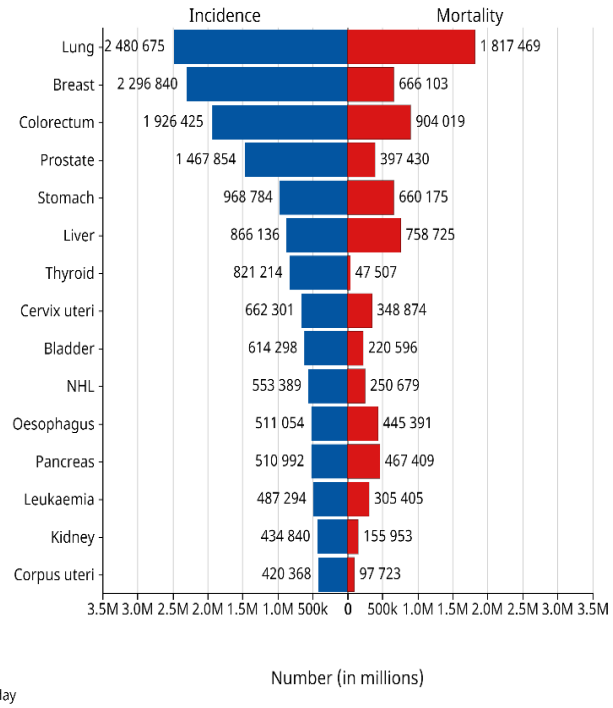
Chapter I: Introduction

1.1 Cancer: A preview

The origin of cancer has a long history intertwined with human existence, dating back to ancient times. Evidence from fossilized bone tumors, Egyptian mummies, and ancient manuscripts showcases early encounters with cancer [1]. The history of cancer extends back thousands of years. Around 1600 BC, references of cancer were found in Egyptian papyri, particularly in the "Edwin Smith" and "George Ebers" papyri describing a tumor- “like swelling of the breast” [2]. Remarkably, descriptions of conditions similar to cancer were also found in the ancient ayurvedic texts, centuries before the common Era. In the *Suśruta Samhitā*, there is a detailed discussion about a condition called *“arbuda”* that closely resembles the development of cancerous tumor, as well as instances of recurrence (*adhyarbuda*) and metastasis (*dvirarbuda*) [3]. Hippocrates, the "Father of Medicine," used the term "karkinos" to describe cancer around 400 BC. In Greek, “karkinos” was used for the disease cancer, which refers to the word “crab” because the veins displayed finger-like spreading extensions similar to the shape of a crab [4].

Cancer is a genetic disease that originates because of mutation in genes which regulates the way our cell’s function, especially how they grow and divide. This causes cells to grow uncontrollably and spread to distant organs through metastasis. Researchers worldwide persistently invest time, effort, and resources in unravelling the complexities of this disease, and discovering solutions to cure cancer. Even with the advancement of modern science, cancer persists as a significant global health challenge and remains a leading cause of death worldwide. In 2019, approximately 18% of global deaths were attributed to cancers [5]. The prevalence and mortality rates of cancer continue to rise globally. In 2022, lung cancer emerged as one of the most prevalent malignancies among the top 15 cancer types, affecting approximately 2,480,675 individuals, with 1,817,469 reported deaths worldwide [6]. Breast cancer ranks second in terms of incidence, affecting approximately 2,296,840 people globally. Breast cancer mortality remains a significant concern, with 666,103 deaths attributed to this disease in 2022. In India, the burden of cancer is substantial, and breast cancer remains a critical public health issue. India reported 192,062 cases and 93,337 deaths in 2022 (**Figure 1.1**) [6]. The cases of breast cancer are one of the most prevalent in the world, representing 15.2% and 13.3% of all reported cancer cases in India and worldwide based on a survey conducted-

Absolute numbers, Incidence and Mortality, Both sexes, in 2022
Continents
(Top 15 cancer sites)



Cancer TODAY | **IARC** - https://gco.iarc.who.int/today
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International Agency for Research on Cancer
World Health Organization

Absolute numbers, Incidence and Mortality, Both sexes, in 2022
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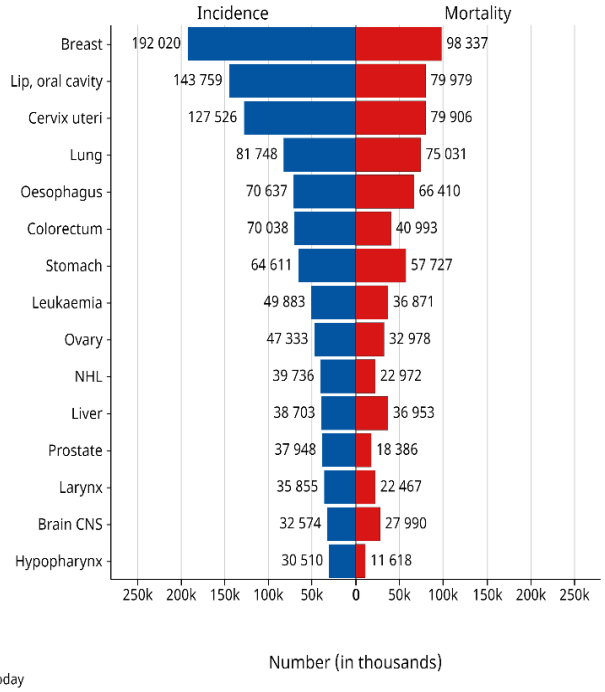

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International Agency for Research on Cancer
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Figure 1.1: Cancer statistics: Absolute number, Incident and Mortality rate around the world and in India (2022). A comparative analysis of the top 15 types of cancer, along with the absolute figures for total incidences and mortality rates, both globally and specifically in India, for the year 2022. ***Image adopted*** *from Cancer Today, Globocan 2022, Refer to [7].*

-in 2022 [7]. The effectors which contribute to the development of cancer include a combination of intrinsic and non-intrinsic factors. Genetic mutations remain the most significant Intrinsic factors, while non-intrinsic factors encompass tobacco exposure, UV radiation, and viral infections [8]. The top five risk factors are tobacco use, dietary risks, alcohol consumption, high body mass index (BMI), and exposure to air pollution [9]. Worldwide, and particularly in low to middle-income countries, the main risk factors for cancer-related deaths have been identified as smoking, alcohol consumption, poor nutrition, exposure to carcinogens in the workplace, substandard air quality, and the sexual transmission of the papillomavirus. In contrast, in high-income countries, the primary contributors to cancer incidence are smoking, alcohol consumption, and obesity [10]. A large number of researchers around the world are involved in understanding the pathophysiology of cancer progression and finding therapeutic solutions to combat the disease. Advances in identifying effective medicines, personalized treatments, and supportive care have contributed to better outcomes for cancer patients. However, continued efforts are essential to reduce mortality rates by understanding the intricacies of the disease.

1.2 Types of Cancer

Based on the site of development in the body or the cellular origin, tumors may be classified into five main types: carcinoma, sarcoma, melanoma, leukaemia, and lymphoma. Carcinoma originates in the skin or the lining of internal organs such as the lungs, liver, breast, or kidneys [11]. Sarcoma is a cancer of connective tissues like bone, cartilage, or muscle [12]. Leukaemia, lymphoma, and myeloma primarily affect the blood and immune system [13]. Leukaemia is characterized by abnormal blood cells and bone marrow. Lymphoma impacts the lymphatic system, whereas myeloma affects plasma cells in the bone marrow [14]. Cancer cells differ from normal tissue by a set of fundamental traits shared by virtually all cancer cells, these hallmarks include “sustaining proliferative signalling, evading growth suppressors, resisting cell death, inducing angiogenesis, activating invasion and metastasis, reprogramming energy

metabolism, and promoting genome instability and mutations" [15,16]. These hallmarks elucidate the mechanisms by which cancer cells grow, survive, metastasize to new sites, and develop into cancer. Oncogenes are the modified forms of normal genes known as proto-oncogenes; upon modification, they undergo gain-of-function activities leading to the development of cancer. Oncogenes become activated through multiple mechanisms, including point mutations, translocations, and gene amplifications. These changes significantly contribute to the alterations known as hallmark features of cancer cells, leading to uncontrolled growth and survival advantages. Despite significant progress in preventing and detecting cancer in the early stage, effective treatment strategies remain a big challenge owing to multiple factors *e.g.* genetic makeup, tumor heterogeneity, drug resistance, etc. [17,18]. Understanding the biochemical processes underlying the oncogenesis and tumor development is essential for designing effective therapies that can counter drug resistance and address tumor heterogeneity.

1.3 Hallmarks of cancer

Cancer cells significantly differ from normal tissue by virtue of unique characteristics. In the year 2000, Hanahan and Weinberg reported six key alterations in cell physiology that drive the change of normal cells into malignant cells [19]. These are "(1) self-sufficiency in growth signals, (2) insensitivity to anti-growth signals, (3) unlimited replicative potential, (4) evasion of apoptosis, (5) sustained angiogenesis, and (6) tissue invasion and metastasis". Collectively these six hallmarks of cancer constitute a framework that helps in differentiating cancer cells from normal cells. A decade later in 2011, Weinberg and Hanahan reported an emerging hallmark i.e. evading immune destruction and deregulating cellular energetics over the preceding knowledge of six original hallmarks [15] **(Figure 1.2)**.

1.3.1 Sustained proliferative signaling

Self-sufficiency in growth signals is a key characteristic observed in cancer cells. Unlike normal cells that rely on external growth signals to proliferate, cancer cells have the ability to produce a majority of these signals on their own, leading to a significant reduction or even elimination of their need for external stimuli. This reduces the dependence of cancer cells on growth factors from their surrounding tissue microenvironment.

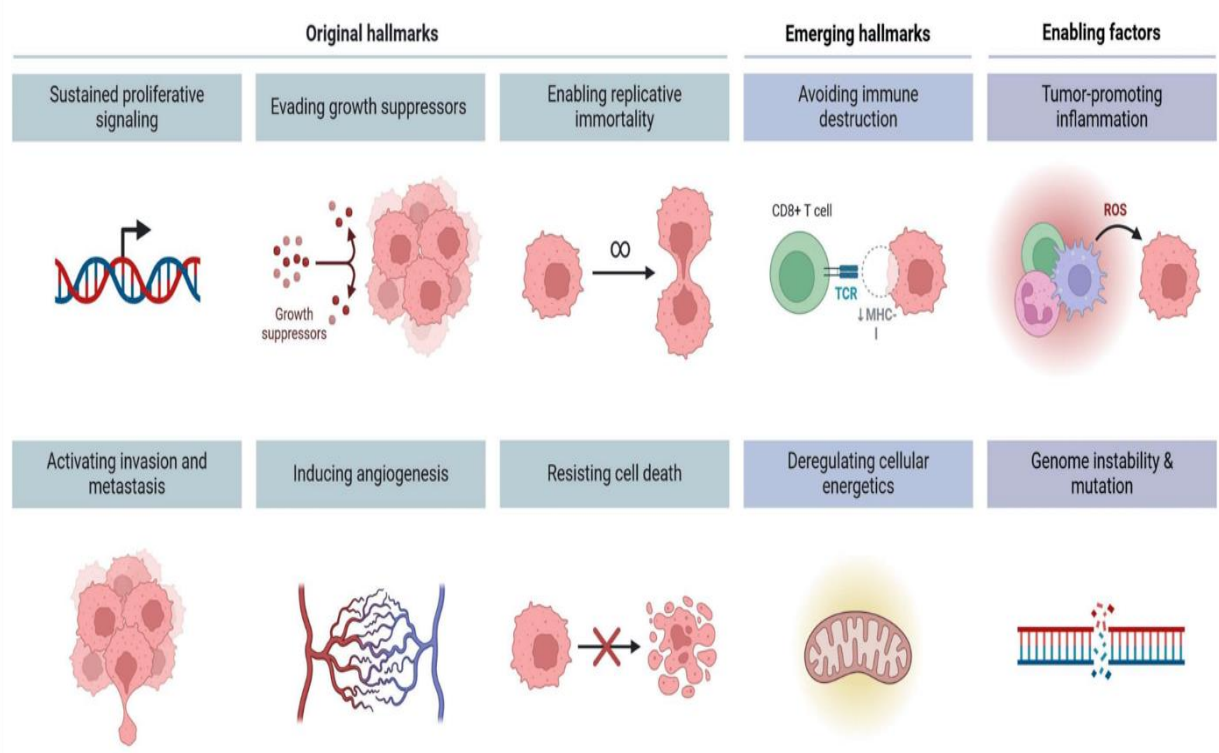

Figure 1.2: Original and emerging hallmarks of cancer. Summary of original and emerging hallmarks of cancer responsible for the transition of normal cells into malignant cells. ***Image adopted*** *from Talib, W. H et.al., Journal: Molecules (2024). Refer to* [20].

Cancer cells utilize the growth factors produced by themselves known as autocrine signalling for their growth creating a positive feedback loop system. Alternatively, cancer cells also secrete factors to recruit immune cells, and vascular cells toward tumor microenvironment (TME) which, in turn, provides growth signals for its proliferation [21].

1.3.2 Evading growth suppressors

To maintain cellular and tissue homeostasis, normal tissues establish multiple anti-proliferative signals e.g. growth inhibitors such as transforming growth factor-β (TGF-β), which prevent the activation of transcription factors responsible for the cell cycle progression [22]. Many tumors achieve insensitivity to the inhibitory signals by disabling components of the TGF-β-mediated signalling pathway. Cancer cells also inhibit the growth-inhibitory signals from crucial tumor suppressor genes to evade growth arrest. This includes genes such as p53, retinoblastoma protein (Rb), and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [15,23].

1.3.3 Enabling replicative immortality

All normal cells have a limited proliferative potential known as the Hayflick limit and eventually stop dividing due to telomere shortening. Telomeres are repetitive DNA sequences located at the ends of each chromosome that become slightly shorter every time cells undergo division. Once telomeres become critically short, cells can no longer divide and may undergo apoptosis or enter a state of senescence. To enable replicative immortality, cancer cells must proliferate indefinitely. Malignant cells achieve immortality overexpressing telomerase enzyme (e.g. hTERT; human telomerase reverse transcriptase), which functions by adding nucleotides to the ends of single-stranded DNA (ssDNA) to maintain their telomere length above a critical threshold, allowing continuous division [24].

1.3.4 Tissue invasion and metastasis

The process of tissue invasion and metastasis is crucial in cancer progression. Cancer cells metastasize from the primary tumor, then spread to new tissue sites, establish themselves, and proliferate. This process significantly impacts disease severity and complicates the treatment strategies. The metastatic process depends on changes in cell interactions with the surrounding microenvironment, facilitated by adhesion molecules such as E-cadherin and integrins, as well as the secretion of matrix-degrading proteases like MMP-2 and MMP-9. The immune cells in TME play a major role in inducing cell invasion and metastasis to new tumor sites by various mechanisms. This includes secretion of pro-angiogenesis-modulating factor Vascular endothelial growth factor C and D (VEGF-C and VEGF-D), microRNAs (miRNAs), thymidine phosphorylase [25] and recruitment of Tumor-associated macrophages (TAM), Myeloid-derived suppressor cells (MDSCs), and T-Regulatory cells (Tregs) in response to TGF-β, CXCL5-CXCR2 that induce immunosuppressive microenvironment [26].

1.3.5 Inducing angiogenesis

Oxygen and nutrients are essential for cell function and survival. These essential factors are supplied to cells by the vascular system. During tumor development, early-stage neoplasms enhance their angiogenic capabilities to grow larger. Angiogenesis, characterized by the formation of new blood vessels, is promoted by three important soluble factors: VEGF, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF). Tumor cells also

secrete cytokines such as TGF-β, TNFα, Interleukins (ILs), and Interferons (IFNs), which act in autocrine and paracrine fashions to regulate tumor angiogenesis. The most prevalent immune cells in tumors, TAMs, also secrete several factors that stimulate angiogenesis, including cytokines like VEGF-A, Epidermal Growth Factor (EGF), Placental Growth Factor (PIGF), Transforming Growth Factor-Beta (TGF-β), Tumor Necrosis Factor-alpha (TNF-α), Interleukin-1 Beta (IL-1β), Interleukin-8 (IL-8) and chemokines like C-C Motif Chemokine Ligand-2 (CCL2), C-X-C Motif Chemokine Ligand-8 (CXCL8), C-X-C Motif Chemokine Ligand-12 (CXCL12) [27]. Attracted by VEGF and TGF-β, mast cells generate matrix metalloproteinases (MMPs) that facilitate the development of new blood vessels by releasing VEGF and Basic fibroblast growth factor (bFGF) from the extracellular matrix [28]. Together in solid tumors, these cells work to promote angiogenesis and tumor growth.

1.3.6 Resisting cell death

Normal cells undergo programmed cell death to maintain cellular balance. The apoptotic cell death program can be executed through two primary pathways. A) Extrinsic Pathway: This is initiated by external signals that activate cell surface receptors known as death receptors. These receptors can bind to specific molecules called death factors or ligands. B) Intrinsic Pathway: Often referred to as the mitochondrial pathway, this pathway is activated by cellular stress signals such as DNA damage and reactive oxygen species. These signals induce alterations in the mitochondria, resulting in the release of pro-apoptotic factors into the cytoplasm. Cancer cells employ various mechanisms to evade programmed cell death, such as apoptosis and pyroptosis. Loss of TP53 tumor suppressor function results in the removal of crucial damage sensors which sense factors for inducing apoptosis [29], suppressing the expression of proapoptotic factors (Bax, Bim, Puma), upregulating the expression of antiapoptotic factors (Bcl-2, Bcl-xL) and survival factors (Igf1/2) [30]. Cells undergoing necrosis can release factors like IL-1α, which aid in recruiting inflammatory cells. These inflammatory cells secrete growth-stimulating factors that support the proliferation of cancer cells [31].

1.3.7 Avoiding immune destruction

Cancer cells avoid destruction from immune cells by reprogramming the machinery necessary for antigen presentation (e.g., PD-1, CTLA-4, LAG-3) to evade killing by cytotoxic T lymphocytes

(CTLs) [32]. This creates an immunosuppressive tumor microenvironment by secreting factors e.g., TGF-β, IL-10, IDO, and modifies its interactions with immune cells [33]. Cancer cells by recruitment of immunosuppressive immune cells, including Tregs and MDSCs actively avoid immune cells mediated killing [15]. In addition, cancer stem cells have immunological characteristics that protect them from the immune responses of healthy counterparts. Cancer cells can also modify naive natural killer (NK) surface markers to reduce immune synapse formation and cytotoxicity [34]. Understanding these immune-cancer interactions informs potential combination treatments, where targeting both metabolic pathways and immune checkpoints could enhance anticancer therapies.

1.3.8 Reprogramming energy metabolism in cancer cells

Normal cells primarily rely on oxidative phosphorylation in mitochondria to process glucose and produce energy. In contrast, cancer cells exhibit a metabolic change known as the Warburg effect, which favours aerobic glycolysis. This glycolytic pathway allows faster ATP production, even in the presence of oxygen. Although less efficient per glucose molecule in terms of ATP production, it supports rapid tumor cell proliferation. Additionally, cancer cells utilize glycolytic intermediates as building blocks for proteins, DNA, and lipids. Some cancer cells even rely on lactate as their primary energy source. However, this metabolic competition impacts the tumor microenvironment, leading to nutrient scarcity and acidosis, which can hinder the normal function of immune cells by shifting to an immunosuppressive state. Stromal and immune cells in TME, increase lactate concentration, leading to TME acidification which in turn affects immune cell infiltration and encourages angiogenesis, survival, and growth of tumor cells [35]. Additionally, lactate in TME inhibits T-cell proliferation and cytokine secretion as well as NK cell and CD8+ T cell-mediated cytotoxicity of tumor cells [36].

1.4 Tumor microenvironment (TME)

Tumor is a dynamic and heterogeneous collection of infiltrating and resident immune cells, extracellular matrix, and secreted factors. The tumor is highly dynamic and keeps on evolving continuously. It consists of tumor cells, adipocytes, extracellular matrix, immune cells, and a network of lymph and blood vessels [37]. Tumor formation and progression involve intricate, dynamic, and multi-step processes influenced by multiple factors including hypoxia, immune

cell infiltration, ionic composition, extracellular matrix, metabolic composition, etc. The tumor microenvironment has unique characteristics different from the surrounding normal tissue such as low pH, hypoxic condition, altered ionic balance, and nutrient availability which contribute to cancer development [38,39]. Additionally, TME significantly influences immune cell behaviour, inducing an immunosuppressive state that aids tumor growth. Within the TME, biological processes such as macrophage polarization, immune suppression, fibroblast recruitment, and extracellular matrix (ECM) degradation facilitate tumor cell invasion and metastasis. Cancer cells actively shape a tumor favouring microenvironment by recruiting and reprogramming stromal, host immune cells and remodelling the blood vessels and surrounding extracellular matrix [40]. These dynamic interactions occur between neo-malignant cells and recruited non-cancerous cells within the TME, thus shaping the outcome of cancer progression.

1.4.1 Inflammation in tumor microenvironment

Immune cells are the most important components of the TME for fighting against cancer. Our immune systems have transformed over millennia through the gradual evolution of innate and adaptive immunity to fight against infection and aberrant cells [41].The acute inflammatory retort acts as the body's first line of defence against tumor cells by activating both innate and adaptive immune responses. To resolve inflammation, innate immune cells such as macrophages, dendritic cells (DCs), and neutrophils phagocytose pathogens and necrotic cells [42,43]. DCs and macrophages function as antigen-presenting cells, delivering antigens to T cells and triggering the adaptive immune response [44]. However, if the acute inflammatory reactions do not subside, it may lead to the development of chronic inflammation resulting in an immunosuppressive microenvironment by transforming immune cells *e.g*. TAMs, MDSCs, Treg cells, etc towards an immunosuppressive state [45] **(Figure 1.3)**. Chronic inflammation in the tumor microenvironment is one of the common reasons for tumor formation in different types of cancer. Chronic inflammation also damages DNA and proteins, activates oncogenes, releases reactive oxygen species (ROS), and alters several signalling pathways, such as NF-κB, K-RAS, and P53, which can lead to malignancy [45]. In human tumors, immune cells constantly infiltrate the tumor tissue [47-49]. The level of immune cells within a tumor is influenced by

multiple dynamic processes *e.g.* its extravasation from blood vessels to its spreading throughout the tumor site (infiltration), proliferation, and retention in TME [50].

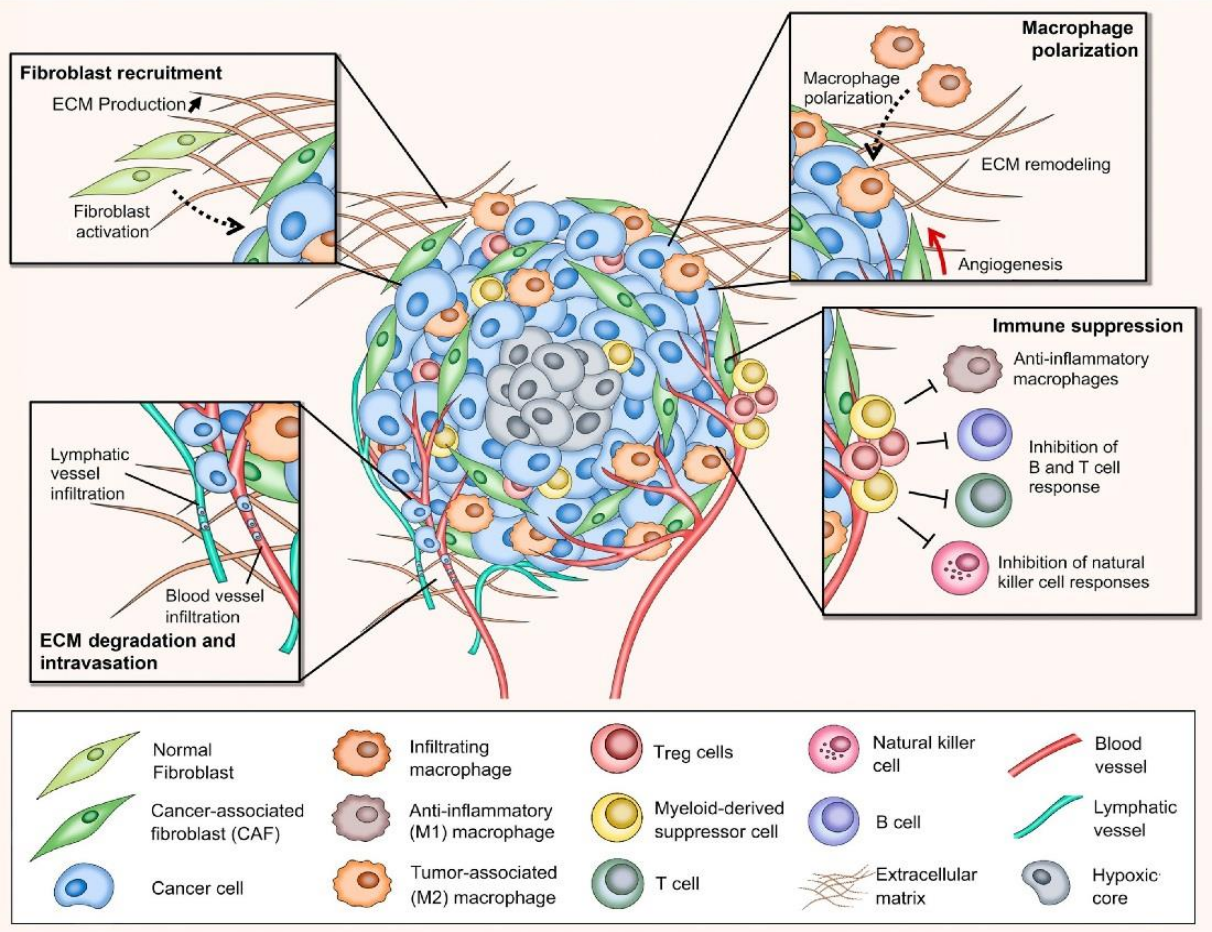

Figure 1.3: Components of tumor microenvironment (TME). Figure highlights the components of tumor microenvironment (TME) detailing the various cell types involved and highlighting key biological processes such as recruitment of fibroblast, polarization of macrophages, immune suppression, and extracellular matrix (ECM) degradation, which facilitate tumor cell intravasation and metastasis. ***Image adapted*** *from J Rodrigues et.al., Journal: Trends in cancer (2021), Refer to* [46].

These infiltrates vary in size and composition across different tumors. This infiltration suggests that the body recognizes the tumor cells and tries to stop their growth. This phenomenon is referred to as immune surveillance [51]. These increased infiltration of immune cells in tumors are known to be associated with improved prognosis and better patient survival [50,52,53]. Immune cells thus can recognize molecular cues of malignant cells and, in most cases, destroy them before they develop to form a cancerous tissue. This is evident from the fact that mice that lack functional components of the immune system are more prone to the development

of spontaneous tumors e.g. (RAG2) mice that do not have functional T cells, B cells, and NK cells are susceptible to spontaneous or chemical induced tumor [54]. Also, increased T-cell infiltration and activation correlate with improved survival in various forms of cancer [55,56]. Further, the level of infiltrating M1 macrophage was associated with improved survival [47].

Nonetheless, cancer cells under the influence of such chronic inflammatory conditions avoid immunosurveillance by the selection of tumour cells that are non-immunogenic by the process of immunoselection and avoiding the immune system by the process of immunosuppression [57]. For example, higher levels of M2 macrophages and Tregs cells were found to be significantly associated with shorter lifespan [58]. On the other hand, breast cancer progression of ductal carcinoma in situ (DCIS) to invasive ductal carcinomas (IDC) was characterized by less no of activated CD8+ T cells, high expression of Programmed death-ligand 1, Cytotoxic T-lymphocyte associated protein 4, and increased infiltration of regulatory T (Tregs) cells [59]. Immune cells generally promote cancer progression through secreted cytokines and chemokines which provide growth stimulatory signals, survival factors, angiogenesis factors, and ECM degrading enzymes to facilitate invasion and metastasis [60,61]. Additionally, the metabolic products of TME, such as hypoxia, glutamine, or potassium ions contribute to this process [62]. Whether cancer arises due to persistent chronic inflammation or an immune-suppressive environment created by the tumor, nearly all malignant neoplasms lead to varying degrees of exclusion of T cells, natural killer (NK) cells, and dendritic cells (DCs) or dysfunction in CD8+ T cells, while simultaneously recruiting myeloid cells like MDSC and M2 macrophages, that foster a tumor-supportive inflammatory environment. Thus, understanding the link between inflammation and tumors can lead to better anti-cancer treatments and improve the effectiveness of immunotherapy, chemotherapy, and radiotherapy.

1.4.2 Components of Tumor microenvironment

The tumor microenvironment (TME) consists of cellular and non-cellular elements. The cellular components consist of tumor cells, fibroblast cells, pericytes, adipocytes, endothelial cells, blood vessels, lymphatic networks, MDSCs, immune cells, and inflammatory cells. Non-cellular components consist of extracellular matrix (ECM) and extracellular vesicles [40]. The cross-talk between cancer cells and the tumor microenvironment (TME) plays significant roles not only

in tumor progression and metastasis but also in treatment outcomes, such as chemotherapy. The immune cell composition and activation state in the tumor microenvironment (TME) vary significantly based on tumor location, intrinsic cancer cell properties, tumor stage, and grade. These components evolve alongside the tumor as it advances. Typically, normal tissue can restrict cancer growth through suppressive mechanisms that involve immune cells, fibroblasts, and extracellular matrix. Nevertheless, under the influence of tumor cells the extracellular matrix (ECM) offers structural support and signalling cues, whereas stromal cells within the TME can take on novel phenotypes to promote tumor invasion and proliferation. Understanding these components and their cross-talk is crucial for developing effective therapeutic strategies targeting the TME to improve cancer treatment outcomes. The role of the components of tumor microenvironments is discussed briefly in the table below **(Table 1.1).**

Table 1.1: Cellular components of tumor microenvironment and their role in tumor microenvironments.

Cell Types	Function in Tumor Microenvironment (TME)	References
CD8+ T cells	Cytotoxic T cells (CD8+) identify abnormal tumor antigens on cancer cells and eliminate them. They recognize cancer cells by binding to MHC peptide complexes via their T cell receptor (TCR). This recognition triggers apoptosis through granzyme and perforin or FASL-FAS-mediated cell death. Additionally, cytotoxic T cells suppress angiogenesis by secreting interferon gamma (IFN-γ).	[63,64]
CD4+ T cells	CD4+ T cells assist CD8+ cells by producing interleukin-2 (IL-2) and interferon gamma (IFN-γ). Elevated Th1 cell levels in the tumor microenvironment correlate with favourable outcomes in various cancers. Conversely, Th2 cells secrete anti-inflammatory molecules that promote tumor growth.	[65,66]
Tregs	Tregs (Regulatory T cells) secrete interleukin-2 (IL-2), which influences the balance and function of natural killer (NK) cells. Consequently, Tregs contribute to tumor growth and progression by suppressing anti-tumor immune responses.	[67,68]

B cells	B cells have dual role in tumor. They play an antitumorigenic role by presenting antigens to T cells, producing anti-tumor antibodies, and secreting cytokines (such as IFN-γ) that enhance cytotoxic immune responses. In addition, B cells can also promote cancer by inducing immune suppressive phenotypes in macrophages, neutrophils, and cytotoxic T cells through the secretion of IL-10 and TGF-β.	[69,70]
Natural killer cells (NK cells)	NK cells survey blood stream seeking for tumor cells and kills them by release perforin and granzymes to kill a target cell. However, tumors cells evade NK cell mediated cytotoxicity via upregulation of inhibitory receptors and activation of immunosuppressive myeloid cells and Tregs.	[71,72]
Macrophages	Macrophages can comprise up to 50% of the total immune infiltrating cells in TME. It can exist either in M1 or M2 phase in TME. M1 macrophages are known to phagocytize and kill malignant cells, while immune-suppressive M2 macrophages participate in tumor progression.	[37,73]
Neutrophils	Neutrophils are known as first line defence against infection and makes up approximately 70% of the circulating leukocytes in the blood. Neutrophils can act to either promote tumor growth, through modification of the extracellular matrix, releasing VEGF, IL-10 and producing matrix metalloprotease (MMP)-9 or supress tumor by release of cytokines and ROS.	[74-76]
Dendritic cells	Dendritic cells are professional antigen presenting cells. They transport tumor-derived antigens to draining lymph nodes and primes naïve T cells into anti-tumor effector T cells. However, cancer cells modulate them to support tumor progression	[77,78]
Myeloid-derived suppressor cells (MDSCs)	MDSCs are crucial immunosuppressive cells within the tumor microenvironment (TME). They actively promote tumor growth and metastasis by hindering anti-tumor immunity. The mechanisms include inhibiting the activation of CD4+ and CD8+ T cells and blocking NK cell cytotoxicity.	[79,80]
Cancer-associated	CAFs are major component of the tumor stroma which actively participate in wound healing. CAFs induce strong	[81,82]

fibroblasts (CAFs)	immunosuppressive microenvironment (via TGF -β) leading to immune evasion and reduced immune cell infiltration. CAFs through ECM remodelling promote cancer invasion.	
Extra Cellular Matrix (ECM)	Extracellular matrix (ECM) is a non-cellular structural component of the tumor microenvironment (TME). It consists of fibrous proteins like collagens, glycoproteins, and proteoglycans. The ECM undergoes continuous remodelling by proteases (such as MMPs) produced by various TME cells. This facilitates tumor cell invasion and metastasis.	[83,84]

1.4.3. Tumor-Associated Macrophages (TAM)

Macrophages are one of the most versatile cells of the innate arm of the immune system and offer the first line of defence against diseases and infections. They are known for their antigen-presenting and phagocytic activity. In addition, these cells secrete various inflammatory cytokines and chemokines during inflammation. Circulating monocytes from blood are recruited at tumor site in response to hypoxia, necrosis, growth factors like CSF-1, VEGF-A, and PDGF, cytokines such as IL-6, and chemokines such as CCL2, CXCR4, and CXCL12 that are secreted by tumor and other cell types in TME [85,86]. CCL2 and CCL5 are important chemokines that direct circulating monocytes to tumors site. In addition, CXCR1 and CXCR2 facilitate monocyte adherence to endothelial cells [87]. Such macrophages under the influence of tumor microenvironments polarize into different phenotypes depending upon the molecular milieu in TME. While monocytes recruited from peripheral blood contribute to the bulk of TAMs, embryonic-derived resident macrophages also contribute as a source of TAMs in certain tumors. However, the role of embryonic-derived TAMs in tumor growth remains to be elucidated. Macrophages are functionally very versatile and can exist in the spectrum of physiological states. At opposite ends of their phenotypic spectrum, macrophages exhibit M1 and M2 polarization states. M1 macrophages, often termed ‘classically-activated,’ produce type I pro-inflammatory cytokines, engage in antigen presentation, and contribute to an anti-tumorigenic environment. In contrast, M2 macrophages, known as ‘alternatively-activated,’ secrete type II cytokines, support anti-inflammatory responses and play pro-tumorigenic roles [88]. Currently, it is not fully understood, how macrophages initially switch from a tumor-

suppressing to a tumor-promoting state, however, research shows alterations in tumor microenvironment such as tumor hypoxia [89], metabolic state [90], and ionic imbalance [91-93] may mediate this transition.

1.4.4. Pro-tumor and Anti-tumor state of Tumor-Associated Macrophages (TAM)

The dynamic tumor microenvironment plays a key role in transforming infiltrating and resident macrophages into tumor-associated macrophages (TAM). TAM can exhibit a dual role in cancer, either as a tumor-promoting M2 phenotype or tumor-suppressing M1 phenotype depending upon the condition in TME. Macrophages being the most predominant immune cells in TME, can sometimes constitute up to 50% of tumor-infiltrating cells [27]. The default function of the infiltrating macrophages is cytotoxic toward tumor cells by producing cytotoxic molecules such as TNF-α, nitric oxide, and reactive oxygen intermediates and by the production of IL-12 [94-96]. M1 macrophages secrete pro-inflammatory cytokines like IL-12 and chemokines such as CXCL9 and CXCL10. These molecules drive Th1 cell polarization and recruitment, amplifying type 1 immune response mediating phagocytosis tumor [97]. Macrophages are one of the important effector cells in TME, they mediate tumor cell clearance by presenting tumor-associated surface antigens to T cells particularly cytotoxic T lymphocytes (CTLs) via MHC-II [98] and enhancing the anti-tumor functions of natural killer (NK) cells via Antibody-Dependent Cellular Cytotoxicity (ADCC) and various other mechanisms [99]. However, macrophages under the influence of factors secreted from tumor e.g. IL-4, IL-10, TGF-β1, and prostaglandin E2 polarize TAMs to alternatively activated or M2 state thereby significantly influencing cancer cell behaviour and support various carcinogenic processes, including tumor growth, angiogenesis, invasion, migration, immune regulation, and chemo-resistance [100,101]. Furthermore, the M1 and M2 phenotypes of macrophages display unique chemokine patterns. Th1 cell-attracting chemokines like CXCL9 and CXCL10 are expressed by M1 macrophages. M1 macrophages also release high concentration of nitric oxide synthase (NOS) enzymes and inflammatory cytokines like TNF-α, IFN-γ, and IL-12 detrimental to cancer cells. [102]. In contrast, M2 macrophages show higher expression of arginase-1 (Arg-1), CD206 / Mannose receptor (MR), and scavenger receptor (SR), and they also express the chemokines CCL17, CCL22, and CCL24. They also release anti-inflammatory cytokines such as IL-4, IL-13, and IL-10 which help in tumor progression. [103]. Furthermore, macrophage displays

characteristics MHC expression based on the site of cancer e.g. TAMs with low MHC-II expression were identified in hypoxic tumor tissues, where they predominantly expressed M2 markers and demonstrated enhanced pro-angiogenic functions In contrast, TAMs with high MHC-II expression were confined to normoxic tumor tissues, expressing M1 markers and antiangiogenic chemokines [85]. These findings highlight the complexity of TAM populations and the importance of understanding their spatial and functional heterogeneity within tumors under the influence of the external milieu.

1.4.5 Targeting TAMs to Combat Tumor

Since tumor-associated macrophages (TAMs) are core to the connection between inflammation and cancer growth, targeting TAMs to fight tumors is an emerging therapeutic technique. Since TAMs are mostly linked to pro-tumoral actions, treating them for anti-cancer therapy could be a lucrative option.

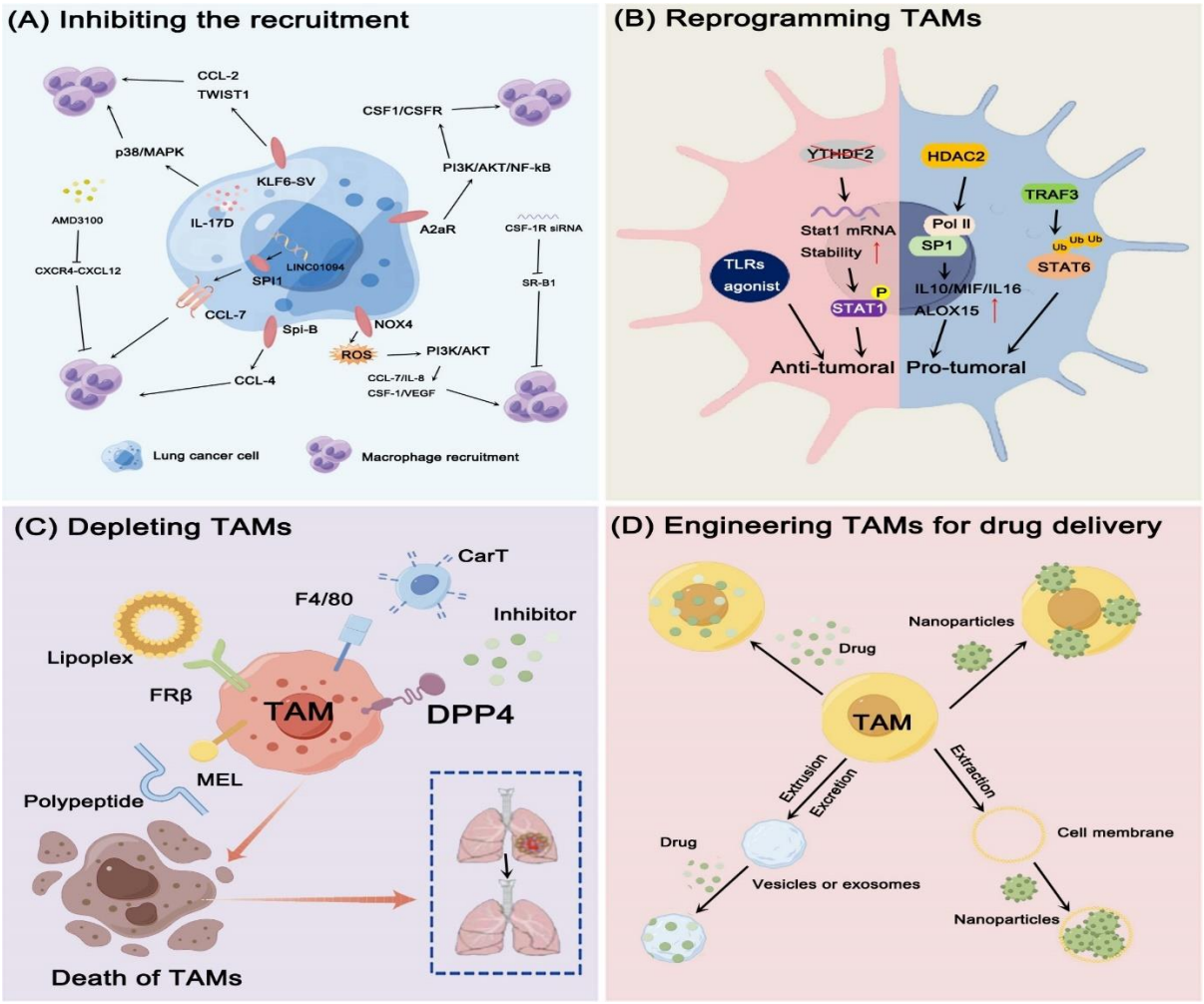

Figure 1.4: Strategies to reprogram Tumor-associated macrophages (TAM) in tumor microenvironment (TME). The figure represents the current strategy used for reprogramming macrophages in the tumor microenvironment (TME) for re-educating the tumor-promoting M2 phenotype to the tumor-killing M1 phenotype that enhances the anti-tumor immune responses. (1) limiting monocyte recruitment to the tumor site; (2) depleting TAMs; (3) reprogramming TAMs; and (4) engineering TAM for site-specific drug delivery. ***Image adapted*** *from L. Liu et.al., Journal: Frontiers of Immunology (2023), Refer to* [104].

Various strategies are evolving to promote the natural function of macrophage cells in tumor microenvironment that shift TAMs from the pro-tumoral M2 phenotype to the tumor-killing M1 phenotype, thereby enhancing their ability to attack tumor cells and support anti-tumor immune responses. Current therapeutic strategies focus on four major aspects— (1) limiting monocyte recruitment to the tumor site; (2) depleting TAMs; (3) reprogramming TAMs; and (4) engineering TAM for site-specific drug delivery [105] **(Figure 1.4)**. Under the influence of TME, infiltrating immune cells undergo polarization which favours tumor growth, colony stimulating factor 1 (CSF1), CCL2, CCL5, and VEGF are known factors responsible for monocyte recruitment. When monoclonal antibodies, receptor antagonists, and cationic nanoparticles (NPs) are used as therapeutic techniques to target those molecules, tumor progression rate, TAM density, and chemotherapy sensitivity decrease in solid tumors. [106-108]. Targeting TAM recruitment also comes with an associated accumulation of immunosuppressive neutrophils. Hence, the original approach to the depletion of TAM was developed using CAR-T cells, which selectively targeted the elimination of FRβ+ TAMs, which resulted in the recruitment of pro-inflammatory monocytes and CD8+ T cells and restrained tumor growth [109]. Strategies are being developed to induce the reprogramming of TAM polarization using antibodies or small molecule inhibitors targeting IL-4, IL-10, or TGF-β. By blocking their signalling pathways, these approaches can reprogram TAMs toward an M1 phenotype and reduce M2 polarization [110]. IFN-γ is known to activate macrophages to produce pro-inflammatory cytokines and enhance their antigen-presenting capabilities. [111,112]. Enhancing pro-inflammatory cytokines like IFN-γ and GM-CSF can promote M1 polarization of TAM. A study revealed that targeting RNA binding protein F2 (YTHDF2) facilitates TAM reprogramming by focusing on IFN-γ/STAT1 signalling. [113]. Reactivating the NF-κB signalling pathway which is impaired in TAMs, through the use of TLR agonists in conjunction with anti-IL-10 antibodies is one method of modifying signalling pathways in TAMs in order to enhance the M1 phenotype [114]. TAMs can also be

reprogrammed from the M2 to M1 phenotype by inhibiting STAT3 and activating STAT1. To this end, small molecule inhibitors or siRNAs that target STAT3 are being investigated [115] . Another approach focuses on the use of pharmacological inhibitors for reprogramming TAM, SHIP1 phosphatase, plays an important role in inducing the M2 phenotype characterized by immunosuppressive functions. Inhibitors of SHIP1 can redirect macrophages towards the M1 phenotype [116]. Indoleamine 2,3-dioxygenase is an enzyme that suppresses T-cell activation by depleting tryptophan. IDO inhibitors, such as methyl-thiohydantoin-tryptophan (MTH-trp), have shown promise in reverting immunosuppression mediated by TAMs which subsequently augment their anti-tumor activities and immune-stimulatory functions within the tumor microenvironment [117]. TAMs exhibit significant homing characteristics, that enable them to travel in a specific direction and gather in tumor tissue by identifying molecular signals in the TME. [118]. The homing and delivery capabilities of TAMs can be leveraged as a vector to deliver nanomedicines at tumor sites. Presently, nanomaterials that selectively target M2-like macrophages, reshaping their phenotype and inducing polarization toward M1-like macrophages, hold promise in inhibiting tumor growth [119-121].

1.5. Breast cancer

Breast cancer is a complex and heterogeneous disease that can occur in different parts of the breast. Breast in women is composed of skin, mammary glandular tissue, fat, and connective tissue such as blood vessels, lymphatic and nerve system [122] . The proportion of this tissue changes with age, body mass index, and hormonal level. The mammary epithelium is of two types: the outer basal epithelium and the inner luminal epithelium. The luminal epithelium cells which form the inner lining of laticiferous duct encircling the lumen, differentiate into milk-producing lobules whereas, the outer layer of mature mammary ducts is differentiated from myoepithelial cells. [123]. Breast cancer is formed by an uncontrolled division of luminal epithelial cells in different parts of the breast, particularly in milk-producing lobules, ducts, and the outer layer of basal cells [124]. Most breast carcinomas are adenocarcinomas, making up more than 95% of breast cancer cases. Ductal carcinoma begins in the milk ducts of the breast. Unlike ductal carcinoma, where cancer cells are confined to the duct lining, invasive ductal carcinoma (IDC) means cancer cells have spread beyond the ducts to other parts of the breast. Invasive ductal carcinoma (IDC) comprises of 80% of invasive breast cancer cases and is the

most prevalent type [22, 23]. Breast tumors are heterogeneous in nature and classified by gene expression patterns and hormone receptor status. Based on the expression of hormone receptors breast cancers are classified into three major classes. Human epidermal growth factor receptor 2 (HER2+), which can be either estrogen receptor-positive (ER+), or estrogen receptor-negative (ER-) [125]. Breast cancers that do not express any of these hormone receptors are known as triple-negative breast cancers (TNBC) [24, 25].

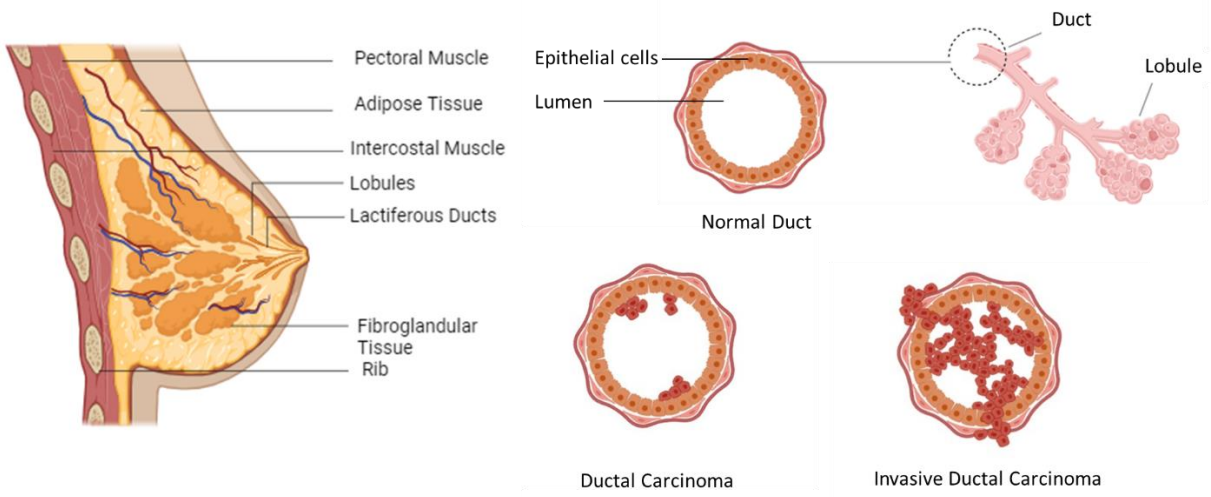

Figure 1.5: Architecture of breast tissue. Anatomy of breast tissue highlighting the structure of the human breast. B. Figure indicating the site of ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC).

1.6 High salt and cancer

Salt (NaCl), commonly known as table salt consists of Sodium and Chloride ions. The use of table salt for human consumption started long before the beginning of recorded history, however the oldest record of use of salt dates back to around 2700 B.C. Most of the salt that we consume comes from the dietary salt that is added to our daily diet. Salt plays a vital role in the regulation of various physiological aspects of the body like maintaining the membrane potential of cells, osmotic balance, and extracellular fluid volume [128]. Although the daily recommended amount required for an average adult according to WHO is 4-5 gm/day, recent data show that populations around the world are consuming much more (3-4 times) sodium than physiologically required [126]. The immune system of the body is regulated by various hereditary, dietary, and environmental factors. Recent studies have demonstrated evidence

for the effect of dietary habits on the immune status and prevalent diseases such as cancer. A preclinical study reported that a high-sodium diet can induce local tissue-specific accumulation of sodium in mice and humans in the skin, thymus, liver, and spleen [127]. Additionally, sodium was also found to be stored non-osmotically in endothelial surface layers of skin, and thus act locally on surrounding cells [128].

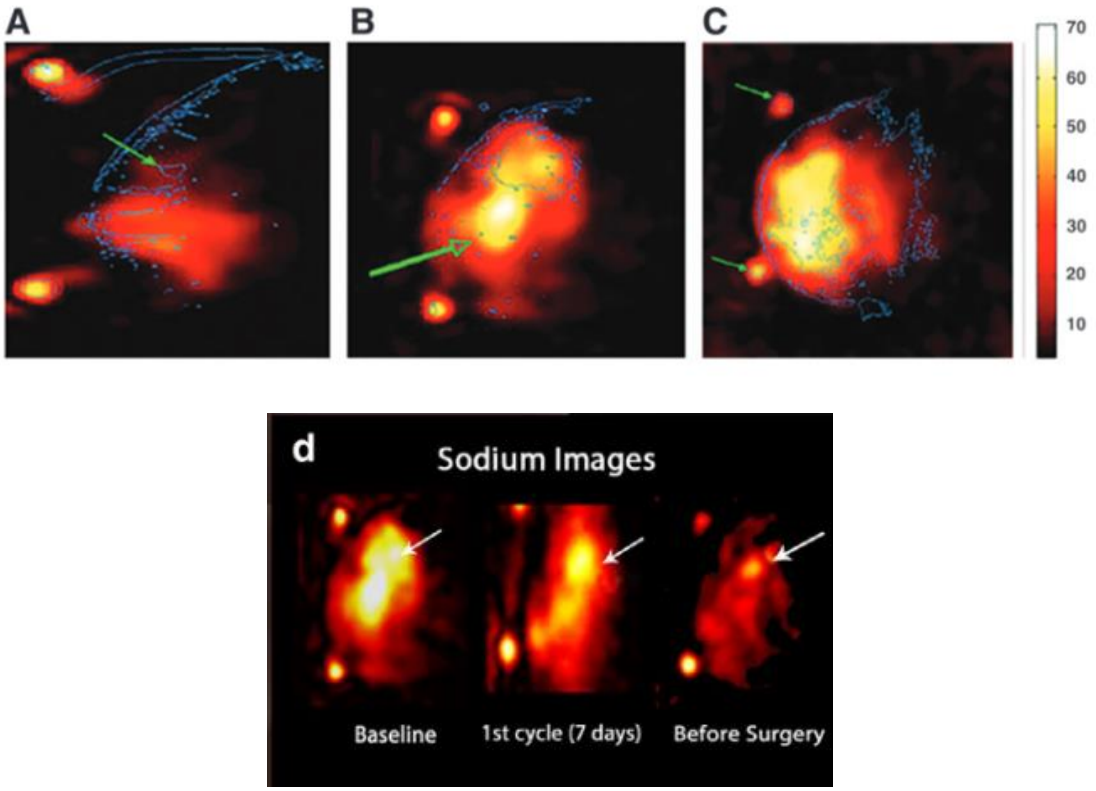

Figure 1.6: ^{23}Na MRI imaging of breast tissues from normal and cancer patients. The ^{23}Na MRI imaging from three different breast cancer patients are shown. (A) benign lesion (B) poorly differentiated ductal carcinoma (C) A large locally advanced breast cancer (D) At baseline contrast-enhanced MRI showed a large tumor volume with markedly increased total tissue sodium concentration using ^{23}Na MRI. ***Image adapted from*** *Poku, L. O et.al., Journal: Journal of Magnetic Resonance Imaging (2021) and Ouwerkerk, R et.al., Journal: Breast Cancer Research and Treatment (2007), Refer to [129] [130].*

The concentration of sodium in the blood is typically maintained within a narrow range of 135–145 mM. In normal tissues, the intracellular sodium concentration (ISC) is much lower, around 10–15 mM, whereas the extracellular sodium concentration (ESC) is higher, typically between 140–150 mM. This sodium balance is tightly regulated by the kidneys and the circulatory system. Hence the total sodium concentration (TSC) which is denoted by ISC + ESC of normal

tissue is approximately 23-26 mM. However, ^{23}Na MRI studies have shown that malignant tissues exhibit a significantly elevated total sodium concentration (TSC) ranging from 47.0 ± 11.1 mM to 53 ± 16 mM in malignant lesions **(Figure 1.6)** [129-131]. This raises questions about the effect of high salt on cancer and immune cells in the tumor microenvironment. The behaviour of cancer, stromal, and invading immune cells can be influenced by the extracellular "ionic tumor microenvironment" in the same way as intracellular concentrations of ions can affect the behaviour of cancer cells. Recent evidence has shown that salt plays a critical role in regulation of immune cells like TH17 and macrophages by skewing it towards a pro-inflammatory phenotype [93,132]. The pro-inflammatory effects of high salt on the T-cells and macrophage cells raise the question of whether high salt conditions could also affect cancer progression and anti-tumor immunity. Immunotherapy is undoubtedly one of the most promising strategies for cancer treatment. However, the highly immunosuppressive environment created in tumors poses a significant obstacle to its success. The phenotype of TAMs is strongly influenced by the surrounding microenvironment. The differences in sodium concentration could have significant functional implications for the tumor microenvironment and the overall outcome of cancer treatment and progression. In literature, the effect of high salt on cancer is debatable, with the majority of reports pointing towards its anti-tumorigenic activity [127,133-135]. In addition, the direct impact of high salt on breast cancer cells remains unexplored. Nevertheless, there is ample scope to study the direct effect of high salt on breast cancer cells and its effect on the regulation of macrophage inflammation that has a direct relationship with the progression of cancer. Considering the gap in the field, the thesis work aimed to study the direct effect of high NaCl treatment on cancer cells and their cross-talk with TAMs.

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