Chapter 1

1.Introduction and Review of literature

Chapter 1

1.1 Breast cancer: Rising global health burden

Breast cancer is a leading concern in the health sector and an increasing global heath challenge amongst the women worldwide. The incidence of breast cancer and death rate have increased in past few decades. In 2020, around 2 million women were detected with breast cancer. Every country worldwide has recorded increase in the cases making it one of the most occurring cancers leading to global burden. According to latest studies, the survival rate for metastatic breast cancer patients is less than 30% [1, 2]. GLOBOCAN data by IARC (International Agency for Research on Cancer) collected from 185 countries have reported 11.7% of breast cancer of all new cancer cases with 6.9% mortality rate recorded in 2020 [3]. WHO established global breast cancer initiative (GBCI) in 2021 and aimed to reduce breast cancer by 2.5% every year which will save 2.5 million lives over 20 years. Breast cancer is not just the highest incident cancer globally, but also the cause of more disability causing cancer than any other malignancy. The risk factors involve age, genetics, poet menopausal hormones, overweight and obesity, alcohol consumption, and avoidable lifestyle factors. The higher income countries record more incidences of breast cancer compared to low income due to increase in globalization and changing lifestyles [4, 5]. Recent statistical study identified the future burden of breast cancer and stated that there will be an 40% increase in the breast cancer cases by the year 2040 [6]. Establishment of essential diagnostics and modalities for treatment should be the major task to be undertaken for early detection in asymptomatic population. ***The current situation emphasizes the need to further understand the pathogenesis of breast cancer from initiation to metastasis and develop tool for early prognosis/diagnosis, treatment and management of the patients.***

1.2 Breast cancer subtypes

Breast cancer is majorly categorized in in situ carcinoma which are localized also called non-invasive or invasive cancer in which the cancer has spread to distant organs or nearby lymph nodes. Invasive breast cancer is further classified based on pathological markers human epidermal growth factor 2 (HER2), oestrogen receptor (ER) and progesterone receptor (PR) [7]. Based on the expression of these receptors the patients are stratified for treatment selections. The five subtypes of breast cancer based on presence and absence of these receptors are namely Her2-enriched, Luminal A, Luminal B, Basal-like and Triple Negative Breast Cancer (TNBC). Studies have shown that the subtypes differ in survival rates and disease

outcome. Luminal breast cancer are 65-70% of all breast cancer tumors. The expression of ER receptors in combination with PR receptors on the cell surface is a distinct feature of Luminal subtype. It is divided in Luminal A and Luminal B subtypes which are highly heterogenous and comprises of distinct gene expression profiles and mutations which leads to difference in responses to treatments [8-11]. HER2 receptor expression is a common feature of both the subtypes of luminal breast cancer. Amongst them Luminal A is more common and shows reduced expression of proliferative genes compared to luminal B subtype [116-117][9, 10]. Multiple cases have identified Luminal B subtypes to have shorter overall survival compared to Luminal A breast cancer [12, 13]. Studies have shown that luminal A has the better prognosis than Basal breast cancer and Luminal B [7]. The overexpression of proliferation markers is considered the main reason for aggressive nature of Luminal B subtype compared to others. Interestingly, an integrated genomic approach identified that both the subtypes have a distinct set of proliferation markers that can be used for sub categorization of breast cancer subtypes and can be targeted for different therapeutic options [14].

HER2 receptor is a mediator of extracellular signaling that initiates processes for cell proliferation, cell survival and cell differentiation. Till date, triple negative and HER2 positive breast cancers are considered highly aggressive with unfavourable prognosis [15]. Basal-like or HER2-only have high Ki-67 levels and correlates to higher pathological condition than a luminal type [16]. HER2 positive tumors metastasize to liver, bone, brain, and central nervous system hence a major concern for breast cancer patients. The dependence of tumor cells on HER2, have made HER2 targeted monoclonal antibodies like: trastuzumab, pertuzumab and tucatinib along with chemotherapy are effective for the subtype and improved survival to 90% in HER2 positive breast cancer patients [17-19].

TNBC subtype tumors lack expression of ER, PR and HER2 on their surface which makes them the most difficult subtype for targeted therapies. TNBC on the other hand have short survival time and mortality rate is around 40% after diagnosis [20, 21]. TNBC are aggressive and have prominent distant metastasis to brain and visceral organs [22]. Highly recurrent lesions and aggressiveness is the key features of Basal like subtypes with poor prognosis and overall survival. Various studies have identified markers associated with basal like subtypes and characterised them based on disease outcome.

Thus, understanding the pathology and molecular basis of evolution, progression, and metabolic differences is important in different subtypes of breast cancers which may help in developing better therapeutic approaches.

1.3 Tumor microenvironment (TME) in breast cancer

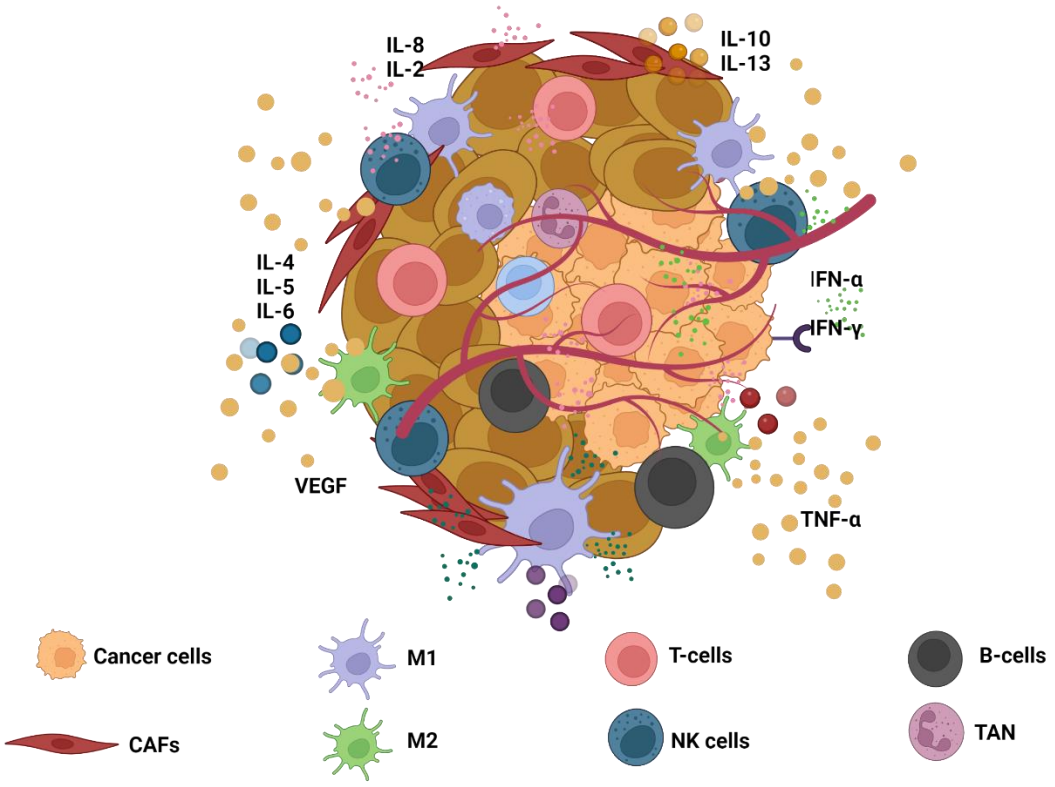


Figure 1: Cellular and acellular components of breast cancer TME

Heterogenous tumor microenvironment consists of cancer cells, macrophages (M1&M2), cancer associated fibroblasts (CAFs), B-cells and T-cells, natural killer cells (NK) and tumor associated neutrophils (TAN). Intercellular interactions amongst the residing cell population promotes oncogenic state and facilitates tumor growth and development. Cancer cells and immune cells secrete cytokines and growth factors that create inflammatory environment which adversely affect the tumor cell proliferation and metastasis

1.3.1 Diversity of cells in TME and their role in Breast Cancer

Tumor microenvironment of breast cancer is highly complex and shows heterogenous population of cells recruited from different origin [23]. The distinct population of cells residing in TME and their cross talk determine the breast tumor progression starting from initiation, adaption, survival, and metastasis [24]. The interactions between breast cancer cells and infiltrating immune cells plays critical role in promoting pro-tumorigenic activities [25]. Host immune system is often suppressed by tumor cells and evade host immune response that also effect targeted immunotherapies [26, 27]. Studies show that tumor-infiltrating B cells, CD4+

and CD8+ tumor infiltrating lymphocytes in TME are highly correlated with primary breast cancer as compared to normal tissue [28]. A study conducted in 3771 patients with TNBC, luminal–HER2-negative and HER2-positive breast cancer highlighted that TILs concentration is correlated with survival and prognosis in breast cancer [25]. Increased TILs concentration showed survival advantages in HER2-positive breast cancer and TNBC. Tumor associated neutrophils (TAN) are shown to reduce CD8 proliferation in TME of breast cancer mouse models [29].

Studies have validated that the immune cells infiltrate vary in cancers of different origin and subtypes including breast cancer. Studies analysing 53 mastectomy specimens showed increased number of T lymphocytes, NK cells, B lymphocytes, CD3+, CD20+ being prominent in ductal carcinoma in situ, benign proliferative breast disease and infiltrating ductal carcinoma compared to normal [30]. Further, a dysregulated secretion of inflammatory cytokines like TNF-α, IL-6 and IL-1β from adipose tissue along with recruitment of macrophages leads to increased risk to breast cancer in individuals with weight gain and obesity [31].

A recent study examined that adipose tissue secretes inflammatory cytokines and adipokines such as TNF-α, IL-6, IL-1, IL-8 and attract macrophages which has proinflammatory and proliferative properties [32, 33]. Cancer associated fibroblast (CAFs) is another cell type which also contributes significantly to inflammation in TME. Studies have revealed that CAFs in breast cancer promote the malignancy by secreting factors like chemokines that promote breast cancer invasion in TNBC [34, 35]. CAFs were shown to secrete IL-6 which activates STAT3 signaling pathways and induce breast cancer cell growth and radio resistance in TME. As a result of such heterogeneity, breast tumors are complex and needs further study. Understanding the dynamics of intercellular communications between tumor cells and immune cells is important for restoring the effective immune response against the developing tumor and achieve long lasting responses.

Thus, in depth investigation of tumor microenvironment associated factors are important to understand the tumor cell evolution, progression and migration.

1.3.2 Cytokines and chemokines in TME of breast cancer

Chemokines and cytokines secreted by tumor cells or immune cells recruited in TME can alter different cellular processes of tumor cells driving tumor cell proliferation and progression. Cytokines have been reported in major processes related to cancer pathogenesis and disease progression. A study in syngeneic tumor models showed that different cytokine combinations

injected in tumor microenvironment can induce strong antitumor response. Murine tumor models including colon carcinoma cells, Lewis lung carcinoma cells, breast cancer cells were injected with chitosan/IL12 + GMCSF+IL2 and showed significant regression of tumor lesions [36]. Cytokines and growth factors like TNF-α, IFN-γ, IL-8, IL-6 appears to be playing a major role in survival, EMT transition, metastasis, and invasion of the breast cancers of different subtypes. Growth factors and cytokines like IL-1, IL-6, IL-4, IL-8, TNF-α, VEGF and TGF-β in modulation of breast cancer growth and metastasis and provides basis for development of adjuvant drugs for the disease control and spread to distant organs in patients. Recent studies characterized IL-6 as a serum marker with recurrence in HER2 negative breast cancer patients [37]. Polymorphisms of IL-6 promoter is being associated with relapse off HER2+ breast cancer and elevated levels of IL-6 in having role in resistance to trastuzumab in pre-clinical models [38].

TNBC cells are well characterized for secretion of inflammatory cytokines and promote angiogenesis and metastasis. Analysis of databases have identified 32 inflammatory genes increased in TNBCs and 10 were identified to be critical for anchorage-independent growth [39]. Pro-inflammatory cytokines like IL-6 and TNF-α are elevated in metastatic breast cancer patients [40]. Breast cancer cells when supplemented with IL-6 and TNF-α shows significant increase in adhesion on E-selectin coated surfaces [41]. Such studies highlight the need to target cytokines along with adhesion molecules to reduce the metastatic loads in TNBC.

Targeting IL-8 with humanized anti-IL-8 tocilizumab, showed impaired neovascularization in breast orthotopic tumor xenografts that can be targeted against angiogenesis [42]. Interestingly, VEGF-C play a vital role to promote metastasis and tumor growth via promoting crosstalk between EMT-Epithelial Breast Cancer cell [43]. Secretion of VEGF-C by the breast cancer cells that undergo EMT can promote paracrine mediated increase in proliferation, invasion and migration via GLI-signaling of epithelial breast cancer cells. Further, bioinformatic analysis have revealed a distinct expression pattern of chemokines in breast cancer that play important role in trafficking of immune cells and stromal development, induce inflammation, and have prognostic significance [44]. The above cited studies indicate that cytokines and chemokines have potential biomarker features, and it is essential to understand their effects on breast cancer biology.

Thus, persistent presence of inflammation in TME leads to breast cancer development and emphasise the need to understand every component of inflammatory system to understand the complex milieu of solid tumors and its implication in tumor progression.

1.4 TNF-α: Signaling and implication in breast cancer

1.4.1 TNF-α: Critical cytokine for tumor progression

Tumor necrosis factor alpha (TNF-α), named after its antitumor responses, has been studied and been shown to act as a multifunctional cytokine which plays indispensable roles in cell proliferation, survival, apoptosis, and immunity. It was isolated almost three decades ago and from then it has been associated with multiple diseases including multiple cancers. In addition, invasion of pathogens leads to release of TNF-α by the macrophages and T-cells in the body [45]. It is produces either as a membrane bound (mTNF) or proteolytically cleaved soluble form (sTNF) [46]. TNF-α activates signalling pathways in different cell types leading to enhanced level of secondary cytokines and chemokines and causes leukocyte attraction at the site of infection [47]. TNF-α may also lead to activate acquired immunity in prolonged infection and cause antigen presentation in immune response [48]. TNF-α treated cells showed rapid MHC class I reconstitution and increased average half-life at the surface. TNF-α exerts its effects upon binding the TNF-R1 and TNF-R2 receptors. TNF-α is a pleotropic cytokine with dual functions leading to survival or death depending on the pathophysiological conditions [49]. TNFR1 is expressed mostly on all the cell types but expression of TNFR2 is highly restricted to specific cell types. TNF-α binding to its receptors triggers various signalling pathway like IKK, JNK, MAPK, NF-κB and AP-1 [50]. These pathways play a major role in regulating cellular homeostasis and managing various pathological conditions. Studies have showed its involvement in neuronal remyelination, cardiac remodelling, cartilage regeneration and protecting macrophages to the intense effects of inflammatory tolerization [51]. TNF-α promotes inflammation by expression of inflammatory genes and inducing cell death, initiating immune responses and disease development [50]. High TNF-α levels were related to poor prognosis in HCC patients who were given adjuvant sorafenib after surgery. Inhibiting TNF-α along with ulinastatin significantly increased the anti-tumor effects on HCC cells with high TNF-α expression [52].

Recent studies have shown that anti-TNF antibodies reduces risk and act as tumor suppressor in TME of colorectal cancers. Administration of anti-TNF mAb led to decrease in colorectal cancer in orthotopic transplant mouse model [53]. Additionally, decreased angiogenesis, tumor growth, increase in apoptosis, and increased tumor immunity were identified using gene ontology analysis and immunohistochemistry. TNF-α is associated with different stages of

Pancreatic ductal adenocarcinoma (PDAC) and serves as a marker for initiation and poor survival in patients [54]. The anti-TNF treatment led to decreased cell viability in PDAC tumor cells and inhibition in inflammatory TME. Combination of anti-TNF therapy and chemotherapy caused PDAC tumor cells to overcome chemoresistance and further increase survival in mouse model.

Anti-TNF treatment in mice model reduces tumor cell viability and tumor promoting inflammatory microenvironment *in vivo*. TNF-α transforms adipocytes at the tumor site in formation of cancer associated adipocytes to promote invasion, migration and proliferation of cancer cells [55]. Desmoplasia is a phenomenon that takes place in carcinoma where cancer cells are responsible in expansion of cellular and molecular stroma causing strong fibrotic response. Adipose cells occur as main fatty tissue in the breast that undergo such changes in breast cancer. Expression of C/EBP alpha and PPAR gamma generated by breast cancer cells along with exogenous TGF-beta 1 and TNF-α were shown to induce conversion of adipose cells to fibroblastic phenotype [56].

These evidences suggest that TNF-α is pleiotropic cytokine and its enhanced level can effect different steps of cancer development in variety of cancers including breast cancer however mechanisms which evolves in metabolic adaptations in tumor cell is not well understood.

1.4.2 TNF-α induced NF-κB signaling

Recent evidence using computational analysis and single cell transcriptomics have identified a variety of intercellular signaling networks that function in the TME [57-59]. Paracrine signaling through release of cytokines, growth factors, chemokines are inevitable for intercellular communications and can directly or indirectly influence the remodelling of TME [60, 61].

TNF-α induced NF-κB pathway is one such pathway that is involved in activation of genes related to inflammation under normal as well as various physiological stress conditions and infections [62, 63]. Inflammatory diseases like Crohn's disease and rheumatoid arthritis are often triggered by dysfunctional NF-κB signalling lead by TNF-α under chronic inflammatory conditions. TNF-α induced inflammation via NF-κB have been associated with heart failure and cardiomyopathies [64, 65]. sTNF and receptor levels are found to be increased in patients serum with hyper cardiomyopathies in larger clinical studies [66]. It is also suggested that NF-κB may play a crucial role in atherosclerosis and diabetes due to expression of genes encoding cytokines and chemokines [67, 68].

NF-κB contains includes a family of proteins which act as transcription factors in a dimeric form and are major inducers of gene transcriptions for cell survival and cell death [69]. NF-κB/Rel family consists of five members namely NFKB1, NFKB2, c-Rel, RELA and RELB. The Rel proteins contains a RHD domain which is responsible for protein dimerization, translocation to the nucleus, and binding to DNA [70].

TNF-α induced canonical NF-κB pathway is activated by binding of TNF-α to its receptor TNFR1. This interaction recruits TRADD, TRAF2 and RIP1 complex and TRAF2 further forms IKKα/IKKβ/NEMO complex. IKKβ gets activated which phosphorylates IκBα which undergoes proteasomal degradation. This results in a rapid nuclear translocation of NF-κB members majorly p50/RelA and p50/c-Rel dimers. RELA has been highlighted as a critical gene in many diseases such as Crohn's disease, lung cancer, acute pancreatitis, rheumatoid arthritis [71-74].

1.4.3 TNF-α and NF-κB; Implication in breast cancer:

The presence of inflammatory cytokines in TME secreted either by tumor cells or the infiltrating immune cells have been associated with cancer severity [75]. TNF-α is one such cytokine that participates in survival, proliferation, cell death and metastasis of cancer cells thus serving as a major target for therapeutics. TNF-α induced NF-κB signalling is usually high in Inflammatory breast cancers and TNBC.

A crosstalk between p53 mutations and NF-κB is observed in TNBCs which leads to larger breast tumor size and enhanced metastasis to vital organs like brain [76]. Mutations in p53 is an important factor in development of breast cancer and p53 is master regulators of EMT such as Snail, Slug, Twist1 in breast cancer.

IKKe expression increases NF-κB activity in both breast cancer cell lines and tumors derived from patients. Increased expression of IKKe leads to increase in clonogenic ability and capacity to form mammosphere [77]. Further, TNBC tumors were found to be enriched in IKKe expression and thus highlighting its therapeutic potentials in breast cancer.

An activated NF-κB signalling creates a tumor promoting environment which is the result of increased cytokine release and DNA damage and increased immune invasion.

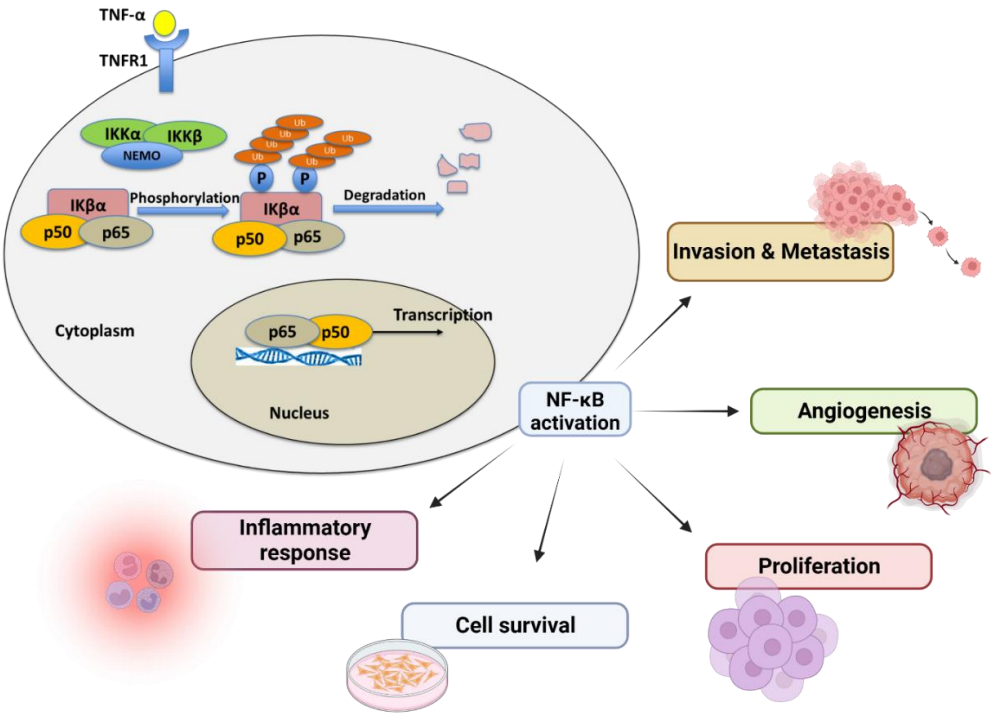


Figure 2: Regulation of TNF-α-induced NF-κB pathway and its role in cancer

NF-κB pathway activation by TNF-α via TNFR1 receptor converges on the IKK complex through adapter complex TRADD-TRAF2. IκBα degradation is achieved by activated IKK by promoting its phosphorylation which leads to its ubiquitination mediated proteasomal degradation through UPS. This results into nuclear translocation of p65 and p50 dimer and is responsible for transcription of NF-κB responsive genes. NF-κB pathway activation is important for regulating inflammation, cancer cell proliferation and survival, angiogenesis, invasion and metastasis of cancer

Breast cancer development is associated with inflammation by an enhanced cytokine release in the TME and activation of inflammatory pathways like NF-κB [78]. It was shown that TNF-α regulated NF-κB is essential for upregulation of Twist1 and induce EMT transition.

Other studies have shown decrease in the growth factors or cytokines like IL-6 and IL-8 in a dysfunctional NF-κB signalling and affect inflammation, cell survival, proliferation, and apoptosis [79]. Inhibition of NF-κB with Dehydroxymethylepoxyquinomicin (DHMEQ) showed decreased invasion and migration in MDA-MB-231[80]. Another study further validated that DHMEQ inhibited invasion of breast cancer cells along with inhibition of IL-6 and matrix metalloproteinase (MMP) [81]. NF-κB is important in expression of IL-6 in breast cell lines and IL-6 stimulates HIF-1α and STAT3 [82]. Knockdown of NF-κB in breast cancer stem cells have shown reduced IL-8 secretion in mammosphere. NF-κB/IL-8 signaling was

blocked using sulconazole, an antifungal medicine which further reduced breast cancer stem cell formation [83].

NF-κB also regulate cell cycle related proteins like cyclin D1 and CDK2 which are responsible for cell cycle progression and proliferation of breast cancer cells [84]. The role of NF-κB in breast cancer is not limited to the initiation and progression but it is also involved in driving the aggressiveness of the breast cancer cells and promoting malignancies. NF-κB dependent genes like MMPs, IL8, CXCR4 are responsible for cellular invasion in breast cancer [85]. EMT is an essential event in breast cancer progression. Various EMT markers like Snail and Twist1 have been reported to be induced by NF-κB activation. NF-κB activation is positively correlated with upregulation of metastasis promoting factors like N-cadherins and Vimentin and antiapoptotic genes like Bcl-2 [80]. Breast cancer stem cells (BCSCs) often overexpress NF-κB components and higher NF-κB activity. Overexpression of NIK in breast cancer stem cells is known to enhances tumorigenicity[86]. Inhibition of NIK through shRNA reduces CSC markers and impairs clonogenicity and tumorigenesis. Further, NF-κB signalling and its cross talk with other transcription factors and signalling pathways in breast cancer results in the disease progression. Studies have shown that pathways like STAT3 regulated expression of TNFRSF1A, that encodes for transmembrane receptor for TNF-α which is responsible for NF-κB activity in breast cancer [87]. Patient data showed that TNFRSF1A transcript levels correlated with STAT3 activation.

Thus, it is important to understand the mechanism by which TNF-α induced NF-κB promotes breast cancer and the overlap of this pathway with other inflammatory axis that can synergistically regulate breast cancer.

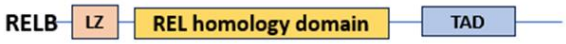
NF-κB subunit	Implication in breast cancer
	Breast cancer tissues have higher levels of p65 [88], Higher RELA expression shows poor prognosis [89], Promotes breast cancer growth and invasion [90,91]
	Suppresses senescence-like growth arrest [92], Breast cancer stem cell formation via IL6 [93], RelB deficiency impairs tumor growth in nude mice and inhibits lung metastasis [94]
	Induce mammary tumors [95] Higher expression in breast tumors, proliferation, survival, and more invasive [97],
	Contributes to metastasis in IBC, Higher expression in malignant breast cancer tissue
	Enhances tumorigenic potential of breast cancer cells, Development of breast cancer [96]

Table 1: NF-κB subunits and their role in breast cancer

NF-κB family consists of five subunits RELA, RELB, cREL, p50 and p51 that forms various homo or heterodimers. All five subunits consist of a DNA binding domain and dimerization domain known as REL homology domain (RHD). RELA, RELB and cREL also consists of transactivation domain (TAD). NF-κB pathway is mainly regulated by its subunits and determine the growth, proliferation, survival, invasion and metastasis of breast cancer [88-99]

1.5 TNF-α and Autotaxin (ATX): Role in breast cancer

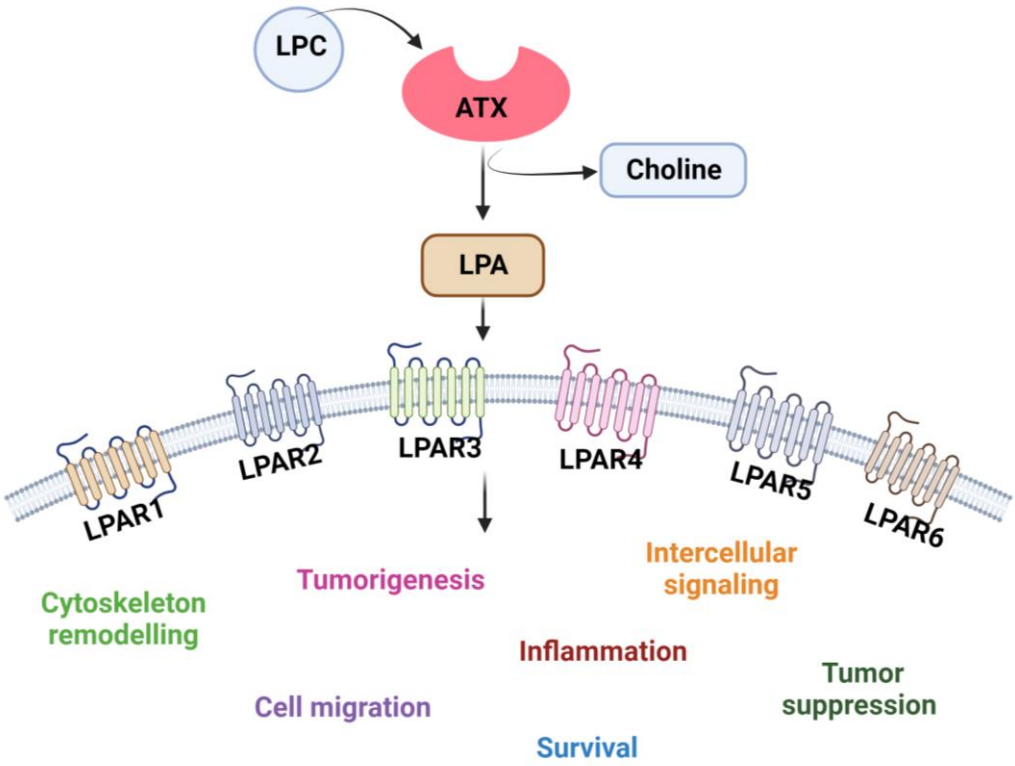


Figure 3: ATX signaling in cancer

ATX enzyme hydrolyses extracellular LPC and converts into LPA and choline. The LPA generated signals through six G protein coupled receptors LPAR1, LPAR2, LPAR3, LPAR4, LPAR5 and LPAR6 to stimulate various cellular processes including cell survival, migration, intercellular signaling, cytoskeleton remodelling, tumorigenesis and inflammation in cancer

1.5.1 ATX in cancer

ATX-LPA axis was first identified almost 30 years ago as a physiological signalling response in wound healing that exerted its effect by platelet aggregation, enhanced angiogenesis, regulating blood pressure, immune regulation, and establishing homeostasis after inflammation at the site of injury. Interestingly, its role in malignancies was also discovered and had been implicated in regulating invasion, cell proliferation and migration. ATX-LPA signaling have profound effects on resistance to chemotherapy and radiotherapy by stimulating prosurvival pathways and altered apoptosis [100]. Studies have shown that LPA and their receptors play vital role in breast, ovarian, prostate, head and neck cancers [101]. Melanoma cells that secrete ATX reduce the infiltration of lymphocytes and circulating CD8+ cells and increase tumor growth [102]. ATX is expressed in tumor sphere-forming cancer stem cells and primary

ovarian CSC from patients [103]. LPA significantly elevated in CSCs and increase in sphere forming, tumorigenic potential and resistance to anticancer drugs. Significantly elevated LPA levels can transform resident pancreatic cells into activated CAFs [104]. ATX-LPA activity was shown to promote PDAC cell proliferation and migration and autotaxin inhibition led to suppressed tumor growth.

Inflammatory mediators like IL6, IL8, TNF-α, CCL2 may act synergistically with LPA to induce malignant transformation in thyroid tumor. Autotaxin levels were four to ten folds higher in metastatic deposits compared to benign or normal thyroid tissue [105]. Patients with hepatocellular carcinoma show increased ATX, LPA and LPA receptor transcripts along with elevated serum ATX levels. ATX-LPA axis is critical role in liver tumorigenesis [106]. ATX-LPA axis is identified to induced EMT transition and increase angiogenesis to accelerate HCC metastasis. TNF-NF-κB pathway is closely associated with ATX-LPA axis in regulation of liver tumorigenesis [107]. The study evaluated 38 human HCC and 10 control subjects and showed that overexpression of ATX was associated with inflammation and liver cirrhosis. TNF/ NF-κB axis promoted the expression of ATX in Huh7 and Hep3B cells which increased lysophospholipase-D activity. In HCC, there is remarkable increase in LPAR1 expression and is responsible for hepatocarcinogenesis by recruiting and activating fibroblasts in TME [108]. Local LPA production have shown positive correlation with tumor angiogenesis in early colorectal cancer [109]. Local LPA production correlated with depressive lesion formation and angiogenesis in colorectal cancers.

Taken together, these studies confirmed the importance of ATX and LPA in initiation and development of various cancers and its implication in breast cancer progression and metastasis is emerging and needs to be further investigated.

1.5.2 ATX: Role in breast cancer

Tumor cells have shown to exploit this mechanism to overcome drug treatments and enhance cancer cell proliferation and metastasis. ATX signalling enhances production of cytokines in particularly breast cancers and activate pathways responsible for cell survival and proliferation [110]. Inflammatory cytokines like TNF-α or interleukin 1β produced by breast cancer cells induce Autotaxin production in TME [111-113]. ATX is also one of the top 40 genes upregulated in metastatic breast tumors. Studies have demonstrated that autotaxin mediated LPA production also leads to vicious cytokine production including TNF-α [114]. Inhibiting

LPA production reduced stimulatory effects of LPA in TNBC and Luminal A breast cancer cells.

LPA treated MDA-MB-468 breast cancer cell line showed increase in cytokine secretions like IL-8, IL-6 and TNF-α. LPA induced transmigratory effects on MDA-MB-231 which was decreased on inhibiting the LPA receptor [115]. The ATX-LPA axis showed differential effects on subtypes of breast cancers. TNBC, stimulated by LPA showed increased tumor promoting functions compared to other breast cancer cell lines which can be further exploited for subtype targeted therapies.

ATX and LPA were identified as novel markers for breast cancer [116]. ATX activity can be easily targeted by inhibitors and thus has potential as efficient therapeutics in breast cancer. ATX and LPA inhibitors like GLPG1690, BBT-877, and BLD-0409 are under clinical trials.GLPG1690, a competitive inhibitor, in phase III trials showed decreased tumor growth and metastasis to lungs and liver in orthotopic mouse model of breast cancer.GLPG1690 in combination with irradiation decreased the percentage of Ki-67 positive cells, IL9, macrophage colony-stimulating factor, IL12p40 and IFNγ in adipose tissue near to the tumor and further showed increase in efficacy of chemotherapy and radiotherapy in a syngeneic orthotopic mouse model [117]. Further, another ATX inhibitor named ONO-8430506, showed promising effects in reducing breast tumor growth and decreasing breast cancer metastasis to liver and lungs [113]. ONO-8430506 decreased the autotaxin activity by >60% in syngeneic orthotopic mouse model of breast cancer.

ATX-LPA signaling proteins show difference in their expression pattern at different metastatic sites of metastatic breast cancer. LPA1 expression was higher in metastatic tumors breast cancer to liver and lung metastasis [118]. Expression of ATX from adjacent adipose tissue to breast cancer produce significant amount of ATX which is stimulated by tumor derived inflammatory cytokines. This further enhances the inflammatory responses and is responsible for tumor growth and metastasis [119]. LPA2 and LPA3, proteins in ATX-LPA signaling are shown to be expressed in breast cancer with adipose stroma and is associated with immune cell infiltration [120].

***This evidence suggests that better understanding to ATX signaling and its association with inflammatory factors like TNF-α act synergistically in breast cancer determine the tumor progression and invasion*.**

1.6 TNF-α induced metabolic adaptation: Implication in breast cancer

TME of solid tumors have complex milieu of cytokines and can alter the expression of several genes and specifically affect the metabolism to rewire for anabolism for highly proliferating tumor cells. Cytokine mediated cellular changes have been targets for many diseases. Cytokine mediated reprogramming of tumor cells metabolism is considered an essential process underlying various biological aspects of cancer. Immunometabolism lies at the intersection of immunity and metabolism and have gained importance in specifically evading immune response and further tumor progression. Several mechanisms of inflammation and its effects on metabolism have been explored. Still the understanding of inflammation induced metabolic reprogramming in cancer cell subtypes remains elusive and needs further investigation.

1.6.1 Reprogramming of metabolism: Implication in cancer progression

To meet the increasing metabolic demands of highly proliferating tumor cells, various metabolic pathways are rewired for bioenergetics as well as anapleotric demand of highly proliferative cells. Metabolic reprogramming, tumor-promoting inflammation and immune evasion are considered major hallmarks of cancer. The metabolic reprogramming is a major contributor for tumor initiation and metastasis. Studies have shown how cancer cells often alter pathways like glycolysis, TCA and oxidative phosphorylation for cell survival. Initially, the basic understanding of Warburg's effect was an increased glycolysis and defects in oxidative phosphorylation of cancer cells has been a topic of debate since decades [121]. In the multicellular tumor microenvironment, Warburg effects may be a source of advantage for the growing cancer cells in comparison to residing non-malignant cells [122]. Studies have identified that solid tumors are highly acidic compared to normal tissue and such acidosis correlates with ability of breast cancer cells to metastasis to lungs and become a potential clinical tool for detecting metastasis [123].

Studies have identified glycolysis related genes like PGK1, LGHA, GLUT1, GAPDH, PSAT1, PGLS, and LDHC to be differentially expressed in breast cancer, renal cell carcinomas, hepatocellular carcinomas and possessed prognostic importance [124, 125]. Glycolytic genes are also upregulated via major redox transcription factor NRF2 via metabolic reprogramming in human breast cancer cells and patients [126]. Downregulation of major glycolytic enzyme like Hexokinase 2 (HK2) have shown improvement in radiosensitivity of breast cancer suggesting their involvement in interfering treatments [127]. Utilization of glucose molecule by aerobic glycolysis is a slower way of generating ATP in comparison to mitochondrial OXPHOS [128]. However, rate of glucose metabolism by aerobic glycolysis is 10-100 times faster in comparison to mitochondrial pathway in most of the cancer cells [129]. In colorectal

cancer, glycolysis tends to generate more lactic acid in TME which stimulates angiogenesis, rapid growth, tumor progression and metastasis [130]. Human and animal studies have shown that the metabolic and nutritional status of solid tumors is very dynamic. Further studies showed that glycolysis is majorly responsible for metastasis under hypoxic environment in prostate cancers

Glucose is a limiting factor in the TME of solid tumors and hence cancer cells rely on other energy sources like lactate that give survival advantages under glucose deprived conditions [131]. Estrogen related receptor alpha (ERRα) regulated this lactate utilization by cancer cells and thus induce resistance to PI3K/mTOR inhibitors. The acidification caused by lactate secretion in tumor microenvironment is a benefit to proliferating cancer cells by promoting proliferation, angiogenesis, migration, chemotherapy resistance and reducing pH. In clinical studies, lactate and pyruvate metabolism is linked the matrix dissociation and increased invasion with increased blood lactate and LDH levels were found in patients with metastatic compared to non-metastatic colorectal cancers [132]. Further the study concluded that elevated LDH and serum lactate may act as prognostic markers in patients with metastatic colorectal cancers. Strategies of inhibiting lactate dehydrogenase A (LDHA) and blockade of pyruvate influx in mitochondria have shown reduced invasion and migration, in renal cell carcinoma and prostate cancer [133, 134]. Major mitochondrial enzymes defects like SOD1, SOD2, LDH, FH have been associated with development of paraganglioma, renal cell carcinoma, hepatocellular carcinoma and lymphoma [4, 135-137]. Recent contrary studies using in vivo isotope tracing methods have identified the need of ETC in glutamine and glucose utilization by tumor for multiple nutrition [138].

The de novo synthesis of building blocks like nucleotides, lipids and proteins for the rapidly proliferating cells is a major reason for altered metabolic pathways in cancer cells. Many oncogenes and tumor suppressors are responsible for activating de novo synthesis and supporting cancer cell growth and proliferation. The protooncogene c-MYC, which is upregulated in 50% of human cancers, is known to regulate genes that are associated with nucleotide synthesis and metabolism [139]. Studies show oncogenic K-RAS, which is upstream of c-Myc, maintains high nucleotide levels in cancers like pancreatic ductal adenocarcinomas [140]. It has been demonstrated that nucleotide de novo synthesis can increase tumor cell stemness and metastasis of breast cancer cells to lungs which was rescued by blocking the synthesis process [141]. The mammalian target of rapamycin (mTOR), which regulates many cellular processes like metabolism, immune functions, is also dysregulated in

colon, neck, head, lung, breast, bone cancers and is targeted for its role in biomass production and promoting nucleotide synthesis [142, 143].

Tumor cells can alter the metabolic profile of adjacent cells lying in their vicinity to benefit from the outcomes of better nutrient availability and resistance to immune surveillance. Cancer associated fibroblast (CAFs), which form the major component of stromal cells are result of metabolic alterations caused by tumor cells on the adjacent fibroblasts [144]. Tumor cell derived TGF-β activate fibroblasts and enhance tumorigenesis which cell type. Interestingly, such metabolic alterations also transform adjacent adipocytes in the breast cancer to Cancer associated adipocytes favouring invasion, proliferation, and metastasis [145, 146]. Cancer associated adipocytes secretes leukemia inhibitory factor (LIF) and induces migration in breast cancer cells through STAT3 signaling pathway. Thus, altered metabolism is a promising targeted therapy and further investigations may help in discovering new therapeutic strategies based on tumor stage and metastatic sites.

Therefore, the complexity underlying the metabolic traits in cancers and their reprogramming for proliferative capacity of tumor cells need detailed understanding. Thus, it is important to investigate the potential metabolic targets in different subtypes of breast cancer and their implication in tumorigenic potential.

1.6.2 Metabolism in breast cancer:

Targeted therapies are options for 70-80% of breast cancer cases with positive receptor status [147, 148]. Major challenge lies for the remaining 15-20 % which are TNBCs where conventional chemotherapy, radiation therapy or surgery are the only treatment options available for these cases [149, 150]. Acquired resistance to majority of the therapies is the ultimate challenge faced in the current scenario of breast cancer therapeutics [151, 152]. One way identified for such resistance is the rewiring of the breast cancer cell metabolism that interfere in the current treatments available and is the important issue that needs to be addressed with deeper understanding [153, 154]. Metabolic approaches have increased the understanding of tumor microenvironment in detail and design strategies for developing personalized therapies. Altered metabolic pathways in breast cancer effects the metabolites levels leading to difference in the nutrient and energy availability to subtypes of breast cancer which defines their survival and metastatic potential. Metabolites levels can vary in ER+ and ER- tumor tissues [155]. Glutamate to glutamine ratio were associated with ER status and tumor grade. ER+ tumor tissue and ER- tumor tissues showed 56% and 88% glutamate enrichment as compared to normal tissues. Recent study identified exosomal metabolites as markers for

neoadjuvant chemotherapy outcomes in breast cancer patients. Plasma exosomes showed increased levels of succinate and lactate in patients with residual disease compared to pathological complete response [156].

The difference lies in the metabolic variations that persist in breast cancer subtypes and in the primary and metastatic tumor cells [157]. A large cohort of 330 TNBC samples and 149 normal breast tissue revealed that metabolomics can serve as a major target for precision treatment of TNBCs [158]. Single-cell RNA sequencing and spatial transcriptomics have identified dynamic changes in the metabolism of primary and metastatic tumors in patients with breast cancer [159]. Integrated analysis of genomics, metabolomics and single cell transcriptomics revealed that breast tumors have different metabolic features. Significant increase in glycolysis activity and decrease in survival rates were associated with cluster 1 whereas cluster 2 showed increase in FAO and glutaminolysis [40].

The study categorized three subgroups based on metabolites by the increase in fatty acids and ceramides; metabolites of oxidation reaction; and the ones having the low-level dysregulated metabolism. Analysis of 2752 metabolic genes reported that identified metabolic subtypes of groups which were sensitive to immunotherapy and targeted drug sunitinib using The Cancer Genome Atlas-Breast Cancer and 2010 (YAU) [160]. This study designed a theoretical method to identify immunotherapy and drug sunitinib sensitive groups.

Metabolomic profiling on MDA-MB-231 and its metastatic subclones identified differential metabolic profile between parent and subclone population. The study identified one carbon (1C) and mitochondrial serine unit pathway to be increased in metastatic subclones which are essential for their faster proliferation through increased de novo purine biosynthesis in mice model. A positive correlation is observed in some metabolomic studies between aggressiveness of breast cancer and levels of metabolites related to energy generating pathways like glycolysis, TCA and OXPHOS. Recent evidence suggests that TNBC have different metabolic adaptions depending on the site of metastasis that is beneficial for coping with the local environment [161]. It was identified that genes related to EMT, metastasis and metabolic pathways were significantly increased in metastatic tumors in liver, lung and brain compared to primary breast tumor. This highlights the need to design metabolic inhibitors that are specially targeting different metastatic sites and are not solely a general approach to metastasis.

1.6.3 Mitochondria: Center of metabolic rewiring in cancer

Mitochondria is a key organelle essential involved in energy production, metabolism and cellular signalling for cancer cell survival. In depth understanding of mitochondrial functions

in recent times have highlighted their importance not only in ATP synthesis but also in generating macromolecule required for rapidly growing cancer cells.

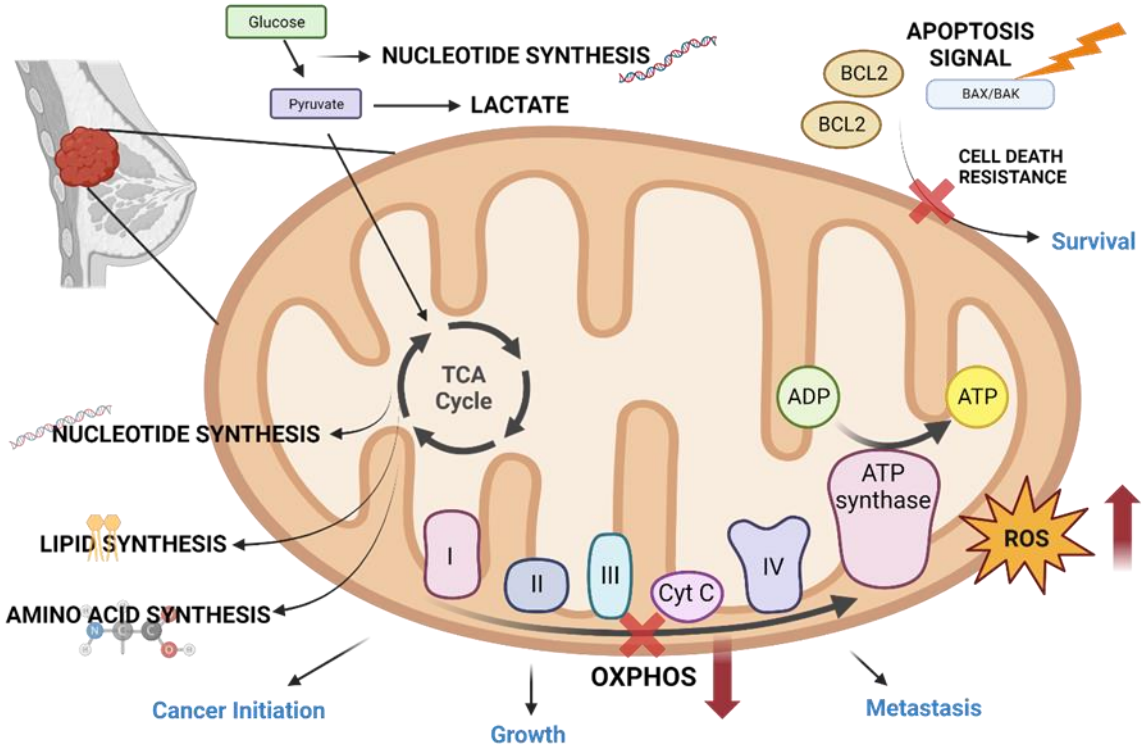


Figure 4: Mitochondria and its role in breast cancer

Breast cancer cells undergo distinct metabolic adaptations that involve majorly reprogramming of mitochondrial metabolic pathways. Defects in identifying apoptotic signals and promoting survival and cell death resistance is major event in breast cancer. Reprogramming of TCA and OXPHOS can generate macromolecules essential for generating building blocks for breast cancer cell growth and proliferation. Defective mitochondrial OXPHOS leads to generation of ROS which supports cancer initiation and metastasis

1.6.4 Mitochondrial OXPHOS: Implication in breast cancer

Highly proliferating cancer cells rewire their mitochondrial metabolism and fuel their energy requirements for cell division, migration and invasion. TME has a strong influence on the mitochondrial functions which plays important role in deciding the tumor energetic status. NADH and FADH2 generated during TCA cycle are reducing equivalents and can donate electrons to ETC complexes I to complex IV. Complex V generates ATP with O_2 acting as a final electron acceptor [162]. OXPHOS pathway can drive tumor cell proliferation by providing ATP as well as carbon fuel generated via biosynthetic intermediates.

Studies targeting inhibition of ETC complex I (ROT and MET) and complex III (AMA or MYXO) have shown restricted cell growth and clonogenicity in liver cell lines, mouse liver organoids and murine xenografts and proved its therapeutic potentials in cancer [163]. Systemic analysis of metabolic genes in 20 different cancers have linked downregulation of mitochondrial genes with poor clinical outcome and further correlated it with EMT markers [164]. Studies related to overexpression of oncogenic transcription factor FoxQ1 in breast cancer cells revealed an increase in mitochondrial complex I proteins NDUFS1 and NDUFV1 [165]. This increase in mitochondrial proteins were correlated with increase in mitochondrial activity as well as assembly of complex I. Further, complex I deficiency was linked to enhanced aggressiveness in human breast cancer cells. Interestingly, the study identified that loss of NDUFS9 promoted MDA-MB-231 cells migration due to elevated mtROS and disturbed NAD+/NADH balance. Comparative studies between parental breast cancer cell line and highly metastatic cancer cell line showed that downregulation of NDUFB9 was linked to increased breast cancer metastasis [166]. Loss of NDUFB9 promoted migration and proliferation in MDA-MB-231 along with elevated mtROS and reduced mtDNA. Further, studies also revealed that epithelial to mesenchymal transition was promoted due to reduced expression of SDHC in breast cancer [167]. Lower expression of SDHC was also shown to have prognostic impacts in patients with basal subtype compared to non-basal tumors.

These evidences suggests that mitochondrial OXPHOS capacity is differentially regulated in highly proliferating and metastatic breast cancer cells subtypes hence this property of breast cancer cells needs to be further explored.

1.6.5 Mitochondrial ROS: Driving breast tumorigenesis:

Reactive oxygen species (ROS) are highly reactive molecules responsible for cellular functions under moderate levels [168]. Mitochondria are major source of ROS and are contributors to pathological conditions including cancer. Mitochondrial enzymes such as pyruvate dehydrogenase and a-ketoglutarate-dehydrogenase are also major inducers of ROS and play important roles in tumorigenesis [169].

Recent study with 1,903 breast cancer patients from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) showed that high ROS was associated with tumor heterogeneity, mutation rates, homologous recombination defects and neoantigens and with aggressiveness [170]. The upregulation of NRF2, an antioxidant transcription factor can control ROS and is shown critical for the recurrence of dormant breast cancers [171]. mtROS can influence tumor initiation to metastasis by altering nuclear and mitochondrial DNA, apoptosis

and also metabolic reprogramming[170]. As mtDNA encodes for proteins which are integral part of complexes of OXPHOS, mutation in mtDNA leads to defects in electron transport resulting in ROS. Studies have shown that accumulation of unfolded mitochondrial proteins leads to excessive ROS production which facilitates carcinoma progression in breast cancer [172].

Mitochondria and its defects as identified as critical aspects of tumor growth that support metabolic needs by undergoing drastic changes. Such reprograming are triggered by elevated ROS that is majorly generated in the inner mitochondrial membrane. Mitochondrial ROS within a certain range is known to act in a Mito protective manner which is termed as mitohormesis [173]. The mitohormesis mediated mtUPR is shown to be important in breast cancer cell invasiveness and metastasis. Studies have identified that UPRmt-HIGH patients have significantly worse clinical outcomes [174]. Recent studies targeting HSP60 and ClpP, two major components of UPRmt and their interaction have demonstrated its importance in prostate cancers. Inhibiting UPRmt have disrupted this interaction and further showed decreased PCs growth and progression [175]. Further, cisplatin, one of the toxic chemotherapy drugs used for solid tumors, have shown to increase the levels of SIRT3 mediated UPRmt in MCF-7 and MDA-MB-231 cells [176]. SIRT3 was shown to protect breast cancer cells from cisplatin. Silencing SIRT3 showed reduced proliferation and invasion and increased the sensitivity of breast cancer to chemotherapy. These targeting mitochondrial biology in cancer might be beneficial in providing precise medicine for cancer.

1.6.6 Mitochondria and metabolites in cancer:

Major contribution of the metabolic reprogramming is to generate NADH/NAD ratio via alteration of mitochondrial function and provide metabolites for production of lipids and amino acids for rapidly growing cancer cells [177]. Accumulation of oncogenic metabolites due to defects in enzymes are major contributors to the initiation and progression of cancer [178]. Oncometabolite like citrate, which is critical component of TCA cycle, has been associated with increased cell migration, invasion and metastasis in triple negative breast cancer cells and mice xenografts. Interestingly, recent study showed that anti-inflammatory TAMs secrete TGF-β when in contact with breast cancer cells. Further this interaction and secretion leads to decrease in STAT1 and metabolic enzyme succinate dehydrogenase (SDH) resulting in succinate accumulation leading to enhanced angiogenesis [180]. Along with mtDNA mutations, mutations in nuclear DNA encoding major enzymes like succinate dehydrogenase, isocitrate dehydrogenase and fumarate hydratase leads to altered metabolite levels and

enhanced cellular proliferation. Further, pyruvate, the end product of glycolysis, crosses the mitochondrial membrane and gets converted to acetyl-CoA [181]. Studies have shown that transient blockade of pyruvate into the mitochondria increased glycolysis in the prostate cancers compared to non-carcinoma prostate tissue stating metabolic differences that are important to exploit [182]. Aspartate is also considered essential for nucleotide synthesis and important for cell proliferation which is majorly governed by mitochondrial metabolism. Studies have shown that inhibition of mitochondrial metabolism causes aspartate limitation for tumor growth and cancer cell survival [183].

Therefore, limitations or availability of metabolites in TME can be targets for cancer therapies. ***This also highlights how alterations in the mitochondrial metabolic pathways leads to accumulation of oncometabolite which contributes to contribute to malignant traits hence important to understand differential mitochondrial functions in different subtypes cancer including breast cancer.***

1.6.7 TNF-α: Critical cytokine in metabolism

TNF-α is a classic pro-inflammatory cytokine is predominantly present in the solid tumors of different origin including breast cancer. TNF-α signaling plays significant role in metabolism of bone tumor which is a major event acting as obstacles in anti-TNF treatments [184]. Further, studies have shown that TNF-α significantly increased glycolysis in breast cancer cells and use of anti-inflammatory agents like curcumin reversed such effects [178]. Such observations suggests that TNF-α in the TME can directly influence metabolic pathways like glycolysis and play important role in metabolic adaptations in cancer cells. Previous studies from the lab have reported the role of TNF-α in regulating activity of OXPHOS in NLRX1, a mitochondrial NOD family receptor protein, depleted breast cancer cells and in determining their proliferation and migration ability [185].

NF-κB has a pivotal role to play in the metabolic adaptions that occur in the tumor cells that are present in nutrient deficient TME [186, 187]. Such changes are established by targeting metabolic programs like glycolysis, OXPHOS, glutaminolysis and aids in sustaining the dynamic tumor microenvironment for growth and proliferation. Cancer cells modulates metabolic pathways for adaption to stress and nutrient deprived environment with mechanisms that are not well understood. Glutamate dehydrogenase 1 (GDH1) is a major enzyme responsible for glutaminolysis that converts glutamate to α-ketoglutarate (α-KG). Studies have shown that under low glucose conditions, RelA and IKKβ interacts with GDH1 and results in α-KG that increase uptake of glucose and tumor cell survival by activating IKKB and NF-κB

signalling. Such evidence provides unique mechanisms of metabolite-mediated NF-κB activation in brain tumor development [188].

Elevated glucose levels in tumor are also responsible for increasing the transcriptional activity of p65 and c-Rel to modulate the hexosamine biosynthetic pathway that promotes tumor growth. Studies have identified Pyruvate kinase muscle (PKM), which is a major rate limiting enzyme in the final step of glycolysis is regulated by NF-κB. TNBC tissues and breast cancer cells showed higher PKM expression and its knockdown significantly reduced cell proliferation, migration via NF-κB [189]. Interestingly, drugs like Metformin are known to reverse the breast cancer cells mesenchymal phenotype through STAT3/NF-κB pathway activation [190]. A combinational treatment of IL-6 and Metformin reduced the expression of Vimentin and SNAIL along with decreased STAT3/NF-κB and increased E-cadherin compared to IL-6 alone in breast cancer cells.

Nutrients like palmitate act as promoter of pre-metastatic niche that enhances metastatic growth in breast cancer. Accumulation of palmitate in lung and liver increases RelA acetylation and pro-metastatic NF-κB signalling which enhances metastatic growth in breast cancer patients [191]. Further, studies in colorectal carcinoma patients identified NF-κB as a major driver of carboxylesterase 1 (CES1), a lipase that fuels fatty acid oxidation in overweight CRC patients and correlates with worse outcomes [192]. Cancer cell proliferation is energetically and metabolically demanding process. The active reprogramming is achieved by coordinating many signalling processes that drive proliferation during highly inflammatory tumor microenvironment. Apart from producing energy by OXPHOS, mitochondria also contribute to cellular homeostasis, controls metabolism, calcium homeostasis, apoptosis, cell cycle, and proliferation. It serves as a critical metabolic hub which converges catabolic and anabolic processes [193].

NF-κB is known to impart balance between the glycolysis and mitochondrial respiration that involves changes in the metabolic enzyme and proteins in cancer cells. Many studies have highlighted the role of NF-κB in driving mitochondrial dynamics and energy production in normal and cancers cells [194]. Recent study identified a multifactorial interaction between damaged mitochondria and NF-κB signaling. The study showed that NEMO, an essential regulator of NF-κB pathway is recruited onto the damaged mitochondria and initiates NF-κB signalling along with upregulations of inflammatory cytokines. Such finding suggests that NF-κB and mitochondrial signaling can show crosstalk at several cellular stress conditions [195]. NF-κB also stimulates of oxidative phosphorylation by upregulating cytochrome c oxidase 2 and established its role in metabolic adaptation in normal and cancer cells [194].

An increase in oxidative respiratory metabolism and decrease in glycolysis was observed when NF-κB activity was decreased due to overexpressed IκBα super repressor in sarcoma cell lines [196]. Further, the study identified a glycolytic gene called hexokinase 2 as a transcriptional target for NF-κB and knockdown of HK2 decreased aerobic glycolysis and overexpression led to rescue of metabolic shift. Further, use of metformin, for mitochondrial dysfunction resulted in activation of AMPK/SIRT1/ NF-κB signalling to induce caspase3 induced pyroptosis in cancer cells [197]. A recent study demonstrated that Rel depletion in melanoma bearing mice reduced tumor growth and antitumor effect due to diminished OXPHOS flux and mitochondrial ATP production [198].

Thus, these reports revealed a crosstalk between NF-κB pathway and mitochondrial functions in metabolic adaptations during tumor development. The differential role of NF-κB in determining the progression of breast cancer subtypes, OXPHOS regulation and patient survival is not well understood and needs further investigation.

1.7 Anti-TNF therapies in cancer:

TNF-α has been associated with multiple cancers and immunomodulatory effects of TNF-α blockers and their therapeutic potential in human cancers have been explored. TNF-α regulate many cellular responses which initiates and promotes tumor cell proliferation, higher malignancy, and is also associated increased metastasis and worse prognosis. This highlights the potential characteristics of TNF-α as an attractive therapeutic target that needs further investigations. Major emphasis has been laid on developing monoclonal antibodies (Adalimumab, Infliximab, golimumab, certolizumab) and fusion proteins (etanercept) to reduce the soluble and transmembrane TNF-α activity in the system [199-202].

In vitro and *in vivo* studies targeting inflammation in solid cancers like pancreatic ductal adenocarcinoma by inhibiting TNF-α with infliximab and etanercept showing strong antitumoral effects. Infliximab and etanercept reduced the liver metastasis and volumes of recurrent tumors representing a promising role in therapy [203]. Further, the effect of Infliximab on metastatic osteosarcoma was investigated *in vitro* and showed that reducing TNF-α greatly reduced cell motility, osteosarcoma aggressiveness and pulmonary metastasis by inhibiting expression of chemokine receptors [204]. TNF-α is also associated with inflammatory responses in colorectal cancer. Adalimumab, a monoclonal antibody was used to inhibit the expression of MACC1, a crucial oncogene in CRC metastasis via p65 and c-Jun in CRC cells [205]. Anti-TNF monoclonal antibody (ibi303) was used to neutralize the effects of

secreted TNF-α and showed a remarkable efficiency in psoriasis by downregulating NF-κB (p65) expression and blood vessel formation as it inhibits both inflammation and angiogenesis [206]. Clinical trails with 41 patients who received infliximab showed that Infliximab was well tolerated with no dose limiting toxic effects and had no evidence of disease relapse in any patient [207]. Phase I trial of an Anti-TNF antibody certolizumab pegol, a pegylated (polyethylene glycol) antigen-binding fragment (Fab) of a recombinant humanized anti-TNF-α monoclonal antibody, with a combination of chemotherapy exerted anti-metastatic effects in liver metastatic lung carcinoma model and demonstrated inhibition of cytokines [208].

There have been several attempts where TNF-α signalling have been targeted in breast cancer. The inhibition of TNF-α with Etanercept, a TNF inhibitor; the drug acts as a soluble TNF-α receptor and binds TNF-α and TNF-β. MDA-MB-231 cells showed decrease in the cell survival, caused cell cycle arrest and apoptosis *in vitro* and inhibited NF-κB activation with Etanercept [209]. Further co-culturing the MDA-MB-231 cells with macrophages inhibited the effects of Etanercept by NF-κB activation lead by the macrophage secretome in the system. A study investigated the role of Infliximab that inhibits binding of TNF-α to its receptor on bone metastasis of breast cancer cell line MDA-MB-231 in vitro and in vivo. Study showed that infliximab reduces cell motility and bone metastasis by inhibiting expression of chemokine receptors [210]. TNF-α blocking therapies have been poorly explored in breast cancer.

Thus, agents blocking TNF-α signalling are efficient promising tools in the breast cancer patient and in combination with current cancer therapies can act as a future therapeutic option to be explored in depth.

1.8 Therapies targeting metabolism in cancer:

Proliferating cells maintains a balance of catabolic and anabolic processes to duplicate the cell mass and alter metabolic program to meet the demands as compared to normal tissues. Metabolism of proliferating cells has provided potential targets and has been extensively studied to develop treatments for cancer cells [211, 212]. Cancer cells can also scavenge nutrients from the surrounding tumor microenvironment and promote the different aspects of metastasis [213]. Interestingly, study utilizing metabolomics, have identified metabolites in tumor interstitial fluid and plasma of murine lung adenocarcinoma and pancreatic to be different based on the type of tumor, location and diet [214].

Studies using radiotracers have highlighted the non-invasive methods of staging and diagnosis and further monitoring the cancers of almost all kinds that interestingly targets metabolites [215]. PET tracers like [^{18}F] F-FDG that are used to study breast cancer glycolysis and

metabolism are under clinical trials. Advance studies have identified such tracers as better predictor of disease specific survival in metastatic breast cancers [216]. Magnetic resonance spectroscopy is also one of the non-invasive molecular imaging for many oncometabolites that is used to identify concentrations of specific metabolites in the tumor microenvironment of breast cancer [1]. Hexosamine biosynthesis pathway, a shunt pathway for glycolysis had been targeted in pancreatic cancer by glutamine analog which enhanced sensitivity to immune therapies and showed tumor regression and prolonged survival[217]. This pathway acts as a metabolic node in cancer cells that increase hyaluronan synthesis in ECM which plays critical role in immune evasion. Glutamine acts a major substrate for the rate limiting enzyme of this pathway. Further, glutamine blockade was also identified to alter the metabolic program and help in overcoming tumor immune evasion [218].

Apart from being prescribed to diabetes patients, Metformin has gain attention as a complex I inhibitor and reduces ATP levels, diminishes oxygen consumption and disturbs NAD+/NADH ratio [219, 220]. A clinical study conducted showed that metformin decreases levels of mitochondrial metabolites in breast cancer patients [221]. Metformin treatment to tumors having upregulation of OXPHOS genes showed increased proliferation and resistant to metformin treatment. Further drugs like Phenformin have shown better potency than metformin and therefore have advantages in cancer therapy [222]. Further, evaluation of phenformin as anti-cancer agent in pancreatic cancers have shown effective suppression of tumor growth in patient derived PDAC xenograft models [223]. Drugs like Atovaquone that inhibits complex III by targeting CoQ10-dependence of the complex have anti-cancer property in CSCs [224]. MCF-7 cells treated with Atovaquone showed inhibition of oxygen consumption and induced aerobic glycolysis [224]. Interestingly, HER2 positive cells and tumors have shown increased assembly of mitochondrial super complexes and complex I activity [225]. MitoTam, a novel derivative of tamoxifen that inhibits complex I and disrupts SCs have shown suppression of HER2 high breast cancer tumors without any toxicity.

Therapies targeting metabolism and immune system are offering new approaches to cancer therapy. However, the dynamics and complexity of the tumor microenvironment makes it a challenging task to accomplish and needs further in depth understanding. Studies have shown combinational therapies involving metabolic inhibitors along with immune checkpoint blockade, radiation, chemotherapy, diet have progressed and shown potential the field cancer therapeutics. But still oncogenic changes in the tumor cell metabolism acts as hindrance and leads to resistance hence it is important to further investigate to develop effective combination therapies for treating different types of cancer.

Studies targeting PD-L1 blocked, one of the major components of immune checkpoint system that limits the effects of antitumor immunity in cancer by vaccine immunotherapy and metformin showed decrease in PD-L1 compared to tumor models that did not respond to metformin alone [226].Expression of TNFR2 receptor was shown high in both breast tumor tissue and infiltrating T-cells which was targeted via TNFR2 antagonist antibody in combination with anti-PD-1. The combinatorial treatment showed effective antitumor activity than the monotherapy [227]. Metformin reduces TCA cycle intermediate through complex I inhibition [228]. Increased glucose and increase in the transcription of OXPHOS genes are the two metabolic adaptations identified in the integrated pharmacodynamic analysis as an effective mechanism in breast cancer patients using metformin [221]. Immuno-metabolic adjuvant and immune checkpoint inhibitors are also the promising targets for metabolically complex cancers like breast cancers. Studies using pH dependent co-delivery nanoplatform to introduce Met and siRNA targeting fibrinogen protein FGL1, enhances PD-L1 degradation and anti-tumor immunity [229]. Met induces programmed death-ligand 1 (PD-L1) degradation and further inhibits signals of PD-L1. As a result, nanoparticle-based delivery of siFGL1 can effectively silence the FGL1 gene, promoting T-cell-mediated immune responses.

Targeting metabolism in cancer therapy is still in early stage and requires extensive study in breast cancer and other solid tumors. Thus, understanding and targeting inflammation and metabolism through combinational therapies may prove beneficial for cancer treatment including breast cancer.