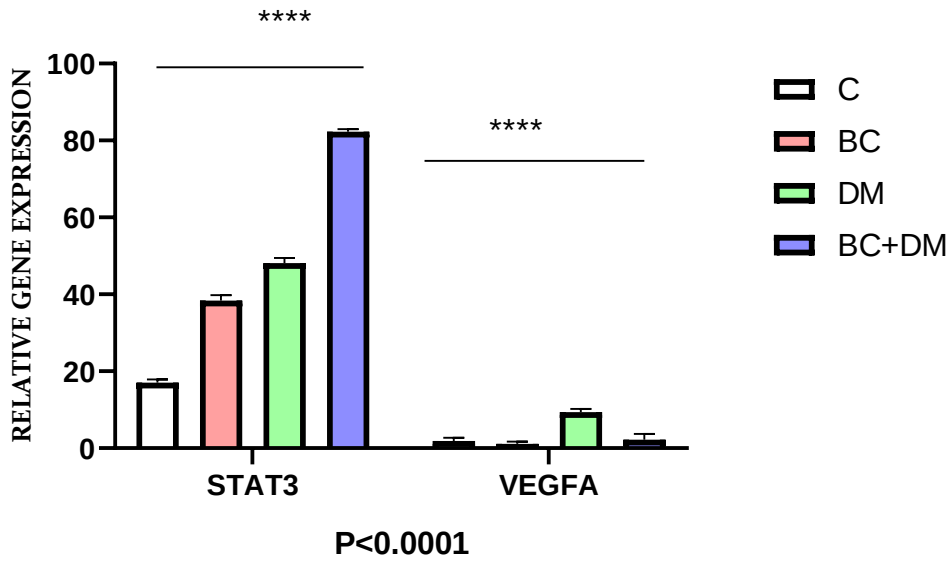

**Graph 4.5. Indicates high expression of STAT3 in BC+DM patients as compared to control group with p value<0.0001 and n=3.**



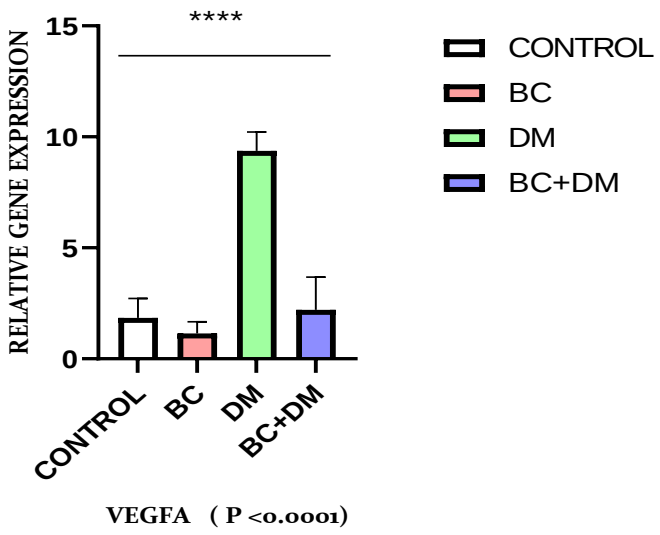

**Graph 4.6. Indicates high expression of STAT3 in BC patients as compared to control group with p value<0.0001 and n=7.**




**Graph 4.7. Indicates high expression of STAT3 in BC+ DM, DM, BC patients respectively as compared to control group with p value<0.0001 and n=3 in each group.**




**Graph 4.8. Indicates high expression of STAT3 in disease patients as compared to VEGFA (p value<0.0001).**




**Graph 4.9. shows comparative expression analysis of disease sample with control for VEGFA.**

# DISCUSSION

The two-stepped quantitative real-time RT-PCR (RT-qPCR), which is also acknowledged as real-time RT-PCR, or quantitative fluorescent RT-PCR have become process used for gene expression determination through the last few years. The expression of genes STAT3 and VEGFA is determined through RT PCR to identify their possibility of a biomarker for Diabetes Mellitus type 2 and Breast cancer. These two morbities are leading diseases exponentially growing all around globe affecting millions of lives.

The interplay of certain risk factors between two diseases are remarkably understudied and need more focus. The global occurrence of type 2 Diabetes Mellitus has been expected to rise for more than 10% in 2035 from 8.3% in 2013. T2DM affects various organs including women Breast a unusual condition called as Diabetic mastopathy which is uncommon multiplication of tissues in parenchyma of Breast developing nodules. There are evidences of T2DM involvement in Breast cancer risk.

This study aimed to identify biological markers involved in breast cancer and diabetes mellitus type 2 and find an interplay between them because almost 16% BC patients that have T2DM has been in danger of 10-20% excessive risk of Breast cancer.

Also, altered glucose tolerance can worsen the prognosis of Breast cancer. Environmental factors and genetic factors both influence the disease trigger and pathogenesis. These factors may act independently or synergic way for hormonal, metabolic disturbance ultimately leading to severity of diseases. There are certain conditions like hyperglycemia and hyperinsulinemia which have consequences on the cancer condition.

The upsurge in the level of insulin or IGF-1, cytokines like IL-6 or STAT3 and VEGFA in circulation cause stimulation of hypoxia-inducible factor 1 $\alpha$. This is followed by further rise in free radicals that can harm DNA and lipids equally indirectly and directly to encourage oxidative stress to further increase inflammation (Wolf et al., 2005).

It is also previously reported that there are certain medications of diabetes like Metformin that have an association with decrease risk of cancer because of indirect action causing inhibition of

insulin signaling and hepatic gluconeogenesis. That is why decrease in insulin amount in T2DM cancer patients whose tumorigenesis growth is influenced by insulin suggests using metformin in the particular subset of patients. Specific risk assessment measures should be developed to screen the spread of oxidative stress and its implications developing site specific cancers and diabetes mellitus type 2. Oxidative stress is one key mediator that is involved not only in mutagenesis but also develops to carcinogenesis, also responsible for redox variation of cancer cells which become resilient to anti- cancer mediators.

That is why patients with both diseases showed higher expression of both genes as the oxidative stress was more in them. Apart from cellular and molecular risk factors establishing link between two diseases there are some genetic factors that are also crucial for the malignancy and insulin resistance. The study's aim was to identify STAT3 and VEGFA part in pathogenesis of Breast cancer and T2DM.

It is found that Signal transducer and transcription factor 3 (STAT3) helps in pathogenesis by cell transformation through multiple protein like tyrosine kinases, viruses, oncogenes that activates the STAT3. It also actively participates in survival and cell proliferation.

Cyclin D1 and Myc is required for the G1 phase of interphase regulation and it is observed that constitutive expression of STAT3 causes upregulation of both of these genes promoting cellular progression. It is also studied that STAT3 signaling provides survival signal causing suppression of death in cancer cells. Its result is obtained by the instigation of Bcl2, and Bcl xL. STAT3 also negatively regulates p53 gene that has role in the inhibition of cell propagation and inducer of apoptotic process (Panis et al., 2012).

It is also found in literature that STAT3 plays role in cellular invasion that is important for metastasis and growth of tumor formation that is also proved by this study reporting higher expression of STAT3 in diseased samples as compared to control ones. It is also observed by biologists that STAT3 overexpression is correlated with enhanced invasion to extracellular matrix, metastasis in squamous cell carcinoma. Stat3 also plays part in cellular migration especially in pathologic condition.

The development of novel blood vessels from already existing vasculature is key point for development of tumor and metastasis. One of the utmost significant angiogenic component is

Vascular endothelial growth factor VEGFA. VEGFA is released from cancerous cells and attaches to transmembrane receptor tyrosine kinases of endothelial cell and participate in neovascularization.

STAT3 is activator of VEGF. The increased expression of STAT3 upregulates VEGFA expression ultimately leading to enhanced angiogenesis in melanoma cells. It is also observed by scientist that targeting STAT3 would also inhibit VEGFA and angiogenesis. Stat3 inhibition also affects the migration and vessel formation. STAT3 also induces expression of hypoxia-inducible factor-1$\alpha$ (HIF1$\alpha$), which is additional mediator for angiogenesis.

In oxidative stress condition, STAT3 and hypoxia-inducible factor-1$\alpha$ (HIF1$\alpha$) binds to VEGF promoter causing full transcriptional activation of VEGF protein leading to angiogenesis and metastasis enhancement. VEGFA has a role to cause permeability to provide to vascular leakage that highly affects indisposition of diabetic retinopathy. Diabetes is associated with various complications that impact microvasculature and microvasculature. VEGFA and STAT3 is found to be mis regulated in diabetic situation and many other micro-vascular related disorders. VEGFA and insulin signaling are very crucial for islet vessel maintenance, blood flow and tight glucose control (Mantovani et al., 2008).

JAK/STAT pathway not only participate in progression of Diabetes Mellitus Type 2 but also in elevated glucose induced damage to umbilical vein endothelial cells, in fact referred to be associated with endothelial cell dysfunction. The symbol of T2DM is insulin signaled glucose metabolism defect that is also referred as insulin resistance. In this pathway GSK-3β plays as negative controller of insulin signaling through suppressing two targets of insulin: glycogen synthase and insulin receptor substrate (IRS-1). It is found that STAT3 contributes to insulin signaling by sensitizing it through negative regulation of GSK-3β. STAT3 leads to increase in hepatic gluconeogenic genes and insulin resistance that are attributed to disturbance of IL-6 signaling directly or through brain mediated discharge of IL-6 from liver.

This study also helped through this knowledge in proving that both STAT3 and VEGFA have high expression in cancer and diabetic patients as compared to normal individuals. This project explored the role of STAT3 and VEGFA in four groups to find the correlation between two diseases so that common biomarker could be searched. Relative expression of four study groups was done by real time PCR in my project. The processed samples gave result in support to previous literature.

STAT3 had shown high expression in each group i.e., breast cancer, T2DM, and the group with both of the diseases. But the expression was fluctuating in different samples suggesting age difference, disease duration, stage, and medication they were taking. The fold change was higher of STAT3 and VEGFA in females of age greater than 45 than those of age below 45. It is also observed that those patients of lower age than 45 with higher stage of breast cancer showed higher expression of STAT3 and VEGFA as compared to higher age and lower stage of Breast cancer indicating the stage of cancer to be deciding factor of gene expression. Moreover, the duration of disease and family history is also critical for determining the expression of genes. Those diabetic patients having longer duration of the disease also showed greater gene expression as compared to slightly lower duration of gene expression (Ricciardiello *et al*., 2018).

The expression of STAT3 is more in BC+DM patients as compared to only with BC or Diabetes Mellitus Type 2 patients. The expression of VEGFA is more in DM patients as compared to in breast cancer or Diabetes mellitus type 2+ breast cancer patients. This correlates with literature that occurrence of two diseases causes aberrant expression of genes also. The expression of STAT3 and VEGFA is more in disease sample as compared to healthy individuals indicating upregulation of both these genes causing malignancy, inflammation, and differentiation of cells.

The In-Silico analysis done by STRING Analysis also indicated that two genes (STAT3+VEGFA) imported in this database showed interaction in metabolic and genetic pathways supported by text mining, curated databases, and experimentally determined studies. However, evidence supporting the co-occurrence and co-expression of these genes were not available on the software.

The data obtained from STRING indicated that STAT3 has role in the cancer progression by activating signaling pathway like the p13k, JAK/STAT that result in uncontrolled differentiation, metastasis, cellular growth, and resistance to apoptosis leading to tumor spread. Evidence supporting that constitutive phosphorylation of STAT3 in skeletal muscles rises accumulation of fatty acids that results in insulin resistance.

It is usually inactive at basal level but the phosphorylation of STAT3 by cytokines induced activation of tyrosine kinases in return activates STAT3. It also has different roles in different cells, in liver the phosphorylation of STAT3 inactivates GSK-3B and sensitize the insulin resistance. Its exact role in the proliferation of T2DM is still unclear. But major studies report high expression of this gene in T2DM and also in BC patients.

In the In-Silico analysis done by ENRICHR different pathways were studied and the role of STAT3 and VEGFA was analyzed in transcription pathways, ontologies, diseases, and drugs etc. Significant data supported the involvement of STAT3 and VEGFA in KEGG pathway where three other genes apart from my genes were imported in this database showed implication of these genes in Breast cancer and T2DM crosstalk supported by KEGG pathway 2019. Top ten enriched pathways for my genes were shown among which were Hif-1 signaling and AGE-RAGE diabetic complication signaling.

Cancer cell line encyclopedia (CCLE) from ENRICHR was also analyzed that showed the enrichment of STAT3 and VEGFA proteins in the Breast cancer cell lines, data obtained from text mining.

PANTHER analysis showed the function of genes and their involvement in several biological methods like biological management of cellular processes, developmental courses, cellular processes, immune system, localization, metabolic processes and signaling. The function of genes in pathways like angiogenesis, EGF receptor signaling, gonadotrophin discharging hormone receptor signaling, inflammation facilitated by chemokine and pathway of cytokine signaling, interleukin signaling, JAK/STAT signaling pathway, PDGF signaling pathways, RAS pathway and VEGF signaling pathways was also mentioned.

The In-Silico analysis and RT PCR result both conclude that STAT3 and VEGFA have roles in proliferation of Diabetes Mellitus type 2 and Breast Cancer. It is also critical to note that the patients who already have Diabetes Mellitus type 2 are more prone to develop cancer in later stage of the age. And similarly, the patients who have Breast cancer they can likely develop T2DM as compared to normal healthy individuals.

The possible reason behind the cause could be the activation of triggering situation like DM, obesity, stress, and pancreatic defect that can activate the transformation elements like elevated levels of AGE'S DNA damage, increase in ROS that can lead to DNA break, mutations that can cause the aberrant expression of genes, aberrant regulation of mRNA and post-translational modification dysregulation.

Any alteration at RNA, DNA, Protein can lead to cancer development. Similarly, the stressed cancerous cells need more glucose uptake and with high expression of genes that will exert more

stress on beta cells to release insulin and increased stress can lead to damage of the pancreatic beta cells and thus resulting in diabetes mellitus type 2.

It is also interesting to note that certain physiological factors like age, smoking routine, duration of disease and weight influenced the clinical course of disease. The patients with age greater than 45 are more likely to have worse condition and implication of both diseases could lead to life threatening situation. The weak immune system and slow working cells could be cause that they have developed both the diseases especially in women with age above 45 who have breast cancer they have developed T2DM as well.

The association of both genes in the interplay of Breast cancer and Type 2 diabetes mellitus is crucial for understanding as it involves certain risk factors like hyperglycemia that causes amplified cancer cell propagation, repressed cancer cell death and elevated cancer metastasis. In diabetes mellitus, the hyperglycemia is caused because of the increase in insulin resistance. Many of the studies support the direct link in breast cancer initiation, impaired glucose tolerance, invasiveness due to circulating levels of IGFs/insulin by high inflammatory cytokines like IL-6 and TNFα by production of reactive oxygen species through platelet activation.

Insulin-like growth factors are found to play role like anti-apoptotic effects in cancerous cell and the development in this consequence caused by elevated insulin especially in insulin-resistant individuals. The effect of certain treatments on these individuals is also interesting to note. Insulin as a treatment many-a-times cause high plasma concentration of insulin in liver.

Despite the adverse roles of insulin on breast cancer cells no evidence for significant association between the cancer proliferation and diabetes is not discovered. Many of oral hypoglycemic agents like metformin and phenformin causes decrease in insulin concentration thus causing increase in insulin sensitivity. This effect is found to be useful in the breast cancer treatment.

The effectiveness of the metformin and other adjuvant action for early breast cancer patients has entered in phase 2 trials. Until now, no significant data support any association between hypoglycemic agents and Breast cancer.

It is also thought-provoking to mention that elder age women with diabetes should be suggested for mammography to understand the hidden symptoms or clinical pathologies for early diagnosis of any underlying morbidity.

The treatments used for breast cancer can also get affected by the presence of type 2 diabetes mellitus in patients. Many complications of diabetes such as nephropathy, heart diseases, neuropathy and weakened wound curing, vulnerability to infection could hinder in cancer therapies.

It is reported that diabetes is linked to higher risk of difficulties next to surgery for patients of breast cancer. The examination of disease patient indicated that diabetes and wound infection are associated after breast surgery. Diabetes is linked not lone with instant problems but too with ipsilateral upper arm fault 5 years after mastectomy. It is also seen that patients with both diseases have high chances of early and late complication after radiation therapy. In elderly patients on radiation, diabetes can severely affect the cancer condition.

Most of the literature data recommend that type 2 diabetes mellitus might be linked with 10-20% of increased danger of Breast cancer development and it can have serious damaging effects on usual history, treatment, and diagnosis of breast cancer. Direct effects of diabetes biologically on individual's with breast cancer are hard to define chiefly for the reason that of certain confusing factors like the old age, comorbidity, obesity, and alterations in screening usage or in the treatment allocation. These are the factors that can cause undertreatment or worsen the consequence for patient with breast cancer.

Various human cancer like breast cancer is vulnerable to therapeutic effect of inhibitors of STAT3. The uncontrolled expression of STAT3 signaling causes carcinogenesis and progression of tumor through dysregulation of many downstream gene which controls propagation, angiogenesis, immunosurveillance, and malignant transformation. The downstream genes are Bcl2, Bcl-XL, c-Myc that causes proliferation, survival, and inhibition of tumor cell apoptosis.

The transcription factors work together to enhance the inflammation mediators like the pro inflammatory cytokines that are involved directly in varied stages of carcinogenesis which are promotion, progression, and initiation.

Stat3 and VEGFA are activated by the JAK/STAT signaling pathway that upregulate angiogenic proteins in tumor microenvironment. The coordinated expression of several cytokines like CCL2, TNFα and IL-6 in cancer indicate that these acts to promote that relapse of disease. These genes

cause the production of estrogen metabolism that led to increase in DNA-adduct side products which associate to further mechanism for breast cancer promotion associated with inflammation.

# Conclusion

It is concluded that both STAT3 and VEGFA have vital roles in the pathogenesis of Diabetes and Breast cancer as these genes are dys-regulated. The high expression of STAT3 induces activation of VEGFA and causes its high expression worsens the prognosis of disease leading to chemoresistance. The relative expression difference of STAT3 and VEGFA through RT-PCR in patient and control samples shows it to an indicator and biomarker for the disease situation. The diabetic patient is more prone to get cancer while cancer patient is also at high risk of getting other microvasculature diseases like Diabetes mellitus type 2. Greater the age and stage of the patients more are the chances of high expression of the genes. Similarly, more the duration of the disease, causes high expression of genes because of dysregulation of oxidative stress and hyperglycemia condition.

# Future Prospects

In future the expression of these genes could be done on more patients of different ethnicities and could be confirmed by ELISA or immunohistochemistry. Also, more genes could be compared to see the expression to select best possible biomarker. The drugs could be designed according to the target and tested clinically. These genes could become biomarkers for their potential relevance and high expression in disease condition. Additional studies are still due to clarify and deeply investigate the signaling pathway through which both diseases interact and pave their ways into each other so that the severity or development of one or two of them could be restricted before damage.

## References

Devesa, S.S., Blot, W.J., Stone, B.J., Miller, B.A., Tarone, R.E., Fraumeni J.F. (1995). Recent cancer trends in the United States. *J Natl Cancer Inst*, 87: 175–82.

Wolf, I., Sadetzki, S., Catane, R., Karassik, A., Kaufman, B. (2005). Diabetes mellitus and breast cancer. *Lancet Oncol*; 6:103–11.

Michels, K.B., Solomon, C.G., & Hu, F.B. (2003). Type 2 diabetes and subsequent incidence of breast cancer in the Nurses' Health Study. *Diabetes Care*; 26: 1752–58.

Cohut, C. (2017). 'Alarming' rise in cancer rates driven by diabetes, obesity. *Medical News Today*.

Baron, J.A., Weiderpass, E., & Newcomb, P.A. (2001). Metabolic disorders, and breast cancer risk (United States). *Cancer Causes Control*;12: 875–80.

Vigneri, P., Frasca, F., Sciacca, L., Pandini, G., & Vigneri R. (2009). Diabetes and cancer. *Endocr Relat Cancer*, 16(4), 1103-23.

Shikata, K., Ninomiya, T., & Kiyohara Y. (2013). Diabetes mellitus and cancer risk: review of the epidemiological evidence. *Cancer Sci*, 104(1):9-14.

Boyle, P., Boniol, M., Koechlin, A., Robertson, C., Valentini, F., & Coppens, K. (2012). Diabetes and breast cancer risk: a meta-analysis. *British journal of cancer*, 107(9):160817.

Smith, U., and Gale, E.A. (2010). Cancer, and diabetes: are we ready for prime time*? Diabetologia*, 53(8):1541-4.

Hoy, A.J., Balaban, S., & Saunders, D.N. (2017). Adipocyte-Tumor Cell Metabolic Crosstalk in Breast Cancer. Trends Mol Med, 23(5):381-92.

Vona-Davis, L., Rose, D.P. (2009). Angiogenesis, adipokines and breast cancer. *Cytokine Growth*

*Factor Rev*, 20(3):193-201.

Delort, L., Rossary, A., Farges, M.C., Vasson, M.P., Caldefie-Chezet, F. (2015). Leptin, adipocytes, and breast cancer: Focus on inflammation and anti-tumor immunity. *Life Sci*, 140:37-48.

Baumann, J., Sevinsky, C., Conklin, D.S. (2013). Lipid biology of breast cancer. *Biochim Biophys Acta*, 1831(10):1509-17.

Santos, C.R., Schulze, A. (2012). Lipid metabolism in cancer. *The FEBS journal*, 279(15):2610-23.

Monaco, M.E. (2017). Fatty acid metabolism in breast cancer subtypes. *Oncotarget*, 8(17):29487-500.

Luo, X., Cheng, C., Tan, Z., Li, N., Tang, M., & Yang, L. (2017). Emerging roles of lipid metabolism in cancer metastasis. *Mol Cancer*, 16(1):76.

Maruthur, N.M., Bolen, S., & Brancati, F.L. (2009). Obesity and mammography: A systematic review and meta-analysis. *J Gen Intern Med*, 24:665-677.

Kim, S., Lee, Y., & Koo, J.S. (2015). Differential expression of lipid metabolism-related proteins in different breast cancer subtypes. *PLoS One*, 10(3): e0119473

Carracedo, A., Cantley, L.C., & Pandolfi, P.P. (2013). Cancer metabolism: fatty acid oxidation in the limelight. *Nat Rev Cancer*, 13(4):227-32.

Dalamaga, M., Diakopoulos, K.N., & Mantzoros, C.S. (2012). The role of adiponectin in cancer: a review of current evidence. *Endocr Rev*, 33(4):547-94.

Chen, X., Wang, Y. (2011). Adiponectin and breast cancer. *Med Oncol*, 28(4):1288-95.

Kujawski, M. (2008). Stat3 mediates myeloid cell dependent tumor angiogenesis in mice. *J. Clin. Invest*, 118, 3367–3377.

Mantovani, A., Allavena, P., Sica, A., & Balkwill, F. (2008). Cancer-related inflammation. *Nature*, 454, 436–444.

Jawad, F., & Maqsood, A. (2007). Prevalence of diabetes in Pakistan. Diabetes research and clinical practice, 76 (2): 219-222.

Krentz, A. J. and Bailey, C. J. (2005). Oral antidiabetic agents. Drugs, 65 (3): 385-411.

Lorincz, A.M., & Sukumar, S. (2006). Molecular links between obesity and breast cancer. *Endocr Relat Cancer*,13: 279 – 292.

Pollan, M. (2010). Epidemiology of breast cancer in young women. *Breast Cancer Res Treat*, 123: 3 – 6.

Zou, C., & Shao, J. (2008). Role of adipocytokines in obesity-associated insulin resistance. *J Nutr Biochem*, 19: 277 – 286.

Cusi, K. (2011). The role of adipose tissue and lipotoxicity in the pathogenesis of type 2 diabetes. *Curr Diab Rep*,10: 306– 315.

Anderson, G.L., & Neuhouser, M.L. (2012). Obesity and the risk for premenopausal and postmenopausal breast cancer. *Cancer Prev Res (Phila)*,5: 515 – 521.

Mantovani, A., Allavena, P., Sica. A., & Balkwill, F. (2008). Cancer-related inflammation. *Nature*, 454: 436 – 444.

Halliwell, B., & Gutteridge, J.M. (2007). Free radicals in biology and medicine. *Oxford, UK: Clarendon Press.*

Reuter, S., Gupta, S.C., Chaturvedi, M.M., & Aggarwal, B.B. (2010). Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med*, 49: 1603– 1616.

Panis, C., Victorino, V.J., Herrera, A.C., Freitas, L.F., De Rossi, T., & Campos, F.C. (2012). Differential oxidative status and immune characterization of the early and advanced stages of human breast cancer. Breast Cancer Res Treat, 133: 881– 888.

Kundu, J.K., & Surh, Y.J. (2012). Emerging avenues linking inflammation and cancer. *Free Radic Biol Med*, 52: 2013– 2037.

Portela, A., & Esteller, M. (2010). Epigenetic modifications and human disease. *Nat Biotechnol*, 28: 1057 – 1068.

Rodriguez-Paredes, M., Esteller, M. (2011). Cancer epigenetics reaches mainstream oncology. *Nat Med*, 17: 330 – 339.

Matsuura, F., Oku, H., Koseki, M., Sandoval, J.C., Yuasa-Kawase, M., Tsubakio-Yamamoto, K., Masuda, D., Maeda, N., Tsujii, K., & Ishigami, M. (2007). Adiponectin accelerates reverse cholesterol transport by increasing high density lipoprotein assembly in the liver. *Biochem. Biophys.Res. Commun*, *358*, 1091–1095.

Rose, D.P., Komninou, D., & Stephenson G.D. (2004). Obesity, adipocytokines, and insulin resistance in breast cancer. *Obesity Reviews* 5 153–165.

Bradley, R.L., & Cheatham, B. (1999). Regulation of obesity gene expression and leptin secretion by insulin and dexamethasone in rat adipocytes. *Diabetes*, 48; 272–278.

Perera, C.N., Chin, H.G., Duru, N., & Camarillo, I.G. (2008). Lepin regulated gene expression in MCF-7 breast cancer cells: mechanistic insights into leptin-regulated mammary tumor growth and progression. *Journal of Endocrinology*,199: 221–233.

Bartella, V., Cascio, S., Fiorio, E., Auriemma, A., Russo, A., & Surmacz, E. (2008). Insulin-dependent leptin expression in breast cancer cells. *Cancer Research*. 68: 4919–4927.

Cirillo, D., Rachiglio, A.M., la Montagna, R., Giordano, A., & Normanno, N. (2008). Leptin signaling in breast cancer: an overview. *Journal of Cellular Biochemistry*, 105: 956–965.

Vona-Davis, L., & Rose, D.P. (2007). Adipokines as endocrine, paracrine, and autocrine factors in breast cancer risk and progression. *Endocrine-Related Cancer*, 14: 189–206.

Barone, I., Catalano, S., Gelsomino, L., Marsico, S., Giordano, C., Panza, S., Bonofiglio, D., Bossi, G., Covington, K.R., & Fuqua. (2012). Leptin mediates tumor–stromal interactions that promote the invasive growth of breast cancer cells. *Cancer Research*,72: 1416–1427.

Lumeng, C.N., & Saltiel, A.R. (2011). Inflammatory links between obesity and metabolic disease. *J. Clin. Investig, 121*: 2111–2117.

Kershaw, E.E., Flier, J.S. (2004). Adipose tissue as an endocrine organ. *J. Clin. Endocrinol. Metab*, *89*: 2548–2556.

Hauner, H. (2004). Secretory factors from human adipose tissue and their functional role. *Proc. Nutr. Soc*, *64*: 163–169.

Halberg, N. (2008). Wernstedt-Asterholm, I.; Scherer, P.E. The adipocyte as an endocrine cell. *Endocrinol. Metab. Clin. N. Am, 37*: 753–768.

Perseghin, G., Ghosh, S., Gerow, K., & Shulman, G.I. (1997). Metabolic defects in lean nondiabetic offspring of NIDDM parents: A cross-sectional study. *Diabetes, 46*: 1001–1009.

Acharya, S., Xu, J., Wang, X., Jain, S., Wang, H., & Zhang, Q. (2016). Downregulation of GLUT4 contributes to effective intervention of estrogen receptor-negative/HER2-overexpressing early-stage breast disease progression by lapatinib. *Am J Cancer Res*, 6(5):981-95.

Ricciardiello, F., Votta, G., Palorini, R., Raccagni, I., Brunelli, L., & Paiotta A. (2018). Inhibition of the Hexosamine Biosynthetic Pathway by targeting PGM3 causes breast Cancer growth arrest and apoptosis. *Cell Death Dis*, 9(3):377.

Veikkola, T., & Alitalo, K. (1999). VEGFs receptors and angiogenesis. *Seminars in Cancer Biology*, 9: 211–220.

Semenza, G.L. (2000). HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J. Appl. Physiol*,88: 1474–1480.

Wacker, A., and Gerhardt, H. (2011). Endothelial development taking shape. *Curr. Opin. Cell Biol*, 23: 676–685

Kerbel, R.S. (2008). Tumor angiogenesis. *N. Engl. J. Med*, 358: 2039–2049.

Jain, R.K. (2014). Antiangiogenesis strategies revisited: from starving tumors to alleviating hypoxia. *Cancer Cell*, 26: 605–622.

Xiong, A., Yang, Z., Shen, Y., Zhou, J. and Shen, Q. (2014). Transcription Factor STAT3 as a Novel Molecular Target for Cancer Prevention. *Cancers* (*Basel*), 6: 926-957.