Original Literary Work Declaration

Declaration from PhD candidate:

I, Meron Yohannes Nigussie, declare that this dissertation is my original work. I have written and submitted to AAU-CHS, Department of Microbiology, Immunology and Parasitology never to any other institution in any form for evaluation. All the information here is dully acknowledged and I have never used any other source except those cited ones.

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I hereby declare that I have advised this Ph.D. work, the dissertation has been prepared under my supervision and has been submitted with my approval.

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Abstract

Background; Breast cancer remains a leading cause of morbidity and mortality among Ethiopian women. It is a heterogeneous disease, both in its molecular characteristics and immune response. Recent evidence demonstrates the importance of incorporating immune cell composition into tumor classification, as the immune landscape plays a crucial role in determining prognosis and therapeutic response. Additionally, emerging studies suggest that tumor immune response is influenced by intratumoral microbiota, which can affect both anti- and pro-tumor immune activities. Despite these insights, there remains a significant gap in understanding the immunological landscape of Ethiopian breast cancer, its prognostic relevance, and its interaction with the mammary microbiota.

Objective; This study was aimed to explore the host immune profile—both local and systemic—its role in predicting disease prognosis, and its interaction with the mammary microbiota in Ethiopian breast cancer patients.

Methodology: A combination of prospective and retrospective follow-up study designs was employed to address the different study objectives. A prospective follow-up study was conducted to investigate the systemic immune repertoire of BC patients and to examine correlations between mammary microbiota and local immunity. Patient/disease characteristics at diagnosis were collected through structured, pretested questionnaires. Blood from BC patients and healthy controls were analyzed by flow cytometry to assess the phenotype and function of immune cell subpopulations. To explore microbiome-immune associations, fresh frozen tissue (FFT)/Formalin fixed paraffin embedded (FFPE) tissue was used for molecular characterization of the microbiota, by targeting 16S rRNA genes (Illumina MiSeq) and for immune gene expression analysis.

Additionally, a retrospective follow-up study was conducted to characterize the tumor immune microenvironment (TIME) and its prognostic role in BC. Archived FFPE samples were collected, subjected to RNA extraction, and analyzed using nCounter to assess immune gene expression. Differentially expressed genes (DEGs) were determined using ROSALIND®. Immune cell population abundance was assessed using Nanostring's profiling module, while tumor infiltrating lymphocytes (TILs) were analyzed via hematoxylin and eosin (H&E) staining. In addition, the PIK3CA gene was genotyped using qPCR. Five-year follow-up data were retrieved from patient medical records, with overall survival serving as the endpoint assessed via follow-up phone calls. Log-rank test was performed to compare the prognostic relevance of immune subgroups. Additionally, data from The Cancer Genome Atlas (TCGA) were included as an independent validation cohort.

Written informed consent was obtained from participants for the prospective study, while a waiver was granted by the IRB for the retrospective study.

Result: Hierarchical clustering of immune gene expression analysis revealed four distinct immune phenotypes (IP1-IP4), each characterized by unique patterns of immune activation, inhibition and immune cell infiltration. Among these IPs, IP2was associated with luminal tumors, and exhibited downregulation of immune genes and the lowest rates of immune cell infiltration. IP4, in contrast, displayed an active tumor microenvironment (TME) with upregulated cytotoxic genes and the highest abundance immune cell, independent of intrinsic subtype. Notably, PIK3CA gene mutations weren’t linked to a specific immune phenotype. Importantly, IPs with an immune active TME demonstrated better overall survival compared to those with suppressed immune genes expression. This highlights the prognostic significance of immune activity in breast cancer.

Systemically, blood immunophenotyping highlighted a tumor-specific activation in breast cancer patients, characterized by the presence of exhausted $CD4^+$ T cells and senescent $CD8^+$ T cells, with an inverted CD4/CD8 ratio observed in approximately 50% of cases. Additionally, there was an expansion of immunosuppressive cell populations, including myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs). Enhanced frequencies of γδ T cells were also noted; however, their precise role in BC immunity remains unclear. These findings highlight the complex interplay between immune activation and suppression in the systemic immune response to breast cancer.

In addition to the local and systemic immune profiling of BC, we conducted further analysis to explore the correlation between mammary microbiota and the TIME. Our findings revealed a positive correlation between the relative abundance of *Delftia* and both tumour-infiltrating lymphocytes (TILs) and the expression of various immune-related genes. Similarly, *Corynebacterium* exhibited a strong trend of positive correlation with TIL percentages. Nominal positive correlations were also found between the phylum *Actinobacteria* and specific immune-related genes, as well as between *Cyanobacteria/Chloroplast* and genes involved in antigen processing and presentation. While overall bacterial diversity did not differ significantly across TIL categories, specific microbial taxa were notably enriched in certain groups. These results suggest that specific components of the mammary microbiota may influence immune infiltration and gene expression within the TIME, highlighting potential interactions between microbiota and host immunity in BC.

Conclusion: In summary, this study identified four distinct immune contextures within the breast cancer TME of Ethiopian patients, each characterized by unique immune gene expression

profiles and immune infiltration patterns. These findings offer valuable prognostic insights and highlight the complexity of immune responses in this population. Systemically, we observed a breast cancer-specific immune response accompanied by immune escape mechanisms. Targeting these mechanisms, such as T cell exhaustion and expansion of immunosuppressive cells, may offer strategies to enhance clinical outcomes and improve survival rates for Ethiopian breast cancer patients. Furthermore, our microbiome analysis suggests a potential immunomodulatory role for specific intratumoral microbiota within the TME, indicating a possible avenue for therapeutic intervention. Future studies should focus on validating these findings and exploring potential therapeutic interventions targeting the identified immune and microbial modulators.

Budget: The study was supported by the Susan Komen GTDR16378013 given to Martin-Luther –University Halle-Wittenberg, Medical Faculty.

Keywords: Immune Phenotypes, Tumor immune microenvironment, Immune profiling, Immune escape, Mammary microbiota, Ethiopian patients, Prognostic biomarkers.

Dedication

I dedicate this dissertation to the **Almighty God** and **the Virgin St. Mary**, for giving me the courage, strength, and faith in times when things seemed unbearable.

It is also dedicated to my parents. **Emama (Emahoy Woletetsadik)**, not only did you raise me with faith and nourishment, but you have also cared for my children during my frequent travels to Germany, even when I am home but too busy to handle my kids. Thank you for being a better mom than I am. **My mom, Almaz Menbere**, you have always been the best mom anyone could ask for—I know I can always rely on you. **My dad, Yohannes Nigussie**, you have always been a supportive father and the reason I am here today. Thank you for instilling in me a passion for knowledge. I will never forget the "lecture sessions" at home during my childhood, even though I wasn't the biggest fan back then.

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Abbreviations

ACT	Adoptive cell therapy
ADCC	Antibody-dependent cytotoxicity
AJCC	American Joint Committee on Cancer
ANXA	Annexin-1
APCs	Antigen-presenting cells
ARG	Arginase 1
BC	Breast cancer
CALR	Calreticulin
CAR	Chimeric antigen receptor
CCL	C-C motif chemokine ligands
cDCs	Conventional DCs
cDNA	Complementary DNA
CEA	Carcinoembryonic antigen
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
CTL	Cytotoxic T lymphocytes
CTLA-4	T-lymphocyte-associated protein-4
CXCL1-17	C-X-C motif chemokine ligands
DAMPs	Danger-associated molecular patterns
DC	Dendritic cells
DCIS	Ductal carcinoma in situ
DEA	Differential expression analysis
DEIGs	Differential expression of immune genes
DFS	Disease-free survival
DMSO	Dimethyl sulfoxide
E-ACTs	Engineered adoptive T-cell therapies
ECM	Extracellular matrix

EMT	Epithelial-to-mesenchymal transition
ER^+	Estrogen receptor positive
EVs	Extracellular vesicles
FBS	Fetal bovine serum
FDR	False discovery rate
FFPE	Formalin fixed paraffin embedded
FLT3-L	FMS-like tyrosine kinase-3 ligand
FVS	Fixable Viabilty stain
GATA3	GATA-binding protein 3
GEP	Gene-expression profiling
GlyCAM-1 molecule-1	Glycosylation-dependent cell adhesion
GM-CSF	Granulocyte macrophage colony-stimulating factor
H&E	Hematoxylin and eosin
HER2	Human epidermal growth factor receptor 2
HMGB1	High-mobility group protein-1
HNF3A	Hepatocyte nuclear factor 3 alpha
HR+	Hormone receptor positive
HRT	Hormone replacement therapy
hTERT	Human telomerase reverse transcriptase
ICAM-1	Intercellular adhesion molecule-1
ICI	Immune check point inhibitors
IDC	Invasive ductal carcinoma
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
IFN-γ	Interferon-gamma
IHC	Immunohistochemistry
IL-2	Interleukin-2
ILC	Invasive lobular carcinoma

ILT2	Immunoglobulin-like transcript 2
IP	Immune phenotypes
ISGs	Interferon-stimulated genes
ITAMs	Immunoreceptor tyrosine-based activation motifs
KD	Knockdown
KIR2DL4	Immunoglobulin-like receptor 2DL4
LAG-3	Lymphocyte activation gene 3
LCIS	Lobular carcinoma in situ
LMICs	Low- and middle-income countries
LPBC	Lymphocyte predominant breast cancer
LPS	Lipopolysaccharide
MadCAM-1	Mucosal addressin cell adhesion molecule-1
MARCKS	Myristoylated alanine-rich c-kinase substrate
M-CSF	Macrophage colony-stimulating factor
MDSC	Myeloid derived suppressor cells
MFS	Metastasis-free survival
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MUC1	Mucin 1
NCDs	Non-communicable diseases
NGS	Next generation sequencing
NK cells	Natural killer cells
NOS2	Inducible nitric oxide synthase
OTU	Operational taxonomic units
PAM50	50-gene prediction analysis of microarray
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline Solution
pCR	pathologic complete response

PD-1/L1	Programmed cell death ligand 1
pDCs	Plasmacytoid DCs
PDGF	Platelet-derived growth factor
PD-L1	Programmed death protein 1 ligand 1
PGE 2	Prostaglandin E2
PIK3CA	Phosphoinositide 3-kinase CA
PR	Progesterone receptor
PRRs	Pattern recognition receptors
PTEN	Phosphatase and tensin homolog
SOPs	Standard operating procedure
STING	Stimulator of interferon genes
TAAs	Tumor-associated antigens
TAM	Tumor associated macrophages
TASH	Tikur Anbessa Specialized Hospital
TCGA	The Cancer Genome Atlas
TCR	T cell receptor
TIL	Tumor infiltrating lymphocyte
TIM-3	T cell immunoglobulin domain and mucin 3
TIME	Tumor immune microenvironment
TLRs	Toll like receptor
TMAO	Trimethylamine N-oxide
TME	The tumor microenvironment
TNBC	Triple negative breast cancer
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor-alpha
T-reg	T-regulatory cells
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

WT1	Wilms tumor gene
XBP1	X-box-binding protein 1

Chapter: Introduction

1.1 Background Overview

Cancer encompasses a broad group of diseases marked by the uncontrolled growth and spread of abnormal cells. Under normal circumstances, cell division is tightly regulated through mechanisms that enforce cell cycle arrest (Diori Karidio and Sanlier 2021). However, mutations in critical genes, such as proto-oncogenes or tumor suppressor genes, can disrupt these regulatory systems, leading to unchecked cellular growth and survival. While environmental factors, including smoking, radiation, and exposure to carcinogens, are well-known contributors to these genetic mutations, they represent only part of the broader spectrum of cancer causes (Dakal et al. 2024). In addition to genetic mutations, recent research has increasingly emphasized the role of epigenetic alterations in cancer development. These changes, such as DNA methylation, histone modifications, and microRNA regulation, can modify gene expression without altering the underlying DNA sequence, contributing to the silencing of key proteins involved in regulating the cell cycle (Prabhu et al. 2024).

Cancer is named after part of the body where the tumor originates. Breast cancer (BC) originates in the breast tissue, primarily in the milk-producing lobules and the ducts that connect them to the nipple (Feng et al. 2018). It is a highly heterogeneous disease, both at histological and molecular levels. The most common histological types are invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC), with IDC accounting for approximately 80% of cases and ILC about 10%. Less common histological types include inflammatory breast cancer, Paget's disease, mucinous carcinoma, and phyllodes tumors (Barroso-Sousa and Metzger-Filho 2016). At the molecular level, BC is classified into four intrinsic subtypes—luminal A, luminal B, HER2-enriched, and basal-like—based on hormone receptor (ER/PR) and HER2 expression, along with genomic profiling (Perou et al. 2000). While this classification has improved risk assessment and treatment selection, it does not fully capture the disease's complexity, as patients within the same molecular subtype often exhibit different clinical outcomes. This highlights the need for additional biomarkers to enhance prognosis and therapy strategies (Abe et al. 2011; Curtis et al. 2012; Dawson et al. 2013).

Growing evidence exists that not only tumor-intrinsic, but also extrinsic factors, such as the composition of the tumor microenvironment (TME), can influence tumor growth and progression. The TME, consisting of cancer cells, stromal tissue, immune cells, extracellular

matrix and soluble mediators has recently emerged as an important factor influencing BC prognosis and therapy response (Li, Tsang, and Tse 2021). In this context, the immune cells that populate the TME have emerged as a key determinant of clinical outcomes (Badr, Berditchevski, and Shaaban 2020). Different effector immune cells including cytotoxic T lymphocytes (CTLs), CD4+ T cells, dendritic cells (DC), natural killer (NK) cells and M1 macrophages recognize malignant transformed cells leading to their direct or indirect elimination. CTLs are involved in direct tumor cell killing via secretion of effector molecules (Raskov et al. 2021). CD4+ cells can support and help the CD8+ T population during the anti-tumor response via the secretion of a wide range of effector cytokines. Another important effector cells of the innate immunity are NK cells which are capable of direct tumor killing without prior sensitization while DCs serve as an essential link between innate and adaptive immunity (Bruno et al. 2021).

However, tumor cells can also highjack various immune regulatory mechanism(s) to protect themselves and suppress an ongoing anti-tumor immune response (Hao et al. 2021; Hao *et al.*, 2012). They downregulate the expression of HLA class I molecules making them invisible to effector cells (Wang et al. 2017). Elevated expression of inhibitory checkpoints including cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and programmed death receptor 1/programmed cell death ligand 1 (PD-1/L1) is another important mechanism of immune escape (Zhang et al., 2023). Engagement of such inhibitory receptors with their ligands results in impaired proliferation and differentiation, reduced effector cytokine production, increased apoptosis in T lymphocytes, and diminished degranulation and cytotoxicity in NK cells (Lin et al., 2024). Tumors also create an immune suppressive TME, which displays changes in the number and function of different immune cell subpopulations including regulatory T cells (Tregs), myeloid derived suppressor cells (MDSC), tumor-associated macrophages (TAM) and cancer-associated fibroblasts (CAFs) (Ohue and Nishikawa 2019; Hao et al. 2021).

Since tumor-induced changes in the immune cell repertoire occur not only in the TME, but also systemically (Hiam-Galvez, Allen, and Spitzer 2021), immune-profiling of patients using peripheral blood is also crucial to better understand the disease immunology for identification of relevant blood biomarkers and therapeutic targets. The immune system undergoes significant alterations in response to the presence of the tumor, with immune cells in peripheral blood reflecting the dynamic interplay between the tumor and host immune defenses (Schnell *et al.*, 2020; Ohue and Nishikawa 2019.

Given the crucial role of the immune system in combating tumorigenesis, cancer immunotherapy has emerged as an important approach for the treatment of cancer by boosting the existing immune response or reversing underlying immune escape mechanisms (Adams et al. 2014). Various approaches have been explored for breast cancer immunotherapy, such as immune checkpoint inhibitors (ICIs), which target inhibitory molecules like PD-1/PD-L1 and CTLA-4 to activate T-cells. Notably, pembrolizumab (Keytruda) gained FDA approval in 2021 for TNBC, demonstrating significant improvements in overall survival, underscoring the potential of ICIs in BC treatment (Schmid et al. 2024). Adoptive cell therapy (ACT), including tumor-infiltrating lymphocyte (TIL) therapy and CAR-T cell therapy, involves engineering or expanding immune cells to enhance anti-tumor responses (Du et al. 2023). Cancer vaccines, such as peptide-based, dendritic cell-based, and nucleic acid-based vaccines, aim to stimulate immune recognition of tumor antigens (Fatima, Fatma, and Saraf 2023).

The human microbiota, consisting of bacteria, viruses, parasites, and fungi, plays a crucial role in maintaining gut homeostasis by supporting immunity, metabolism, and protection against pathogens (Sun et al., 2023). A balanced microbiota is essential for health, but its disruption, known as dysbiosis, has been linked to various diseases, including cancer. In the context of cancer, the microbiome, particularly the gut microbiota, has been increasingly recognized for its dual role in both tumor suppression and promotion, partly through immune-mediated mechanisms (Degruttola et al. 2016). Emerging evidence highlights its influence on immune surveillance, tumor immunity, and responses to both immunotherapy and conventional cancer treatments, underscoring its potential as a key factor in cancer progression and therapy outcomes (Sun, Chen, and Wu 2023).

This growing understanding of the gut microbiota's role in cancer has paved the way for exploring the impact of microbiota beyond the gut, particularly within the TME. Recently, the intratumoral microbiota has emerged as a significant element within the TME, forming the tumor microbe microenvironment (Jiayao Ma et al. 2021). Studies have revealed that intratumoral microbiota plays an important role in both tumor suppression and promotion (Chun-Ming and Lam 2024) the mechanism of which is partly attributed to remodeling of the immune landscape. Anti-tumor immune responses can be triggered by microbial antigens or their metabolites, which activate innate and adaptive immune cells leading to an immune-activated microenvironment potentially capable of combating the tumor. This immune activation may also involve cross-

reactivity against shared epitopes between microbes and tumor cells, enhancing the overall anti-tumor response (Hoskinson, Jiang, and Stiemsma 2023). Microbiota are also capable of activating the signaling pathway: stimulator of interferon genes (STING) which induces type I interferon (IFN-I) in immune effector cells such as macrophages and DCs triggering anti-tumor immunity in the TME (Lam et al. 2021). Conversely, intratumoral microbiota also recruit immune suppressive cells such as tumor associated macrophages (TAMs), tumor-associated neutrophils and Tregs to the TME via secretion of different chemokines. In addition, upregulation of check point molecules, reduced infiltration of immune effector cells and secretion of tumor promoting cytokines are also among the mechanisms to pro-tumorigenic immunity *(Zhang et al., 2023;* Liu et al., 2024; M. Wang et al., 2023).

Although the immune response plays a crucial role in breast cancer outcomes, no study has assessed its impact on clinical outcome or its correlation with microbiota in the Ethiopian context. This study was aimed to address this critical gap in knowledge by focusing on these three interconnected themes: the immune landscape of the TME and its correlations with the intratumoral microbiota, as well as peripheral immunity and immune escape mechanisms in Ethiopian breast cancer patients. By integrating these aspects, we sought to contribute to a comprehensive understanding of breast cancer in Ethiopian patients for better management of the disease.

1.2 Statement of the problem

Comprehensive cancer surveillance and population-based cancer burden assessments are still lacking in Ethiopia. According to GLOBOCAN (2022), the country recorded an estimated 80,334 new cancer cases and 54,698 cancer-related deaths across all types. Among these, BC accounted for 16,904 new cases and 9,626 deaths. The estimated age-standardized incidence and mortality rates for breast cancer were 40.8 and 22.4 per 100,000, respectively (Global Cancer Observatory, 2022). The first cancer registry in Ethiopia was established in 2011, and data from 2011–2013 indicated that breast cancer constituted 31.5% of all registered cancers, making it the most frequently diagnosed malignancy. The annual age-standardized incidence rate was estimated at 40.6 per 100,000 (Timotewos et al. 2018). A systematic review reported that breast cancer constitutes 15.2–26% of all cancers and 29.3–37% of female cancers in Ethiopia, with a significant proportion of patients presenting at advanced stages (57% to 71.2%) (Dandena et al. 2024).

Breast cancer outcomes in Ethiopia are strongly influenced by stage at diagnosis and delays in accessing care. A study from southern Ethiopia reported overall survival rates of 83.0%, 73.2%, and 63.1% at one, two, and three years, respectively. Among early-stage breast cancer patients, the two-year survival rate was 89.9%, compared to 63.8% for advanced-stage cases (73.4% for stage III and 44.3% for stage IV). Independent predictors of mortality included advanced disease at diagnosis and delays in seeking treatment, with an average delay of 23 months from symptom onset to medical consultation (Shita et al. 2023). Similarly, a large cohort study of 1,070 Ethiopian breast cancer patients diagnosed between 2005 and 2010 reported metastasis-free survival (MFS) rates of 74% and 46% at two and five years, respectively. In early-stage patients (stages I–II), five-year MFS was 72%, whereas stage III patients had a significantly lower MFS of 33% (Kantelhardt et al. 2014).

These findings underscore that while clinical and epidemiological factors such as stage and treatment delay are critical determinants of survival, the underlying biological and immune mechanisms driving these outcomes remain poorly understood in Ethiopia (Kantelhardt et al. 2014; Shita et al. 2023; Dandena et al. 2024), highlighting an urgent need to better elucidate the immunological and biological aspect of the diseases within the Ethiopian context.

In line with this need, emerging evidence suggests that breast cancer classification has to be refined by including information on the composition of the immune cell infiltrate, as an increasing number of studies highlight the crucial role of immune landscape profiling in predicting prognosis and guiding treatment decisions (Hendrickx et al. 2017; Tekpli et al. 2019; ;Larsson et al. 2022; Massa et al. 2020). In addition, there are a growing number of studies globally, emphasizing the role of microbiota in shaping immune response to the tumor with both anti and protumorigenic roles described. Despite these advancements, there is a significant gap in research on the immunological landscape of BC in Ethiopia, as well as potential correlations between the mammary microbiota, local immune response, and their prognostic implications. This gap in knowledge limits the development of context-specific prognostic markers and targeted therapeutic strategies suitable for Ethiopian breast cancer patients. So, this study was aimed to explore the immune profile of breast cancer patients, both in the tumor microenvironment and systemically, assess the role of intratumoral microbiota in modulation of the tumor immune microenvironment in Ethiopian BC patients.

1.3 Significance of the study

BC remains a significant public health burden in Ethiopia, with limited research on its immunological landscape. Understanding the immune response within the TME, systemic immunity, and microbiome interactions is essential for identifying novel biomarkers, improving therapeutic strategies, and tailoring interventions to Ethiopian BC patients.

This study revealed the TME of Ethiopian BC patients exhibit varying immune activation states and possess prognostic relevance. Additionally, it examines how clinicopathologic features and genetic mutations, such as PIK3CA mutations, influence immune activation within the TME. Understanding these factors can help identify potential immunotherapy targets and use of immune parameters in stratification of patients in to different prognostic and treatment groups (immunotherapy, chemotherapy and radiotherapy).

Beyond the TME, cancer affects the broader immune system, leading to systemic immune dysregulation. This study revealed the presence of tumor specific immune response and various immune escape mechanisms expanding our understanding of how tumors evade immune surveillance, which is essential for identifying therapeutic targets and biomarkers of tumor. Furthermore, it reported the impact of surgical intervention on circulating immune effector and suppressor cells, providing insights into post-surgical immune milieu.

Recent evidence suggests that the microbiome plays a crucial role in modulating immune responses in cancer. This study demonstrated the presence of specific microbiome components which influence immune effector or suppressor gene expression. It also reported that distinct microbiota profiles correlate with TILs. Understanding these interactions could pave the way for microbiome-targeted therapies that enhance anti-tumor immunity and improve patient outcomes.

All in all, this research provides a comprehensive understanding of the immune landscape in Ethiopian BC patients, addressing key gaps in knowledge regarding immune interactions in the TME, systemic immune responses, and microbiome influences on immunity. Exploring the biology of this common tumor is essential for designing therapeutic approaches effective in controlling this disease.

1.4 Research questions

1. Does the tumor microenvironment exhibit varying immune activation states?
2. Does the expression of immune effector and suppressor genes correlate with the prognosis of breast cancer?
3. Does the expression of these genes vary with intrinsic subtypes of breast cancer?
4. Does the clinico pathologic features of BC exhibit distinct immune activation of the TME?
5. Does PIK3CA mutation correlate with immune activation in the TME?
6. Is the systemic immune response in breast cancer patients distinct from that of healthy individuals (is there a tumor specific immune response)?
7. Does surgical intervention impact immune effector and suppressor cells in the circulation?
8. What are the immune escape mechanisms in Ethiopian BC patients?
9. Does the systemic immune profile of Ethiopian BC patients vary with clinicopathologic features?
10. Does a specific microbiome component induce immune suppressor or effectors genes?
11. Does a distinct microbiota profile correlate with tumor-infiltrating lymphocytes?

1.5 Objectives of the research

1.5.1 General objective

The general objective of this study was to characterize the tumor immune microenvironment and its prognostic role in breast cancer, investigate the systemic immune repertoire of breast cancer patients, and examine correlations between mammary microbiota and local immunity.

1.5.2 Specific objectives

- to identify BC subtypes (immune phenotypes) via hierarchical clustering of immune gene expression data from the TME
- to determine the prognostic role of immune phenotypes
- to explore possible correlations between PIK3CA mutation and immune activity
- to determine the phenotype and function of immune cell (sub) populations in BC patients in comparison to healthy controls
- to characterize the systemic immune response of BC patients pre- and post-surgical intervention
- to explore microbiome-immune interactions in the TME at both transcriptional and cellular levels of immunity

Chapter 2: Literature review

2.1 Global Epidemiology of breast cancer

Cancer is a leading cause of premature death and disability worldwide, especially in women. According to the 2022 GLOBOCAN database, the global cancer burden was predicted to be 20 million new cases and 9.7 million cancer deaths in 2022 —an increase from 18 million cases and 9.6 million deaths in 2018 (Global Cancer Observatory, 2022). This reveals an alarming increase in both incidence and mortality attributed to both aging and growth of the population, as well as changes in the prevalence and distribution of the main risk factors for cancer, several of which are associated with socioeconomic development (Bray et al. 2018).

BC accounts for 11.6% of all cancer cases, making it the second most commonly diagnosed cancer across both sexes and ranks fourth in terms of mortality (Bray et al. 2024). In 2022 alone, an estimated 2.3 million new breast cancer cases were diagnosed globally, representing nearly one in four cancer cases among women. The disease is the most commonly diagnosed cancer in 157 out of 185 countries and the leading cause of cancer-related deaths in 112 nations (Bray et al. 2024). Incidence rates are highest in high-income regions, including Australia/New Zealand, Northern and Western Europe, and North America (WHO, Global Health Observatory. 2018; (Bray et al. 2024).

However, breast cancer is emerging as a major public health crisis in low- and middle-income countries (LMICs), where the burden of disease has shifted from infectious diseases to chronic non-communicable diseases (NCDs) (Sabetghadam and Sabetghadam 2013). In Africa, precise incidence figures remain challenging to obtain due to the absence of comprehensive cancer registries. Nonetheless, GLOBOCAN estimated that in 2022, approximately 198,553 women in Africa were diagnosed with breast cancer, leading to 91,252 deaths. In several sub-Saharan African countries breast cancer has now become the most commonly diagnosed cancer and the second cause of cancer death in women, a shift from previous decades in which cervical cancer was the most commonly diagnosed cancer in many of these countries (Global Cancer Observatory, 2022). Despite its increasing significance, breast cancer incidence remains lower in Africa (approximately 40 per 100,000 women) compared to Europe and North America (90–120 per 100,000 women) (Brinton et al., 2018).

Although incidence rates are highest in developed regions, breast cancer mortality is disproportionately high in less developed countries. For example, Western Europe has an annual incidence rate exceeding 90 per 100,000 women, compared to just 30 per 100,000 in Eastern Africa. However, despite the stark difference in incidence, mortality rates in both regions remain similar (approximately 15 per 100,000), highlighting disparities in early diagnosis and treatment access (Bray et al. 2024). A prospective cohort study in sub-Saharan Africa has also found that the three-year overall survival rate was only 50%, compared to a five-year survival rate exceeding 85% in most European countries (McCormack et al. 2020; Dafni, Tsourti, and Alatsathianos 2019).

2.2 Risk Factors for the Development of Breast Cancer

Many factors are proposed to be the risk factor for breast cancer; increasing women's life time probability of developing the disease. This includes female sex, older age, genetics, lack of childbearing or lack of breastfeeding, higher levels of estrogens, early onset of menarch, going through menopause later in life, hormonal replacement therapy, certain dietary patterns, exposure to radiation, positive family history of breast cancer and obesity.

The risk of developing breast cancer increases with age. As most of the data is compiled from developed countries, data from US has shown that approximately 80% BC arises in women over age of 50yrs. The 10 years probability of developing BC for age 40 is 1.5% while this figure increases to 3% at age 50 and over 4% by age 70. This is especially true for hormone receptor positive (HR+) BC (Benz 2008). However, emerging data from studies on African and African American women has shown that the average age of diagnosis of breast cancer in this population tends to be younger (Shah, Rosso, and Nathanson 2014; Martin et al. 2010).

Earlier on set of menarch or going through menopause later, not having children or having children later in life, lack of breastfeeding and obesity increases women's life time exposure to an endogenous estrogen. In addition, exogenous hormone exposure like hormone replacement therapy (HRT) and hormonal birth controls also increase the level of circulating hormone in a women's body. High levels of circulating estrogens are associated with increased risk of estrogen receptor positive (ER^+) BC development (Obeagu and Obeagu 2024). It is hypothesized that estrogen might play a role in breast cancer progression and development. Two major hypotheses attempt to explain the tumorigenic effects of estrogen: (i) genotoxic effects of estrogen

metabolites via generation of radicals (initiator) and (ii) the hormonal properties of estrogen inducing proliferation of cancers as well as the premalignant cells (promoter*)* (Miziak et al. 2023).

Genetic factors play a significant role in the risk of BC. Among the various genetic mutations linked to BC risk, those in the tumor suppressor genes BRCA1 and BRCA2 are the most prominent. The BRCA genes are expressed in tissues such as breast and ovary and mutations in these genes is associated with increased breast and ovarian cancer risk (Gilkes, Inês Godet 2017). Inherited mutations (genetic alterations) in BRCA1 and BRCA2, genes, account for 5%-10% of all female breast cancers, an estimated 5%-20% of male breast cancer, and 15%-20% of all familial breast cancers (Kabel and Baali 2015). The average life time probability of women with BRCA1and BRCA2 mutation developing the disease is estimated to be between 55%-65% and 45%, respectively (Gilkes, Inês Godet 2017). Less common mutations associated with increased BC risk include p53 (Li–Fraumeni syndrome), phosphatase and tensin homolog (PTEN) Cowden syndrome), and serine–threonine kinase (STK11) (Peutz–Jeghers syndrome) accounting for small proportion of hereditary BC cases (Kabel and Baali 2015).

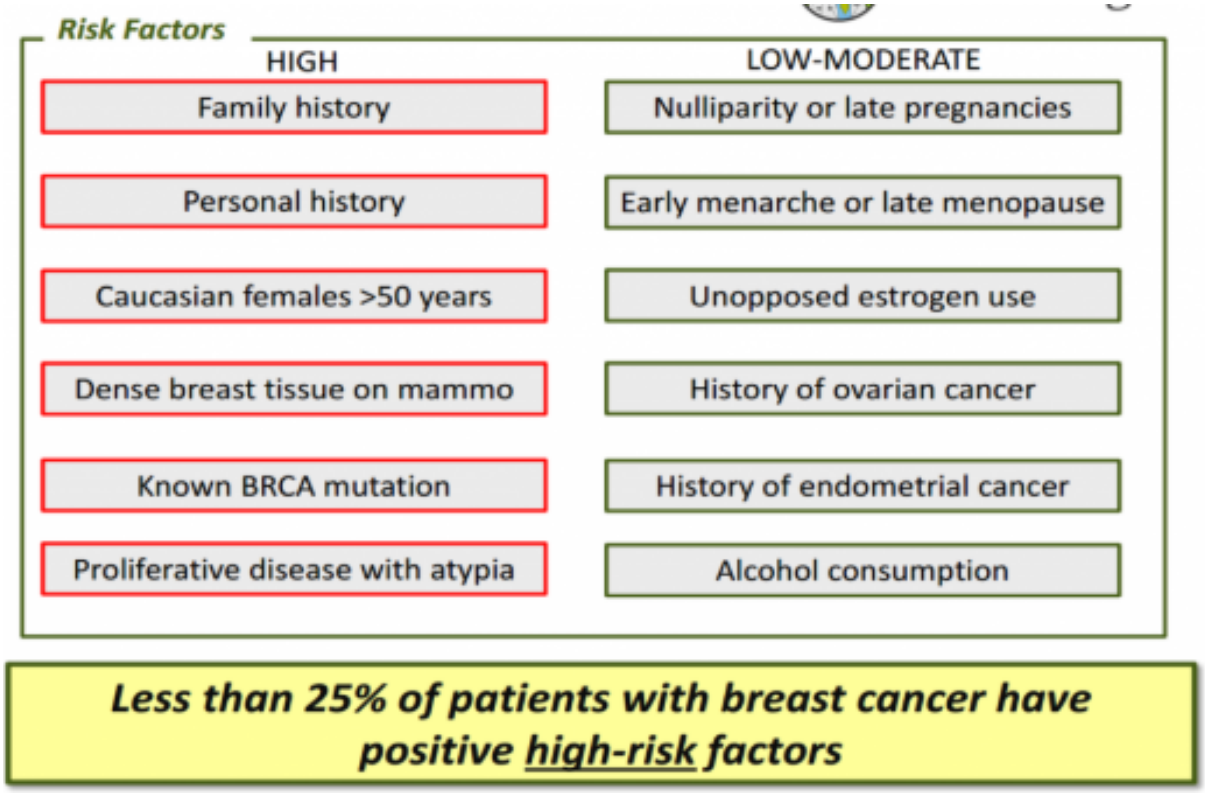


Figure 1. Risk factors for the development of BC

2.3 Intrinsic classification of breast cancer

Gene-expression profiling (GEP) with the use of DNA microarrays allows measurement of thousands of messenger RNA (mRNA) transcripts in a single experiment. Results of such studies have confirmed that breast cancer is not a single disease with variable morphologic features and biomarkers but, rather, a group of molecularly distinct neoplastic disorders (Sotiriou et al. 2003).

The pioneering study by Perou et al. utilized complementary DNA (cDNA) microarrays to analyze gene expression profiles, identifying four distinct groups of breast cancer samples potentially linked to different molecular characteristics of mammary epithelial biology: ER+/luminal-like, basal-like, Erb-B2+, and normal breast (Perou et al. 2000). Shortly after, Sørlie et al. refined this classification by incorporating a larger sample size and evaluating its clinical relevance. Consequently, breast tumors were categorized into five intrinsic subtypes with distinct clinical outcomes: luminal A, luminal B, HER2-overexpressing, basal-like, and normal-like tumors (Sørlie et al. 2001). This classification was later refined into four subtypes, which are currently in clinical use to provide important prognostic and predictive information for breast cancer.

2.3.1 Luminal subtypes

The name "luminal" derives from similarity in expression between these tumors and the luminal epithelium of the breast; they typically express luminal cytokeratins 8 and 18, ER, progesterone receptor (PR) and genes associated with ER activation such as LIV1and CCND (Kabel & Baali. 2015). At least two subtypes exist within luminal-like tumors, i.e., luminal A and luminal B. The luminal A and luminal B tumors each represents the [ER+|PR+] HER2- (tumors with ER or PR positivity and HER2 negativity) and [ER+|PR+] HER2+ (tumors with ER or PR positivity and HER2 positivity) subtype, respectively (Sørlie et al. 2003).

Luminal tumors are the most prevalent subtype of breast cancer, with luminal A being the most common among them. Luminal A tumors are characterized by high expression of luminal-specific genes (e.g. ER, GATA-binding protein 3 [GATA3], hepatocyte nuclear factor 3 alpha [HNF3A], X-box-binding protein 1 [XBP1]), low expression of the HER2 cluster of genes, and low expression of proliferation-related genes (e.g., cyclin B1, proliferation-associated antigen Ki-67) (Dai et al. 2015; Sørlie et al. 2001). This kind has the most favorable prognosis of all breast cancer subtypes and could be adequately treated with endocrine therapy. Whereas luminal

B tumors exhibit lower, though still present, expression of ER-related genes, variable expression of the HER2 cluster, and higher expression of the proliferation cluster. As a result, luminal B tumors have a poorer prognosis than luminal A and carry a high risk of recurrence (Bediaga et al. 2016).

2.3.2 HER2 over-expression tumors

The HER2-enriched subtype (previously defined by IHC as HER2+/ER- subtype) is characterized by high expression of the HER2 and proliferation gene clusters, along with low expression of luminal-associated genes. The HER2 over-expression tumors are characterized by over-expressing other genes in the HER2 amplicon such as GRB7 and PGAP3. 40% to 80% of these tumors harbor TP53 mutation. This subtype comprises only about half of clinically HER2-positive breast cancer. The rest have high expression of both the HER2 and luminal gene clusters and fall in a luminal subtype (Dai et al. 2015; Perou et al. 2000). HER2 overexpression breast tumors carry a poor prognosis but can still benefit from anthracycline and taxane-based neoadjuvant chemotherapy and targeted therapy: antiHER2 monoclonal antibody, trastuzumab (Perou et al. 2000) .

2.3.3 Basal-like tumors

The basal subtype consists of ER-, PR-, and HER2-negative (triple-negative) tumors and shares a gene expression profile similar to that of basal epithelial cells in other tissues and normal breast myoepithelial cells. (Sørlie et al. 2003). Such expression patterns include lacking or low expression of hormone receptors and HER2, and high expression of basal markers (such as keratins 5, 6, 14, 17, EGFR) and proliferation related genes (Dai et al. 2015). Basal-like tumors are often mixed up with triple negative tumors; the key distinction lies in the classification method; GEP for basal-like and IHC for TN (Sotiriou et al. 2003). Basal-like tumors are more probable to have low BRCA1 expression and harbor TP53 mutation. Clinically, they tend to follow an aggressive course, are often grade 3, and are more frequently diagnosed in younger patients and African-American women. Due to their triple-negative receptor status, basal-like tumors do not respond to conventional targeted breast cancer therapies, leaving chemotherapy as the primary treatment option (Dai et al. 2015).

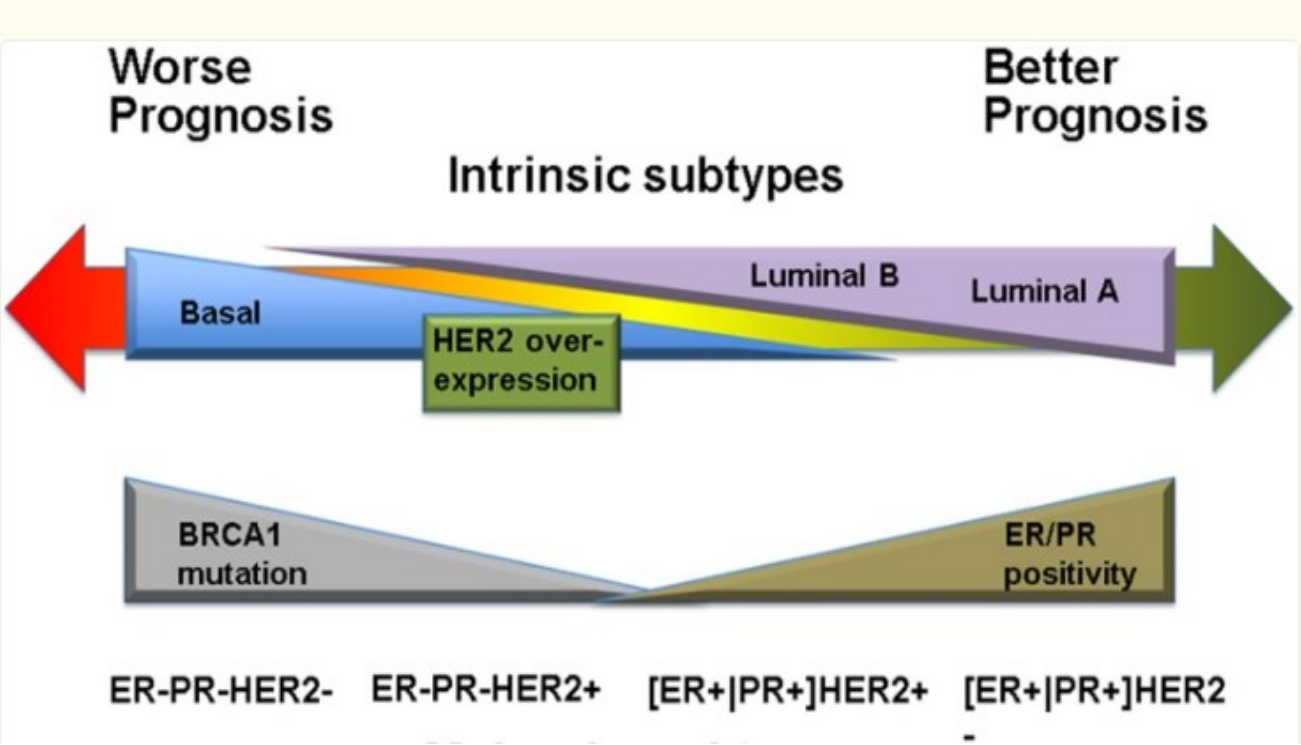


Figure 2. Molecular subtypes of BC. Adopted from (Dai et al. 2015)

2.4 Breast Cancer Immunology

2.4.1 Cancer immune editing and immune surveillance

The concept of cancer immune surveillance, initially proposed by Ehrlich in 1909 and later refined by Burnet in 1970, suggests that emerging tumors are continuously recognized and eliminated by the host immune system before clinical symptoms appear. However, the occurrence of malignant tumors in individuals with fully functional immune systems indicates that immune surveillance alone is insufficient (Muenst et al. 2016). Consequently, over the past 15 years, this concept has evolved into the broader theory of 'immune editing,' which encompasses both tumor elimination and immune system adaptation (Kim, Emi, and Tanabe 2007).

The current concept of cancer immune editing, which progresses from immune surveillance to immune escape, involves three key phases: (1) elimination, (2) equilibrium, and (3) escape. Elimination, central to the original idea of cancer immune surveillance, refers to the immune system's ability to successfully eradicate emerging tumor cells. Transformed cells express stress ligands and antigens that are recognized by both innate and adaptive immune cells, resulting in their destruction, mainly through CD8+ T cells and NK cells (Mittal et al. 2014; Kim, Emi, and Tanabe 2007).

When an evolving tumor is completely destroyed, it marks the end-point of immune editing through the elimination phase. However, if the tumor is not eradicated, it progresses to the equilibrium phase, where immune effector cells continuously eliminate transformed cells, but more resistant tumor variants selectively survive, maintaining a balance between the immune system and the tumor. In this phase, tumor cells persist in an immunocompetent host, remaining dormant under immune control (Mittal et al. 2014; Muenst et al. 2016). While demonstrating the equilibrium phase has posed technical challenges, some transplant studies have provided evidence for its existence. For instance, tumors can emerge following the unintentional transplantation of cancer from an organ donor with no clinical history of malignancy to an immunosuppressed recipient, leading to tumor outgrowth (Teng et al. 2008).

The mechanisms that disrupt the balance between the immune system and tumor cells, triggering the transition to the escape phase, remain unclear. In the final phase of immune editing, tumor cells exploit various immune escape mechanisms to evade immune control and proliferate uncontrollably, resulting in clinically detectable tumors (Wang et al. 2017; Mittal et al. 2014).

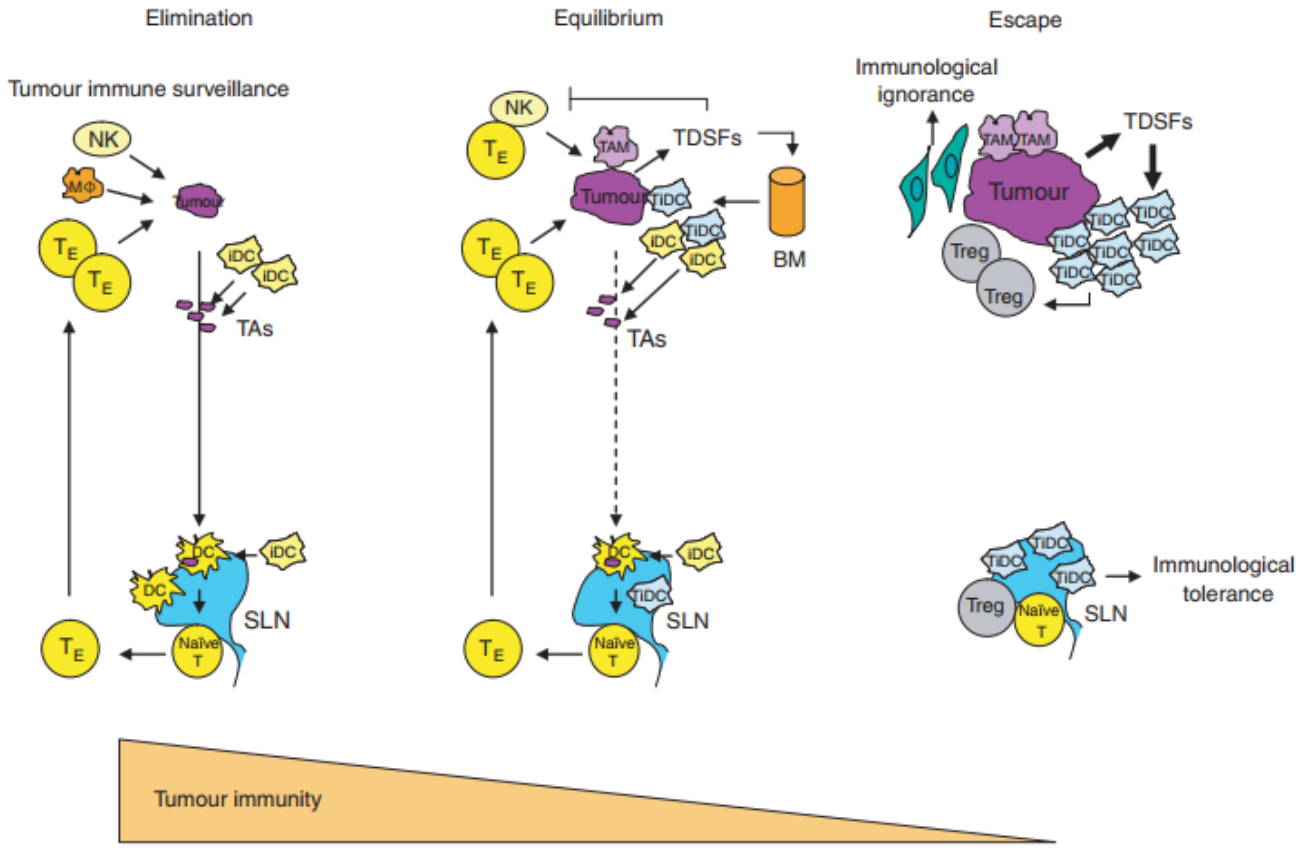


Figure 3. Schematic representation of cancer immune editing 3 phases: *iDC, immature dendritic cell; M/, macrophage; NK, natural killer; TE, effector T cell; TAs, tumour antigens; SLN, sentinel lymph node; TiDC, tumour-associated iDC; TAM, tumour-associated macrophage; TDSFs, tumour-derived soluble factors; Tregs, regulatory T cells; BM, bone marrow. Adopted from (*Kim, Emi, and Tanabe 2007)

2.4.2 Immune response to Breast cancer

2.4.2.1Immunological recognition of BC

The immune composition of normal breast tissue primarily consists of both myeloid and lymphoid immune cells, including CD8+ and CD4+ T lymphocytes, macrophages, and CD11c+ dendritic cells. These immune cells are predominantly localized within the lobular structures of the breast, where they play a critical role in protecting against potential pathogens and in monitoring for cellular abnormalities as part of immune surveillance (Goff and Danforth 2021).

During carcinogenesis however, the immune landscape of the breast undergoes significant changes. The immune landscape becomes heavily influenced by the surrounding TME marked by an influx of immune cells, which are recruited in response to inflammatory signals associated with disruptions in tissue homeostasis. This recruitment is primarily driven by soluble factors produced by either tumor cells or surrounding stromal cells. These factors include various chemokines, such as C-C motif chemokine ligands (CCL) and C-X-C motif chemokine ligands (CXCL1-17), which mediate the migration of innate immune cells into premalignant and malignant tissues (Stoll et al. 2018).

Immune cells within the TME recognize tumor-derived antigens, including tumor-associated antigens (TAAs) and neoantigens. TAAs are proteins that are produced by normal cells but are typically expressed at higher levels in tumors. In breast cancer, several TAAs have been identified, such as the human epidermal growth factor receptor 2 (HER2), mucin 1 (MUC1), carcinoembryonic antigen (CEA), human telomerase reverse transcriptase (hTERT), and the Wilms tumor gene (WT1) (Varn et al. 2017). Neoantigens, on the other hand, arise from unique somatic mutations. While BC is generally characterized by a lower mutational burden compared to melanoma and other cancers, making it to be historically characterized as ‘cold tumor’, a number of somatic mutations with BC specific neoantigens are currently being described. Neoantigens are particularly immunogenic because they result from mutations that generate proteins foreign to the body, eliciting a stronger immune response than TAAs, which resemble normal self-proteins (Sueangoen et al. 2024; X. Zhang et al. 2017).

In addition to these antigens, tumor cells also express danger-associated molecular patterns (DAMPs), such as adenosine triphosphate (ATP), annexin-1 (ANXA1), calreticulin (CALR), F-actin, and high-mobility group protein-1 (HMGB1), which make them visible to immune cells. The release of DAMPs is primarily driven by the metabolic and hypoxic stress within the

growing tumor, which induces necrotic cell death. Additionally, tumor cells can release DAMPs through stress-related pathways (Hernandez C, Huebener P. 2016; Stoll et al. 2018).

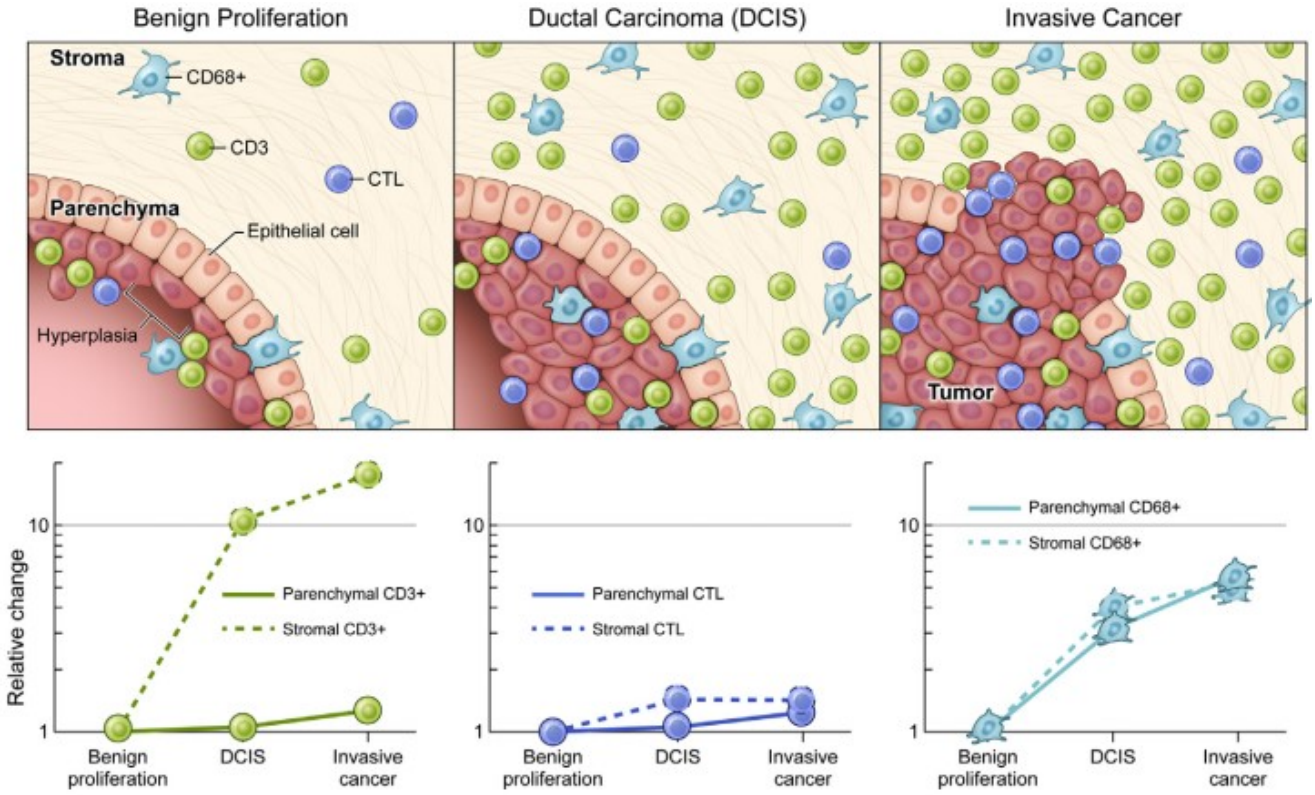


Figure 4. Immune cell content of benign, DCIS and breast cancer: *Adopted from (Goff and Danforth 2021)*

2.4.2.2. Immune cells in the TME and their anti-tumor role

Immune cells that traffic to solid tumors can exert profound influences on the clinical behavior of cancer. Tumor-infiltrating immune cells such as cytotoxic T lymphocytes (CTL), T-helper (TH) cells, natural killer (NK) cells and dendritic cells (DC) are generally known to effect anti-tumor immune responses that can limit tumor growth and progression, while others such as T-regulatory cells (T-reg), tumor associated macrophages (TAM) and myeloid derived suppressor cells (MDSC) are associated with pro-tumorigenic functions that disable anti-tumor immunity and facilitate cancer invasion and metastasis (Hernandez C, Huebener P. 2016).

DCs

DCs are specialized antigen-presenting cells (APCs) that act as a link between innate and adaptive immunity. In their immature state, DCs present self-antigens to promote tolerance. However, upon encountering an antigen, immature DCs become activated and mature, triggering their migration (Fucikova et al. 2019). Mature DCs detect tumor-derived antigens or DAMPs through pattern recognition receptors (PRRs). They then process these antigens and present them as peptide-major histocompatibility complex (MHC) complexes to naïve T cells in lymphoid tissues, effectively priming T cells (Marciscano AE 2021).

DCs are highly heterogeneous, and their immune responses depend on the distinct subsets of infiltrating DCs. Among these subsets, conventional DCs (cDCs) play a prominent anti-tumor role. The differentiation of cDCs from precursors is driven by the growth factor FMS-like tyrosine kinase-3 ligand (FLT3-L), produced by NK cells, while their recruitment to the TME is mediated by NK cell-derived chemokines, such as XCL1 and CCL5 (Marciscano AE 2021). cDCs execute their anti-tumor functions primarily by capturing and processing tumor antigens, which are cross-presented on MHC-I molecules to prime tumor-specific CD8+ T cells. Furthermore, cDC1 subsets enhance NK cell-mediated tumor killing by secreting cytokines like IL-12 and IL-15, demonstrating a key role in innate immunity (Fucikova et al. 2019). The importance of DCs in anti-tumor immunity has been demonstrated in mouse models. For instance, fibrosarcoma models lacking CD8α+ DCs exhibited impaired tumor rejection mediated by CD4+ and CD8+ T cells, highlighting the role of DCs in tumor immunity (Gonzalez, Hagerling, and Werb 2018; Lu et al. 2023). Clinically, increased levels of cDC1 in TNBC correlated with favourable responses to chemotherapy and combination therapy with immunotherapy (Zhang et al. 2021).

Plasmacytoid DCs (pDCs) on the other hand, are primarily involved in antiviral immunity but can contribute to immune tolerance in malignancies. This is achieved through the induction of programmed cell death protein 1 ligand 1 (PD-L1) or granzyme B expression and the regulation of Tregs within the TME (Marciscano AE 2021). The presence of pDCs has been linked to poor clinical outcomes, such as shorter overall survival and relapse-free survival, as well as more aggressive cancer subtypes, in breast cancer (Szpor et al. 2021; Treilleux et al. 2004).

NK cells

NK cells are innate lymphocytes that recognize and kill tumor targets directly upon activation with our prior sensitization. Downregulation of the MHC class I molecule by transformed cells is the initial trigger for activation of NK cells. Further binding of activating NK cell receptors; NKG2D, NKp46, NKp30, NKp44, and CD16 to transformed cells leads to their full activation (Guillerey, Huntington, and Smyth 2016). For example, attaching B7H6 and BAG to NKp30, NKG2D can recognise stress-induced ligands on tumour cells, MICA and MICB (MHC class I chain-related protein A or B), which are related to MHC I. MICA or MICB ligand interaction with NKG2D is a potent activating signal for NKs, that can lead to NK activation and consequently lysis of transformed cells via several mechanisms (Mamessier et al. 2011; Banu et al. 2020). NK cells release granules containing granzymes and perforin that induce apoptosis on target cells. Activated NK cells also mediate their anti-tumour role via death signal-associated receptors such as FAS and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), which induces lysis of transformed cells upon binding with its ligand on tumour cells (Razeghian et al. 2022).

A study conducted by Bouzidi and colleagues investigated CD56+ NK cells in 126 BC patients using immunohistochemical staining. The results showed that higher levels of CD56+ cells were significantly positively correlated with higher tumor grade, more aggressive breast cancer subtypes (such as TNBC and HER2+), as well as increased proliferation index (KI67%) and higher TIL levels. The median follow-up period was 5.5 years, and the study found that high expression of CD56 was associated with significantly longer OS and DFS compared to patients with low CD56 expression (Bouzidi et al. 2021). Additionally, another BC study reported that the downregulation of genes associated with NK cell surface receptors (NCR1/Nkp46, KIR, KLRC) and effector molecules (granzyme B and perforin) was significantly linked to resistance to taxane–anthracycline chemotherapy (Mariel et al. 2018).

T cells

T cells recognize TAA and neoantigens expressed by BC cells via MHC molecules on the surface of APCs or directly in tumor cells. The engagement of T cell receptor (TCR)-antigen-MHC leads to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the intracellular domains of the signaling subunits, CD3 complex resulting in signal transduction. Since full activation of T cells requires a comstimulatory signal, the interaction of CD28 on CD8+ or CD4+ T cells with CD80/B7.1 or CD86/B7.2, on APCs results in activation of T cells (Raskov et al. 2021).

Once activated, T cells exit the lymph nodes through the circulation to the TME to do their effector function. Successful trafficking of T cells to the tumor involves a cascade of events. Up on activation, T cells express the chemokine receptors; CXCR3 and CCR5 enabling them to respond to chemokine gradients. The chemokines CXCL9, CXCL10, and CXCL11, secreted by immune cells and stromal cells within the TME, play a pivotal role in directing T cell recruitment (Raskov et al. 2021). Binding of these chemokines to their respective receptors on T cells initiates the activation of integrins. Engagement of activated integrins with adhesion molecules expressed on tumor endothelial cells, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), and mucosal addressin cell adhesion molecule-1 (MadCAM-1), facilitates firm adhesion to the endothelium. This firm interaction consequently results in the extravasation of lymphocytes into the TME, enabling their infiltration into tumor tissue (Tian et al. 2024; Sackstein et al. 2017).

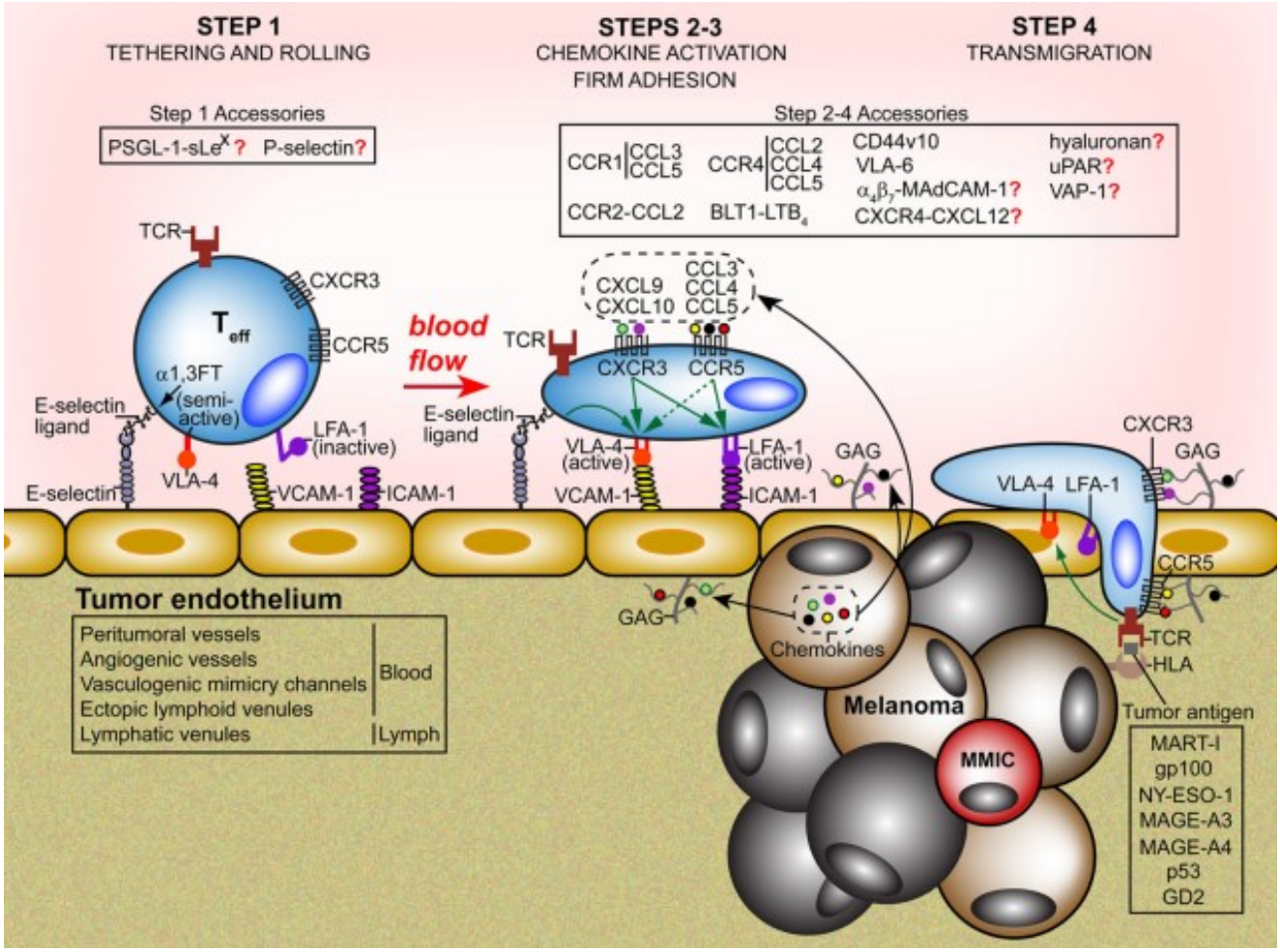


Figure 5. T cell homing to the tumor microenvironment. *Adopted from (Sackstein et al. 2017)*

CD4+ T cells

CD4 cells are central coordinators of the adaptive immune response being involved in anti-tumor immunity either directly or indirectly. As helper T cells, CD4+ T cells provide help to innate and adaptive immune cells. CD4+ T cells stimulate cells of innate immunity including NK cells, macrophages and eosinophils supporting anti-tumor immunity (Kravtsov et al. 2022). In adaptive immunity, CD4+ cells induce activation of CTLs via direct and indirect mechanisms. They directly enhance CTL function through interleukin-2 (IL-2) production, while indirectly induce and maintain cross-presenting DCs, which provide essential activation signals to CTLs. Moreover, CD4+ T cells are crucial for B cell maturation, providing CD40 ligand-mediated signaling that drives class switching and differentiation into plasma cells. Additionally, these cells secrete key cytokines, including interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), which exert direct anti-tumor effects (Tay, Richardson, and Toh 2021).

While CD4+ T cells are traditionally recognized for their supportive role in immune responses, emerging evidence suggests they also possess intrinsic cytotoxic capabilities, contributing directly to tumor cell elimination (Cenerenti et al. 2022). In a murine melanoma model, Quezada et al demonstrated the expansion of tumor-specific cytotoxic CD4+ T cells capable of recognizing tumor cells that upregulate MHC class II. These cells displayed elevated levels of granzyme B and acquire granzyme-dependent cytotoxicity. Notably, the combination of these cytotoxic CD4+ T cells with CTLA-4 blockade and radiation therapy led to effective tumor rejection (Quezada et al. 2010).

In a separate study, Cachot and colleagues performed an analysis of publicly available single-cell RNA sequencing data and identified cytolytic CD4+ T cells in various cancer types, including BC. Using hierarchical clustering of transcriptomic profiles, they identified four distinct CD4+ T cell subsets, one of which was associated with effector and cytolytic functions, characterized by the expression of perforin and granzymes (Cachot et al. 2021).

Additionally, another analysis of transcriptomic data from 1,083 BC patients in The Cancer Genome Atlas (TCGA) focused on five genes that define active CD4+ CTLs. Higher expression of CD4+ CTLs related genes were observed in younger patients, those with ductal carcinoma, and non-luminal subtypes. Further correlation analysis of immune cell infiltration revealed a positive correlation between CD4+ CTLs and CD8+ T cells, while an inverse relationship was found with M2 macrophages. A higher CD4+ CTL signature score was significantly associated with improved disease-free survival (DFS) and overall survival (OS) (Shohdy et al. 2022).

CD8+ T cells

The major effector cells of the immune system, CTLs are capable of directly inducing death in tumor cells via a number of effector molecules. CD8+ T cells play a pivotal role in antitumor immunity by directly targeting and eliminating malignant cells. They achieve this through the release of cytotoxic molecules such as perforin and granzymes, which induce tumor cell lysis. Additionally, CD8+ T cells secrete IFNγ, which enhances tumor cell susceptibility to immune attack by upregulating MHC class I expression and amplifies the activity of other immune cells involved in tumor surveillance (Maimela, Liu, and Zhang 2019; Giles et al. 2023).

A meta-analysis by Sun et al assessed the prognostic significance of CD8+ T cells in BC its correlation with pathological features in BC patients. The analysis included 14 studies involving 22,222 patients. The findings revealed that higher levels of CD8+ T-cell infiltration were strongly linked to improved overall survival and disease-free survival in breast cancer patients. Additionally, elevated CD8+ T-cell infiltration was associated with lower expression of hormone receptors, specifically ER and PR, and an increase in Her2 expression. No significant relationship was observed between CD8+ T-cell infiltration and clinicopathologic variables including age, tumor size, or lymph node involvement in breast cancer patients (Sun, Ke, and Li 2023).

In the largest study evaluating T cells as a tumor marker, Ali and colleagues enrolled 12,439 women with BC. They quantified the absolute numbers of immunoreactive intratumoral and stromal CD8+ T cells. In ER- tumors, specifically TNBC and HER2+ cases, the presence of both intratumoral and stromal CD8+ T cells was associated with a 28% and 21% reduction in breast cancer-specific mortality, respectively. Additionally, it was demonstrated that in ER-negative tumors, those with CD8+ T cells showed a greater benefit from chemotherapy, particularly anthracyclines, compared to CD8-negative tumors. In ER+ HER2+ tumors, the presence of intratumoral CD8+ T cells resulted in a 27% reduction in the hazard of dying from breast cancer (Ali et al. 2014).

Furthermore, a study investigating the function of CD8+ T cells in both BC and peripheral environments assessed the expression of checkpoint molecules such as PD-1, TIGIT, 2B4, BTLA, TIM-3, LAG-3, and CD160, as well as their degranulation capacity in antigen-experienced (CD45RA-) CD8+ T cells. The results revealed that effector CD8+ T cells predominantly expressed PD-1, TIGIT, and 2B4, but they retained their polyfunctional abilities, continuing to produce effector cytokines and perform degranulation in both the TME and the periphery (Egelston et al. 2018).

γδ T cells

In addition to conventional T lymphocytes, there exists a rare subset of T cells known as γδ T cells, which possess a distinct T cell receptor (TCR) composed of γ and δ chains. Unlike typical T cells, γδ T cells do not express the CD4 or CD8 co-receptors. These cells recognize antigens in a manner that is independent of MHC molecules and express the NK cell receptor, NKG2D

(Lafont et al. 2014). γδ T cells have been extensively studied for their anti-tumor roles. They can directly target tumor cells through the perforin-granzyme pathway and death ligands such as TRAIL and FasL. Additionally, they exert indirect anti-tumor effects by producing cytokines like IFN-γ and TNF-α, or by expressing CD40L, which promotes DC maturation. γδ T cells also play a role in activating other anti-tumor immune cells, including NK cells (Yijing Zhao, Niu, and Cui 2018; Schönefeldt et al. 2021; Lafont et al. 2014).

However, more recent studies have highlighted their protumorigenic potential as well. γδ T cells have been identified as major sources of IL-17, a cytokine involved in angiogenesis and the recruitment of MDSCs to the TME. Under certain conditions, such as the presence of TGF-β and IL-15, γδ T cells can be polarized into FOXP3+ γδ Treg cells. These FOXP3+ γδ Treg cells function similarly to αβ Treg cells, suppressing anti-tumor immunity and facilitating tumor immune evasion (Yijing Zhao, Niu, and Cui 2018).

In the context of BC, γδ T cells have been linked with both anti-tumor and protumorigenic immune responses. A study by Song and colleagues examined the relationship between peripheral γδ T cells and clinicopathological features of BC. They found a significant correlation between reduction in γδ T cell population and poor clinical outcomes; lower numbers of γδ T cells in higher histological grade tumors, estrogen receptor-negative (ER-) BC, increased Ki-67 proliferative index, and lymph node metastasis (Song, Wei, and Li 2022a). In another randomized, double-blind phase II GeparNuevo trial involving 63 TNBC patients, the role of γδ T cells in response to neoadjuvant chemotherapy and immunotherapy was investigated. Notably, the expansion of γδ T cells in peripheral blood was significantly associated with a complete pathological response to the checkpoint inhibitor durvalumab (Massa et al. 2024).

In contrast, Coffelt and colleagues demonstrated the tumor-promoting function of intratumoral γδ T cells in mammary tumor-bearing K14cre; Cdh1F/F;Trp53F/F (KEP) mice. They found that depletion of γδ T cells significantly reduced pulmonary and lymph node metastasis in these mice. Further functional analysis revealed that γδ T cells produce IL-17, which induces systemic expansion and polarization of CD8+ T cell-suppressive neutrophils, contributing to tumor progression (Coffelt et al. 2015).

Clinical implication of Tumor infiltrating lymphocytes (TIL)

Although the immune system may not fully eliminate clinically detectable tumors, the presence of immune cell infiltration in the TME suggests the potential for immunological memory to control residual disease. Evidence supports the idea that lymphocytic infiltration serves as a marker of the host's anti-tumor immune response (Ino et al. 2013). Numerous studies highlight the clinical importance of TILs in predicting treatment response, including chemotherapy and immunotherapy, as well as overall prognosis. Immune cell infiltration, particularly by lymphocytes, has been associated with improved survival outcomes in several cancers, such as colon, lung, ovarian, and breast cancer (Valenza et al. 2023; Stanton and Disis 2016).

Although breast cancer was once thought to be an immunocold tumour, it has now been shown that tumour-infiltrating lymphocytes are an invariable feature of BC, with immune cells present in all breast cancers examined (Carsten Denkert et al. 2010). However, the extent of immune infiltration and its clinical significance varies greatly within the BC subtypes. Triple-negative and Her2+ BCs are characterized by an abundance of tumour-infiltrating immune cells and the highest clinical benefit. Breast cancers with >50 % lymphocytic infiltrate are known as lymphocyte predominant breast cancer (LPBC) and exhibit the greatest clinical benefit. LPBC is more common in both TN and HER2+ breast cancers (Stanton and Disis 2016)

The initial evidence of TILs' role in predicting treatment outcomes comes from Denkert et al., who analysed 1,058 samples from two prospective, randomized clinical breast cancer trials. They assessed the predictive role of infiltrating lymphocytes to neoadjuvant chemotherapy (anthracycline/taxane). In a multivariate analysis of all other parameters suggested to be predictors of therapy response, the level of lymphocyte infiltration was found to be an independent predictor of pathologic complete response (pCR). LPBC had pCR rates of 42% whereas tumors without any infiltrating lymphocytes had pCR rates of 3% (Carsten Denkert et al. 2010).

A meta-analysis conducted by Wang et al., involving 13,100 patients with primary invasive cancer, demonstrated a correlation between TILs and high histological grade. Regarding the histological subtypes of breast cancer, TILs exhibited an inverse correlation with ER and PR expression, while no significant association was found with HER2 expression. Additionally, high TIL expression was identified as a strong predictor of pCR. Subtype analysis revealed that CD8+

TILs were significantly associated with improved pCR rates, whereas CD4+ TILs and the Foxp3 subgroup showed no such correlation. Furthermore, elevated TIL levels were significantly linked to improved DFS (K. Wang et al. 2016).

By subtype analysis, Denkert et al demonstrated that higher levels of TILs are associated with better response to neoadjuvant chemotherapy and improved survival in TNBC. Research from the German Breast Cancer Group found that patients with higher TIL levels had a greater likelihood of achieving pCR; pCR achieved in 80 (31% of 260 patients) 117 (31% of 373) and 136 (50% of 273) with low, intermediate and high TILs respectively. Additionally, TILs were identified as a predictive factor, with an increase in TILs correlating with prolonged DFS and OS (C. Denkert et al. 2018). Similarly, TILs had both a predictive and prognostic role in Her2+ tumors. Patients with stromal TIL (sTIL) > 60% (LPBC phenotype) had higher pCR rates compared to the non-LPBC phenotype. Additionally, an increase in sTIL levels was linked to improved OS. However, the clinical relevance of TIL quantification in HR+ HER2- tumors remains uncertain (Valenza et al. 2023).

2.4.3 Immune Escape Mechanisms in Breast Cancer

In spite of rigorous immune surveillance, the immune system doesn't always protect from carcinogenesis and at times might be involved in it. Breast cancer cells utilize multiple and complex mechanisms to fully escape immune surveillance.

2.4.3.1 Abnormal expression of major histocompatibility complex class I

An important mechanism BC cells utilize to surpass immune recognition is via defective antigen processing and presentation such as down regulation of the expression of MHC class I complex, and other proteins involved in antigen processing, loading, transport, and presentation which is necessary to generate a robust immune response (Wang et al. 2017).

A study conducted by Fang et al. analysed TCGA and GSE32646 breast cancer datasets and found that low MAL2 expression was associated with enhanced CD8+ T cell cytotoxicity in TNBC with high tumor-infiltrating T cell levels. MAL2, a transmembrane protein involved in protein endocytosis, was identified as a suppressor of CD8+ T cell activity, as its in vivo knockdown (KD) led to increased cytolytic granule (GZMB) and cytokine (IFN-γ, TNF-α) production, while overexpression (OE) reduced these responses. Further investigations using confocal imaging and membrane protein analysis demonstrated that MAL2 downregulates MHC-

I expression, with KD increasing MHC-I presence on the cell surface and OE leading to its suppression. The study identified the underlying mechanism of reduced CD8 cytotoxicity as posttranslational downregulation of MHC-I through endocytosis-mediated degradation, emphasizing the role of MAL2 in tumor immune evasion (Fang et al. 2021). In another study, Pedersen et al evaluated the expression of differentially expressed proteins between recurrence versus recurrence-free TNBC patients followed for 10 years. They found that downregulation of MHC class I-related proteins, including TAP1, TAP2, CALR, HLA-A, ERAP1, and TAPBP, was enriched in patients with recurrence. This downregulation was associated with recurrence and negatively correlated with RFS and OS (Pedersen et al. 2017).

In addition, aberrant expression of human leukocyte antigen-G (HLA-G), a nonclassical HLA class I antigen, has been observed in various malignancies, including BC, and is strongly associated with tumor immune escape. HLA-G could lead to tumor evasion by inhibition of immune cell cytolysis, inhibition of cytokine production, induction of immune cell apoptosis, expansion of suppresser cells and by impairment of chemotaxis (Lin and Yan 2015). Several HLA-G receptors have been identified on innate and adaptive immune cells, such as immunoglobulin-like transcript 2 (ILT2), ILT4/CD85d, killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4), CD8, and CD160. Engagement of these receptors with HLA-G leads to immune cell inhibition (Jeong et al. 2014). In Her2+ breast cancer, the binding of HLA-G to KIR2DL4 impairs the cytotoxicity of NK cells in the TME, resulting in decreased IFN-γ production. Furthermore, the HLA-G/KIR2DL4 interaction can contribute to resistance to trastuzumab in vivo by preventing NK cell-mediated killing (Zheng et al. 2021). In another study, soluble HLA-G (sHLA-G) levels in BC were correlated with clinicopathologic features predicting a worse prognosis. Serum sHLA-G levels were significantly higher in BC patients than healthy controls. Significantly elevated sHLA-G levels were also observed in advanced pathologic stages and metastatic patients (Jeong et al. 2014).

2.4.3.2 Defective IFN signaling

Three types of interferons have been identified: Type I IFN (IFN-α and IFN-β), Type II IFN (IFN-γ), and Type III IFN (IFN-λ). Type I and Type II interferons are particularly well-known for their anti-tumor properties. Type I IFN is primarily produced by various innate immune cells, while Type II IFN (IFN-γ) is produced by NK cells and T lymphocytes (Shi et al. 2022; Saleiro and Platanias 2019). When these interferons bind to their respective receptors, they activate the

JAK-STAT signaling pathway, leading to the expression of interferon-stimulated genes (ISGs). These ISGs are responsible for the effector functions of interferons, which directly combat tumors by inducing apoptosis, necroptosis, and growth arrest through senescence. IFNs also upregulate the expression of MHC I in tumors increasing their immunogenicity. Additionally, interferons promote tumor elimination indirectly by stimulating both innate and adaptive immune cells to enhance their anti-tumor activity (Han, Wu, and Liu 2023).

However, defects at various molecules in the IFN signaling pathway can hinder the effectiveness of IFNs in tumor suppression. This was demonstrated in BC, where the expression of five ISGs was significantly downregulated in the peripheral blood of BC patients compared to healthy controls. To pinpoint the location of the defect, levels of pSTAT1 (the phosphorylated form of STAT1) were measured in peripheral blood through Phosflow following IFN-α and IFN-γ stimulation. The results showed that pSTAT1 induced by IFN-α was reduced in T, B, and NK cells, while pSTAT1 induced by IFN-γ was reduced specifically in B cells but not in T or NK cells. This study further revealed that the downregulation of ISGs and pSTAT1 was not stage-dependent, suggesting that the dysregulation of IFN signaling occurs early in carcinogenesis, potentially affecting tumor suppression before the later stages of cancer progression (Critchley-Thorne et al. 2009).

In ER+ BC, Cao et al. found that ER+ tumors exhibited significantly lower ISG scores compared to ER- tumors, based on TCGA BRCA data analysis. In a BC cell line, they demonstrated that overexpression of ERα inhibited IFN-β-induced STAT1/2 activation, leading to a downregulation of ISGs like *IFIT1* and *IFI44*. In contrast, ERα deficiency promoted the transcription of downstream genes in response to IFN-β. To further explore how ERα inhibits type I IFN-induced signaling, they identified genes induced by ERα signaling, with H2A.Z expression emerging as a key factor. H2A.Z restricts the engagement of ISGF3 with the ISRE, thereby limiting IFN signaling. Additionally, the study showed that ERα directly interferes with IFN signaling by binding to STAT2 instead of STAT1 and IRF, disrupting the formation of the ISGF3 complex. Finally, they demonstrated that an ERα antagonist, FUL, could restore the antitumor effects of type I IFNs. In the presence of FUL, type I IFN was able to suppress tumor growth in both a BC cell line and a xenograft model (Cao et al. 2023).

2.4.3.3 Upregulation of Immune check points

Immune checkpoints are inhibitory receptors expressed under physiological conditions to maintain self-tolerance, prevent autoimmunity, and regulate excessive immune responses, thereby minimizing tissue damage. Immune checkpoints of clinical relevance in BC include cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), programmed death receptor 1/programmed cell death ligand 1 (PD-1/L1), lymphocyte activation gene 3 (LAG-3), T cell immunoglobulin domain and mucin 3 (TIM-3) (Zhang et al., 2023).

CTLA-4 (CD152) is predominantly expressed on activated T cells, where it interacts with its ligands, CD80 (B7-1) and CD86 (B7-2), on APCs. CTLA-4 shares these ligands with CD28 but exhibits a significantly higher binding affinity, allowing it to outcompete CD28 and inhibit T-cell activation. This competitive interaction suppresses lymphocyte proliferation by limiting co-stimulatory signaling. Additionally, CTLA-4 actively mediates immune inhibition by facilitating the removal of CD80 and CD86 from the APC surface through reverse endocytosis (Zhang et al. 2023). In regulatory T cells (Tregs), where CTLA-4 is constitutively expressed, its engagement with ligands further enhances their immunosuppressive function (Jama, Tabana, and Barakat 2024).

In vitro studies have demonstrated that co-culturing BC cell lines expressing CTLA-4 with APCs leads to the downregulation of key immune-activating molecules, including HLA-DR, CD83, CD40, CD80, and CD86 (Chen et al. 2017). However, compared to malignancies such as melanoma, the clinical implications of CTLA-4 in BC remain less well-characterized. For instance, a study by Erfani et al. reported significantly elevated serum levels of soluble CTLA-4 (sCTLA-4) in BC patients compared to healthy controls, suggesting its potential clinical utility (Erfani, N, Razmkhah, and Ghaderi, M 2010). Additionally, according to a systematic review by Kern et al. many studies indicated increased CTLA-4 expression in tumor-resident Tregs and associated lower CTLA-4 levels with better OS and DFS, highlighting its prognostic significance (Kern and Panis 2021). In contrast, Montoyo-Pujol et al outlined a significant positive correlation between high CTLA-4 expression and longer DFS in Her2+ BC (Montoyo-Pujol et al. 2024).

Another inhibitory receptor that tumor cells exploit to evade immune destruction is PD-1. Under normal physiological conditions, PD-1 is expressed on lymphocytes, where it prevents excessive T-cell stimulation. Its ligand, PD-L1, is typically expressed at low levels in most cells. However,

tumors hijack this mechanism by upregulating PD-L1 expression. Studies have shown that BC cells express high levels of PD-L1 (Lin et al., 2024; Steven & Seliger, 2018). The interaction between PD-1 and PD-L1 exerts multiple immunosuppressive effects on T and NK cells, including impaired proliferation and differentiation, reduced effector cytokine production, increased apoptosis in T lymphocytes, and diminished degranulation and cytotoxicity in NK cells (Lin et al., 2024). Furthermore, PD-1/PD-L1 engagement has been shown to induce regulatory T cells (iTregs) and enhance their suppressive potential (Francisco et al. 2009).

A study by Liu et al. analyzed 2,994 BC cases from the TCGA and METABRIC datasets, revealing a significant association between PD-1 expression and clinicopathologic variables. PD-1 was correlated with hormone receptor-negative (HR-) BC in both cohorts and with HER2+ BC in the METABRIC cohort. Additionally, PD-1 expression strongly correlated with immune cell populations in the TME, including T cells, B cells, NK cells, monocytic lineage cells, and myeloid dendritic cells. The study also reported co-upregulation of PD-1 with other immune checkpoints, such as PD-L2, CD274 (PD-L1), CTLA-4, IDO1, and LAG-3, indicating the presence of multiple inhibitory pathways in T-cell activation. While the prognostic role of PD-1 in BC has been debated, PD-1 was identified as an independent predictor of survival after adjusting for other prognostic factors (Liu et al., 2020).

In another study, immunohistochemical analysis of PD-1 and PD-L1 expression in primary tumors and metastatic lymph nodes revealed a significant correlation between PD-L1 expression in lymph nodes and markers of aggressive disease, including a high proliferation index (Ki-67%), an increased number of metastatic lymph nodes, and advanced pathological stage. However, no significant association was observed between primary tumor PD-1/PD-L1 levels and poor prognostic features. Furthermore, PD-1 and PD-L1 expression did not significantly differ among BC intrinsic subtypes (Yuan et al. 2019)

2.4.3.4 Immune suppressive cytokines and molecules

Tumor growth factor-Beta (TGF-β)

TGF-β are a large family of cytokines widely expressed across various cell types, playing a crucial role in regulating cell proliferation, differentiation, apoptosis, and immune responses (Deng et al. 2024). Its role in cancer is particularly complex, as it has been implicated in both tumor suppression and promotion acting as a double edge sword, a phenomenon known as

“TGF-β paradox”. In early phases of carcinogenesis, it is anti-tumorigenic: induces apoptosis via modulation of Bcl2, enhances cell cycle arrest and autophagy in tumor cells. It also activates tumor suppressor genes, limiting malignant progression. Later in carcinogenesis however, it promotes tumor development via a number of immune and non-immune mechanisms. It aids in cell proliferation, epithelial-to-mesenchymal transition (EMT), degradation of extracellular matrix (ECM), promotion of metastasis and modulation of immunity (Zarzynska 2014; Baba et al. 2022).

A key aspect of TGF-β’s impact on cancer progression is its ability to suppress anti-tumor immunity. Tumor-derived TGF-β, as well as that produced by Tregs, has been linked to immune evasion mechanisms. Slattery et al. demonstrated that NK cells from patients with metastatic BC exhibited impaired function and metabolism, characterized by decreased cytokine production, particularly IFN-γ, and reduced cytotoxicity. Interestingly, these defects were reversed when NK cells were co-cultured with anti-TGF-β antibodies, highlighting TGF-β’s role in dampening immune surveillance (Slattery et al. 2021). Additionally, in a mammary tumor model, TGF-β derived from Tregs was found to upregulate IL-17RB, with further analysis showing a correlation between IL-17RB expression and tumor burden in mice, reinforcing its clinical relevance (Huang et al. 2017).

Beyond NK cell dysfunction, TGF-β also contributes to CD8+ T cell exhaustion, further weakening anti-tumor immunity. Extracellular vesicles (EVs) containing TGF-β type II receptor (TβRII+) derived from metastatic breast cancer cell lines were shown to induce CD8+ T cell dysfunction in a mouse model. When compared to EVs lacking TβRII, wild-type EVs significantly reduced TIL cytotoxicity, leading to lower IFN-γ and GZMB expression. Additionally, exhaustion of CD8+ T cells was demonstrated in mouse bearing wild-type EVs. These cells displayed increased inhibitory receptors such as PD-1, TIM-3, and LAG-3, alongside diminished proliferative capacity. Further mechanistic analysis revealed that TGF-β/SMAD signaling activated the transcription factor TCF-1, which in turn induced the expression of exhaustion-associated genes in T cells (F. Xie et al. 2022).

Interleukin-10 (IL-10)

IL-10 is an immunoregulatory cytokine essential for maintaining immune homeostasis. Its role in many cancers including breast remains controversial, as it predominantly exerts immunosuppressive effects while also displaying anti tumor and anti-angiogenic properties (Sheikhpour et al. 2017; Al-Ameri et al. 2020). IL-10 is produced by immune regulatory cells including Treg and TAM which mediate their pro-tumor role partly via secretion of IL-10 in to the TME. IL-10 has been linked to induction of DC dysfunction by antagonizing the expression of CD-80 and CD-86, impairment of the effector function of T lymphocytes via inhibition of production of IFN-γ, TNF-α, inhibition of NK cells and induction of Tregs (Mirlekar 2022; Al-Ameri et al. 2020). On the other hand, IL-10 has been associated with anti-tumor effects, such as promoting B cell differentiation into antigen-producing plasma cells and enhancing antigen-specific proliferation of CD8+ T cells (Fujii et al. 2001; Chang et al. 2021).

This dual nature of IL-10's function is reflected clinically in BC, where elevated levels of IL-10 have been associated with conflicting outcomes in patients. For example, a study by Lv et al. found that BC patients exhibited higher serum IL-10 levels compared to healthy individuals, with IL-10 levels being particularly elevated in patients with hormone receptor-negative tumors and advanced clinical stages, linking it to poor prognostic features (Lv et al. 2018). In another study, IL-10 produced by macrophage was shown to inhibit IL-12 production by DC. DCs have been displaying elevated 1L-10 receptor and its blockage with monoclonal antibody resulted in significant tumor suppression in tumor bearing mice during treatment with the chemotherapy, paclitaxel. Additionally, using BC TCGA data, they showed that increased IL-12 expression was associated with chemotherapy response, supporting the tumor-suppressive role of IL-10 (Ruffell et al. 2014).

Conversely, in a study including 1,380 early-stage invasive BC patients, IL-10 was correlated with lower tumor grade, lower Nottingham Prognostic Index (NPI) values, hormone receptor positivity, and Her-2 negativity. Analysis of survival outcomes indicated that high IL-10 expression was associated with a longer DFS, with a mean DFS of 166.1 months for patients with high IL-10 levels, compared to 150.3 months in those with lower IL-10 expression. However, IL-10 expression was not found to be an independent prognostic factor for DFS (Ahmad et al. 2018).

2.4.3.5 Recruitment of immune suppressive cells to the TME

Myeloid derived suppressor cells

MDSC are a population of early myeloid cells that are expanded in various disease states including cancer and are capable of suppressing the immune response. These cells originate from bone marrow progenitors and exhibit diverse maturation markers influenced by tumor-derived factors such as vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF) (Ochoa et al. 2007). Activated MDSC produce arginase 1 (ARG1), inducible nitric oxide synthase (NOS2), IDO (indoleamine 2,3-dioxygenase), NADPH oxidase and immunosuppressive cytokines that have the potential to inhibit CTLs, DCs, and NK cells as well as expand $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells. This leads to an immunologically permissive tumor microenvironment (Wesolowski, Markowitz, and Carson 2013).

For many cancers, peripheral blood MDSC levels correlate with a higher tumor burden and a worse prognosis (Ko et al. 2009; Gabitass et al. 2011; Montero et al. 2012). For example, Gabitass et al. reported that a higher percentage of MDSCs was linked to an increased risk of mortality. In a multivariate analysis, MDSC levels were identified as an independent prognostic factor for survival. Specifically, a one-unit increase in MDSC percentage corresponded to a 22% higher risk of death in patients with pancreatic, esophageal, and gastric cancers (Gabitass et al. 2011). Similarly, increased peripheral blood MDSC level was demonstrated in patients with BC in a stage dependent manner. In a study by Diaz-Montero et al. (2009) the percentage of whole blood MDSCs in patients with early stage (I/II) breast cancer was 1.96%. In stage III and IV, these levels increased to 2.46% and 3.77%, respectively. MDSC levels greater than 25% were also reported in some patients with stage IV cancers. Similarly, a pilot study of 25 subjects with metastatic breast cancer showed that patients with higher-than-average levels of peripheral blood MDSCs following palliative systemic therapy had shorter overall survival (Markowitz et al. 2013).

T-regulatory cells (T-reg)

Because cancer originates from normal cells, the same immune mechanisms that lead to self-tolerance also restrict the development of an efficient antitumor immune response. These immunoregulatory mechanisms include Tregs as well as expression of immune checkpoints and enable malignant tumors to escape immune control and proliferate. Under physiological circumstances, Tregs protect against autoimmune diseases by suppressing self-reactive T cells while in the TME, the presence of Tregs may result in inhibition of effective antitumor immune responses (Allen and Jones 2011). T-regs expressing CCR4 are recruited to the tumor site by CCL22 released by tumor cells and macrophages. In the TME, Tregs become activated through the recognition of TAAs or self-antigens released by dying tumor cells. Once activated and expanded, Tregs are able to selectively suppress the activation of TAA-specific effector T cells through various mechanisms, including IL-10 and TGF-β production, and consequently prevent tumor destruction (Nishikawa and Sakaguchi 2014; Tanaka and Sakaguchi 2017).

In BC patients, an increase in the number of activated tumor-infiltrating Tregs is shown to be associated to lack of CD8+ T cell response to Her-2. In the non-metastatic setting, patients with low level of Tregs along with elevated CD8+ T cell response to Her-2 had an overall survival rate of 100%. Whereas when both metastatic and non-metastatic patients are considered together, patients with low Treg levels and high a CD8+ T cell response to Her-2 had a significantly increased overall survival than patients with high Treg levels and low Her-2 specific CD8+ T cell count (Bailur et al. 2015). In another study in BC, higher infiltration of Treg in to the TME, was correlated to several prognostic parameters, including high proliferation and negative ER. Additionally, in both univariate and multivariate analysis, Treg infiltration was found to be correlated with breast cancer specific survival (BCSS) and an independent prognostic factor for BCSS. Furthermore, high infiltration of Treg was also correlated with worse 2-year post-recurrence survival PRS (Stenström, Hedenfalk, and Hagerling 2021).

Tumor associated macrophages (TAMs)

In mature adults, macrophages originate from peripheral blood monocytes and differentiate based on their microenvironment and stimuli, leading to distinct functional phenotypes. Two primary subsets have been identified: classically activated (M1) and alternatively activated (M2) macrophages. M1 macrophages are induced by the Th1 cytokine IFN-γ, either alone or in combination with bacterial components like lipopolysaccharide (LPS) or cytokines such as tumor necrosis factor-α (TNF-α). These macrophages produce proinflammatory cytokines, chemokines, and effector molecules. In contrast, M2 macrophages arise in response to different stimuli and can be further classified into M2a, M2b, and M2c subsets. M2a macrophages are activated by Th2 cytokines IL-4 or IL-13, while M2b macrophages are stimulated by immune complexes (ICs), LPS, toll-like receptors (TLRs), or the IL-1 receptor antagonist (IL-1ra). M2c macrophages, on the other hand, are induced by IL-10, TGF-β, or glucocorticoids. These M2 macrophages predominantly exhibit anti-inflammatory properties, producing molecules such as IL-10, TGF-β, and arginase-1 (Qiu et al. 2018; Sindrilaru et al. 2011).

TAMs exhibit an M2-like phenotype, driven by various chemokines and cytokines within the TME. Factors such as IL-4, IL-13, TGF-β, VEGF, platelet-derived growth factor (PDGF), and IL-10, along with chemokines including CCL3, CCL4, CCL5, CCL7, CCL8, and CXCL12, have been reported to promote macrophage recruitment (Hao et al. 2012). It has been shown by various studies that TAMs are causally involved in various aspects of cancer and mediate their effects via both non-immune and immune mechanisms. TAMs suppress anti-tumor immunity by inhibiting M1 macrophage-mediated innate immune responses and impairing T cell activation. Additionally, TAM-derived IL-10 and CCL22 recruit regulatory T (Treg) cells, which further suppress T cell activation. Secretion of prostaglandin E2 (PGE 2) and TGFβ by TAMs further contribute to immune suppression. TAMs also can cause T cell apoptosis through their expression of PD-L1, which binds to its receptor PD-1 on activated T cells (Gabrilovich, Ostrand-Rosenberg, and Bronte 2013).

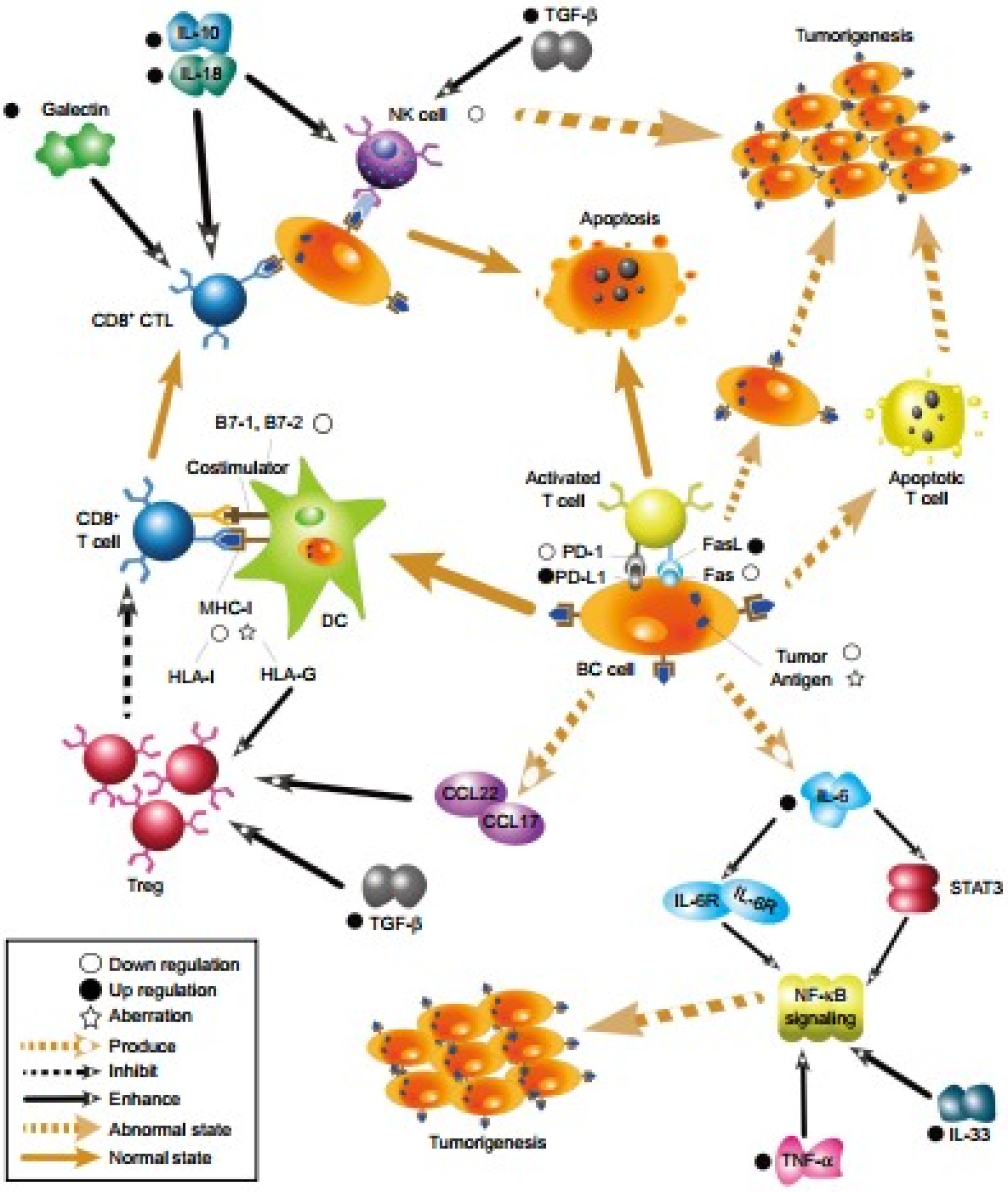


Figure 6. The ifferernt immune escape mechanisisms in breast cancer. *Adopted from (Wang et al. 2017).*

2.4.4 Immune therapeutic approaches for the treatment of breast cancer

The goal of cancer immunotherapy is to activate a patient's immune system or to reverse underlying immune escape to recognize and kill their tumors (Adams et al., 2014). In the past, breast cancer was assumed to be nonimmunogenic, limiting the role of host immune response to the tumor (Naji et al. 2024). However, the data available from studies suggest that breast cancer, especially the more aggressive subtypes of Her2-positive and TNBC, does elicit host antitumor immune responses. Therefore, it is expected that not all BC patients would benefit from the same immunotherapeutic strategy to restore or elicit an anti-tumor immune response (Polk et al. 2018).

2.4.4.1 Immune check point inhibitors (ICI)

Over a decade ago, checkpoint inhibitors were successfully developed for highly immunogenic cancers like melanoma. Breast cancer, which was historically regarded as an "immune cold" tumor, has seen significant advancements in immunotherapy. Recent immunophenotyping and transcriptomic analyses have revealed that breast cancer exhibits considerable heterogeneity in terms of immune response; subtypes such as TNBC and HER2-positive tumors demonstrate higher levels of TILs and increased expression of immune checkpoint molecules. This has facilitated the development of check point inhibitors, especially for TNBC and HER2-positive breast cancer. Currently, antibodies targeting the T-cell inhibitory molecules pD-1 or its ligand, pD-L1 and CTLA-4 are being actively explored for BC treatment.

Among these, PD-1 is the most studied co-inhibitory receptor, and several monoclonal antibodies (mAbs) targeting PD-1 or PD-L1 are in clinical development. Atezolizumab, the first ICI approved for TNBC, demonstrated its potential in improving DFS when combined with chemotherapy in the IMpassion130 trial. However, due to the lack of long-term clinical benefits, its approval was later withdrawn (Debien et al. 2023). Pembrolizumab (Keytruda), an IgG4 monoclonal antibody targeting PD-1, gained FDA approval in 2021 for use in TNBC as a neoadjuvant treatment in combination with chemotherapy, as well as for monotherapy in the adjuvant setting. This approval was based on the positive results from the phase 3 KEYNOTE-522 trial. The final OS results from this trial, with a median follow-up of 75.1 months, showed that patients receiving pembrolizumab in combination with chemotherapy had a statistically significant improvement in OS compared to those receiving chemotherapy alone (Schmid et al. 2024).

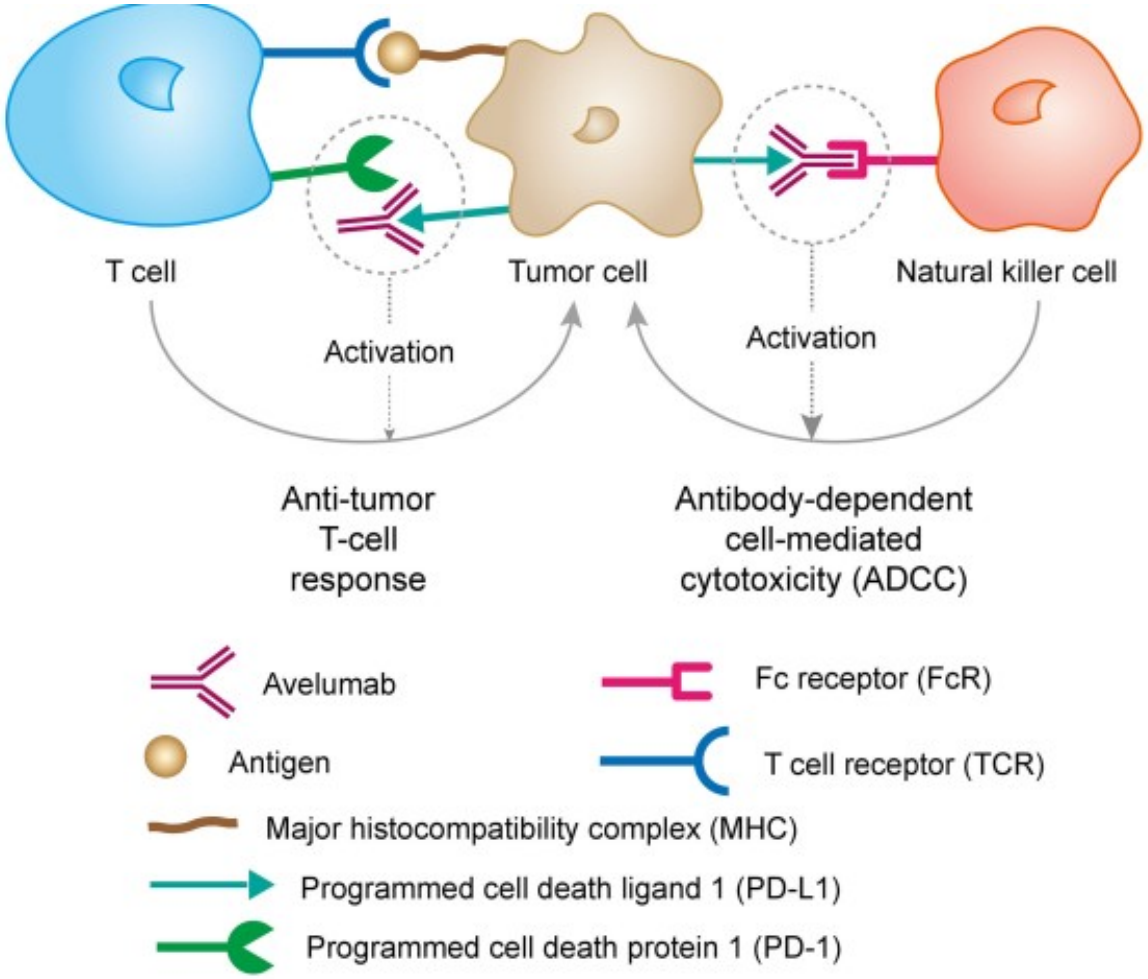


Figure 7. Mechanisism of action of Avelumab. *Adopted from (Zhang et al. 2023)*

Anti-CTLA-4 antibodies on the other hand function by preventing the endocytosis of costimulatory molecules in DCs, thereby enhancing their role in T cell priming. Additionally, these antibodies deplete Tregs and BC cells that express CTLA-4 through antibody-dependent cytotoxicity (ADCC) by macrophages (Jama, Tabana, and Barakat 2024). Ipilimumab and tremelimumab are the primary anti-CTLA-4 antibodies currently being investigated in clinical trials, either as monotherapies or in combination with anti-PD-1 antibodies and chemotherapy. These therapies are being explored in phase I and II clinical trials for various types of breast cancer (Mcarthur et al. 2019; Zhang et al. 2023).

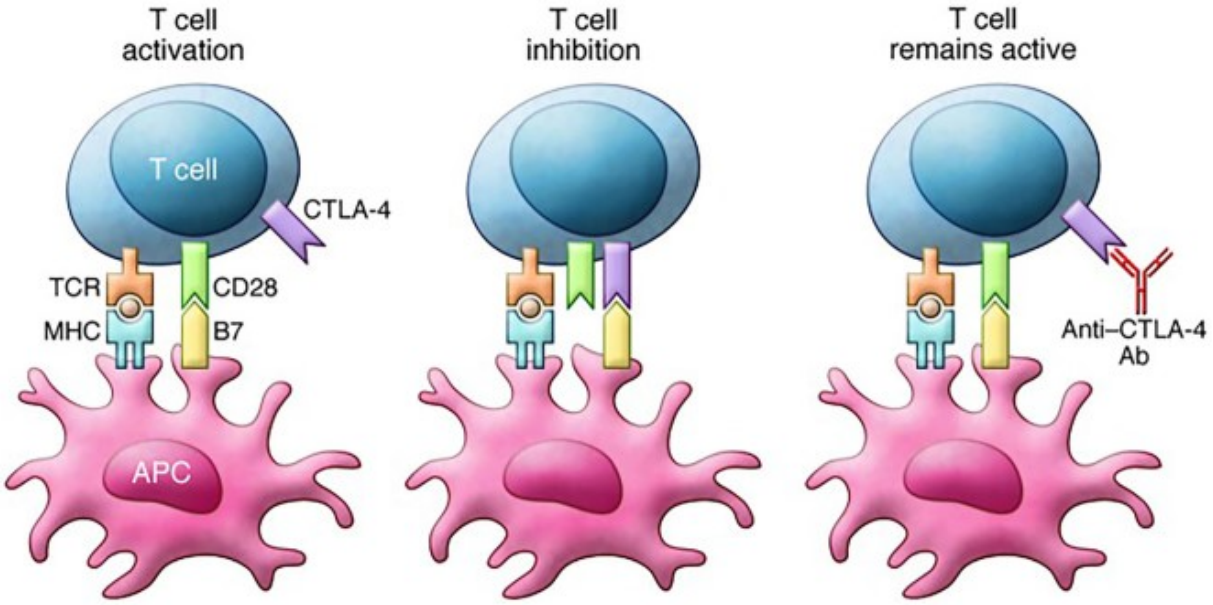


Figure 8. Mechanisism of action of Ipilimumab. *Adopted from (Zhang et al. 2023)*

2.4.4.2 Adoptive cell therapy (ACT)

ACT is a personalized cancer immunotherapy approach that involves isolating and modifying immune cells ex vivo before reintroducing them into the patient. Among ACT strategies, TIL-based therapies utilize a patient's own TILs, activating and expanding them in vivo without altering their TCRs before reinfusion. In contrast, engineered adoptive T-cell therapies (E-ACTs) involve genetic modifications to the TCR, including chimeric antigen receptor (CAR) T-cell therapy and TCR T-cell therapy. Newer approaches involving other immune cells expressing CARs such as CAR-NK therapy and CAR-M cells are also under exploration (S. Du et al. 2023).

TIL infiltration within the TME has been associated with improved survival outcomes and serves as a predictive marker for chemotherapy response in BC (Denkert et al. 2018). However, not all BC cases exhibit strong TIL infiltration. Moreover, even when TILs infiltrate tumors, the immunosuppressive nature of the TME often hinders their survival and proliferation, rendering them ineffective (Kotsifaki et al. 2023). To address this challenge, TIL therapy has emerged as an alternative strategy. This approach involves cultivating tumor-reactive immune cells from the TME, stimulating them with high-dose IL-2, expanding them ex vivo, and reintroducing them into the patient. Whole genome sequencing is used to identify mutations and select TILs that target tumor-specific neoantigens, enhancing the efficacy of this therapy (Fuentes-Antrás et al.

2020). Few clinical trials are currently investigating TIL therapy for BC, with one of the most notable being an ongoing phase II trial evaluating the efficacy of neoantigen-reactive TILs in combination with a short course of pembrolizumab for metastatic BC. Among six patients eligible for adoptive TIL transfer, three experienced objective tumor regression, including one case of complete response (Zacharakis et al. 2022). Three other trials are still ongoing with no result yet (Marei et al. 2025).

In parallel, CAR-T cell therapy represents another avenue of ACT, utilizing synthetic TCRs composed of TCR signaling domains and T-cell costimulatory molecules, enabling MHC-independent antigen recognition. Several clinical trials are assessing CAR-T therapies for BC, with most still in the recruitment phase and others not releasing results (Chamorro, Somes, and Hoyos 2024; Marei et al. 2025). Notable early-phase trials include phase 0 (NCT01837602) and phase I (NCT03060356) studies targeting the cMET antigen, which is highly expressed in BC tumors. These trials demonstrated that cMET-directed CAR-T therapy was well-tolerated and triggered an inflammatory immune response (Shah et al. 2020; Tchou et al. 2017). Another phase I trial (NCT02706392) evaluated CAR-T therapy targeting ROR1 in TNBC, confirming its safety profile (Specht; et al. 2019).

TCR therapy offers a more targeted approach by modifying T cells to express transgenic TCR α and β chains with high affinity (Fuentes-Antrás et al. 2020). However, limited clinical trials have been conducted in BC, and early-phase studies targeting MAGE-A3 and NY-ESO-1 antigens reported significant toxicity without notable efficacy. In contrast, a phase II trial investigating a TCR therapy specific for the p53 R175H mutation in metastatic BC demonstrated tumor regression at metastatic sites, offering promising prospects for future applications (Chamorro, Somes, and Hoyos 2024).

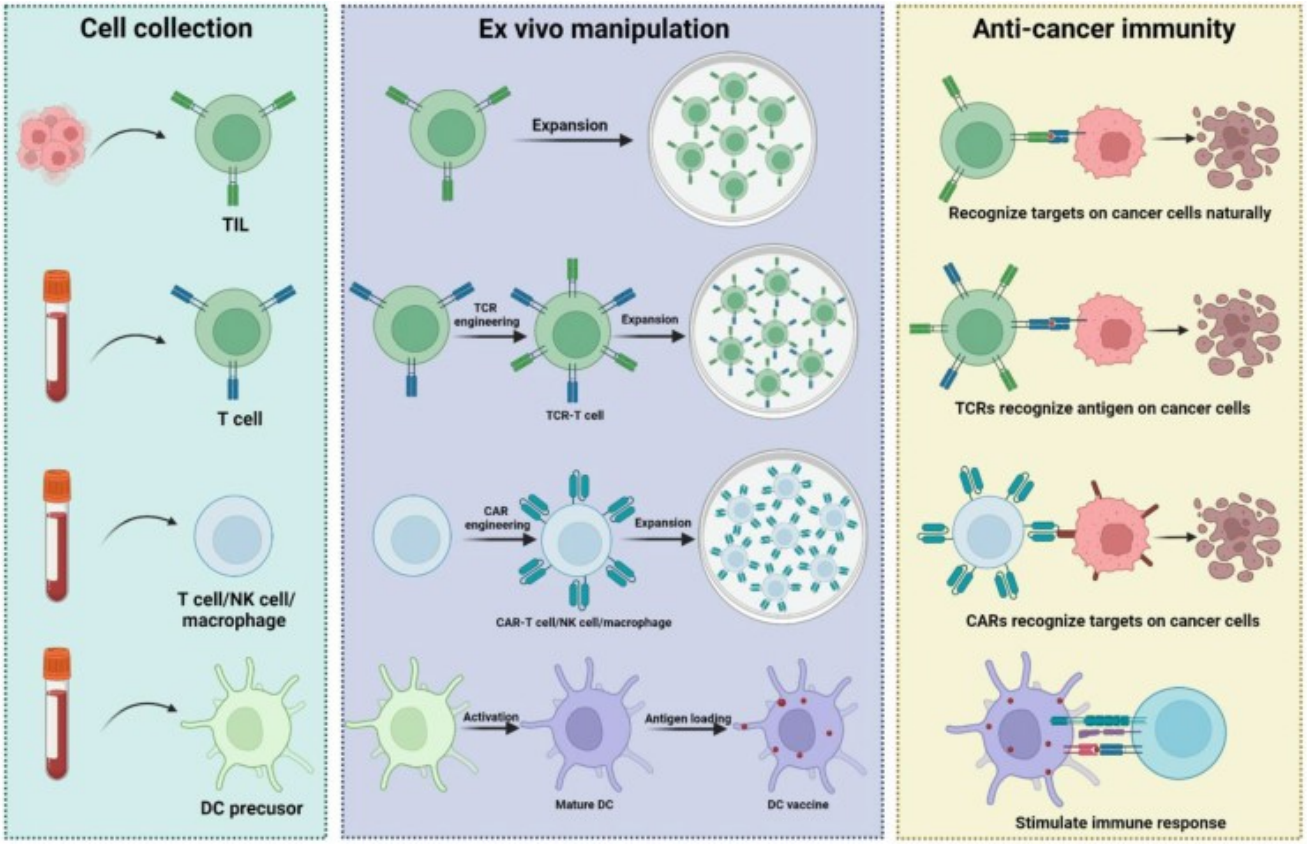


Figure 9. The different forms of Adoptive cell therapy. *Adopted from (Du et al. 2023)*

Table. 1 Comparisison between TCR and CAR T. Adopted from (Chamorro, Somes, and Hoyos 2024).

	TCR T	CAR T
Constructs	Minimally engineered TCR	Fully synthetic receptor
MHC Restriction	Dependent	Independent
Affinity and Sensitivity	Lower affinity, higher sensitivity	Higher affinity, lower sensitivity
Antigens Recognized	Peptides presented within the MHC molecule (proteins)	Cell surface proteins/molecules
Origin of Antigens	Intra-/Extracellular	Cell surface
Co-stimulatory Molecules	Endogenous CD28, 4-1BB	Linked to scFv (CD28, 4-1BB in combination with CD3ζ
Probability of CRS	Lower	Higher

2.4.4.2 Cancer vaccines

In addition, antigen-directed immunotherapies such as cancer vaccines are also being explored as alternative approaches. Cancer vaccines include peptide-based, DC-based and nucleic acid-based vaccines. The first category targets TSAs, such as neoantigens or TAAs, to stimulate immune responses against tumors. Several TAAs are under investigation for breast cancer vaccines, including HER2, Mucin 1 (MUC1), Globo-H, p53 and human telomerase reverse transcriptase (hTERT) (Fatima, Fatma, and Saraf 2023). TAAs are non-mutated self-antigens that are overexpressed in malignancies; however, their immunogenicity is often limited due to central T-cell tolerance. In contrast, neoantigens arise from tumor-specific mutations and bypass central tolerance, making them more effective in eliciting robust immune responses (Xie et al. 2023).

HER2 has been the most extensively studied tumor antigen in clinical trials, with many phase I and II studies demonstrating good safety profiles and promising efficacy (Zhu and Yu 2022). However, subsequent phase III trials failed to show a significant difference in DFS between vaccinated and non-vaccinated groups (Mittendorf et al. 2019). Currently, another phase III BC vaccine trial is underway, targeting the HER2 antigen GP2, administered with GM-CSF. This trial advanced to phase III based on encouraging Phase IIb results, where no recurrences were observed in the HER2+ population after five years of follow-up. However, no preliminary results have been released yet for this phase III trial (Patel et al. 2024).

DC play a crucial role in bridging innate and adaptive immunity making them a valuable target for cancer vaccines. DC-based vaccines are prepared by coculturing PBMCs with GM-CSF and IL4. TNF-α is then added to induce maturation. Once matured, DCs are loaded with TAA, neoantigens or mRNAs to enhance antigen presentation and stimulate immune responses (Zhu and Yu 2022). While phase I and II clinical trials have demonstrated the safety and immunogenicity of various DC-based vaccines, either alone or in combination with chemotherapy or immunotherapy, none have progressed to phase III randomized controlled trials (Qian et al. 2023).

Nucleic acid-based vaccines involve the use of plasmids to transfer RNA or DNA coding the tumor antigens. Such approaches have fallen short in the past because of lack of immunogenicity but the emergence of next generation sequencing (NGS) for the identification of neoantigens has make it a valuable alternative (Fatima, Fatma, and Saraf 2023). Recently, neoantigen DNA vaccine encoding on average 11 neoantigens per patient revealed good tolerability with minimal adverse effects and showed an 87.5% recurrence-free survival rate at a median follow-up of 36 months (Zhang et al. 2021).

Looking ahead, combination immunotherapy is emerging as a key direction in BC treatment. Various strategies are being explored, including combining cytotoxic chemotherapy with immune checkpoint inhibitors, integrating radiation therapy with immune checkpoint blockade, and combining checkpoint inhibitors with hormonal therapy or dual immune checkpoint antibodies. However, challenges such as toxicity and the failure to induce durable immunity remain significant hurdles that must be addressed to enhance the success of these therapies.

2.4.5 Role of Microbiota in Anti and protumorigenic immunity

The term microbiota refers to the entire community of bacteria, viruses, parasites, and fungi that colonize human mucosa surfaces. In a healthy individual, the intestinal microbiota provides numerous health benefits including providing protection against invading pathogens, support for nutrition and metabolism and immune modulation (Sun et al., 2023; Flint et al. 2012). A delicate balance is maintained between beneficial and harmful microbes. This dynamic interplay ensures gut homeostasis. However, when this balance is disrupted, a condition known as dysbiosis, it has been linked to various human diseases including cancer (Degruttola et al. 2016; Vimal, Himal, and Kannan 2020).

In the context of cancer, the microbiome within mucosal surfaces has been increasingly recognized for its dual role in tumor suppression and tumor promotion, with immune-mediated mechanisms playing a significant part. The most studied microbiota in this context is the gut microbiota. Growing evidence supports the importance of the gut microbiome in control of immune surveillance, anti and pro tumor immunity and tumor responses to therapy; immunotherapy as well as conventional treatments (Sun, Chen, and Wu 2023).

However, there exists increasing evidence that bacteria found within tumors themselves (intra-tumoral bacteria) may impact carcinogenesis, immune modulation, and therapeutic responses. Although breast tissue was originally considered to be sterile, it is now well established that it is populated with a distinct local microbiota (Lam et al. 2021). Differences in the composition of breast microbial communities have been reported between breast of cancer patients compared to healthy individuals. Differences in microbiome were also apparent between benign and malignant disease, between nipple aspirate fluid of healthy individuals and breast survivors and among the different subtypes of BC (Parida and Sharma 2019).

One proposed mechanism through which the microbiome contributes to anti-tumor immunity is via augmented antigenicity, where bacterial antigens share structural similarities with tumor antigens, leading to cross-reactive immune responses. It has been suggested that T cell epitopes might be shared between bacteria and tumor cells (Vétizou et al. 2016; Routy et al. 2018). This cross-reactive T cells primed against bacterial antigens might exert anti-tumor effects either via induction of CD4+ T cells or direct killing by CD8+ T cells. In a preclinical study, adoptive transfer of *B. fragilis*-reactive CD4+ T cells conferred enhanced tumor control and restored anti-CTLA-4 efficacy in germ free (GF) mice (Vétizou et al. 2016). Microbiota can also activate the STING signaling pathway, inducing type I interferon (IFN-I) in immune effector cells, like macrophages and promoting DCs' cross-priming thereby promoting anti-tumor immunity (Lam et al. 2021). Microbiota in the TME ae intracellular and such intratumoral microbiota-derived antigen presented to CD8+ T cells also induces secretion of anti-tumor cytokines including IFN-γ.

In a quantitative survey of breast microbiota by (Xuan et al.), they have shown that breast tumor tissue had significantly reduced amounts of bacteria compared to both paired normal tissue from breast cancer patients and healthy breast tissue from healthy individuals. In addition, they have shown a reduction in bacterial load in advanced stage breast tumours. The decrease in bacterial load has further been associated with a reduced expression of one-third of antibacterial response genes surveyed. Notably, key innate immune sensors including TLR 2, 5 and 9 and antimicrobial response effectors IL- 12A, BPI and MPO were expressed at lower levels in tumors than healthy breast tissue. The observed bacterial-dependent immune cell stimulation might contribute to an anti-tumour immune response. The study also identified a strong association between a specific bacterium, *Sphingomonas yanoikuyae (S. yanoikuyae)* and normal breast tissue and the dramatic

reduction in its abundance in the corresponding tumor tissue suggesting its possible probiotic functions in the breast (Xuan et al. 2014). In support of this finding, S. yanoikuyae express glycosphingolipid ligands, which are potent activators of invariant NKT (iNKT) cells and it can be directly recognized by NKT cells (Honey 2005). iNKTs are important mediators of cancer immunosurveillance and have been reported to have an integral role in controlling breast cancer metastasis (Terabe and Berzofsky 2007; Hix et al. 2011).

In another study involving a cohort of 360 TNBC patients, bacterial genera within the order *Clostridiales* and their metabolite TMAO were linked to an activated tumor immune microenvironment. Higher plasma TMAO levels correlated with improved immunotherapy response, as patients with elevated TMAO showed longer progression-free survival and a better therapeutic outcome compared to those with lower TMAO levels. In vivo experiments further supported these findings, where tumor-bearing mice received daily TMAO injections, either alone or in combination with anti-PD-1 therapy. Notably, mice treated with both TMAO and anti-PD-1 antibodies exhibited greater tumor suppression than those receiving anti-PD-1 therapy alone. Additionally, TMAO treatment was associated with increased CD8+ T cell infiltration and elevated IFN-γ expression, suggesting an enhanced anti-tumor immune response (Wang et al., 2022).

To the contrary, certain commensal bacteria may suppress anti-tumor immunity by skewing immune subset balances towards suppressive phenotypes such as Tregs and MDSCs. For example, for colorectal cancer, direct molecular interaction between *Fusobacterium* Fap2 protein and TIGIT, inhibitory receptor present on all human NK cells and on various T cells, contributes to tumor immune evasion; this interaction was shown to inhibit activation of NK cell and limited killing of colon adenocarcinoma cells. It was further demonstrated that tumor-infiltrating lymphocytes expressed TIGIT and that T cell activities were also inhibited by *F. nucleatum* via Fap2. (Gur et al. 2015). In BC, *Fusobacterium was* correlated with tumor grade and shown to promote BC metastasis and progression mainly via prevention of infiltration of cytotoxic T cells to the TME in which depletion of the bacteria with antibiotics reversing *Fusobacterium* mediated BC progression (Chun-Ming and Lam 2024).

Another important human commensal, Enterotoxigenic *Bacteroides fragilis* (ETBF), is shown to promote tumorigenesis via modulation of the immune system. It is a potent initiator of IL-17-dependent colon tumorigenesis in mice. IL-17 in colonic epithelial cells promoted the differentiation of monocytic (M)-MDSCs which selectively upregulated *Arg1* and *Nos2*, produced NO and consequently suppressed T cell proliferation (Orberg et al. 2017). *Bacteroides fragilis* was enriched in the TME of BC but their anti or pro tumor immunity has yet to be determined (Chun-Ming and Lam 2024).

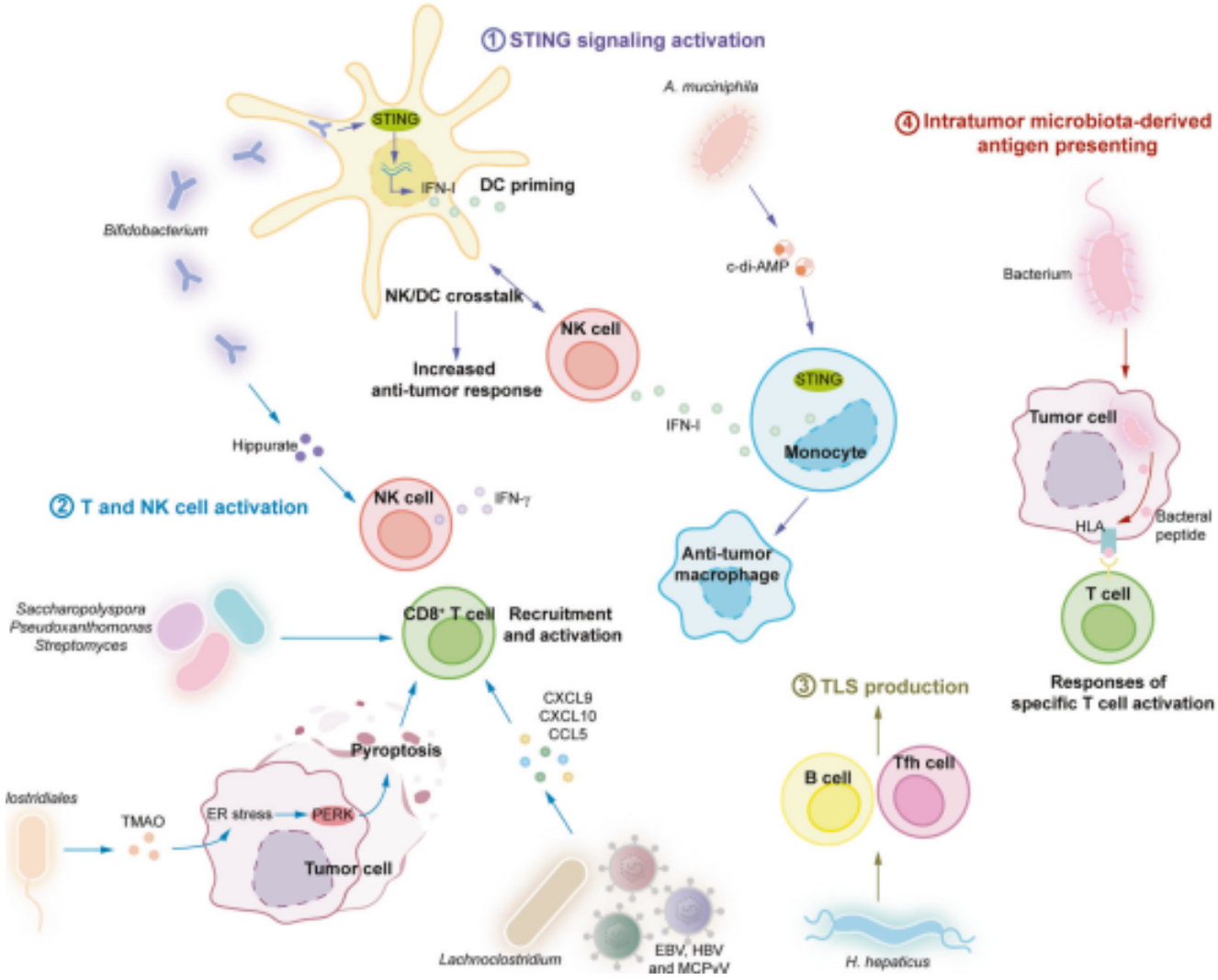


Figure 10. Role of intratumoral microbiota in anti tumor immunity. *Adopted from (Yang et al. 2023)*

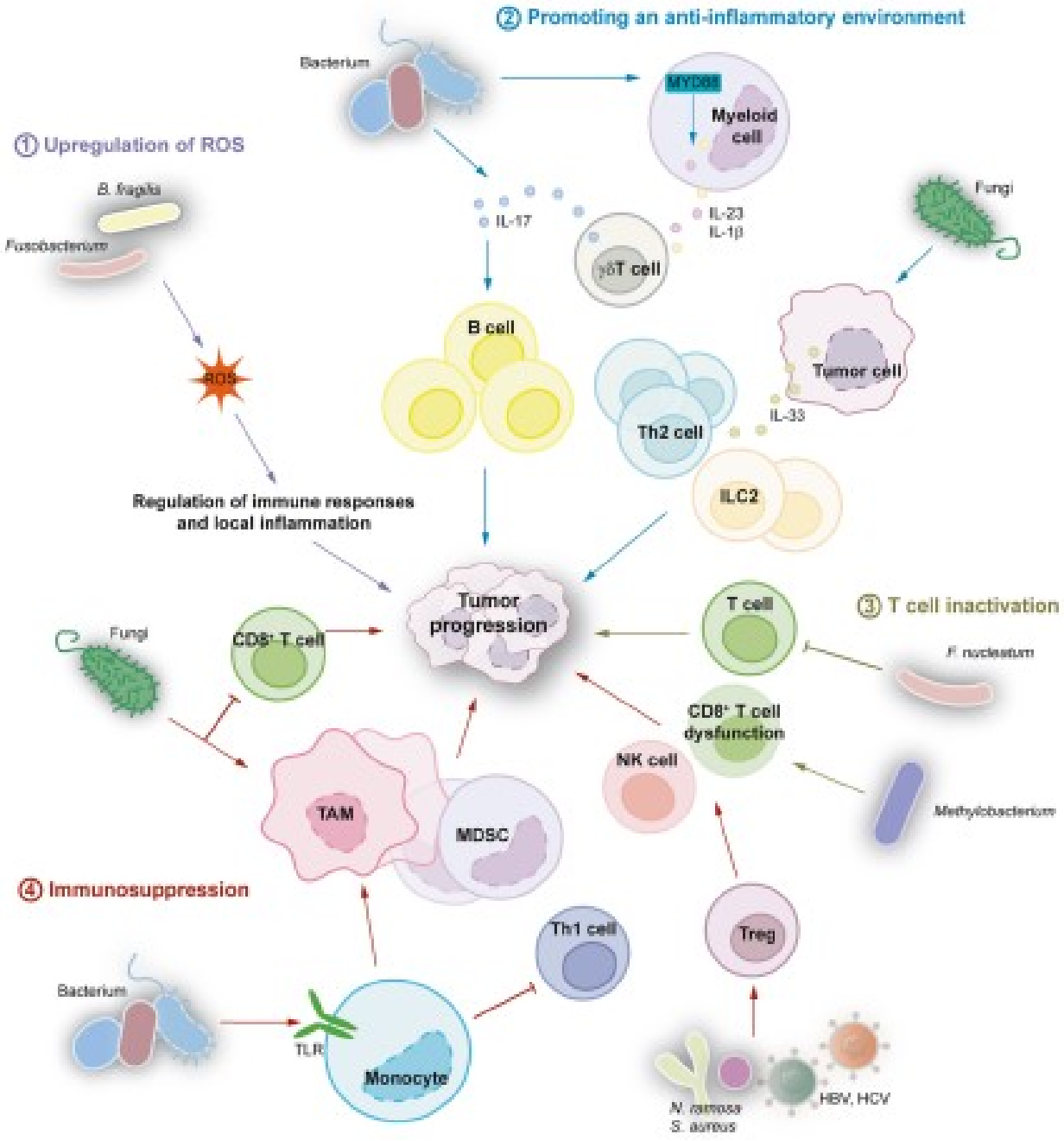


Figure 11. Role of intratumoral microbiota in pro-tumorigenic immunity. *Adopted from (Yang et al. 2023)*

Chapter 3: Materials and methods/Methodology

3.1 Study area and period

The study was conducted in four public hospitals and one private hospital. The public hospitals included Tikur Anbessa Specialized Hospital (TASH), Zewditu Memorial Hospital, and Yekatit-12 Hospital Medical College, all located in Addis Ababa, as well as Aira Hospital in Western Ethiopia. Additionally, Bethzatha Hospital, a private facility in Addis Ababa, was included in the study. The intended research project was part of a big project which has been reviewed and approved by department ethical review committee (DERC), College of Health Sciences-institutional review board (CHS-IRB), and National Research Ethics Review Committee (NRERC) 10/193/2018) started November 2018.

3.2 Study design and population

The study included various study designs to address the specific objectives, involving two cohorts and publicly available validation cohort.

3.2.1. Retrospective follow up

A retrospective follow-up study design was employed to determine the prognostic role of immune phenotyping of the TME in Ethiopian breast cancer patients. Patients who were diagnosed with pathologically confirmed breast cancer from 2013 to 2015 G.C and ended up in TASH for pathology service and oncological care were included in this study group. Five years follow up data from the time of diagnosis were extracted from patient card. Due to a significant number of patients lacking follow-up information regarding recurrence, metastasis and overall survival from the medical records, survival status was determined through follow-up phone calls and served as the endpoint measure for this study. Additionally, socio demographic and clinico-pathologic variables like age at first diagnosis, tumor size, status of axillary lymph nodes, histologic type of the tumor, histological grade, hormone-receptor status, clinical stage and pathologic stage (TNM) were also retrieved from medical card and biopsy report using structured questionnaire by the attending resident (Annex XIII).

3.2.2 Prospective follow up

A prospective cohort study was conducted to examine the abundance and functionality of systemic immune cells in Ethiopian breast cancer patients before and after surgery. The study included 65 newly diagnosed breast cancer patients (diagnosed between 2018 and 2021) and 10 control individuals with no known disease. Data on socio-demographics, medical history, and clinical and pathological information were collected through patient card reviews and interviews conducted by surgical nurses using a structured, pre-tested questionnaire (Annex XIV).

3.2.3 Publicly available validation cohort

A validation data set obtained from The Cancer Genome Atlas (TCGA) was utilized as a validation cohort (https://www.cbioportal.org/study/summary?id=brca_tcga). The TCGA BC data set encompasses RNAseq data of 177 African American BC patients with available clinical and pathological data, such as stage, immunohistochemical receptor expression and patients' survival outcome.

3.3. Inclusion and exclusion criteria

Inclusion criteria

- Pathologically confirmed Breast CA
- Who are >18 years of age
- Who are able to provide consent

Exclusion criteria

- Severely ill patients
- Patients with incomplete data; follow up data, pathologic data
- Patients with previous mastectomy or excisional biopsy
- Patients who had taken neoadjuvant chemotherapy
- Patients who had taken antibiotics 3 months prior to surgery

3.4 Sample size, sampling method and procedure

For the prospective study cohort, consecutive sampling technique was employed to include participants to the study; All patients who fulfilled the inclusion and exclusion criteria and visit the study sites within the study period were included. Whereas for the retrospective cohort, all confirmed breast cancer cases diagnosed from 2013 to 2015 G.C and had complete five year follow up data with their corresponding formalin fixed paraffin embedded (FFPE) blocks were included.

3.5 Variables

3.5.1 Independent variables

- clinical and histopathological parameters: age at diagnosis, simple pathologic stage (T1/T2: early; T3/T4: advanced), histological grade.
- IHC (ER, PR, HER2 and Ki-67status)
- molecular BC subtypes (Luminal, Her2 & basal)
- relative abundance of microbial taxa
- alpha diversity (Shannon and Simpson diversity indices)
- PIK3CA mutation status

3.5.2 Dependent variables

- immune gene expression
- TIL infiltration level
- immune phenotypes
- relative percentage of immune cells
- overall survival

3.6 Specimen collection and processing

3.6.1. Blood Collection and PBMC isolation

Fifteen milliliter (15ml) peripheral blood was collected from 65 patients prior to and 2-3 weeks after surgery. The blood was collected by vein puncture using heparinized tubes. The collected blood was used for the isolation of peripheral blood mononuclear cells (PBMCs).

PBMC was separated shortly after the blood was collected by density gradient centrifugation on Histopaque®-1077 (Sigma) using the standard procedure. In brief, in a 50-ml conical centrifuge tube, 10ml HISTOPAQUE®-1077 was added and 10 ml whole blood was carefully layered on to it and centrifuged at 1900rpm for 20 minutes at room temperature. After centrifugation, the buffy coat which contains the PBMCs was carefully aspirated using Pasteur pipet and transferred to a clean conical centrifuge tube. 10 ml Isotonic phosphate buffered saline solution (PBS) was added to the aspirated buffy coat and mixed by gentle aspiration. It was then centrifuged at 1800 rpm for 10 minutes and the supernatant was aspirated and discarded.

After PBMCs were isolated and washed, cell concentration and viability were determined using an Improved Neubauer hemocytometer and trypan blue exclusion method. A 10 µL aliquot of the PBMC suspension was mixed with an equal volume (10 µL) of 0.4% Trypan Blue solution (1:1 dilution). After gentle mixing, 10 µL of the mixture was loaded into the hemocytometer. Cells were counted under a light microscope at 20× magnification. Unstained cells were recorded as viable, while blue-stained cells were considered non-viable. The average cell number from four large corner squares was calculated, and total cell concentration (cells/mL) was determined using the formula:

$$\text{Cells/mL} = (\text{Average count per square}) \times (\text{Dilution factor}) \times 10^4.$$

Finally, the PBMC were directly cryopreserved in 10% v/v dimethyl sulfoxide (DMSO Sigma-Aldrich UK) and 90% v/v fetal bovine serum (FBS, Fisher Scientific) and kept in liquid nitrogen until flow cytometry was performed.

3.6.2. Fresh frozen tissue

A total of sixty-six fresh frozen tissue (FFT) samples were collected from BC patients The attending physician excised approximately 3-4 mm of the surgically resected tumor specimen. Each sample was cut in to two pieces and put it in to cryovials. The samples were then snap

frozen in liquid nitrogen and stored at -80ºC till RNA/DNA extraction was carried out for downstream immune gene expression assays.

3.6.3. Formalin fixed paraffin embedded (FFPE) tissue

FFPE blocks (n=140) were obtained from both retrospective and prospective cohorts through the TASH pathology laboratory. These blocks were collected from patients who underwent surgery at TASH, Yekatit Hospital, and Zewditu Memorial Hospital, as these institutions use the central pathology laboratory at TASH. FFPE blocks from Bethezatha Hospital and Ayra Hospital were retrieved from their respective pathology laboratories.

3.7 Specimen storage and transportation

The PBMC was cryopreserved in 10% v/v dimethyl sulfoxide (DMSO Sigma-Aldrich UK) and 90% v/v fetal bovine serum (FBS, Fisher Scientific) and kept in – 80^0C freezer at DMIP laboratory until it was transported to Germany. The fresh frozen biopsy was stored in – 80^0C freezer whereas the FFPE block was kept at room temperature protected from direct sunlight, dust and moisture. All samples were then transported to Germany, where the analysis was done, via sufficient amount of dry ice in a tightly packed Styrofoam whereas the biopsy sample collected for the microbiome analysis was shipped to Canada.

3.8 Analysis of specimen

3.8.1 Tumor infiltrating lymphocyte (TIL) analysis and grouping

Hematoxylin and eosin (H&E) staining was performed according to standard protocols initially in Ethiopia and subsequently double checked in Germany. Histopathological diagnoses of BC were made according to the WHO classification of Breast Tumors 5th edition while histopathological grading was performed according to Elston and Ellis (Nottingham Grading) System. Scoring of TILs was performed by an experienced pathologist by visual inspection of hematoxylin and eosin-stained tissue sections following recommendations by International Immuno-Oncology Biomarker Working Group on Breast Cancer. Percentage of TILs was quantified as a percentage of area occupied by TILs per total stromal area. Accordingly, tumors with TILs percentage of < 10% were classified as low, 11-49% intermediate and > 50%, as highly infiltrated (Locy et al. 2022).

3.8.2 Immunohistochemistry staining of ER, PR, Her2 and Ki-67

To determine hormone receptor (ER, PR) status, Her2 overexpression and Ki-67 proliferation index of BC, immunohistochemistry (IHC) staining was performed. Briefly, paraffin-embedded tissue sections were first deparaffinized and rehydrated. To block endogenous peroxidase activity, the slides were incubated with 3% H_2O_2 solution. Formalin fixation often masks antigen epitopes by creating strong protein cross-links, making them inaccessible to primary antibodies. To restore antigen accessibility, the slides were placed in preheated retrieval buffer (sodium citrate, pH 6.0) and incubated at 89°C. Next, the sections were incubated with the specific primary antibody (Table 1). After washing, the enhancement reagent was applied and incubated (except for HER2). A second wash was followed by the application of the HRP-polymer, and any unbound HRP-polymer was thoroughly removed by washing. For HER2 staining, instead of the enhancement reagent and HRP-polymer, a visualization reagent was applied and incubated. Any excess unbound visualization reagent was then washed away. The chromogenic substrate, diaminobenzidine (DAB), was added to initiate the enzymatic reaction of peroxidase, leading to color precipitation at the site where the primary antibody was bound. The slides were then counterstained with hematoxylin, followed by dehydration and mounting.

A tumor was classified as hormone receptor-positive (ER or PR) if the immune reactive score (IRS) was >0. HER2 positivity was defined as IRS ≥3, while cases with IRS = 2 were considered equivocal and subjected to chromogenic in situ hybridization (CISH) for confirmation. Tumors were classified as low Ki-67 expression if <20% of tumor cells stained positive and high Ki-67 expression if ≥20% of cells showed staining.

Table 2: Antibodies used for IHC staining of ER, PR, Her2 and Ki-67

name	clone	host	order nr.	company	dilution
Estrogen receptor (ER)	Ab-11	mouse	MS-354-P1	Thermo Scientific	1:150
Progesterone receptor (PgR)	PgR 636	mouse	M3569	Dako	1:100
Ki-67	SP6	rabbit	RM-9106-S	Thermo Scientific	1:250
name	clone	host	order nr.	company	dilution
human epidermal growth factor receptor 2 (HER2)	NA	rabbit	SK001	Dako	rtu

3.8.3 Extraction of RNA from FFPE tissue sections

Purification of RNA from FFPE tissues was carried out by Qiagen miRNeasy FFPE Kit (Catalog no. 217504) (Qiagen, Germany). Up to four sections of FFPE material each with a thickness of

10 µm were used. Depraffnaztion was carried out by treating samples with xylene, 100% Ethanol and 70% ethanol. Following deparafinization, samples were incubated with lysis buffer containing proteanase K which results in release of RNA from sections. All samples were subsequently treated with DNase in order to eliminate contaminating DNA. Buffer RBC and ethanol were then added to the lysate and the resulting mixture was finally loaded to RNeasy MinElute spin column in which purified RNA remains attached till subsequent elution of RNA by RNase-free water (Annex VII).

3.8.4 Purification of RNA from Fresh frozen tissue

RNA was extracted from fresh frozen tissue using the Qiagen miRNeasy Mini Kit (Catalog No. 217004) Qiagen, Germany. Cryostat sections (10 µm thick) were used as the starting material. To disrupt the cells, 700 µl of QIAzol lysis reagent was added, and the sample was mixed thoroughly by vortexing, followed by incubation at room temperature (15–25°C) for 5 minutes. 140 µl of chloroform was then added to the homogenate, and the tube was securely capped and shaken vigorously. The sample was centrifuged at 4°C for 15 minutes, and the upper aqueous phase was carefully transferred to a new collection tube. 100% ethanol were then added and mixed thoroughly. Up to 700 µl of the sample was pipetted into an RNeasy Mini spin column and centrifuged for 15 seconds at room temperature. The column was then washed twice with 500 µl Buffer RPE to ensure purity. The RNeasy Mini spin column was transferred to a new 1.5 ml collection tube, and 30–50 µl of RNase-free water was directly applied to the membrane. The column was centrifuged for 1 minute to elute the RNA. Finally, on-column DNase digestion was performed, followed by a final elution with 40 µl of RNase-free water to obtain purified RNA (Annex VIII).

3.8.5 RNA Expression assay

RNA concentration and quality was measured using Nanophotometer and RNA with an A260/A280 ratio of 1.7–2.3 and an A260/A230 ratio of 1.8–2.3 were used for subsequent expression assay. Expression of genes was determined by nCounter MAX/FLEX platform (Nanostring Technologies, Seattle, WA, US). The system is based on direct molecular bar coding of target RNA molecules using colour coded probe pairs; a reporter probe, which carries the signal on its 5’ end, and a capture probe which carries a biotin on the 3’ end. Hybridization of the probe pairs with RNA was done by incubating both (3µL of reporter Code Set, 5 µL of

hybridization buffer, 5 µL of 400ng RNA, and 2 µL of Capture ProbeSet) overnight resulting in binding of individual RNAs with the probes. A subsequent washing step was applied to remove excess unbound probes. Finally, the RNA/probe complexes were eluted off and immobilized in the cartridge for data collection and counts were read with 555 fields of view (FOV) by nCounter® Digital Analyzer (Annex X).

Intrinsic sub typing of breast cancer was carried out using the research-based, 50-gene prediction analysis of microarray (PAM50) subtype predictor. PAM50 was calculated from the nanostring expression data.

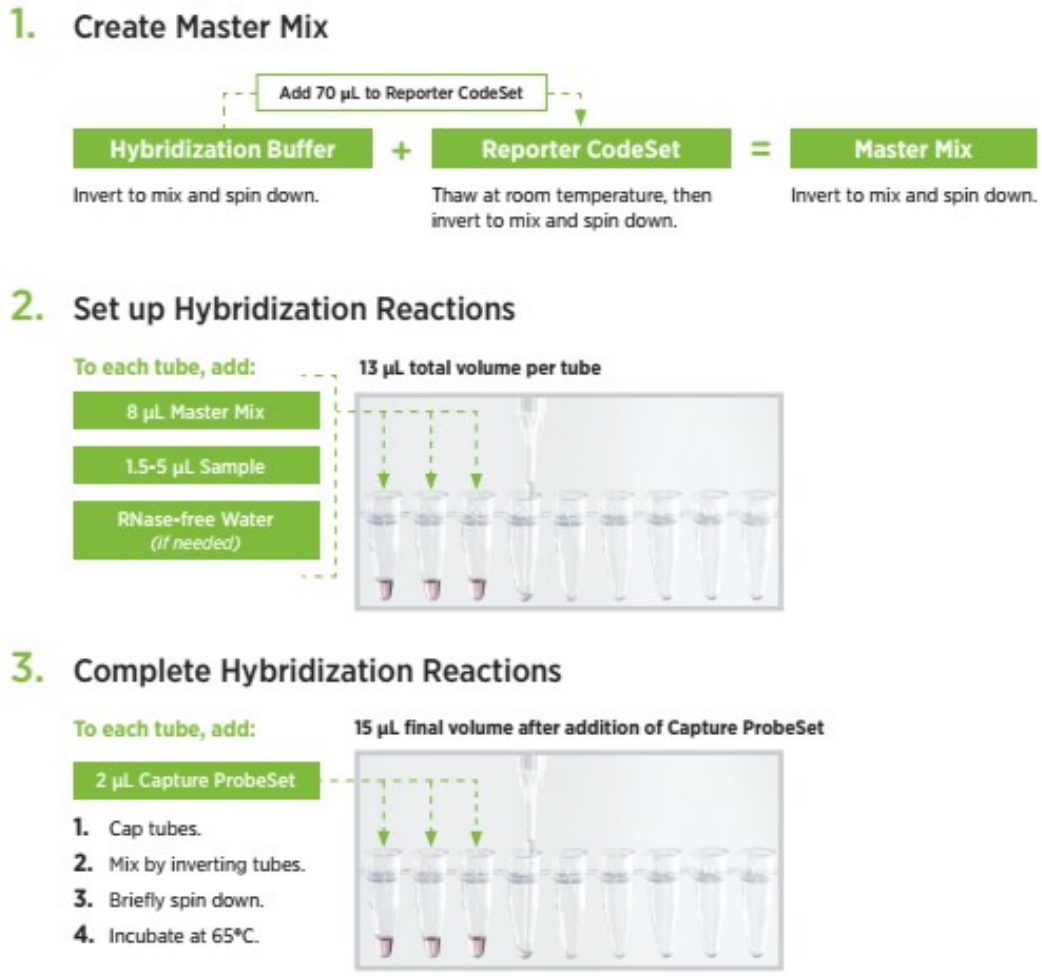


Figure 12. Work flow of the gene expression assay on MAX/FLEX nCounter (Nanostring). *Adopted from nCounter® XT Assay User Manual.*

3.8.6 Extraction of DNA from FFPE and Fresh frozen tissue for Mutational analysis

DNA extraction from FFPE was done using QIAamp® DNA FFPE Tumor Tissue kit (Qiagen, Hilden, Germany) while genomic DNA from frozen specimens was isolated using a NucleoSpin Tissue kit (Clontech, Mountain View, CA, USA) for the mutation assay. The quality of genomic DNA was measured using the UV absorbance (A260/A280) and high-quality DNA with A260/A280 ratio >1.7 was used for further analysis. The extracted DNA was stored at -80ºC to perform mutational analysis.

3.8.7 Genotyping of PIK3CA Hotspot Mutations Using TaqMan® Assays

Phosphoinositide 3-kinase CA (PIK3CA) mutations at three hotspot loci; p.E542K (c.1624G>A), p.E545K (c.1633G>A), and p.H1047R (c.3140A>G) were assessed using the TaqMan® Mutation Detection Assay (Applied Biosystems, Carlsbad, CA 92008 USA) in an Applied Biosystems®' real-time PCR instrument (ABI Step OnePlus v2.1).

The genotyping procedure involved two experiment types for ΔCT cutoff determination and mutation detection. Each experiment included detection of both the mutant allele and a corresponding reference gene. PCR mixtures were prepared by mixing 20 ng of gDNA with TaqMan® Genotyping Master Mix and the TaqMan® Mutation Detection Assay in a microcentrifuge tube. Following gentle vortexing, 20 µL of this reaction mixture was added to each well of a PCR plate. The PCR plate was covered with optical adhesive film, briefly centrifuged, and then loaded into the real-time PCR instrument. The instrument was configured according to the universal mutation detection thermal-cycling protocol. Detailed thermal cycling conditions are outlined in the (Annex XII). The data was finally analysed using the real-time PCR system software. The mutation status of the tumor was considered positive, if at least one of the three substitutions could be detected. Accordingly, tumors were considered wild type (wt) only when all three genotyped sites were congruent with the human DNA reference sequence (NCBI RefSeq GRCh38, https://www.ncbi.nlm.nih.gov/refseq/).

3.8.8. Immunophenotypic staining of PBMCs and flow cytometry

Flow cytometry was performed on cryopreserved PBMCs to quantify the frequency and phenotype of immune effector cells, including T cells, B cells, NK cells, monocytes, DCs, and their subpopulations. Additionally, immunosuppressive cells such as Tregs and MDSCs were

analyzed within the total PBMC fraction. Details of the antibodies used are provided in Table 3 below.

In brief, cryopreserved PBMC (1-4 x 10^6) were thawed, resupended in 10 mL of RPMI 1640 medium (Themo Fisher Scientific) at room temperature and washed twice with PBS to remove debris. Cells were then incubated with Fixable Viabilty stain (FVS 700; BD) for 15 minutes and washed with 10ml PBS to remove unbound stain. For membrane staining, 100µl of the cell suspension was then incubated with the respective Ab (Table 2) for 15 minutes in dark. Cells were washed twice and resuspended in 300 µl of PBS prior to acquisition.

For intracellular staining, cells were washed with 2ml PBS and proceeded with permeabilization by adding 1ml of Fix/Perm (BD Bioscience) buffer and incubating samples at 2-8^{0}C for 40-50 min. in dark. Following permeabilization, the cells were washed with 1ml perm/wash buffer (BD Bioscience) and labeled with the Ab. The sample-antibody mixture was then kept in dark at 2-8^{0}C for 40-50 min and washed twice with 2ml of perm/wash buffer. Finally, 300 µl of PBS was added and acquisition was done in LSR Fortessa II (BD Bioscience, CA, USA) flow cytometer (AnnexV).

Table 3. Antibodies used for surface and intracellular staining of peripheral immune cells.

						Intra	Intra	Intra		
Flurochromes	T cell I 1	T cell II 2	DC/Mo/NK 3	T cell III 4	B cell 5	Intra Treg 6	Perforin 7	CTLA 4 8	Cost I 1	MDSC 13
FITC	γδ TCR 25µl	CD45 25 µl	SlanDC 25 µl	tim3 50 µl	CD45 25 µl	CD25 50 µl			CD45 25 µl	CD15 100 µl
PE	CD56 50 µl	CCR7 100 µl	CD56 50 µl	CXCR3 30 µl	CD24 100 µl				CTLA4 100 µl	CD124 200 µl
PerCP-Cy5.5	CD4 12.5 µl	CD4 12.5 µl	CD123 100 µl	CD4 12.5 µl	CD19 15 µl	CD4 12.5 µl	CD4 12.5 µl	CD4 12.5 µl	CD4 12.5 µl	

PE –Cy7	CD128 15 µl	CD45RA 15 µl	CD11c 25 µl	CCR6 70 µl	IgD 25 µl	CCR4 25 µl	CD56 15 µl		CD19 15 µl	CD33 50 µl
APC	CD16 50 µl	CD38 100 µl	CD16 50 µl	CD57 25 µl	CD38 100 µl	CD127 70 µl	CD19 10 µl	CD19 10 µl	Lir-1 50 µl	Cd19+56 10+50 µl
APC-H7	CD8 25 µl	CD8 25 µl	CD3+19+20 Je25 µl	CD8 25 µl	CD20 25 µl	CD45RO 25 µl	CD8 25 µl	CD8 25 µl	CD8 25 µl	CD11b 50 µl
Mix	17.75	27.75	32.5	21.25	29	18.25	6.25	4.75	22.75	42
V450	CD45 2.5 µl		CD14 2.5 µl	CD45 2.5 µl		CD45 2.5 µl	CD45 2.5 µl	CD45 2.5 µl		CD14 5 µl
Brilliant violet 421					CD27 2.5 µl				PD-L1 5 µl	
V500/BV 510		HLA-DR 1 µl	HLA-DR 1 µl	PD1 5 µl		HLA-DR 1 µl			PD1 5 µl	HLA-DR 2 µl
BV605	CD3 0.25 µl	CD3 0.25 µl	CD45 1 µl	CD3 0.25 µl	CD3 0.25 µl	CD3 0.25 µl	CD3 0.25 µl	CD3 0.25 µl	CD3 0.25 µl	CD3 0.25 µl
Intracellular										
FITC							CD3 zeta 5 µl			
PE						FoxP3 5 µl	Perforin 5 µl	CTLA 4 10 µl		

3.8.9 Microbial DNA Extraction from Fresh frozen Tissue

DNA was extracted from the cancerous tissue specimen using Qiagen kit for microbiome profiling (Qiagen, Germantown, MD, USA). The isolated DNA was shipped via dry ice to Microbiome Insights (Vancouver, BC, Canada) for further processing.

The MoBio PowerMag Soil DNA Isolation kit was used for further purification of microbial DNA. Before starting, PowerMag® Bead Solution / RNase A Solution mixture was prepared for a 96 well plate by adding 4 µl RNase A Solution to each 750 µl of PowerMag® Bead Solution. Samples were then carefully added to each well of PowerMag® Bead Plate to avoid cross contamination between samples. PowerMag® Bead Solution / RNase A Solution and PowerMag® Lysis Solution were added to each well respectively. The plate was then transferred to kingfisher robot; programmed according to MoBio's DNA isolation protocol (Detailed steps are Annexed in Annex VI).

3.8.10. PCR amplification and 16S rRNA Gene Sequencing

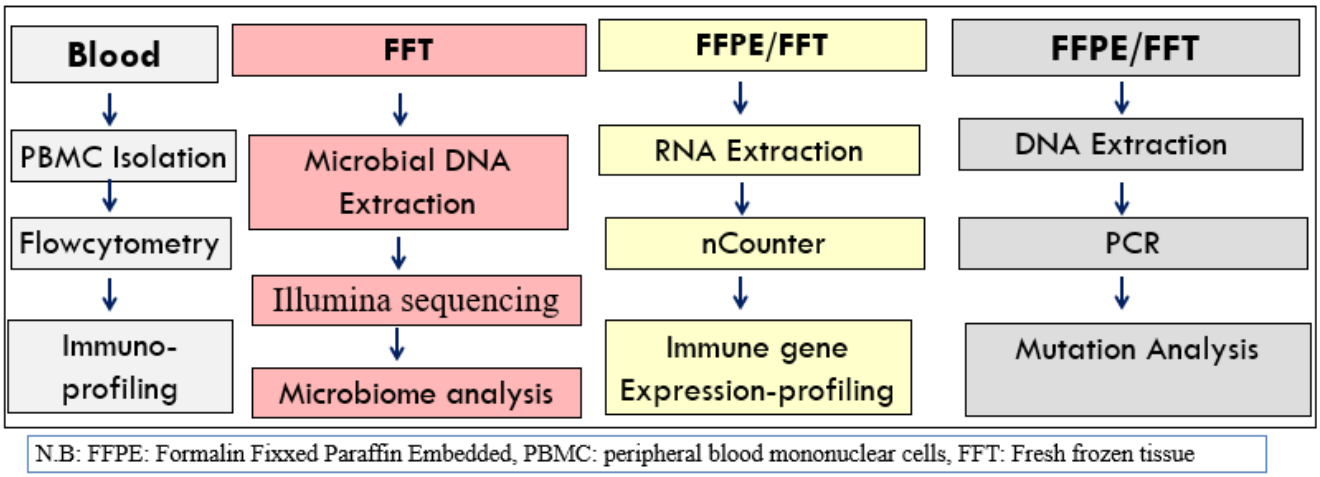
For PCR amplification of bacterial 16S rRNA genes, dual-barcoded primers targeting the V4 region (515F 5'-GTGCCAGCMGCCGCGGTAA-3', and 806R 5'-GGACTACHVGGGTWTCTAAT-3') were selected as per the protocol of Kozich et al. (2013). The PCR reaction mixture was prepared by mixing 2x KAPA HiFi HotStart Ready Mix, forward and reverse primers, DNA template and nuclease free water. This mixture was then aliquoted into a 96-well PCR plate, sealed with Microseal 'A' film, and subjected to thermal cycling.

Post-PCR, amplicons were purified using AMPure XP beads, followed by washes with freshly prepared 80% ethanol and 10 mM Tris (pH 8.5). The Nextera XT Index Kit was used in order to attach dual-index barcodes and Illumina sequencing adapters to the amplified DNA followed by second washing step. Amplicon libraray and PhiX library, serves as an internal control were denatured with NaOH and diluted with hybridization buffer. Pooled library, prepared by combining the amplified library with PhiX library, was heat denatured at 96°C immediately before loading the library into the MiSeq reagent cartridge.

The Nextera XT Index Kit was utilized to append dual-index barcodes and Illumina sequencing adapters to the amplified DNA. A second washing step was performed before proceeding. The amplicon library along with the PhiX library, used as an internal control, was denatured with NaOH and diluted with hybridization buffer. The final library pool, which combined the

amplicon and PhiX libraries, was heat-denatured at 96°C immediately before loading into the MiSeq reagent cartridge for sequencing (Annex XI).

3.9 Work flow of Laboratory procedures



N.B: FFPE: Formalin Fixxed Paraffin Embedded, PBMC: peripheral blood mononuclear cells, FFT: Fresh frozen tissue

Figure 13. Flow chart of laboratory procedures

3.10 Quality Assurance

Immune Gene Expression Analysis; In order to maintain the quality of the immune gene expression data, RNA integrity was maintained during sample collection, transportation & extraction. Biopsy samples were immediately snap frozen in liquid nitrogen and maintained at -80°C until RNA extraction whereas FFPE samples were protected from direct sunlight during shipping and storage. During RNA extraction, all work surfaces were decontaminated with alcohol to eliminate RNase activity. RNA- extraction was carried out by strictly adhering to standard operating procedure (SOPs) and the isolated RNA quality and quantity were assessed using a nanophotometer before proceeding to expression assay. To ensure the quality of the gene expression data obtained from nCounter analysis, a quality control check assessing imaging quality, binding density, positive control linearity and limit of detection was done. Samples with flags for the above metrices were excluded. Read distribution percentages, violin plots, identity heat maps, and sample multidimensional scaling (MDS) plots were also generated as part of the QC step.

Flowcytometric Analysis: In order to ensure the quality of flowcytometric data, blood was collected aseptically by an experienced phlebotomist and processed within 2 hours of collection. Isolated PBMC was kept in liquid nitrogen until processing in order to maintain viability of cells.

In addition, the LSR Fortessa II (BD Bioscience) flow cytometer used for the acquisition of cells underwent weekly performance check and instrument validation with the Cell Signaling Technology (CST) beads (BD Bioscience).

Microbiome Analysis; Biopsy sample for microbiome analysis was collected in to Eppendorf tubes following aseptic techniques. DNA extraction and downstream assays were performed after decontaminating work bench with 70% alcohol to avoid contamination. The potential for contamination was addressed by co-sequencing DNA amplified from specimens and from template-free controls (negative control) and extraction kit reagents processed the same way as the specimens. A positive control from 'S00Z1-' samples consisting of cloned SUP05 DNA, was also included. Operational taxonomic unit (OTU) were considered putative contaminants (and were removed) if their mean abundance in controls reached or exceeded 25 % of their mean abundance in specimens.

In addition, data extraction form was assessed for consistency and completeness and incomplete data was excluded. Moreover, all laboratory work was carried out after an adequate training by senior scientists in a well-established laboratory.

3.11 Data Analysis

3.11.1 Data processing and analysis of gene expression data

Data processing; Data were analysed using ROSALIND® (https://rosalind.bio/) platform (ROSALIND, Inc., San Diego, CA, USA). QC metrics, such as imaging quality, binding density, positive control linearity and limit of detection, were inspected. Normalization of raw counts was performed according to NanoString's guidelines. Eleven housekeeping genes with the lowest coefficient of variation (CV < 0.6) were selected as normalizer probes using the geNorm algorithm (Perkins et al. 2012). Data were then normalized against the geometric mean of the selected housekeeping genes and positive control probes as described (Nanostring Technologies 2017).

Clustering and visualization of multivariate data

Unsupervised hierarchical clustering of the normalized gene expression data was performed using ClustVis, which is a web tool implementing the pheatmap R package (https://CRAN.R-project.org/package=pheatmap). Expression values were centered and unit variance scaling was

applied. Both rows and columns were clustered using the correlation distance and Ward linkage. Heat map was used for visualization of gene expression data.

Identification of differentially expressed genes (DEGs) and cell type profiling; The identification of DEGs between specified groups was performed using ROSALIND®. The Benjamini-Hochberg method was applied for adjustment of p-values in order to correct for multiple comparisons. Genes with $|\log2^{\text{fold-change}}| > 1.5$ and adjusted p-value < 0.05 were defined as DEGs. Abundance of various cell populations was calculated in ROSALIND® using the Nanostring cell type profiling module.

Statistical analysis; If not stated otherwise, data distributions and frequencies were compared among different groups with one-way ANOVAs, as implemented in GraphPad Prism v9 (GraphPad, San Diego, CA, USA). Fisher-Freeman-Halton exact tests of independence were performed for contingency tables larger than 2x2 using IBM SPSS Statistics v26 (IBM Corp., Armonk, NY, USA). Kruskal–Wallis H tests were employed to compare the numbers of TILs among the four IPs using the "stats" library for Scipy/Python (Virtanen et al. 2020). Kaplan–Meier survival analysis was performed to compare the prognostic significance of the immune subgroups and DEGs and log-rank test was employed as statistical a test.

3.11.2 Data processing and Data analysis of flowcytometric data

Gating strategy: Flow cytometry data were analyzed using BD FACS Diva software (BD Biosciences), incorporating compensation and gating. For identifying immune populations and subpopulations, a sequential gating approach was applied. First, cells were gated based on forward scatter-area (FSC-A) versus forward scatter-height (FSC-H) to exclude doublets. Next, FSC-A versus side scatter-area (SSC-A) was used to define the cell population of interest and remove debris. FVS 700 was then applied to exclude dead cells, ensuring that only live cells were analyzed. To identify the different immune cell populations, leukocytes were first gated based on CD45 expression and SSC parameters, segregating them into myeloid cells, lymphocytes, and granulocytes in cases of contamination. Lineage markers were then used to define specific immune cell subsets: T cells (CD3), B cells (CD19), NK cells (CD56), and monocytes (CD14 & CD16). Detailed gating strategies employed to identify the various immune cell subpopulations is indicated below (Figure 15-19).

Since PBMC was used instead of whole blood, absolute cell counts were not determined; instead, data were presented as cell frequencies within PBMCs or specific immune cell subsets.

Statistical analysis; Normality of data was checked by Kolmogorov-Smirnov test and D'Agostino & Pearson test. Unpaired t test or unpaired t test with Welch's correction was performed to compare the means of two independent groups with Gaussian distribution while Mann Whitney test was employed for non-parametric tests. For three or more groups with normally distributed data, ordinary one-way ANOVA or Brown-forythe and Welch ANOVA tests were done, whereas Kruskal-Walli's test was implemented for groups with non-Gaussian distribution. Comparisons between pre- and post-surgery samples were performed using paired T test while non-parametric statistical comparisons were performed using the Wilcoxon matched-pairs signed-rank test. Spearman tests were done for continuous variables. All statistical analyses were performed in GraphPad Prism v9 (GraphPad, San Diego, CA, USA) and p values of less than 0.05 were considered statistically significant.

3.11.3 Data processing and Data analysis of microbiome data

Data processing; Raw sequence data were processed by filtering low-quality reads and trimming the first 10 nucleotides, followed by denoising using the DADA2 pipeline. Chimeric sequences were identified and removed to ensure data integrity. Taxonomic classification was performed using a naive Bayesian classifier with the reference dataset rdp_train_set_18.fa.gz used for assigning sequences to their respective taxonomic categories. Finally, to account for sequencing depth variations, raw OTU counts were normalized to relative abundances by dividing each count by the total number of reads in the corresponding sample which allowed for direct comparison of microbial compositions across samples.

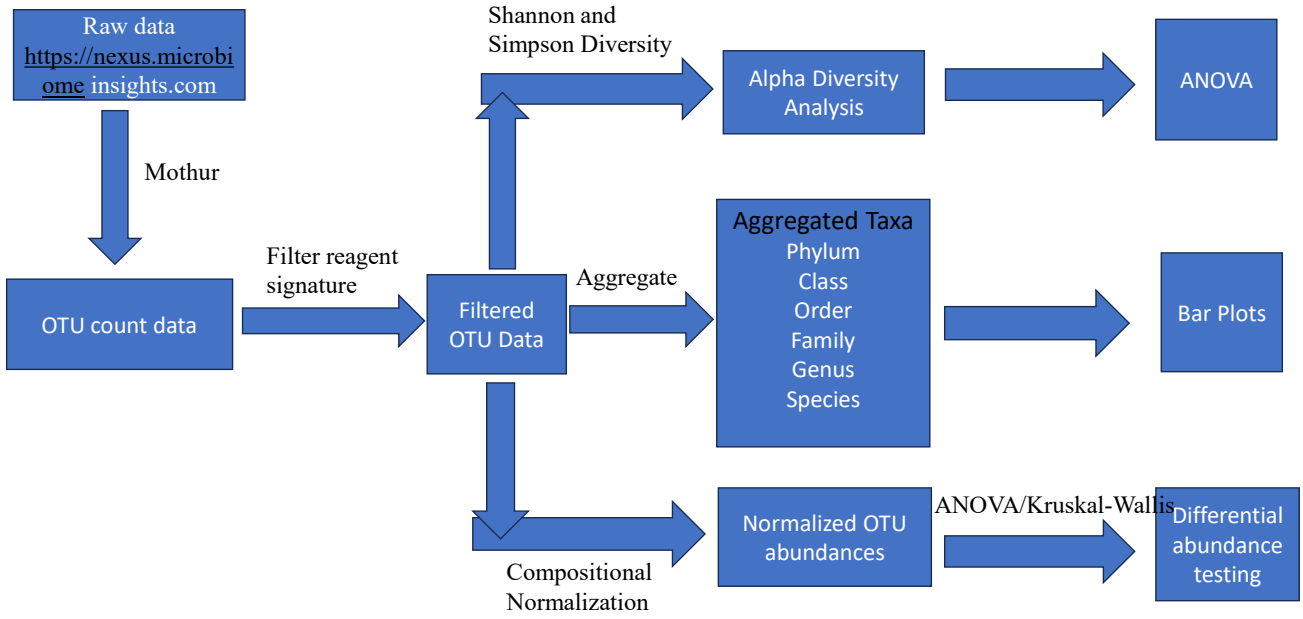


Figure 14. Analytical flowchart of the microbiome data.

Statistical analysis; The phyloseq package in R, was used to import, analyze and graphically display sequenced data which has already been clustered in to OTU. Rank-based correlation (Spearman r) was used to measure the association of relative abundance for each taxon with each gene expression. In order to control the false discovery rate (FDR) associated with multiple comparisons, the Benjamini Hochberg 1995 method was employed. Statistical significance was declared for P value <0.05 and q<0.1. Shanon and Simpson diversity indices were calculated as a measure of bacterial richness. ANOVA was employed to compare the Sahnnon and Simpson index and relative abundance of microbiome between the TIL groups.

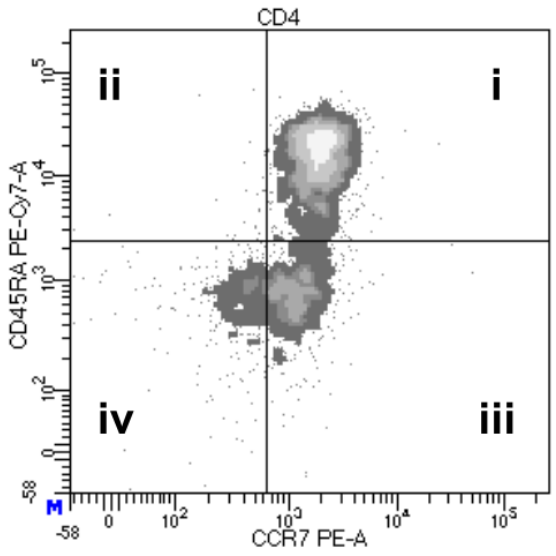


Figure 15. Gating strategy for T cell (CD4 and CD8) memory subsets: *As representatively shown for CD4$^+$ T cells, staining with CCR7 and CD45RA allows to identify (i) CCR7$^+$ CD45RA$^+$ naïve T cells, (ii) CCR7$^-$ CD45RA$^+$ effector cells, (iii) CCR7$^+$ CD45RA$^-$ central memory and (iv) CCR7$^-$ CD45RA$^-$ effector memory T cells.*

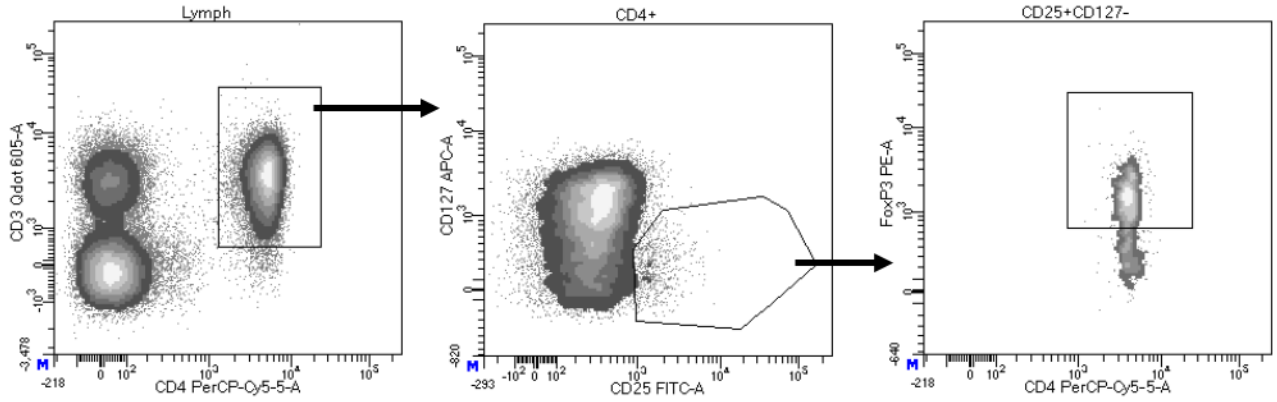


Figure 16. Gating strategy for regulatory T cells*: Tregs were identified among CD4$^+$ T cells as CD25$^+$ CD127^{low} cells expressing the transcription factor Foxp3.*

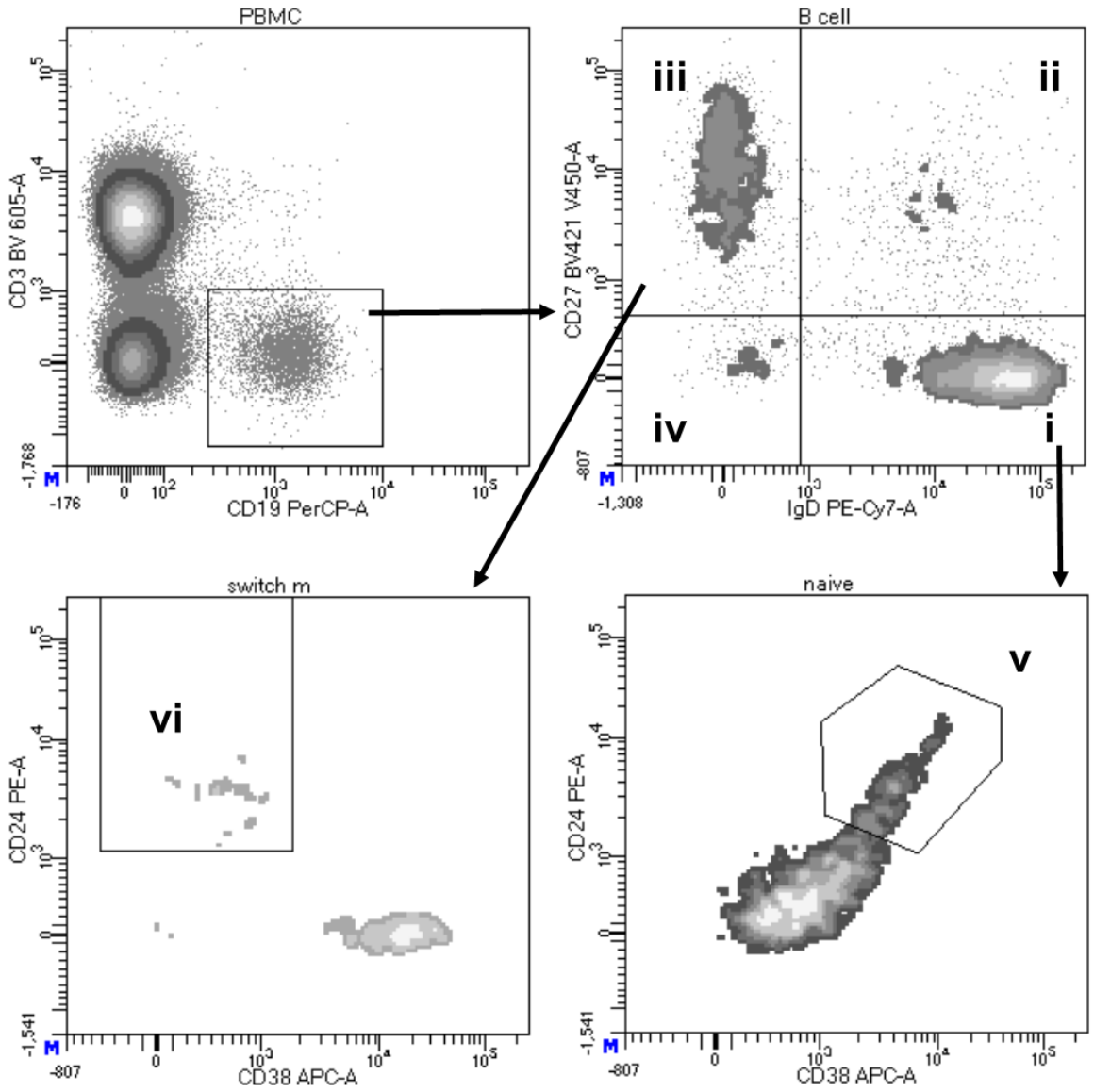


Figure 17. Gating strategy for B cell subpopulations: *B cells, identified as CD19$^+$ cells within the PBMC are classified into (i) IgD$^+$ CD27$^-$ naïve B cells, (ii) IgD$^+$ CD27$^+$ pre switch memory, (iii) IgD$^-$ CD27$^+$ switch memory and (iv) IgD$^-$ CD27$^-$ exhausted memory B cells. Additional gating for the expression of CD24 and CD38 allows to identify the (v) IgD$^+$ CD27$^-$ CD24$^+$ CD38$^+$ transitional B cells within the naïve B cells and (vi) the IgD$^-$, CD27$^+$, CD24$^+$, CD38$^-$ plasmablasts among the switch memory cells.*

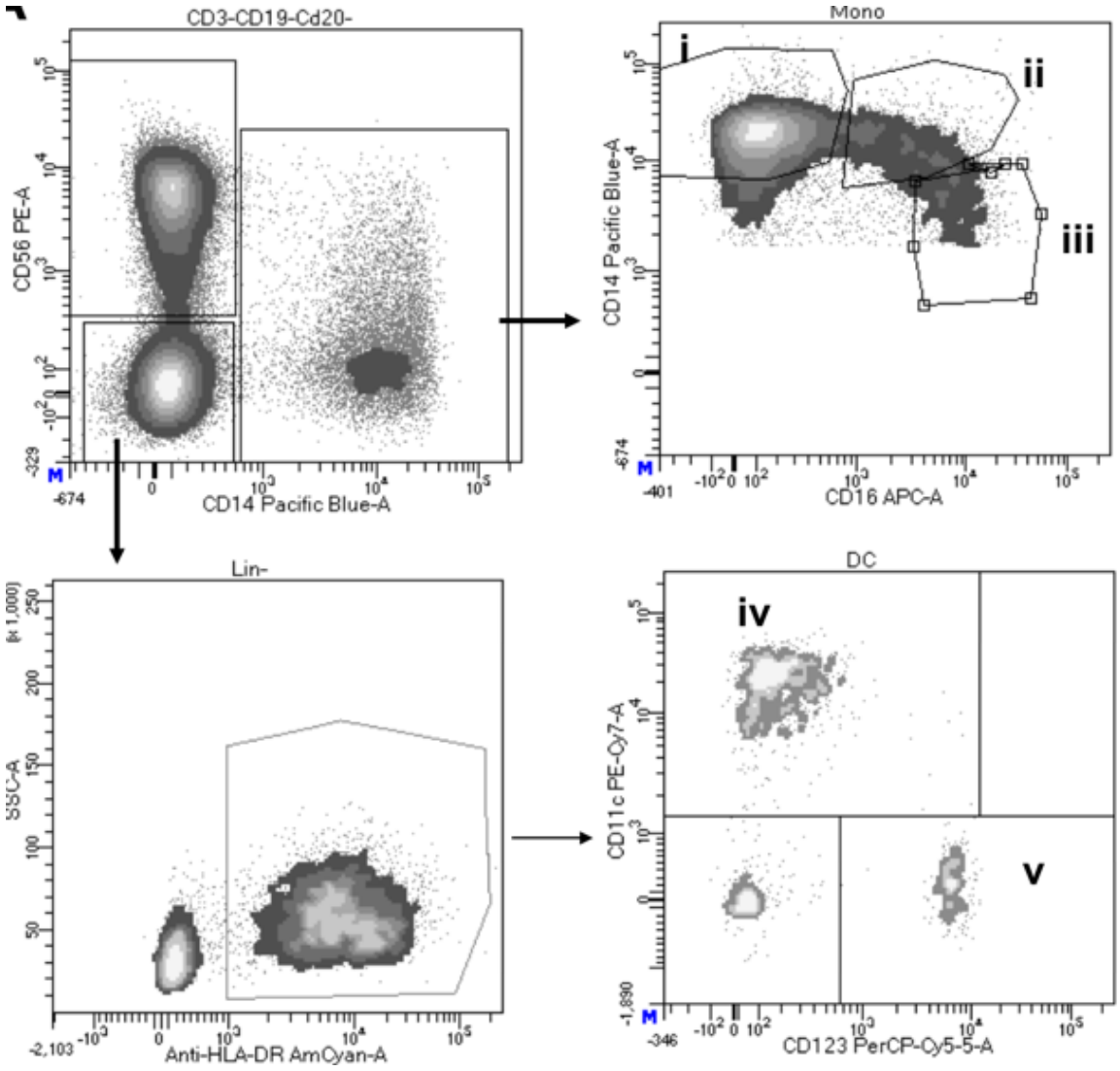


Figure 18. Gating of monocytes and DCs: *A) Among the CD3, CD19 and CD20 negative PBMCs, staining with CD14 and CD56 discriminate the CD14^{+} monocytes which can then be further divided based on the CD16 expression into (i) CD14^{high} CD16^{-} classical, (ii) CD14^{+} CD16^{int} intermediate and (iii) CD14^{low} CD16^{+} inflammatory monocytes. Among the linage negative PBMCs (i.e. CD3, CD19 CD20, CD14 and CD56), DC can be identified as HLA-DR^{high} cells and further divided into (iv) CD11c^{+} myeloid DC and (v) CD123^{+} plasmacytoid DC.*

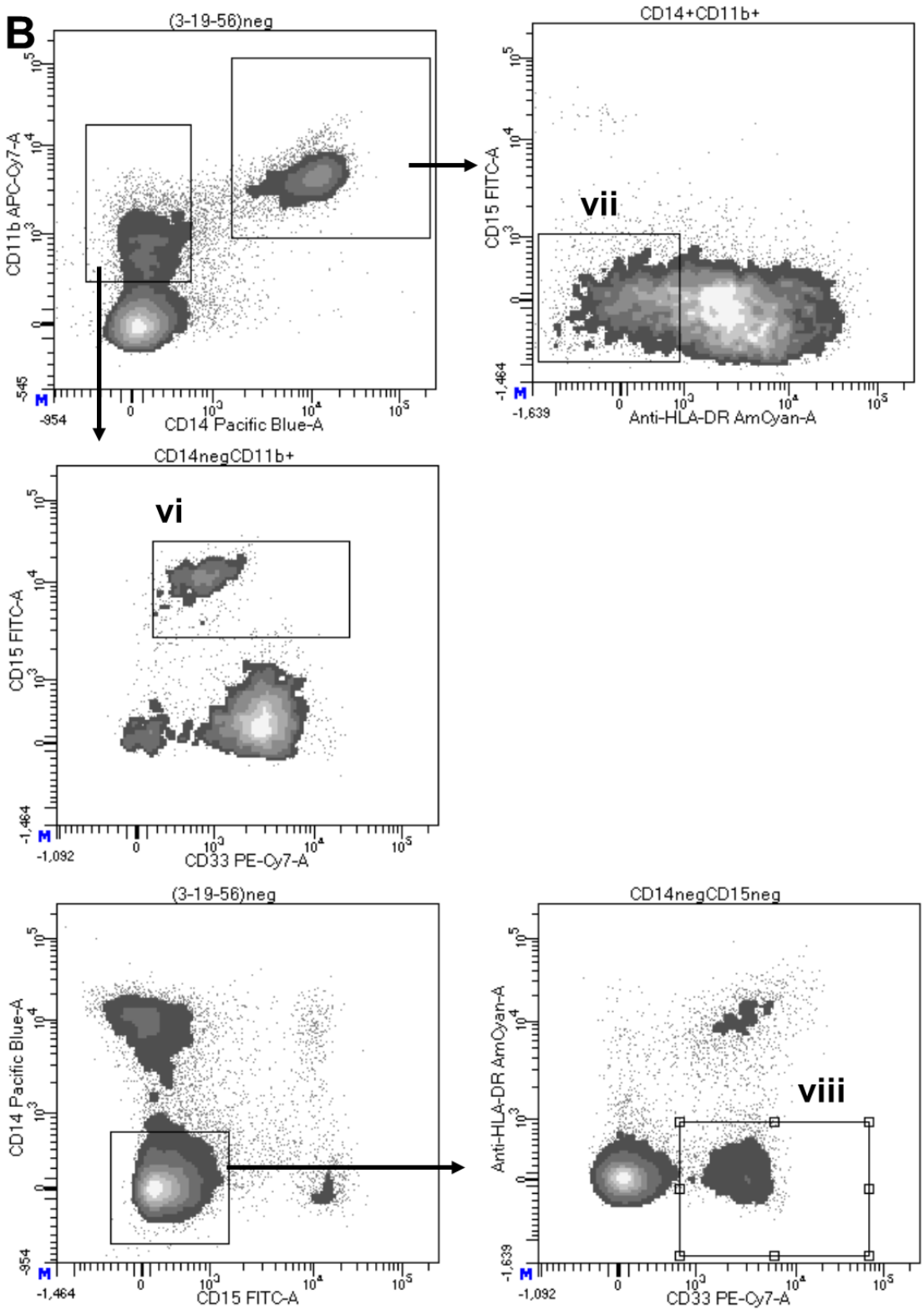

B
(3-19-56)neg
CD11b APC-Cy7-A
CD14 Pacific Blue-A
CD14+CD11b+
CD15 FITC-A
vii
Anti-HLA-DR AmCyan-A
CD14negCD11b+
vi
CD15 FITC-A
CD33 PE-Cy7-A
(3-19-56)neg
CD14 Pacific Blue-A
CD15 FITC-A
CD14negCD15neg
Anti-HLA-DR AmCyan-A
viii
CD33 PE-Cy7-A

Figure 19. Gating of MDSCs: *PBMC negative for CD3, CD19 and CD56 are used as a starting point for the identification of the different subpopulation of MDSC: (vi) the polymorphonuclear (PMN)-MDSC are identified within the $CD11b^+$ $CD14^-$ cells as $CD15^+$ cells; (vii) the mononuclear (M)-MDSC are identified as $CD11b^+$ $CD14^+$ cells expressing low levels of HLA-DR and $CD15^-$; (viii) the early eMDSC are $CD14^-$ $CD15^-$ cells characterized by low levels of HLA-DR and expression of CD33.*

3.12 Ethical consideration

This study was reviewed and approved by department ethical review committee (DERC) of department of Microbiology, Immunology and Parasitology and IRB of College of Health Sciences, Addis Ababa University (IRB protocol #092/17/17) and National Research Ethics Review Committee (NRERC) protocol # MOSHE/RD/2018). Official letter of co-operation was written to each selected Hospital from department of Microbiology, Immunology and Parasitology, College of Health Science, Addis Ababa University. The study population was approached and then details of the study objective, the type of sample to be collected and the anticipated minimal risk related with sample collection was explained in a simple language understandable by the participants. Written informed consent was obtained prior to sample collection. Experienced phlebotomist had been engaged in the study to minimize risk and maintain patient safety. There was no direct benefit in being involved in this study. Confidentiality of patient data was secured via keeping the participant related data and the respective result in a locked cabinet. Any personal identifier was not used. For the retrospective study, because of the difficulty of accessing patients diagnosed 7 and 8 years back, waiver was requested and granted by the IRB.

Chapter Four: Result

4.1 The tumor immune microenvironment; Role in immune subtyping and prognosis of Ethiopian Breast cancer patients

4.1.1 Clinico-pathologic data of participants

In total eighty-two patients with pathologically confirmed BC were included in this study. The participants' age at diagnosis ranged from 20 to 70 years, with a median age of 40 years. Early-stage tumors (stage I & II) were more prevalent in the cohort compared to late-stage tumors (48.8% vs. 33%). The majority of tumors (65.8%) exhibited high histological grade and were classified as grade III. Classification in terms of hormone receptor and Her2 status showed that majority of the cases (76.8%) were hormone receptor positive; HR+ HER2- cases making 50% while HR+ HER2+ constituting 26.8%. from the total cohort. HER2+ and TNBC comprised only 8.6% and 14.6% of the total cohort respectively. PAM50-based subtyping revealed that luminal subtypes were the most common, with luminal A and luminal B accounting for 34% and 18% of cases, respectively. The HER2-enriched and basal subtypes constituted 23% and 24.3% of cases, respectively (Table 4). With regards to concordance between IHC and intrinsic classification, the majority aligned well while some BC cases showed discordant results (Table 4). The HR+ BC cases (luminal-like) constituted the majority of cases (76.8%) in the IHC classification but only 52.4% when using the intrinsic classification.

Table 4. Clinico-pathologic data of participants

Features	N (%)
Age	
younger (<50)	50 (61%)
older (≥50)	19 (23%)
Unknown	13 (16%)
Pathologic Stage	
Early (stage I & II)	40 (48.8%)
Advanced (stage I & II)	27 (33%)
Unknown	15 (18.2%)
Histological grade	
G1 or G2	28 (34.1%)
G3	54 (65.9%)
Intrinsic subtype	
luminal A	28 (34.1%)
luminal B	15 (18.3%)

HER2-enriched	19 (23.2%)
basal-like	20 (24.4%)
IHC Group	
HR^+ $HER2^-$ (luA-like)	41 (50.0)
HR^+ $HER2^+$ (luB-like)	22 (26.8)
HR^- $HER2^+$ (HER2+)	7 (8.6)
HR^- $HER2^-$ (TNBC)	12 (14.6)

4.1.2 Identification of BC immune phenotypes

Hierarchical clustering was conducted on the expression profiles of fifty-four immune-related genes for eighty-two breast cancer samples and normal breast tissue sample pooled from two healthy donors. Analysis of the resulting dendrogram revealed four distinct clusters or immune phenotypes (IP), each displaying unique gene expression patterns. These phenotypes, designated as IP1, IP2, IP3, and IP4, were named based on their order of appearance in the clustering tree (Figure 20). IP1 consists of 9 samples, IP2 of 31, IP3 of 19 and IP4 of 24 samples, respectively.

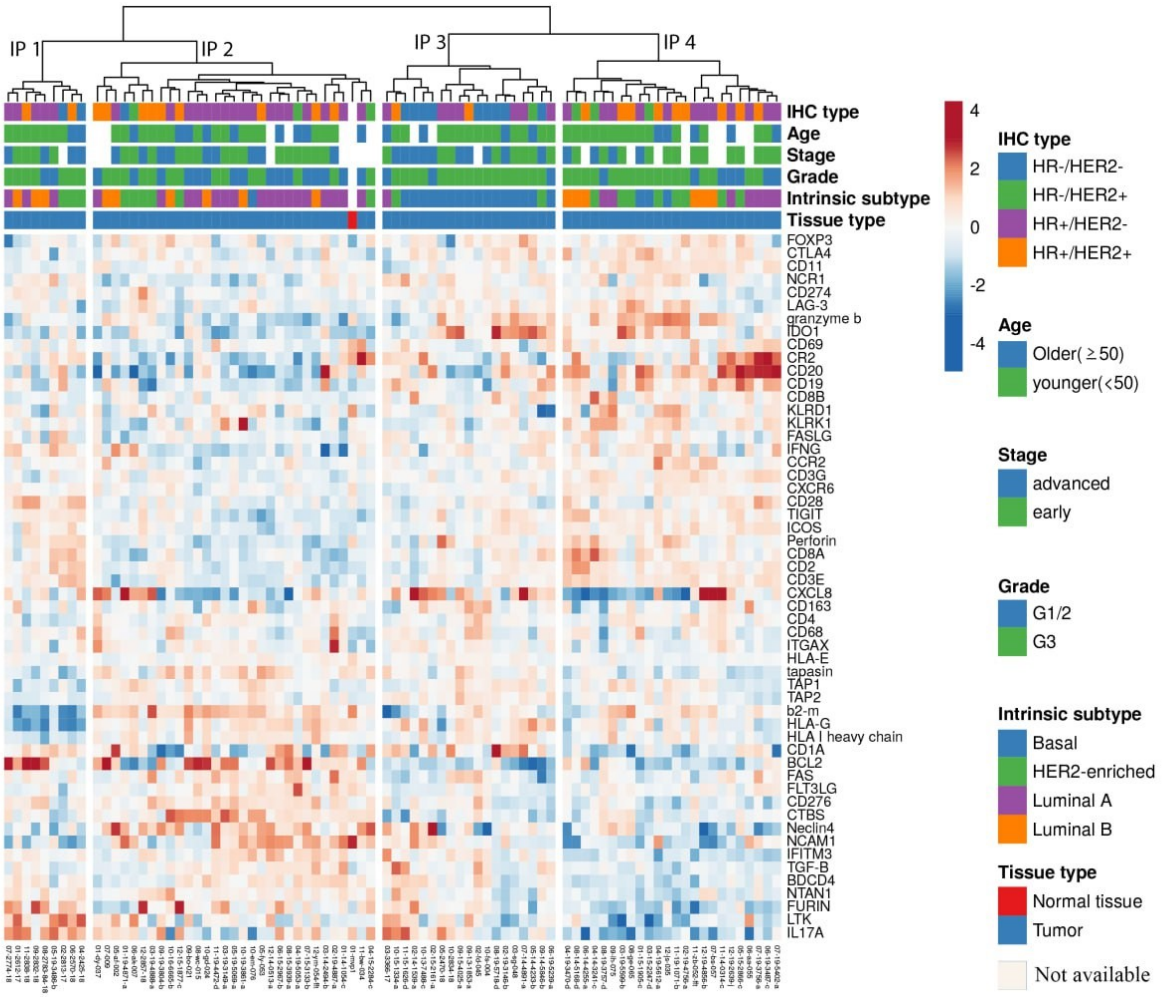


Figure 20. Heatmap of the expression levels of 54 immune-related genes in 82 BC samples: *Unsupervised hierarchical clustering of log-transformed expression levels of immune genes identified the four immunophenotype groups IP1 to IP4. Red marks indicate upregulation, blue marks indicate downregulation of immue-relatetd genes. Sample annotations are included at the top of the heatmap. HER2: human epidermal growth factor 2. HR: hormone receptor.*

4.1.3 Differential immune activation across Immunophenotypes in the TME

To characterize the IPs in relation to the expression of immune activating and immune regulatory genes, a differential expression analysis (DEG) was performed. The analysis demonstrated a different extent of immune activation in the TME between the four IPs (Figure 20 and 21). IP2 represents an immunologically inert phenotype with a consistent downregulation of immune

suppressive as well as immune activating genes. Immune genes encoding effectors cells (CD4, CD8A, CD8B, and CD20), T cell receptor signalling pathway (CD3E, CD3G, CD28, ICOS and CD2), immune modulatory molecules (TIGIT, CTLA4, CD274, HLA-E, IDO1, CCR2), antigen processing and presentation (KLRD1, TAP1, TAP2, CD1A), immune activating molecules or receptors (KLRK1, CXCL8, CXCR6), tumor associated macrophage markers (CD68, CD163) and the regulatory T cell marker (FOXP3) were suppressed in this phenotype (Figure 21).

In contrast, IP4 tumors were marked by highest immune gene expression representing an immunologically active microenvironment, characterized by an up-regulated expression of genes associated with anti-tumoral functions. Genes related to effectors cells (CD4, CD8A, CD8B, CD20), T cell activation (CD69), TCR signalling components (CD3E, CD3G, CD28, ICOS, CD2), antigen processing and presentation (KLRD1, TAP1, TAP2, CD1A) and activating molecules or receptors (KLRK1, CXCR6, FLT3LG) were significantly upregulated in this phenotype compared to the others (IP1-3). Interestingly, next to immune-stimulating molecules, some immune inhibitory molecules, like TIGIT, CTLA4 and CCR2 as well as the Treg marker FOXP3, were also highly expressed in this phenotype.

In addition, two phenotypes with an intermediate level of immune activation were characterized. In IP1, the immune activation genes CD4, NCAM1, CD3G, KLRD1 and the counter-regulatory molecules FOXP3, IDO and HLA-G were differentially suppressed when compared to the other phenotypes. In addition, NTAN1, FURIN and CD276 were upregulated in this phenotype. In the IP3, the majority of the immune genes were normally expressed; genes related with antigen processing and presentation (CD1A, TAP2), inflammation (CXCL8), TCR signalling pathway (CD28) and immune suppression (HLA-G, IDO1) were particularly upregulated in IP3, FGFR4, FLT3LG and CTBS were downregulated.

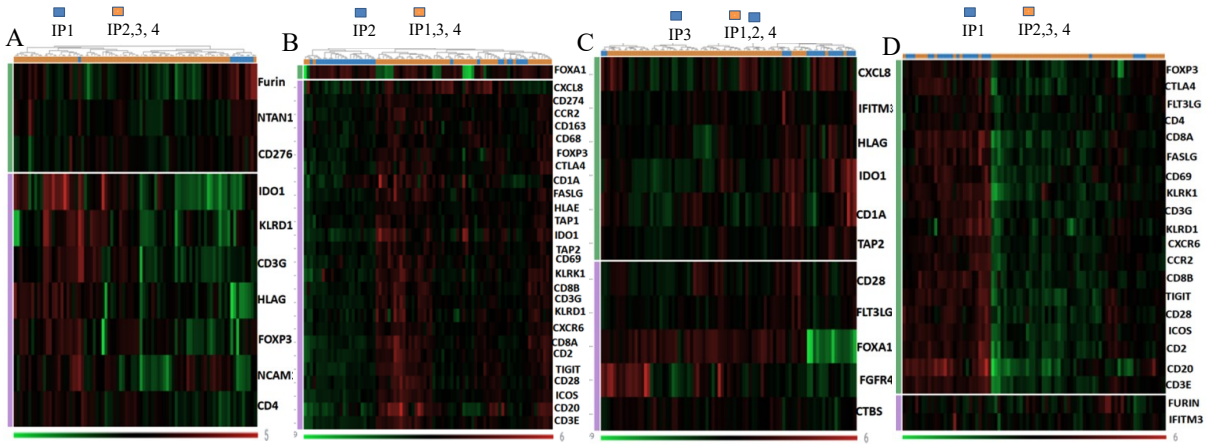
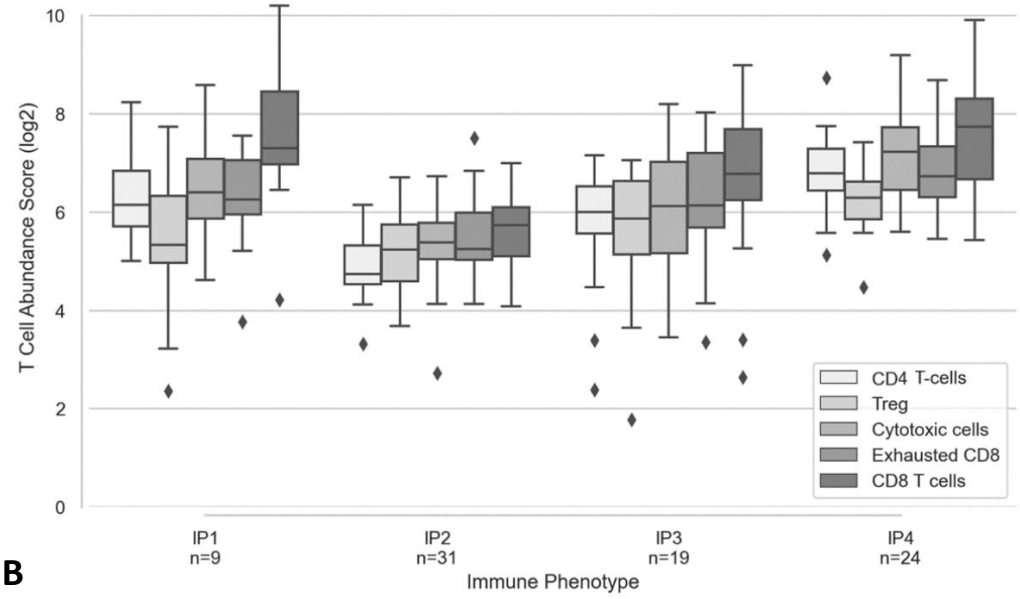


Figure 21. DEG heat map of the distinct immune phenotypes: *A: IP1 versus IP2, IP3 and IP4; B: IP2 versus IP1, IP3 and IP4; C: IP3 versus IP1, IP2 and IP4; D: IP4 versus IP1, IP2 and IP3. Differentially expressed immune genes between the immune phenotypes were shown. Genes with log2 fold change (FC) ≥ 1.5 and false discovery rate (FDR)-corrected p value < 0.05 were presented. Green: downregulated genes, red: upregulated genes.*

4.1.4 Immune cell infiltration of the IPs

The immune infiltration level of the different immune phenotypes was analyzed using cell type-specific profiling, estimated from the gene expression data, and through the quantification of TILs from H&E staining (Figure 22). The immune inflamed phenotype (IP4) displayed the highest log2 abundance score for cytotoxic cells, Treg cells, and exhausted immune effector cells as well as significantly elevated TIL counts ($H = 14.56$, p-value < 0.001), indicating a robust immune response (Figure 22A and 3B). In contrast, IP2 exhibited the lowest log2 abundance score for infiltrating immune cells (CD4+ T cells, CD8+ T cells and immune suppressive cells) along with fewest level of TILs ($H = 8.19$, p-value < 0.01). IP1 and IP3 showed intermediate levels of immune cell infiltration.

A


T Cell Abundance Score (log2)
10
8
6
4
2
0
CD4 T-cells
Treg
Cytotoxic cells
Exhausted CD8
CD8 T cells
IP1
n=9
IP2
n=31
IP3
n=19
IP4
n=24
Immune Phenotype

B

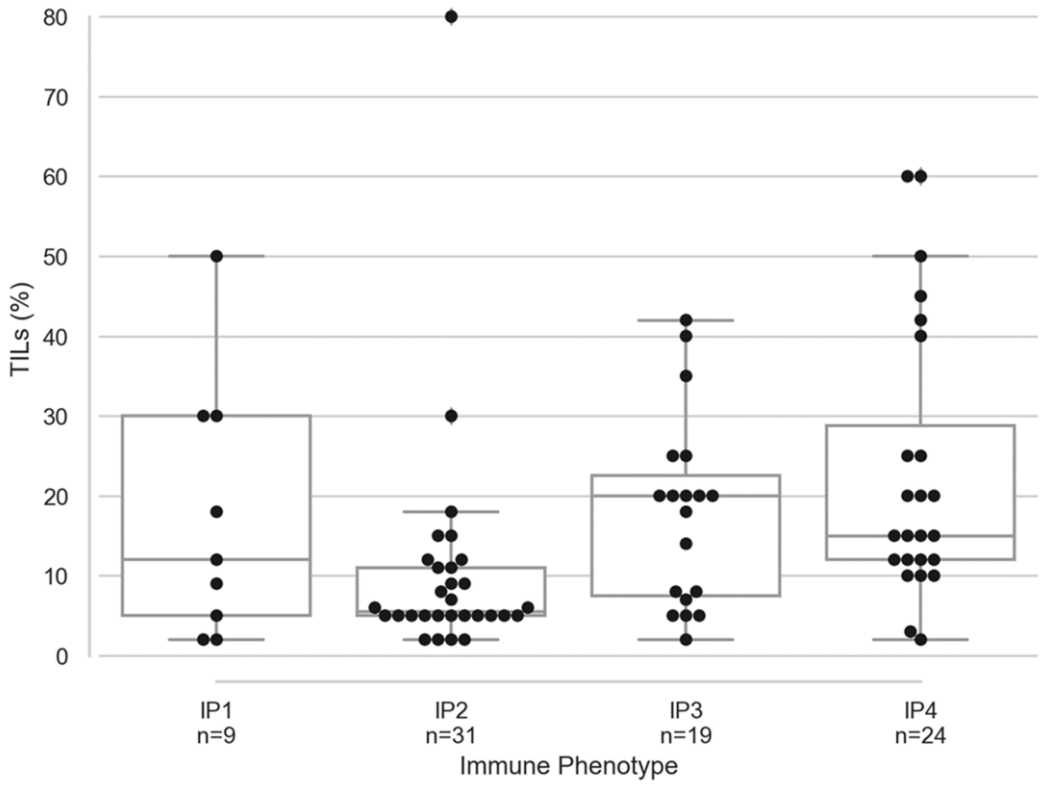

TILs (%)
80
70
60
50
40
30
20
10
0
IP1
n=9
IP2
n=31
IP3
n=19
IP4
n=24
Immune Phenotype

Figure 22. Distinct distribution of the immune cell subpopulations in the distinct immune phenotypes: *A: Log2 abundance score of immune cell subpopulations in IP1-4. Nanostring cell type profiling module was used to assess the immune composition of IPs. B: Frequency of TILs. TILs were counted from H&E-stained slides. Data are presented as % of TIL in the different IPs*.

4.1.5 Immune phenotypes and prognosis

4.1.5.1 Prognostic Role of Immune phenotypes in Ethiopian BC cohort

Since the distinct IPs differ in the activation status of the immune cells, we assessed whether the IPs can predict survival probability. Due to the low number of BC patients the IP1 subgroup was excluded from the analysis. Patients with the IP4 type had a longer, but not statistically significant overall survival (OS) than patients with IP2 and IP3 (Figure 23A). The DEGs from IP4; CD2, CD3E, CD8A, CD8B, CD28, CD69, TAP1, CXCR6, KLRK1 and TIGIT correlated with a higher probability of a longer OS, but due to the limited number of patients involved in each group the difference was not statistically significant. In contrast, among the DEGs a lower expression of CD1A was significantly associated with higher OS (Figure 23B).

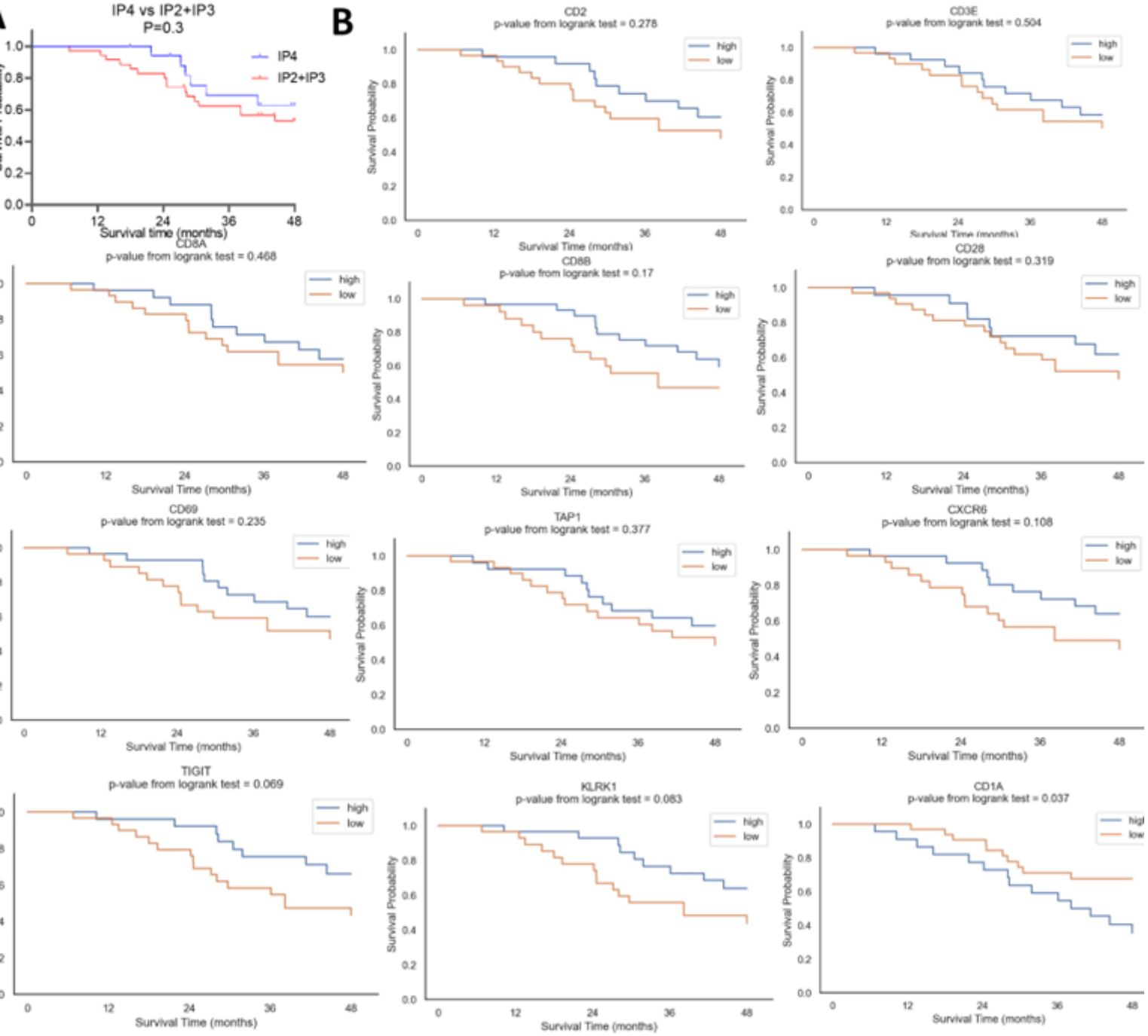


Figure 23. Kaplan-Meier survival curves*: (A) Kaplan-Meier survival curves between IP4 versus IP2 and IP3 (B) differentially up-regulated genes of IP4 and survival probability. Median of the normalized log2 transformed expression data was used to categorize genes in to high and low expression. Patients were followed for 60 months and survival probabilities were determined by calculating p values from log rank test using phyton.*

4.1.5.2 Prognostic Role of Immune phenotypes in the Validation data set

To further validate our findings, the publicly available BRCA data set from TCGA comprising 177 primary invasive BC cases from African American patients was analysed for the expression of 50 out of 54 genes evaluated in our cohort. Hierarchical clustering of these genes revealed a similar pattern with four distinct IPs, each representing a different immune activation status within the TME (Figure 24).

Differential expression analysis (DEA) revealed that IP1 and IP2 exhibited a significantly immunologically active TME with 28 and 35 immune genes, respectively, being upregulated when compared to the other IPs. In contrast, IP3 and IP4 were characterized by a “cold” immune microenvironment with 26 and 29 immune genes downregulated, respectively (Figure 25).

We further investigated the prognostic potential of these IPs. The results demonstrated that IPs with an immune-activated microenvironment (IP1 and IP2) were associated with a significantly improved OS compared to the “cold” immune microenvironment IPs (P = 0.0143) (Figure. 26B). Although IP1 and IP2 showed a trend towards a better OS when stratified by hormone receptor (HR) status (ER or PR), this difference did not reach statistical significance (Figure. 26 C & D).

To refine our analysis, IP1 and IP2 were combined into a single "immune-activated" phenotype, while IP3 and IP4 were classified as "immune-suppressed." This reclassification revealed a significantly higher OS in patients with the immune-activated phenotype compared to those with the immune-suppressed phenotype (P = 0.04). Notably, when stratified by HR status, the immune-activated phenotype was associated with significantly better OS in HR-negative cases (P = 0.0274), but not in HR-positive cases (P = 0.126) (Figure. 26 D, E & G).

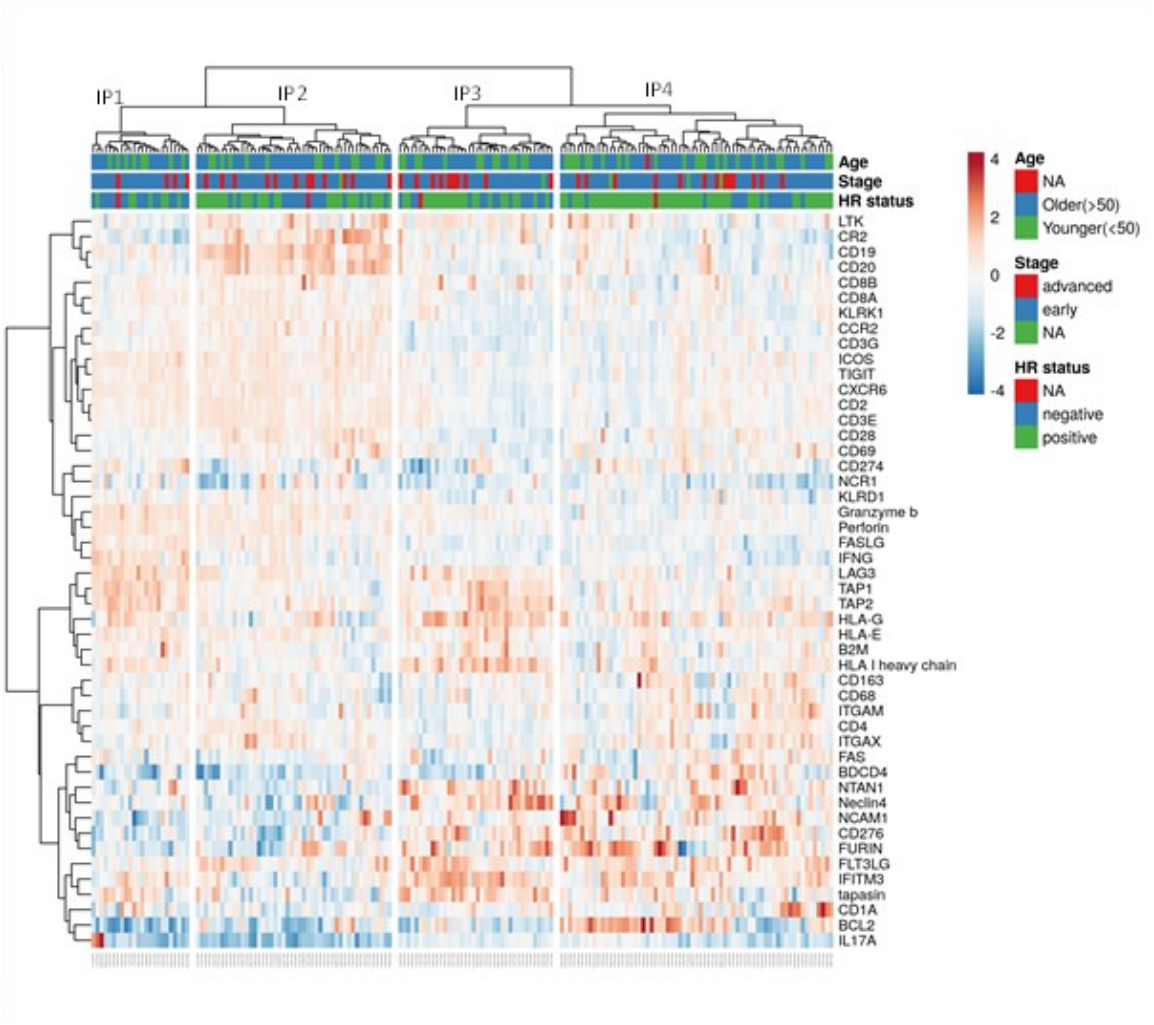


Figure 24. Heatmap of the expression levels of 50 immune-related genes in 177 BC samples from African Americans: *Data were obtained from TCGA database. Unsupervised hierarchical clustering of log-transformed expression levels of immune genes resulted in four immune clusters termed immune phenotypes (IP). Red marks indicate upregulation, blue marks indicate the downregulation of immune-related genes.*

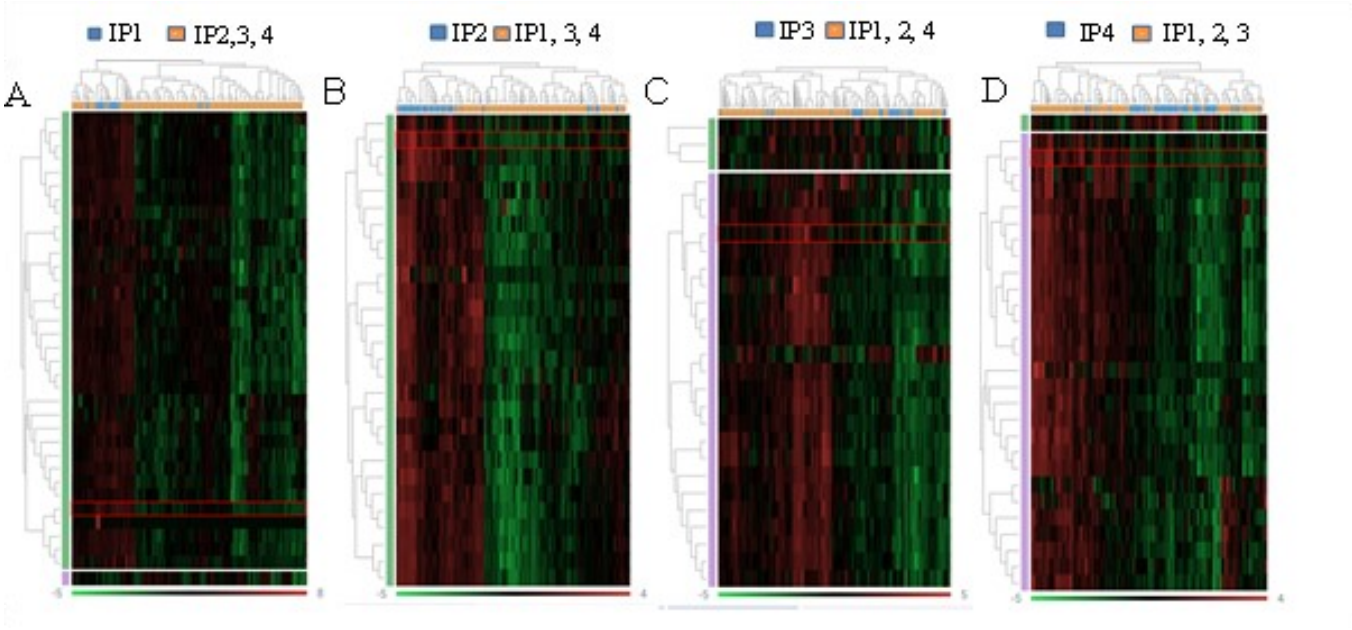


Figure 25. DEG heat map of the distinct immune phenotypes in the TCGA cohort: *A: IP1 versus IP2, IP3 and IP4; B: IP2 versus IP1, IP3 and IP4; C: IP3 versus IP1, IP2 and IP4; D: IP4 versus IP1, IP2 and IP3. Differentially expressed immune genes between the immune phenotypes were shown. Genes with log2 fold change (FC) $\geq$ 1.5 and false discovery rate (FDR)-corrected p value < 0.05 were presented. Green: downregulated genes, red: upregulated genes.*

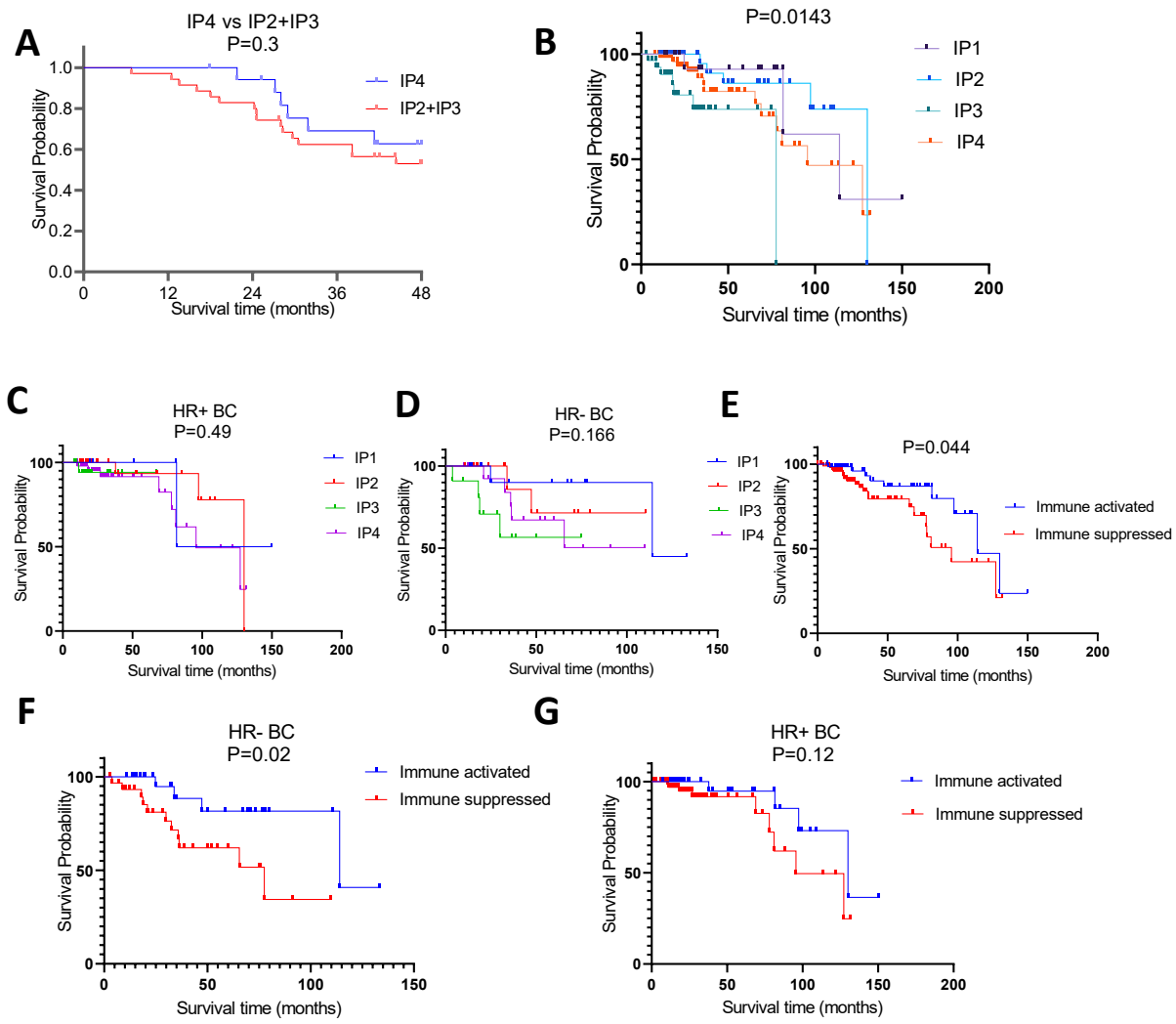


Figure 26. Association of the immune phenotypes with the patients' survival: *A: Kaplan-Meier survival curves between IP4 versus IP2 and IP3. Patients were followed for 60 months and survival probabilities were determined by calculating p values from log rank test. B-G: Kaplan-Meier survival curves derived from TCGA data set of 177 African American BC cases. B: Survival probabilities across the four IPS. C: HR+ BC survival within the four IPs. D: HR- BC survival within the four IPs. E: survival between the two IP groups F: HR- BC survival within the two IP groups. G: HR+ BC survival within the two IP groups. IP1 & IP2 were categorized as "Immune Activated" while IP3 and IP4 were categorized as "Immune Suppressed".*

4.1.6 Clinico-pathologic characteristics and PIK3CA mutation within IPs

The different IPs were analysed in relation to the well-known clinico-pathologic features demonstrating the highest frequency of HER2+ tumors in IP4 (50%) and the lowest in IP3 (15.7%) ($p<0.05$). IP4 and IP2 with 87.5% and 86.6% have the highest proportion of HR^+ tumors, respectively, whereas IP3 has the lowest proportion of HR^+ tumors (47.4%). TNBC were dominant in IP3 (47.4%) (Figure 27A). The analysis of intrinsic BC subtypes revealed that IP1 and IP2 were mainly enriched in luminal subtypes (66.7% and 76.5%, respectively), whereas in IP3 the non-luminal subtypes were dominant with a frequency of 94.7%. Concerning the distribution of intrinsic subtypes, luminal A tumors were the major constituents of IP2 (56.7%), while the basal subtype was predominant in IP3 (84.2%) (Figure 27B). In IP4, the intrinsic subtypes were equally distributed.

Neither the age nor the pathologic stage was related to different IPs ($p= 0.5643$ and 0.199, respectively). In contrast, a higher proportion of high-grade tumors were found in IP1, IP3 and IP4, whereas IP2 had a similar frequency of high- and low-grade tumors *($P<0.05$)* (Figure 27C). The Ki-67 proliferation index indicated a dormancy of the IP2 subtype, while IP3 had the highest proliferation index ($P<0.05$) (Figure 27D).

Since mutations can generate immunogenic neoantigens leading to immune activation (Efremova et al. 2017), it was analyzed whether mutations in the PIK3CA gene are more frequent in the immunologically active phenotype. Analysis of 70/82 BC cases revealed the highest frequencies of PIK3CA-mutated tumors in IP4 (33.3%) and IP2 (31%), respectively without a significant difference between these two extreme IPs. IP3 had a significantly lower proportion of PIK3CA-mutated tumors (6.25%). Interestingly, 75% of the mutations in IP4 occurred within luminal tumors. Mutated tumors have a significantly lower expression of CD8 genes than the wild type BC (Wt mean= 6.3 versus mutated mean=5.6; $p=0.04$), but there was no association with CD4 (Figure 27E, F).

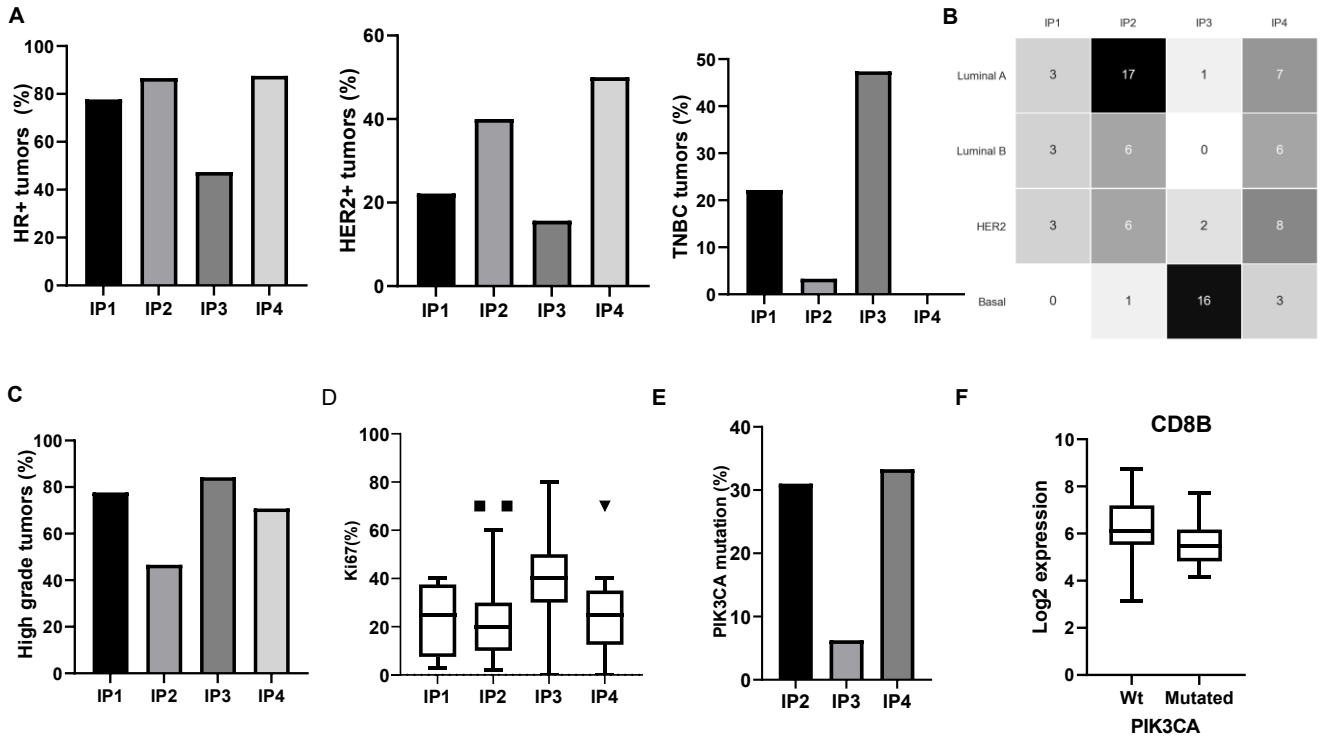


Figure 27. Immune phenotypes and clinic-pathologic features of BC: *A: Proportion of HR+, HER2+ and TNBC tumors. B: Distribution of the intrinsic subtypes within IPs; C: Frequency of high-grade tumors; D: Ki-67 proliferation index; E. Proportion of PIK3CA-mutated tumors: Only 9 tumors were grouped in IP1 and the mutation status could not be determined for the majority of samples (proportion not shown in the figure); F: A box and Whisker plot showing log2 expression value of CD8 in the wt and PIK3CA-mutated tumors.*

4.1.7 Differential expression of immune genes across breast cancer subtypes and clinicopathologic Features

The normal breast tissue control obtained from 2 pooled samples was analysed for immune gene expression and compared to that of BCs. Interestingly, no significantly differentially expressed immune gene (DEIGs) was detected between control and BC tissues, from the 54 immune genes analysed. To make things more fascinating, this pooled control sample clustered within IP2 during hierarchical clustering (Figure 20). This suggests a similarity in the immune microenvironment of the normal breast tissue with the TIME of the immune inert BC subtype.

In order to get better insights whether the TME changes with age in BC, the DEIG signatures between the age groups <50 (younger) versus ≥ 50 (older) were investigated. However, no age-dependent significant DEIGs were detected. Furthermore, analysis of the impact of the expression of immune genes in the TME on prognostic parameters and pathologic features demonstrated that CD20 and GZMB were significantly downregulated in advanced pathologic stages when compared with early BC stages suggesting their use as biomarkers (Figure 28A). In contrast, no significant DEIGs were found between high grade and low-grade tumours.

Since distinct immune responses have been reported in BC intrinsic subtypes (Griguolo et al. 2021; Li, Wu, and Han 2023) , the expression of immune-relevant genes was analysed in the intrinsic subtypes by comparing luminal with non-luminal tumors. Genes associated with antigen processing and presentation (TAP1, TAP2, and CD1A), CTL- and NK cell-mediated cytotoxicity (GZMB, perforin), immune checkpoints (CD274, LAG-3), chemokines, e.g. CXCL8, as well as the immunoregulatory metabolite IDO1 implicated in T cell inhibition (Dill et al. 2018) were upregulated in non-luminal tumors versus luminal tumors (Figure 28B). Whereas a comparison of luminal versus basal-like and HER2+ tumors separately revealed downregulation of IDO1, CD1A, CXCL8, GZMB, TAP2, HLA-G, NCR1, LAG3, TAP1, CTLA4, HLA-A, CD274 and CD163 in luminal compared to basal-like tumors, CXCL8 was the only gene that remained downregulated in luminal tumors when compared to HER2+ tumors (Figure. 28C). Analysis of the differences in the gene expression patterns between luminal A and luminal B tumors demonstrated that IFITM3 was the only significantly differentially expressed gene *(FC=2.1; p=0.00095)*. Comparison within non-luminal tumors revealed an upregulation of CD1A, IDO1, HLA-G and TAP2 and a down regulation of NTAN in basal subtypes when compared to HER-2-enriched tumors.

Immune gene expression analysis across the IHC groups showed minimal variation between the subtypes. Only CD1A was downregulated, while CTBS was upregulated in HR+ BC compared to TNBC. No significant DEIGs were observed when comparing HR+ BC to HER2+ BC (Figure. 28D).

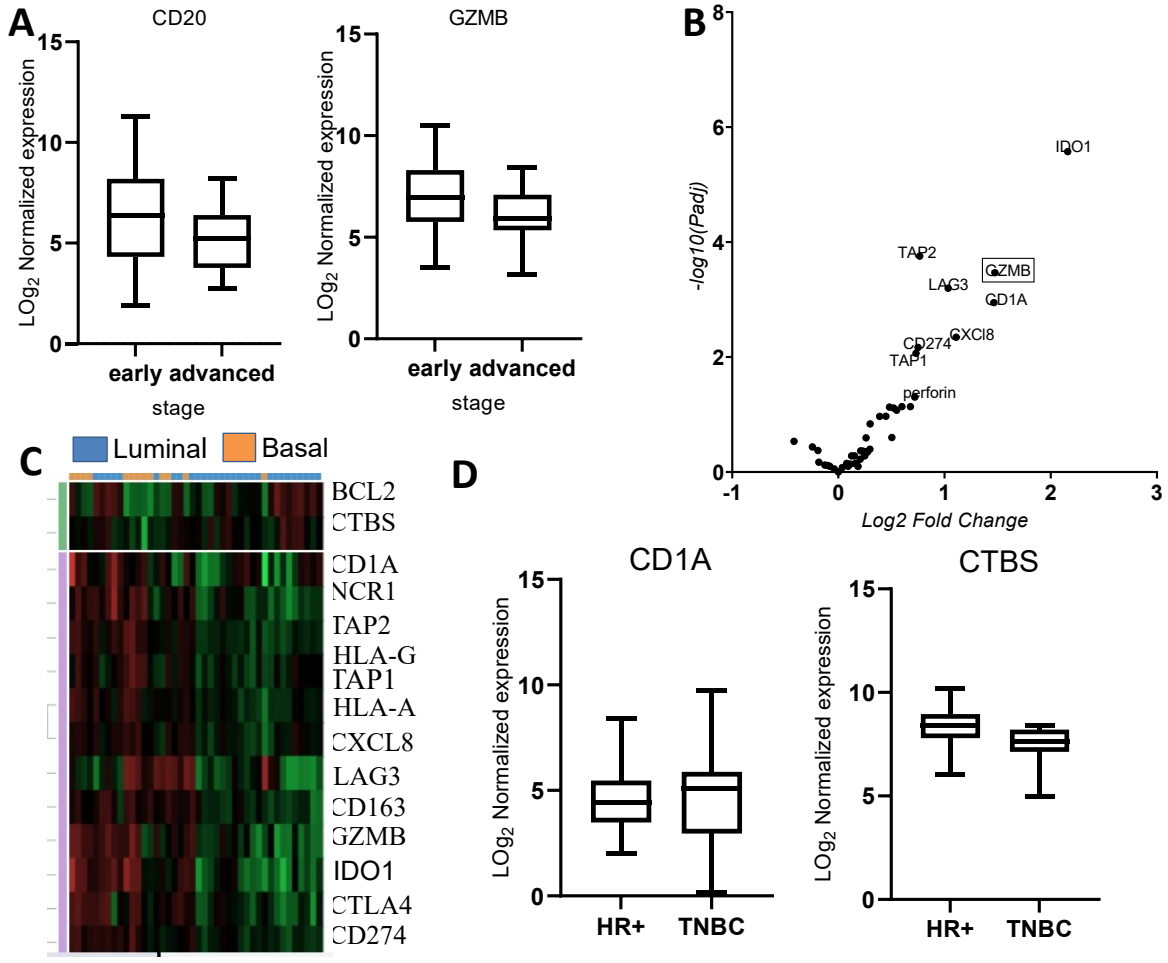


Figure 28. Differentially expressed immune genes in BC subtypes: ***(****A) Box and whisker plot showing a differential expression of CD20 and GZMB between early and advanced pathologic stages. (B) Volcano plot of DEGs between luminal and non-luminal tumors. Volcano plot is plotted using graph pad prism to show differentially expressed immune genes; FC>1.5, adjusted P values<0.05. Each dot represents an immune gene. (C) DEG heat map of luminal versus basal like tumors, green shows downregulation and red shows upregulation. (D) Box and whisker plot showing a differential expression of CD1A and CTBS between HR+ and TNBC. DEG: Differentially expressed genes.*

4.2 Immune profiling of Peripheral blood in Ethiopian BC patients

4.2.1 Clinicopathologic Characteristics of the BC patients and HCs

A total of 65 Ethiopian BC patients with a median age of 41 years (22 - 83) were recruited in to this immune-phenotyping study while the control group comprises ten healthy females (median age 36 years). Pathological staging of tumors was done according to the American Joint Committee on Cancer (AJCC) TNM system. The BC cases were equally distributed between stage 2 and stage 3 tumors with only a few stages 1 cases (n=5, 8%). For further statistical analysis, simple staging was used where stage 1 and stage 2 tumors were considered early-stage tumors whereas stage 3 and stage 4 were labelled as advanced tumors. Histological grading revealed that more than half of the BC lesions were grade 2 and one-third grade 3. With respect to the histological type, most of the tumors were IDC (80%), whereas only 3 cases (4.6%) were ILC and the rest with unknown, mixed or other histology. The majority of the patients were positive for HR (78.5%) and negative for HER2 over-expression (63%) as determined by IHC. The PAM50 intrinsic based subtype classification was performed only on 45 samples due to difficulties in retrieving sufficient RNA from the formalin-fixed samples. Accordingly, almost half of the evaluated patients showed a luminal A subtype (46.7%, corresponding to 32.3% of the total cohort), while the second most frequent type was the basal-like subtype (22.2% of the evaluated patients (corresponding to 15.4% of the patient cohort) (Table 5).

Table 13. Clinico-pathologic characteristics of breast cancer patients

Features	Patient number (%)
Age	
median	41
min	22
max	83
Pathologic Stage	
stage 1	5 (8%)
stage 2	28 (43%)
stage 3	28 (43%)
unknown	4 (6%)
Histological grade	
G1	4 (6%)
G2	37 (57%)
G3	22 (33.9 %)
unknown	2 (3.1%)
Histological type	
Invasive ductal	52 (80%)
Invasive lobular	3 (4.6%)
Mixed (ductal & lobular) _	2 (3.1%)
Others	6 (9.2%)
unknown	2 (3.1%)
HR status	
HR^+	51 (78.5%)
HR^-	8 (12.3%)
unknown	6 (9.2%)
HER2 status	
$HER2^+$	16 (24.7 %)
$HER2^-$	41 (63 %)
unknown	8 (12.3%)
Ki-67	

4.2.2 Immunological differences between BC patients and HC

PBMC from BC patients and HC were stained with the different Ab panels (Table 3) and evaluated for differences in the frequencies as well as phenotype of the major immune cell populations and sub-populations. Since PBMCs were employed, no absolute cell counts were determined and data are presented as cell frequencies within the PBMC or the indicated immune subsets. In the following sections, the results for the different immune subpopulations are presented.

4.2.2.1 T cells and their major subpopulations

T cells were identified phenotypically as CD3+ cells gated from CD45 side scatter (SSC) populations. BC patients exhibited a higher frequency of total CD3+ T cells within PBMCs compared to HCs, primarily due to significantly elevated levels of $CD8^+$ T cells. In contrast, CD4+ T cells showed a non-significant trend toward reduced numbers (Figure 29A).

Functional subsets of CD4+ and CD8+ T cells were classified based on CD45RA and CCR7 expression into naïve (CCR7+CD45RA+), central memory (Tcm, CCR7+CD45RA−), effector memory (Tem, CCR7−CD45RA−), and effector T cells (CCR7−CD45RA+), as illustrated in the gating strategy (Figure 15). The memory phenotype analysis revealed a lower proportion of naïve CD4+ T cells in BC, though not statistically significant (Figure 29B). A more prominent and statistically significant difference was observed within CD8 cells. CD8+ T cells in BC patients had significantly fewer naïve cells and an increased frequency of Tcm cells (Figure 29C).

To assess potential functional impairments, we analyzed activation and exhaustion markers. Both CD4+ and CD8+ T cells in BC patients exhibited significantly higher HLA-DR expression but lower CD38 expression. Despite this, the proportion of double-positive; HLA-DR+CD38+ CD4+ and CD8+ T cells remained similar between BC and HC groups (Figure 32D-E). CD28 expression was unchanged in CD4+ T cells but it was significantly downregulated in CD8+ T cells from BC patients (Figure 29G). CD57, a marker of proliferative potential, showed a trend toward increased expression in CD8+ T cells but not in CD4+ T cells. Functionally, BC patients and HCS had comparable levels of the intracellular signaling molecule CD3ζ in both CD4+ and CD8+ T cells. CD8+ T cells in BC patients displayed significantly elevated perforin expression (32F), with a trend toward higher CD3ζ and perforin co-expression, though not statistically

significant (data not shown). Finally, the evaluation of exhaustion markers showed a significant upregulation of PD-1 and PD-L1 in CD4+ T cells, whereas CD8+ T cells did not exhibit this pattern (Figure 29F). No significant differences were observed in CTLA4, Tim3, or Lir1 expression between BC and HC groups for either CD4+ or CD8+ T cells (data not shown).

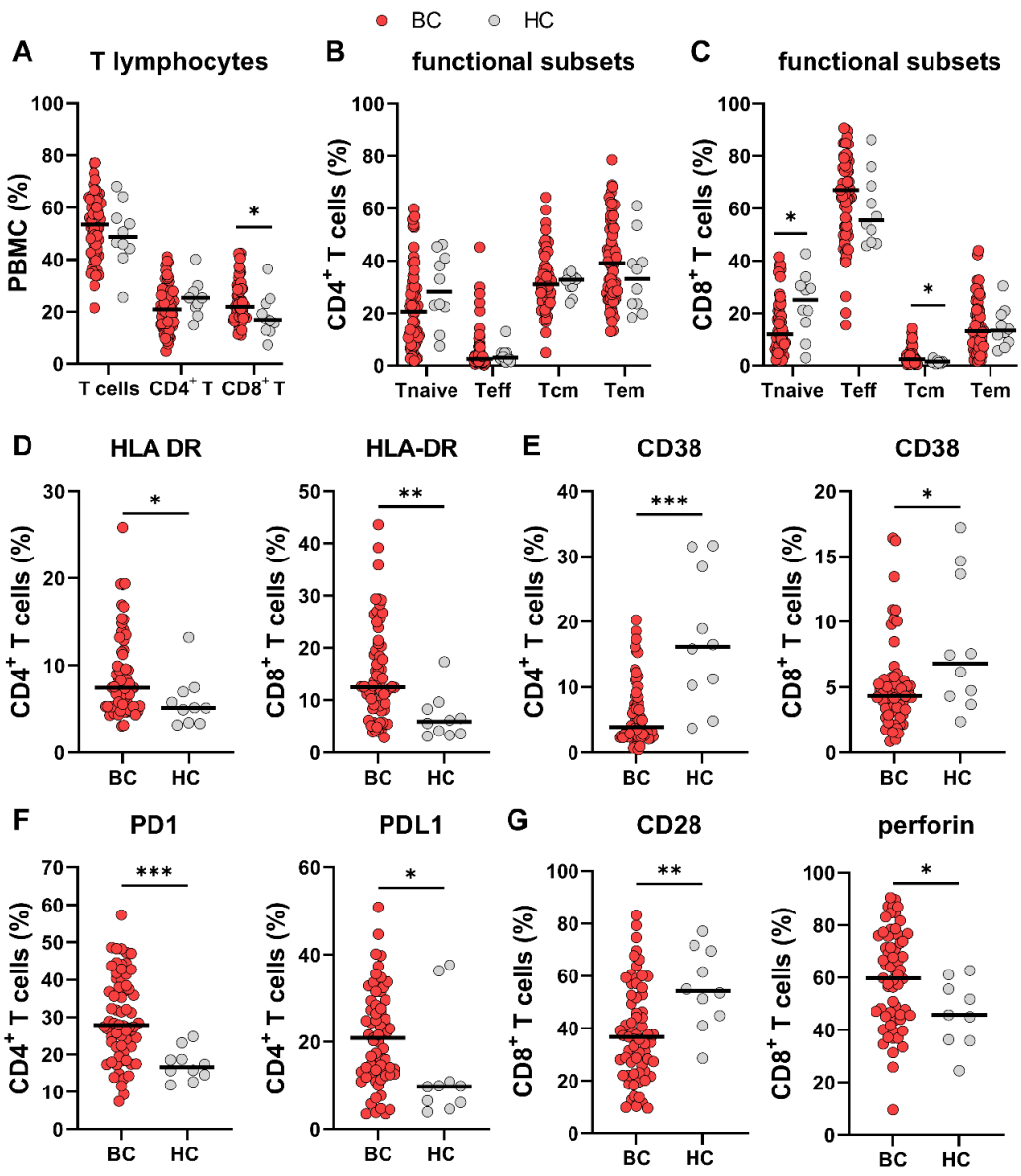


Figure 29. Frequency and phenotype of the major T cell subpopulations: *The percentages of total T cells, CD4$^+$ and CD8$^+$ T cells within the PBMC (A), of the different memory subsets of CD4$^+$ (B) and CD8$^+$ T cells (C) and of CD4$^+$ or CD8$^+$ T cells expressing the indicated markers (D-G) are shown for BC patients and HC as individual values together with their median values. Tnaive; naïve T cells, Teff; effector memory T cells, Tcm; central memory T cells, Tem: effector T cells. Individual data together with their median values are shown. * p<0.05, ** p<0.01, *** p<0.001.*

4.2.2.2 T cell phenotype in breast cancer patients with inverted CD4/CD8 ratios

In addition to the analysis of the activation, memory, and exhaustion markers of CD4 and CD8 T cells, we further compared the CD4/CD8 ratio, prognostic marker used to evaluate the function of the immune system. Using 1 as a cut off value to define inverted CD4/CD8 ratio, The CD4/CD8 T cell ratio was significantly depressed in BC patients compared to HC groups. As a consequence of the enhanced CD8$^+$ T cell frequencies, breast cancer patients had a lower CD4/CD8 T cell ratio than HC, with more than half of the breast cancer patients (n=33, 50.7%) having an inverted ratio (Figure 30A). In order to determine the consequence of such inverted ratio, CD4$^+$ and CD8$^+$ T cells were re-evaluated upon subdivision of the breast cancer patients into BC^{IR} (patient with inverted ratio, CD4/CD8 <1) and BC^{NR} (patient with normal ratio, CD4/CD8 >1). The median age of BC^{IR} and BC^{NR} was 40.5 and 42.5 years, respectively, and statistical evaluation suggested no significant correlation between the CD4/CD8 ratio and patients´ age (p=0.0928; data not shown).

Splitting the breast cancer patients into the BC^{IR} and BC^{NR} subgroups highlighted that the increase in the central memory CD8$^+$ T cells in the total cohort was restricted to the BC^{NR} sub-cohort, whereas the loss of naïve CD8$^+$ T cells was more prominent in the BC^{IR} patients. Moreover, a statistically significant increase in effector CD8$^+$ T cells was specifically found in the BC^{IR} patients (Figure 30B). Regarding the functional phenotype of the CD8$^+$ T cells, the BC^{IR} sub-cohort displayed a more substantial loss of CD28 and a specific increase in perforin and CD57 (Figure 30C). Concerning CD4$^+$ T cells, BC^{IR} patients had significantly less naïve and more effector memory CD4$^+$ T cells than BC^{NR} and HC (Figure 30D). However, other markers of immunosenescence including CD28 and CD57 had a slight trend of being downregulated and upregulated respectively in BC^{IR} than BC^{NR} but without statistical significance. Though the

exhaustion markers PD1 and PD-L1 didn't differ between BC^{IR} and BC^{NR}, they were significantly upregulated in BC^{IR} when compared with HC (Data not shown).

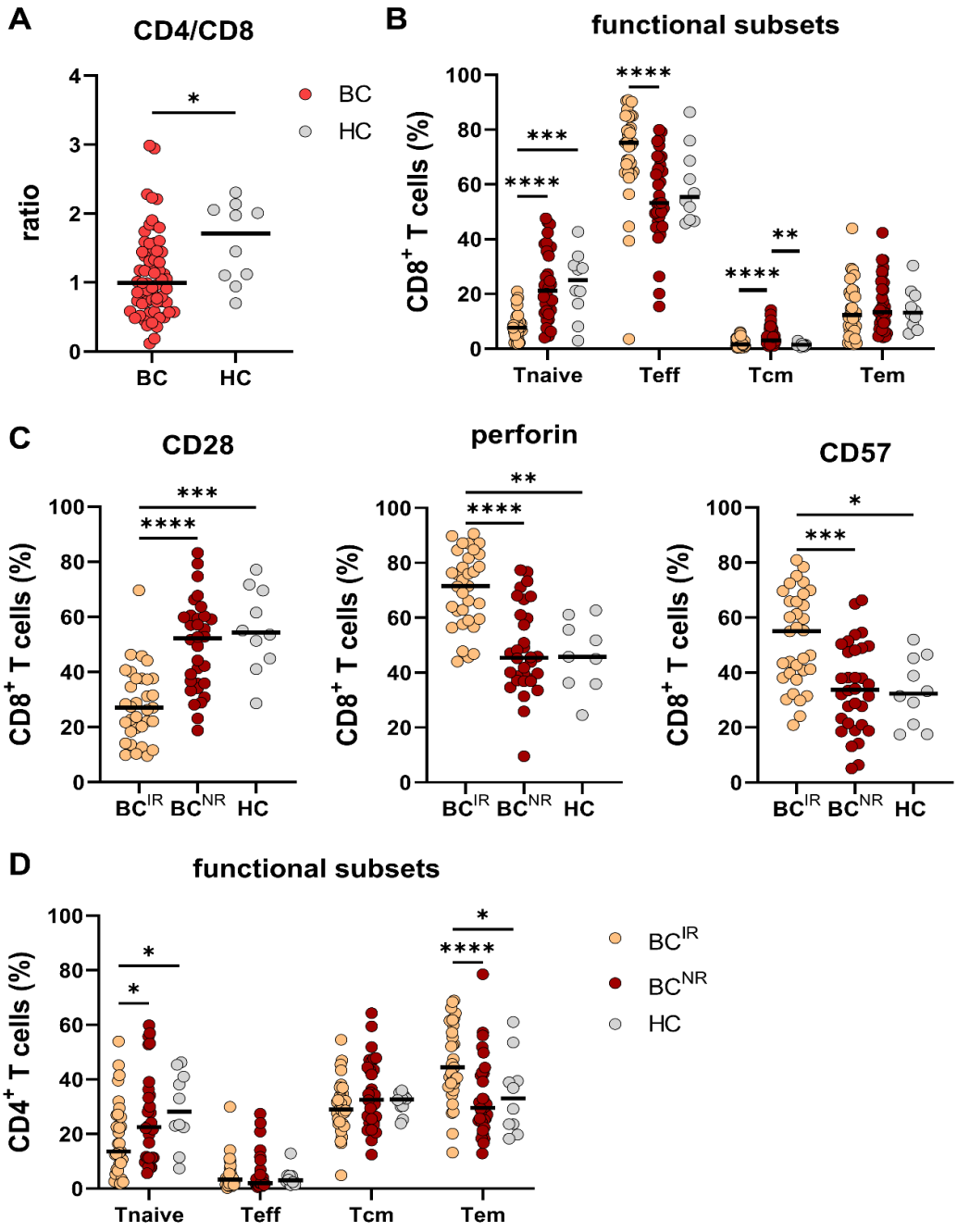


Figure 30. Immune phenotype of BC patients based on their CD4/CD8 ratio: *The ratio between $CD4^+$ and $CD8^+$ T cells in BC patients and HC was calculated (A) and used to distinguish patients with an inverted (BC^{IR}) or a normal (BC^{NR}) CD4/CD8 ratio. The frequencies*

*of the different memory subsets and marker positive cells among CD8$^+$ (B-C) and CD4$^+$ T cells (D) are shown for the BC^{IR} and BC^{NR} patients and HC. Individual data together with their median values are shown. * p<0.05, ** p<0.01, *** p<0.001.*

4.2.2.3 Minor T cell subpopulations

As illustrated in Figure 31A, the number of γδ T cells was significantly increased in the breast cancer patients compared to the HC controls, but whether these cells were promoting breast cancer cohort requires further investigation. In addition, it is noteworthy that the Ethiopian cohort of HC had higher frequencies of γδ T cells (range from 3.2 to 16.3% of all T lymphocytes) than the Caucasian population (range from 0.5 to 5% of T cells) (Hviid et al. 2000).

Tregs identified as CD25$^+$ FoxP3$^+$ CD127^{low} CD4$^+$ cells (gating strategy in Figure 16) demonstrated a non-statistically significant trend toward increased frequencies within both PBMCs and CD4+ T cells in BC patients compared to HC (Figure 31B and data not shown). Since Tregs display a functional heterogeneity, discrimination of different functional subsets based on the staining for the chemokine receptor CCR4 and the activation marker CD45RO highlighted an increase of the highly suppressive double positive subpopulation (Watanabe et al. 2019), which was statistically significant when evaluated among Tregs, but not as percentage of total PBMC (Figure 31C and 31D). The proportion of Tregs expressing HLA-DR was assessed to further evaluate their suppressive activity in BC patients. Although BC patients had a higher frequency of HLA-DR$^+$ Tregs compared to controls, the difference was not statistically significant (data not shown).

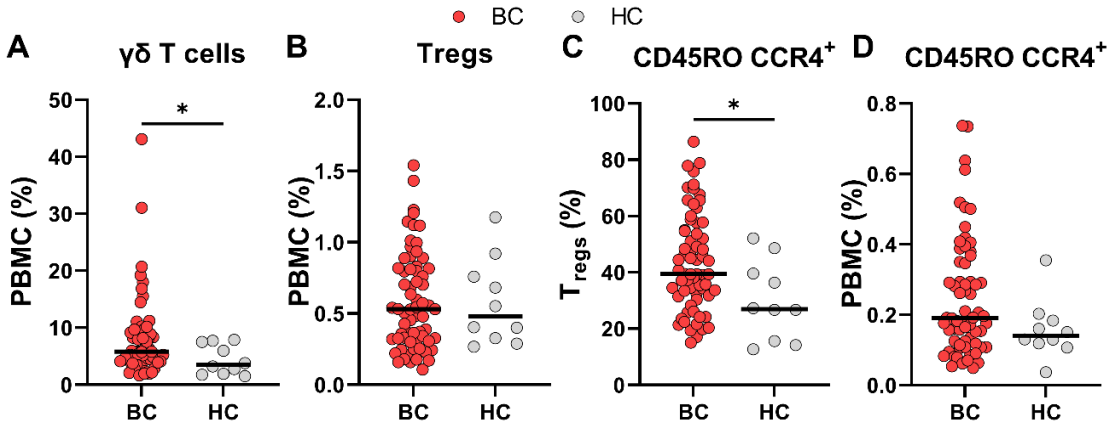


Figure 31. Frequencies of minor T cell subpopulations: *The frequencies of γδ T cells (A) and Treg (B) within total PBMC as well as of the CD45RO CCR4 double positive Treg within the Treg (C) or total PBMC (D) are shown for BC patients and HC as individual values together with their median values. Individual data together with their median values are shown. * p<0.05, ** p<0.01, *** p<0.001.*

4.2.2.4 B cell frequencies and phenotype

The total frequency of B lymphocytes; identified as $CD45^+$ $CD19^+$ cells (gating strategy in Figure 17) was comparable between BC patients and HC (data not shown). Further gating based on IgD and CD27 staining showed statistically significant lower levels of naïve B cells and higher levels of plasma blast in breast cancer patients (Figure 32A). A non-statistically significant trend towards higher frequencies of switch memory B cells in breast cancer patients was also found (Figure 32A). Interestingly, the ICP molecules PD1, PD-L1 as well as CTLA4 were significantly elevated in B cells from BC patients when compared to HC (Figure 32B).

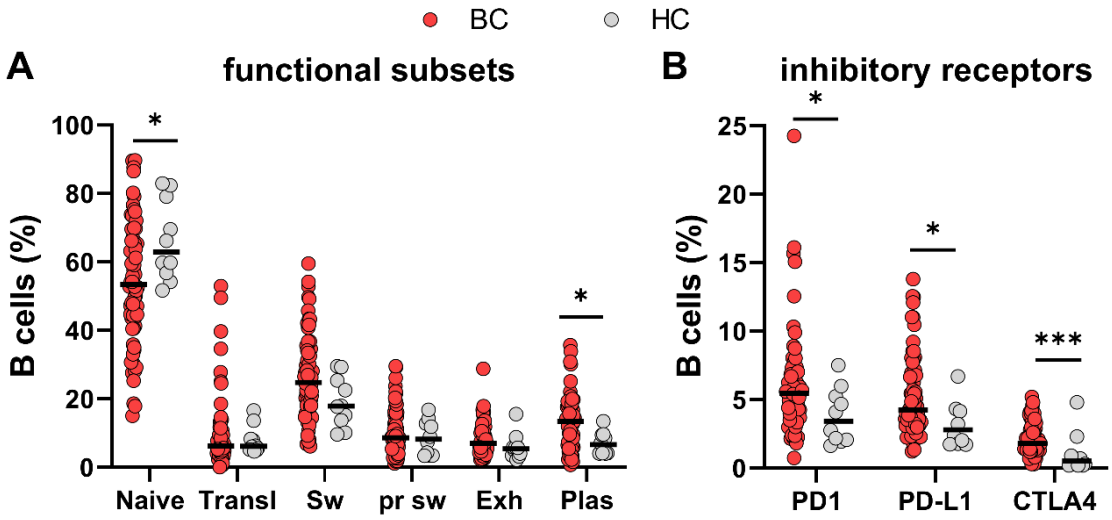


Figure 32. B cell frequencies: *The frequencies of the different functional subsets of B cells (A) as well as of the cells expressing the indicated markers (B) are shown for BC patients and HC as individual values together with their median values. The B cell subsets were identified as following: Naïve (IgD^+ $CD27^-$), translational (Transl, IgD^+ $CD27^-$ $CD24^+$ $CD38^+$); switch memory (Sw, IgD^- $CD27^+$); pre switch memory (pr sw, IgD^+ $CD27^+$); exhausted memory (Exh, IgD^- $CD27^-$) and plasmablast (Plas, IgD^- $CD27^+$ $CD24^+$ $CD38^-$). Individual data together with their median values are shown. * p<0.05, ** p<0.01, *** p<0.001.*

4.2.2.5 Natural killer (NK) cells

NK cells, identified using CD16 and CD56 markers, were analyzed in both BC patients and HCs. The total NK cell count, along with its two subpopulations—CD56bright/CD16bright and CD56dim/CD16bright—showed no significant differences between the two groups (data not shown). Although the proportion and classification of NK cells remained comparable, their functional activity was altered in BC patients. NK cells from breast cancer patients displayed higher expression levels of the CD3ζ chain and perforin than HC, also resulting in higher frequencies of double positive cells (Figure 33).

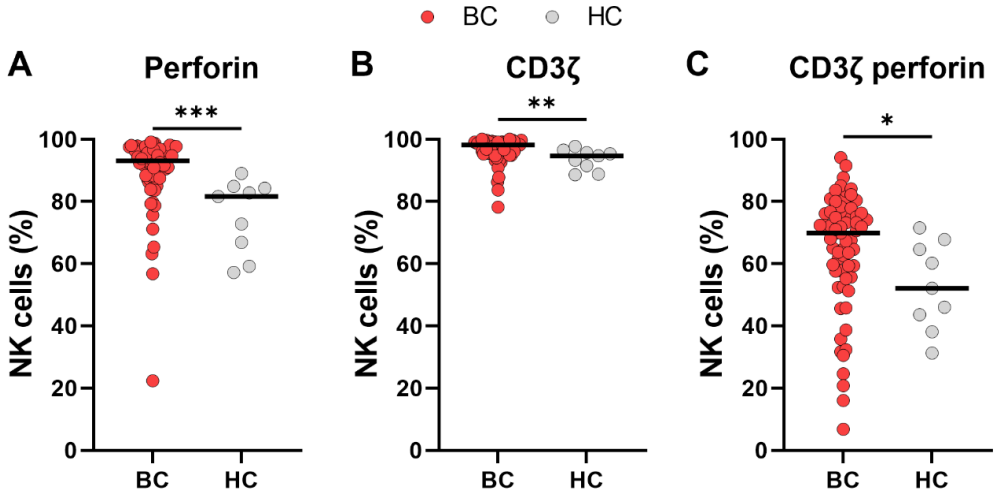


Figure 33. Phenotype of NK cells in breast cancer patients and HC: *Frequencies of NK cells expressing the indicated marker are shown for breast cancer patients (BC) and healthy controls (HC). Individual data together with their median values are shown. * p<0.05, ** p<0.01, *** p<0.001.*

4.2.2.6 Myeloid cells

Additionally, lineage negative (i.e. CD3, CD19, CD16 and CD56), HLA-DR$^+$ DCs were analyzed (Gating strategy in figure 18), revealing a significantly lower presence in BC patients compared to HCs. This reduction was consistent across both myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), which were identified by the markers CD11c and CD123, respectively (Figure 34A**).**

The number of total monocytes tended to be lower in breast cancer patients, but the difference was not statistically significant (Figure 34B). In addition, subdivision into classical, intermediate and inflammatory monocytes based on the expression of CD14 and CD16 did not result in differences between breast cancer patients and HC (data not shown: gating strategy in Figure 18.

MDSC are a heterogeneous population of immature cells which can be subdivided into $CD11b^{+}$ $CD14^{-}$ $CD15^{+}$ polymorphonuclear MDSC (PMN-MDSC), $CD11b^{+}$ $CD14^{+}$ $HLA\text{-}DR^{-/low}$ $CD15^{-}$ monocytic MDSC (M-MDSC) and $HLA\text{-}DR^{-/low}$ $CD33^{+}$ early-stage MDSC (e-MDSC) (gating strategy in Figure 19). The frequencies of the highly immune suppressive PMN-MDSC subpopulation were significantly higher in BC patients than in HC (p value <0.0001) (Figure 34C), whereas only a trend toward expansion in BC patients was found for the M-MDSC. Unexpectedly, e-MDSC were significantly lower in BC patients than in HC.

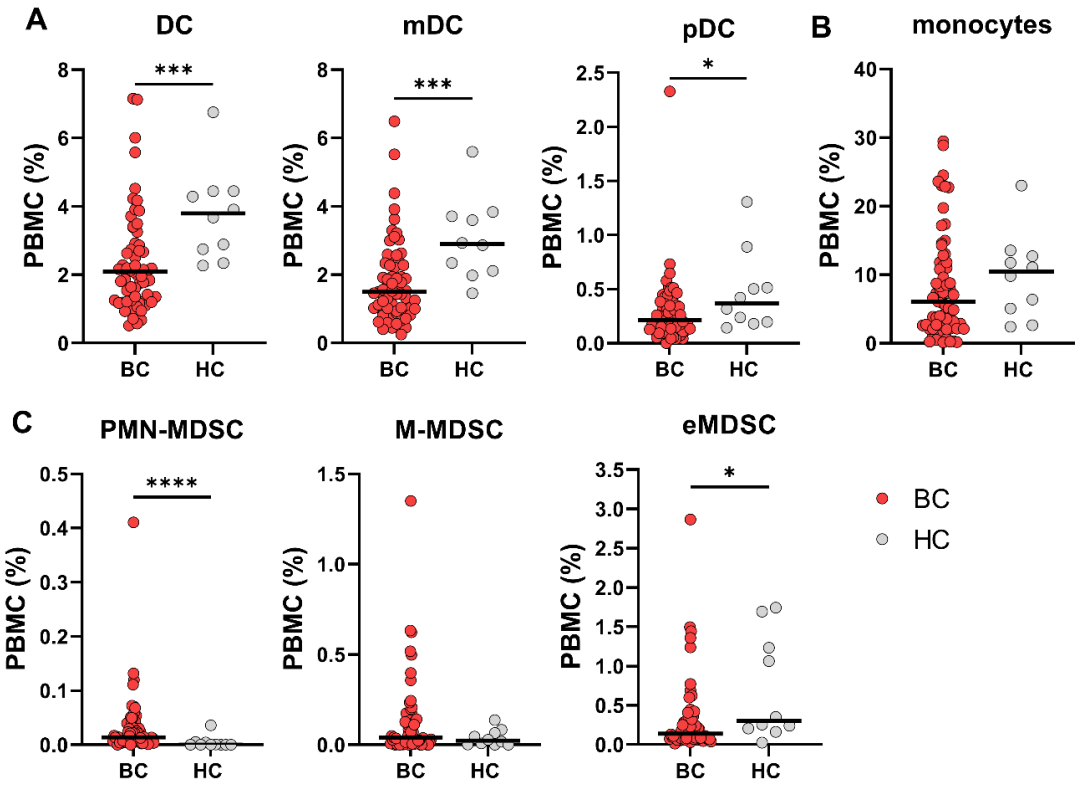


Figure 34. Frequency of myeloid cells: *The frequencies of DC and their subpopulations (A), monocytes (B) and of the different MDSC subpopulations (C) are shown for BC patients and HC as individual values together with their median values. * p<0.05, ** p<0.01, *** p<0.001.*

4.2.3 Clinical relevance of the immune cell composition in breast cancer patients

To evaluate the clinical significance of peripheral blood immune characteristics in BC patients, we analyzed the frequency and phenotype of various immune cell subpopulations in relation to age, tumor grade, pathological stage, histologic type, HR status (ER or PR positivity), HER-2/neu status, intrinsic subtype (PAM50 subtypes), and the proliferation marker Ki-67. Our analysis revealed varying degrees of correlation between immune parameters and factors such as age, pathologic grade, histologic type, intrinsic type. However, no significant correlations were observed between immune parameters and pathologic stage, HR status, or HER-2/neu status, Ki-67.

Aging did not significantly alter the relative abundance of immune cells within the total PBMC fraction, except for monocytes and PMN-MDSCs, which declined with age. Within T cell populations, the proportion of CD8+ T cells showed a positive correlation with aging but not CD4+ T cells. Notably, the frequency of naïve CD4+ and CD8+ T cells significantly decreased with age, while effector memory and effector T cells increased in CD4+ and CD8+ subsets, respectively. Assessment of immune activation, inhibition, and senescence markers in relation to age revealed an increase in CD57-expressing CD4+ and CD8+ T cells, whereas the co-stimulatory molecules CD28 and CD38 significantly declined in CD8+ T cells. Other markers of immune activation or exhaustion showed no correlation with age (data not shown).

Reduced T cell frequencies were detected in tumors with increased grading with a statistical significance reached between the slowly proliferating G1 and the highly aggressive G3 tumor (Figure 35A), but the number of G1 breast cancer cases was very low.

No significant differences among the four intrinsic subtypes were found but in comparison to HC, patients with luminal A tumors displayed significantly higher frequencies of $CD4^+$ T cells expressing PD1 (Figure 35B). Division of the BC patients into luminal (n=29) and non-luminal cases (n=16), showed that luminal tumor associated with higher frequencies of $CD8^+$ T cell, and thus also lower CD4/CD8 T cell ratio (Figure 35C-D) whereas the opposite tendency was found for B cells with enhanced frequencies in non-luminal tumors (Figure 35E).

Despite the limited number of ILC cases, some statistically significant differences were also found with respect to the histologic subtypes. Among the major populations within the PBMC, ILC cases had reduced frequencies of $CD56^{br}$ NK cells (Figure 35F). In contrast, within the $CD4^+$ T cells, ILC had higher frequencies of effector $CD4^+$ T cells whereas central memory $CD4^+$ T cells were significantly reduced (Figure 35G-I). Furthermore, double-positive cells (CD3ζ+ Perforin+), total perforin, CD57+ cells, and LIR+ cells were all significantly more abundant in ILC (Data not shown).

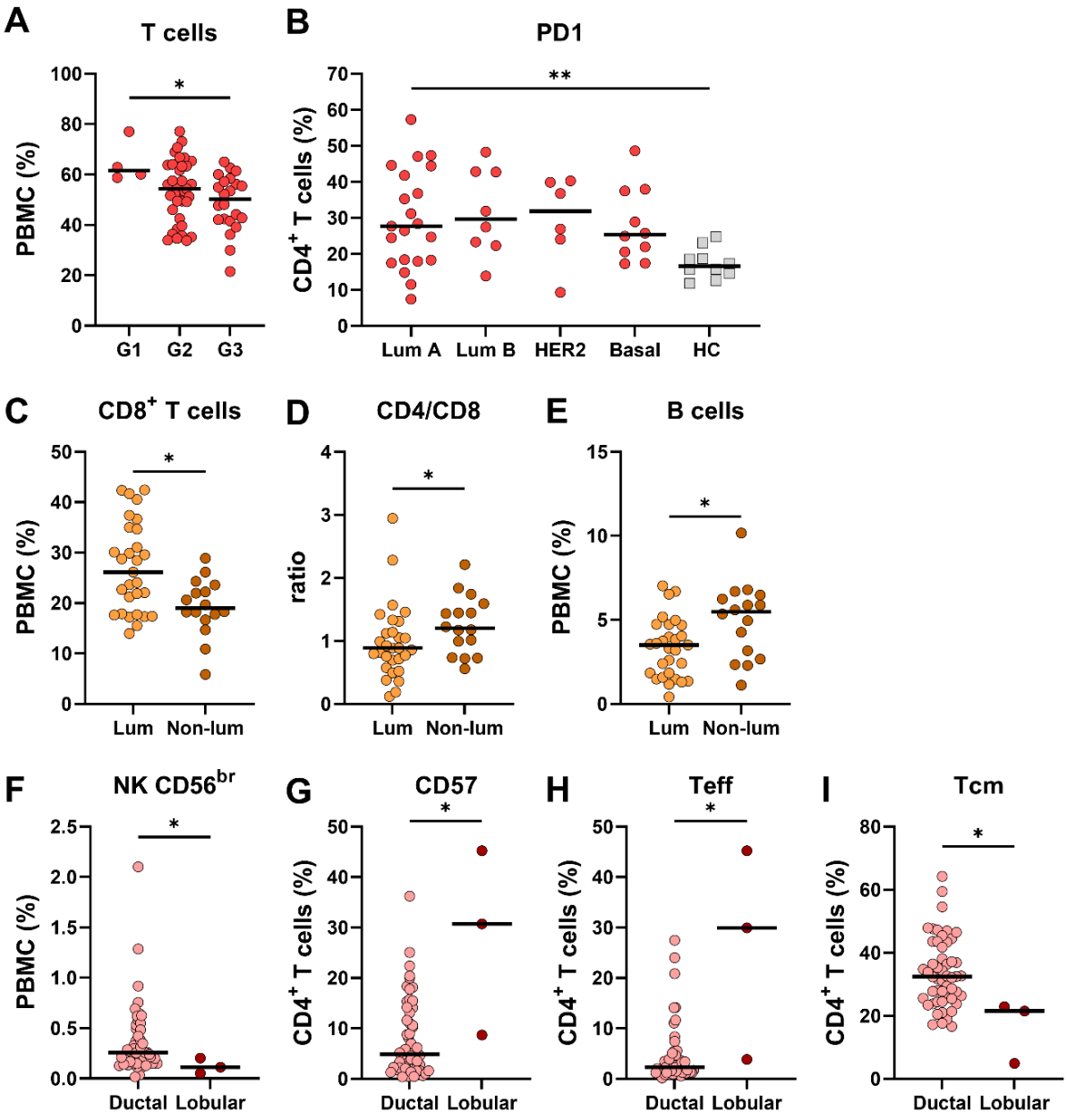


Figure 35. Correlation of immune parameters with breast cancer patients´ clinical characteristics: *(A) Frequencies of T cells within PBMC are shown for the BC patients based on their tumor grading. (B) Frequencies of PD1$^+$ cells among CD4$^+$ T cells in HC and BC patients subdivided by their molecular intrinsic subtypes. (C-E) BC patients were grouped into luminal and non-luminal cases. Shown are the frequencies of CD8$^+$ T cells (C), the CD4/CD8 T cell ratios (D) and the B cell frequencies (E). (F-I) BC patients were grouped into invasive ductal and lobular cases. Shown are the frequencies of CD56^{br} NK cells within the PBMC (F) as well as the frequency of CD57 (G), effector (H) and central memory (I) cells within CD4$^+$ T cells. Individual values are presented together with their median values. * p<0.05, ** p<0.01, *** p<0.001.*

4.2.4 Immune cell populations before and after surgery

The tumor immune microenvironment and systemic immunity undergo significant changes during carcinogenesis. We hypothesized that the removal of tumor burden through surgery would lead to alterations in immune cell populations. To test this, 24 pre- and post-surgery samples were analyzed to assess the impact of surgical intervention on the phenotype and function of immune cells. Notably, the most prominent changes were observed in CD4$^+$ T cells. Post-surgical analysis revealed a decline in effector CD4$^+$ T cells and PD-1$^+$CD57$^+$ double-positive cells, while the expression of the costimulatory molecule CD28 and CD57 increased following surgery (Figure 36A).

In CD8$^+$ T cells, an unexpected increase in the expression of co-inhibitory molecules was observed after surgery. Meanwhile, CD57 expression in CD8$^+$ T cells exhibited a decreasing trend postoperatively, with a borderline statistical significance (P = 0.0526) (Figure 36B). Furthermore, when analyzed as a proportion of total T cells, CD8$^+$ T cells demonstrated a trend towards a lower frequency post-surgery (P = 0.0787) (Figure 36C).

Regarding regulatory T cell (Treg) subsets, the CD45RO$^-$CCR4$^-$ Treg population, which is associated with a loss of immune suppressive function, increased following surgical intervention. However, no significant changes were detected in other Treg subsets (Figure 36D).

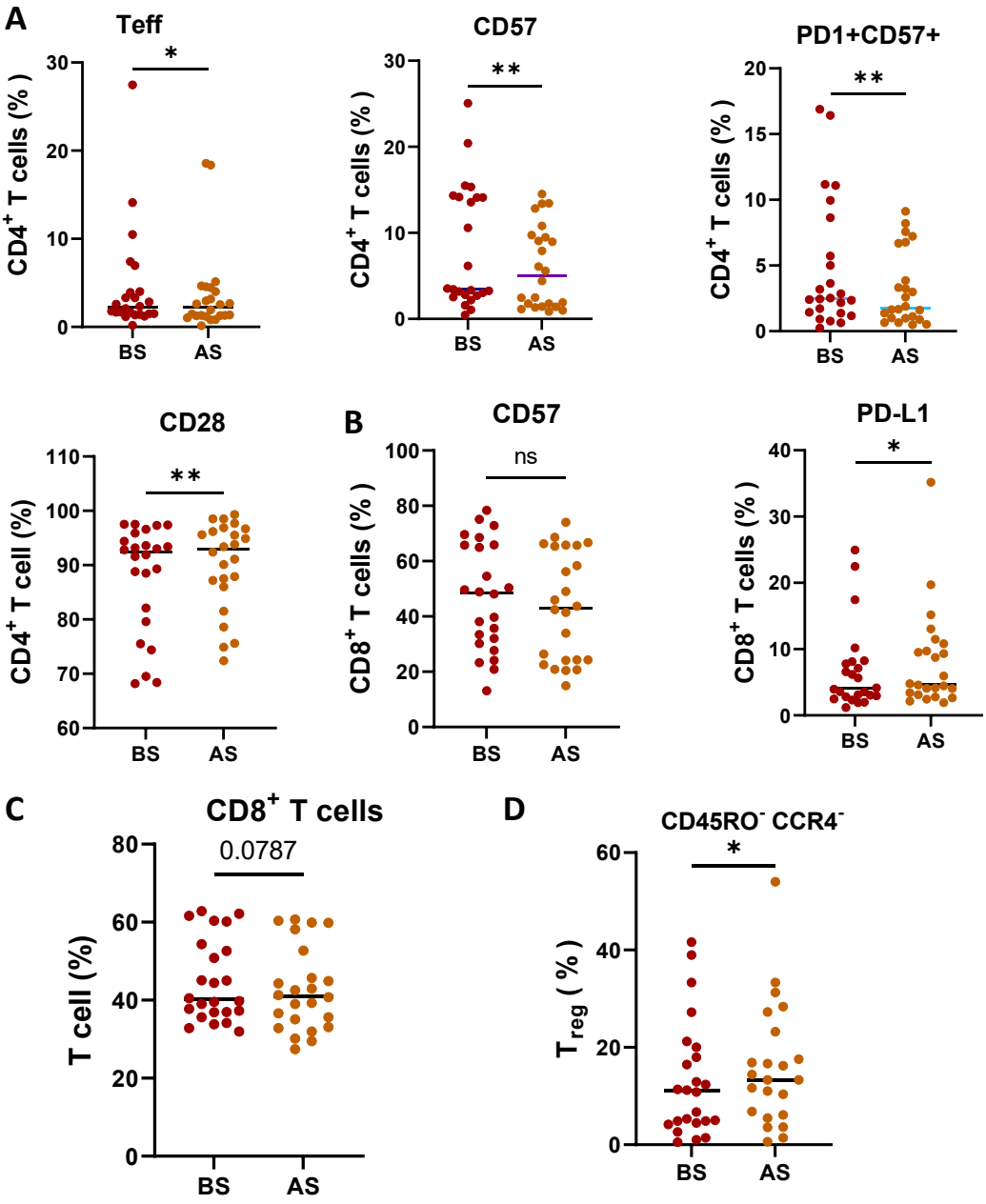


Figure 36. Frequency of immune cell populations and expression of the indicated markers before and after surgery: *Immune parameters altered after surgery in (A) CD4+ T cells (B) CD8+ T cells and (C) Tregs. Individual values are presented together with their median values. * p<0.05, ** p<0.01, *** p<0.001. BS: Before surgery, AS: After surgery*

4.3 Characterizing the interplay between Intratumoral Microbiota and Tumor immune microenvironment

4.3.1 Clinico-pathologic data of participants

A total of 29 women with histologically confirmed BC were included in the study to investigate the relationship between intratumoral microbiota diversity as well as abundance and immune gene expression profiles. Their age ranged from 30 to 75 years, with a median age of 42 years. The majority of the tumors were low grade-G1, G2(65.5%) and early stage (58.6%). In terms of TIL infiltration, the majority of tumors exhibited intermediate infiltration (Table 6).

Table 6: Clinico-pathologic characteristics of breast cancer patients (n = 29) treated at hospitals in Addis Ababa, Ethiopia.

Clinic pathologic variable	**N (%)**
Age	
Max	75
Min	30
Median	42
TIL grouping	
Low TILs (< 10%)	11(37.9%)
Intermediate TILs (11-49%)	16(55.1%)
High TILS (> 50%)	2(7%)
Pathologic Stage	
Early	17(58.6%)
Advanced	7(24.1)
Missing	5(17.3%)
Tumor Grade	
Low grade tumor	19(65.5%)
High grade tumors	10(35.5%)

4.3.2 Correlations between specific taxa abundance, Alpha diversity, and immune gene expression

A substantial body of evidence has unequivocally demonstrated a robust correlation between the gut microbiome, general immunity and antitumor immunity (J. Sun, Chen, and Wu 2023). Since there exists increasing evidence supporting the influence of tissue microbiota on local immunity in cancer, this study aimed to investigate whether genes associated with immune activation and suppression exhibit a positive or negative correlation with the microbiome diversity and relative abundance in cancerous breast tissues. The relative abundance of each taxon at the phylum, family and genus levels as well as alpha diversity (Shannon index) were correlated with the expression of 52 immune related genes. However, alpha diversity analysis in relation to immune gene expression did not reveal any significant findings (data not shown).

4.2.2.1 Correlation at Phylum level

We further examined correlations between phyla abundance and specific immune gene expression profiles (Table 7). Only nominal correlations were observed between the relative abundance of phyla and immune gene expression, which were lost after adjusting for multiple comparisons, likely due to the study's small sample size. Actinobacteria showed positive associations with gene coding for the immune effector molecules (ITGAX & FAS) and TGF-B, while Proteobacteria also positively correlated with TCR signaling components (CD2 and CD3E), the immune inhibitory molecule IDO1 and the immune signaling molecule LTK. Moreover, the presence of the phylum cyanobacteria/chloroplast mainly correlated with genes involved in antigen processing and presentation, such as β2M, TAP, TAP2, HLA class I, heavy chain and HLA-G.

In contrast, some intratumoral microbial phyla exhibit a negative correlation with local immunity. For example, a nominally significant negative correlation was observed between Firmicutes and a number of immune genes including FURIN, LTK, NTAN1, CD274, NCR1 and TGF-B. In addition, Verrucomicrobia presence negatively correlated with CD274, CD68, CXCL8, NTAN1 and TIGIT expression, while Parcubacteria, Plantae and Bacteroidetes demonstrated a negative correlation with the immune genes β2M, CD274 and TGF.B, respectively.

Table 7. Association of relative abundance for each Phyla with each gene expression: *Rank-based correlation (Spearman r) was used to identify correlations. Benjamini Hochberg 1995 method was used to control false discovery rate (FDR) for each gene.*

Phylum	Gene	Spearman.r	Pvalue	BH95.q
Actinobacteria	TILS	0.502	5.53E-03	0.3174
Actinobacteria	BDCD4	0.476	9.05E-03	0.3174
Proteobacteria	ACE2	0.4676	1.05E-02	0.5016
Actinobacteria	ITGAX	0.4432	1.61E-02	0.3174
Actinobacteria	TMPRSS2	-0.4369	1.78E-02	0.3174
Cyanobacteria/Chloroplast	HLA.I.heavy.chain	0.4353	1.83E-02	0.5493
Firmicutes	NTAN1	-0.4343	1.86E-02	0.3197
Firmicutes	ACE2	-0.4319	1.93E-02	0.3197
Bacteroidetes	TGF.B	-0.4297	2.00E-02	0.8938
Firmicutes	NCR1	-0.4278	2.06E-02	0.3197
Verrucomicrobia	CD68	-0.4262	2.11E-02	0.7699
Firmicutes	BDCD4	-0.4233	2.21E-02	0.3197
Firmicutes	FURIN	-0.4218	2.27E-02	0.3197
Actinobacteria	TGF.B	0.4163	2.47E-02	0.3776
Verrucomicrobia	NTAN1	-0.4151	2.51E-02	0.7699
Cyanobacteria/Chloroplast	TAP2	0.4122	2.63E-02	0.5493
Cyanobacteria/Chloroplast	TAP1	0.4002	3.15E-02	0.5493
Actinobacteria	FAS	0.3976	3.27E-02	0.409
Proteobacteria	IL17A	0.3962	3.34E-02	0.5016

Firmicutes	TGF.B	-0.3954	3.38E-02	0.4018
Actinobacteria	ANLN	-0.3487	3.44E-02	0.409
Plantae	CD274	-0.3934	3.47E-02	0.926
Cyanobacteria/Chloroplast	b2.m	0.3883	3.74E-02	0.5493
Cyanobacteria/Chloroplast	KLRD1	0.3832	4.02E-02	0.5493
Verrucomicrobia	CXCL8	-0.3824	4.06E-02	0.7699
Verrucomicrobia	CD274	-0.3815	4.11E-02	0.7699
Parcubacteria	b2.m	-0.3762	4.43E-02	0.774
Cyanobacteria/Chloroplast	HLA.G	0.3743	4.54E-02	0.5493
Firmicutes	CD274	-0.3732	4.62E-02	0.4943
Verrucomicrobia	TIGIT	-0.3706	4.78E-02	0.7699

4.3.2.2 Correlation at lower taxa level-Genus and Family

To further identify microbiome–immune associations at genus level, we performed network analysis between microbiome and immune marker expression (Figure 29). *Delftia* was identified as the most predominant microbial genus with a broad range of influence on the expression of immune transcripts. The relative abundance of *Delftia* was significantly correlated with immune-related genes encoding for effectors (CD8A, CD8B), T cell receptor (TCR) signaling pathway (CD3G), antigen processing and presentation (CD1A, KLRD1, TAP2, tapasin), the immune check point molecule (TIGIT), effector molecules (IFNG, Perforin, IL17A, FASLG), activating molecules or receptors (NCR1, CR2), tumor associated macrophages (TAM) marker (CD68) and other immune genes with involvement in various immune activities (LTK, IFITM3, FURIN) (Figure 37 & table 9).

The *Rhodobacteraceae* family exhibited a significant negative correlation with Nectin-4, while other correlations at the family and genus levels showed only nominal significance which was lost after adjustment for multiple testing. Notable positive trends observed include the family *Corynebacteriaceae* and the genus *Corynebacterium* which showed a positive association with the death receptor FAS and the immunosuppressive molecule TGF-β, while *Sphingomonadaceae* and the genus *Sphingomonas* were positively correlated with NCAM1, a molecule involved in the activation and differentiation of T and NK cells. In contrast, *Bifidobacteriaceae* and the genus *Bifidobacterium* demonstrated a negative correlation with NCAM1. Other notable negative correlations at the family level include *Burkholderiaceae* with HLA-G and HLA-I heavy chain, as well as *Moraxellaceae* with β2M (Table 8 & 9).

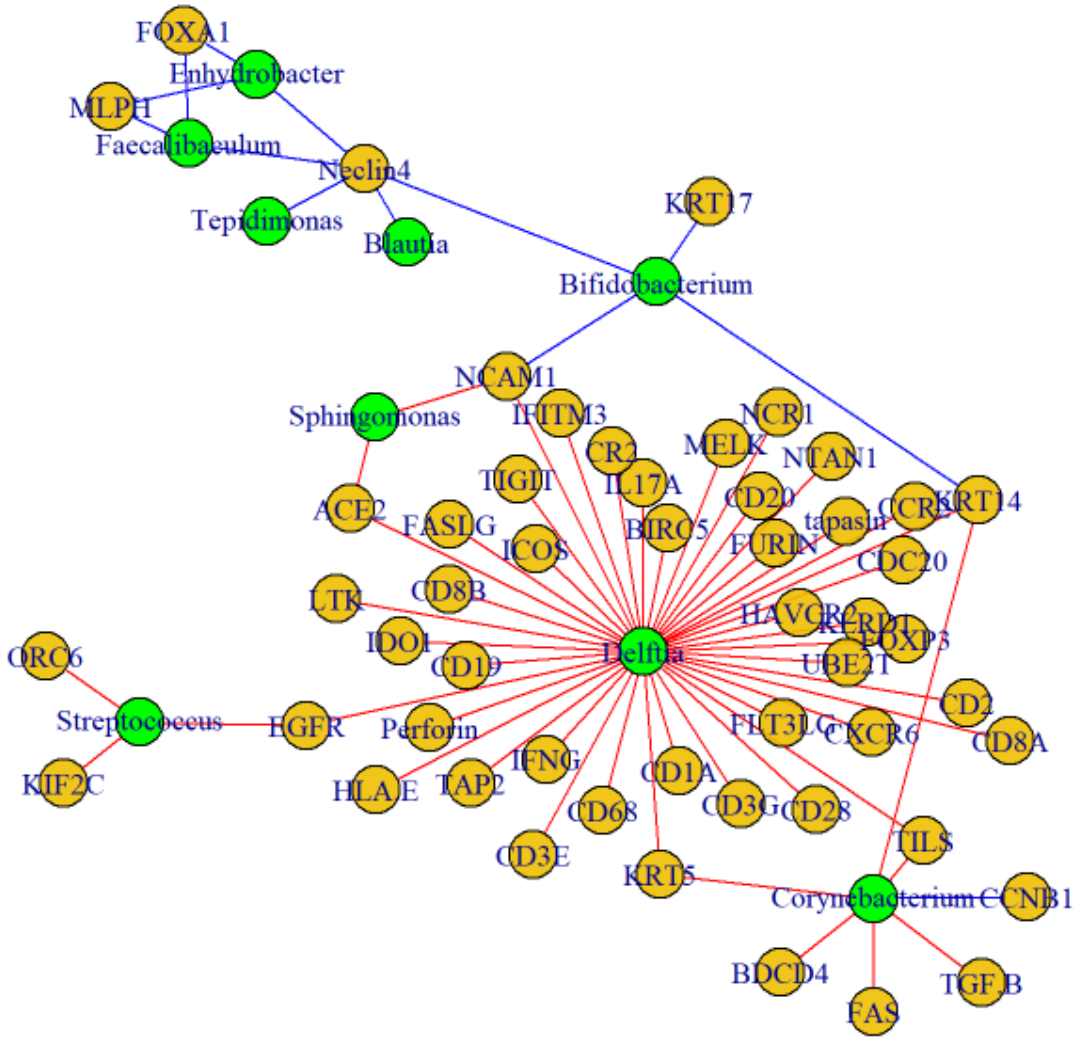


Figure 37. Network analyses showing microbiome–immune associations in breast tissues: *Network analysis was performed on genus abundance and immune gene expression. Significant associations were visualized as edges between nodes, with significance determined by a Spearman correlation coefficient (r) < 0.5 and a false discovery rate (FDR) < 0.2. Positive associations were visualized in red while negative associations were depicted in blue. igraph was used to plot the network.*

Table 8. Association of relative abundance for each Family with each gene expression

Family	Gene	Spearman.r	Pvalue	BH95.q
Rhodobacteraceae	Neclin4	-0.6338	2.23E-04	0.0239
Moraxellaceae	b2.m	-0.5743	1.12E-03	0.1198
Burkholderiaceae	HLA.G	-0.5486	2.06E-03	0.1102
Burkholderiaceae	Neclin4	-0.5286	3.20E-03	0.1141
Corynebacteriaceae	BDCD4	0.5127	4.46E-03	0.1618
Burkholderiaceae	HLA.I.heavy.chain	-0.5069	5.01E-03	0.134
Bifidobacteriaceae	Neclin4	-0.5028	5.44E-03	0.1691
Corynebacteriaceae	TILS	0.4974	6.05E-03	0.1618
Corynebacteriaceae	TGF.B	0.4688	1.03E-02	0.1636
Corynebacteriaceae	FAS	0.467	1.07E-02	0.1636

Table 9. Association of relative abundance for each Genera with each gene expression.

Genus	Gene	Spearman. r	P value	BH95.q
Delftia	FASLG	0.7094	1.64E-05	0.0009
Delftia	IL17A	0.6915	3.26E-05	0.0012
Delftia	NTAN1	0.6097	4.47E-04	0.012
Tepidimonas	Neclin4	-0.5978	6.16E-04	0.0659

Delftia	NCR1	0.5957	6.52E-04	0.014
Delftia	CD68	0.5631	1.47E-03	0.0225
Enhydrobacter	Neclin4	-0.5593	1.61E-03	0.0861
Blautia	Neclin4	-0.5544	1.80E-03	0.1926
Delftia	TILS	0.5388	2.57E-03	0.0273
Delftia	tapasin	0.5307	3.06E-03	0.0273
Faecalibaculum	Neclin4	-0.5278	3.25E-03	0.1159
Sphingomonas	ACE2	0.5263	3.36E-03	0.1798
Delftia	IFITM3	0.5196	3.87E-03	0.0319
Corynebacterium	TILS	0.5175	4.04E-03	0.137
Corynebacterium	BDCD4	0.5113	4.59E-03	0.137
Bifidobacterium	Neclin4	-0.5018	5.55E-03	0.1806
Delftia	ACE2	0.4942	6.43E-03	0.0459
Delftia	FURIN	0.4778	8.77E-03	0.0502
Delftia	TAP2	0.4771	8.87E-03	0.0502
Delftia	Perforin	0.4769	8.91E-03	0.0502
Corynebacterium	TGF.B	0.4649	1.10E-02	0.1743
Delftia	KLRD1	0.4637	1.13E-02	0.0576
Corynebacterium	FAS	0.4632	1.14E-02	0.1743
Delftia	TIGIT	0.4205	2.31E-02	0.0979
Delftia	HLA.E	0.3973	3.28E-02	0.121

4.3.3 Correlations between the intratumoral-microbiota and tumor infiltrating Lymphocytes

To gain a comprehensive understanding of intratumoral microbiota-immune interactions, we investigated the relationship between infiltrating immune cells: TILs and the intratumoral microbiota. First, the percentage of TILs as an indicator of anti-tumor immunity was analyzed followed by the investigation of the microbial richness and relative abundance to identify any potential correlations with TIL levels.

4.3.3.1 Associations between Microbiome diversity and TILs

As shown in Figure 36A and B, the average alpha diversity of the mammary microbiota as measured in Shannon and Simpson diversity indices showed no statistically significant difference between the TIL groups (Figure 36A & B). However, there was a weak, but not statistically significant positive correlation between the number of TILs and the Shannon ($r = 0.2452$; $p = 0.1998$) & Simpson indices ($r = 0.2742$; $p = 0.15$). Although these data suggest a potential trend, further studies with larger sample sizes are needed to confirm this relationship.

4.3.3.2 Microbiome relative abundance at Phylum level correlates with TILs

The relative abundances of the microbiota in each TIL group were analyzed at both higher and lower taxonomic levels. Proteobacteria was identified as the predominant microbial phylum across all TIL groups comprising 80.3%, 63.1% and 63.7% of total microbial phylum in the low, intermediate and high TIL groups respectively (Figure 30C, table 10). In the low and intermediate TIL groups, Proteobacteria was followed by Firmicutes, Actinobacteria, Bacteroides and Verrucomicrobia. Conversely, the high TIL group displayed a slightly distinct microbial profile with Actinobacteria, Firmicutes, Bacteroidetes and Cyanobacteria constituting the remaining phyla (table 10).

Further analysis of differential abundance across TIL groups revealed that Firmicutes ($p = 0.0095$) and Verrucomicrobia ($p = 0.0028$) were significantly enriched in the intermediate TIL group when compared with low and high TIL groups (Figure 37A). A trend toward higher abundance of Actinobacteria in the high TIL group compared to the low and intermediate groups was observed, with a mean relative abundance of 19.2% versus 3.6% and 8%, respectively (Table 10).

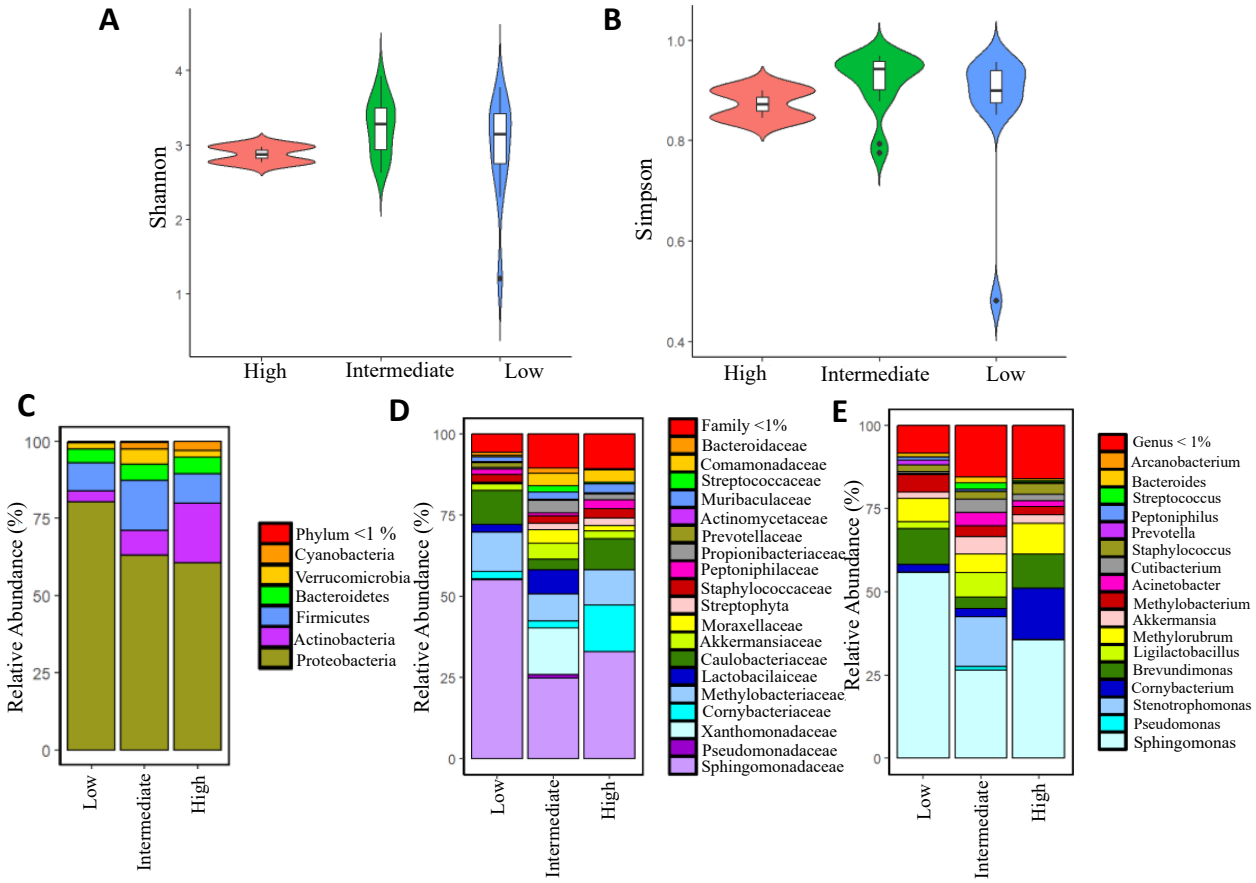


Figure 30. Intratumoral microbiome diversity and TIL abundance: *Violin plots were used to depict alpha diversity measured by the Shannon index (A) and Simpson index (B) for each TIL group. P values obtained from one-way ANOVA test were provided to indicate significant differences in diversity indexes across the TIL groups. Microbiome relative abundance at Phylum (C), family (D) and Genus (E) level in the different TIL groups was shown in the grouped bar chart. Bacteria with relative abundance of <1% were shown in red. TIL: Tumor infiltrating lymphocytes.*

Table 9. Relative abundance of microbial Phyla in the different TIL groups of BC patients:

TIL group	Proteobactera	Actinobactea	Bacteroides	Firmicuts	Verrucomicrobia
Low	80.3%	3.61%	4.42%	9.1%	2.0%
Intermediate	63.1%	8.0%	5.3%	16.1%	4.85%
High	60.8%	19.2%	5.2%	9.6%	2.4%

4.3.3.3 Microbial abundance at Lower taxonomic level versus TILs

At the lower taxonomic levels (family and genus), *Sphingomonadaceae* and *Sphingomonas* were identified as the predominant microbial groups across all TIL categories, though their relative composition varied among the groups. *Sphingomonadaceae* accounted for 55.1%, 25%, and 33% in the low, intermediate, and high TIL groups, respectively, while *Sphingomonas* comprised 55.7%, 26.5%, and 35% in the low, intermediate, and high TIL groups, respectively.

In the low TIL group, additional abundant microbiota families included *Methylobacteriaceae*, *Caulobacteriaceae*, *Lactobacilaiceae* and *Cornybacteriaceae* along with the genera *Brevundimonas*, *Methylorubrum*, *Methylobacterium* and *Cornybacterium*. In BC lesions classified as intermediate TIL groups, a strong association with the families, *Xantomondaoceae*, *methylobacteriaceae*, *Lactobacilaiceae* as well as the genera *Stenotrophomonas*, *Ligilactobacillus*, *Methylorubrum* and *Akkermansia* with decreasing abundance was found. In BC cases with a high TIL frequency, *Cornybacteriaceae*, *Methylobacteriaceae*, *Caulobacteriaceae* were the most abundant families. In addition, microbiota at the genus level *Cornybacterium*, *Brevundimonas*, *Methylorubrum* and *Methylobacterium* were associated with the high TIL BC group (Figure 36 D & E, tables 11 &12).

Differential abundance analysis revealed that families *Lactobacillaceae ($p=0.0283$)* and *Akkermansiaceae ($p=0.0024$)* (*Figure 37B*) and the genera *Akkermansia (0.0029)* and *Cutibacterium ($p=0.0216$)* were most abundant in the intermediate TIL group (*Figure. 37C*). In the high TIL group, genus *Delftia* was the most significantly enriched microbiota ($p < 0.0001$). Although differential abundance analysis did not detect a significant enrichment, possibly due to small sample size, *Corynebacteriaceae* constituted 14.1% of the microbiota in the high TIL group, compared to 2.2% in the low and intermediate groups. Similarly, *Corynebacterium* accounted for 15.3% in the high TIL group, compared to 2.3% and 2.4% in the low and intermediate groups, respectively (Tables 11 & 12).

4.3.4 Correlation between microbial abundance and TIL percentage

We conducted a correlation analysis to assess the relationship between TIL percentage and the relative abundance of microbiota at the phylum, family, and genus levels. At the phylum level, *Actinobacteria* showed a nominally significant correlation with TIL percentage (P= 5.53e-03), but this association didn't remain significant after multiple testing correction (BH95.q=0.31). At the genus level, *Delftia* demonstrated a significant positive correlation with TIL percentage (P = 2.57e-03, BH95.q = 0.0273). *Corynebacterium* showed a borderline positive correlation at both the genus (P = 4.04e-03, BH95.q = 0.1370) and family *(Corynebacteriaceae)* (P = 6.05e-03, BH95.q = 0.1618) levels, with q-values approaching 1, indicating borderline statistical significance. (See Tables 7–9).

Table 11: Relative abundance of microbial Families in the different TIL groups of BC patients. TIL; Tumor infiltrating lymphocytes. BC; Breast cancer.

	Sphingomonadaceae	Caulobacteraceae	Methylobacteriaceae	Corynebacteriaceae	Lactobacillaceae	Akkermansiaceae	Xanthomonadaceae	Comamonadaceae
Low	55.1%	10.6%	12.0%	2.2%	2.3%	1.9%	0.0009%	0.4%
Intermediate	25%	3.3%	8.3%	2.2%	7.4%	4.9%	14.3%	3.9%
High	33.0%	9.6%	10.9%	14.1%	0.00%	2.4%	0.0%	3.8%

Table 12: Relative abundance of microbial Genera in the different TIL groups of BC patients. TIL; Tumor infiltrating lymphocytes. BC; Breast cancer.

TIL	Sphingomonas	Brevundimonas	Methylorubrum	Corynebacterium	Staphylococcus	Ligilactobacillus	Methylobacterium	Akkermansia	Cutibacterium	Stenotrophomonas
Low	55.7%	10.7 %	7.0%	2.3%	2.1%	5.91%	5.2%	2%	0.53%	0.06%
Intermediate	26.5%	3.5%	5.7%	2.4%	2.3%	7.3%	5.7%	5.2%	4.05%	15.0%

High	35.0%	10.3 %	9.2%	15.3%	3.3%	0.00%	2.5%	2.5%	2.0%	0%

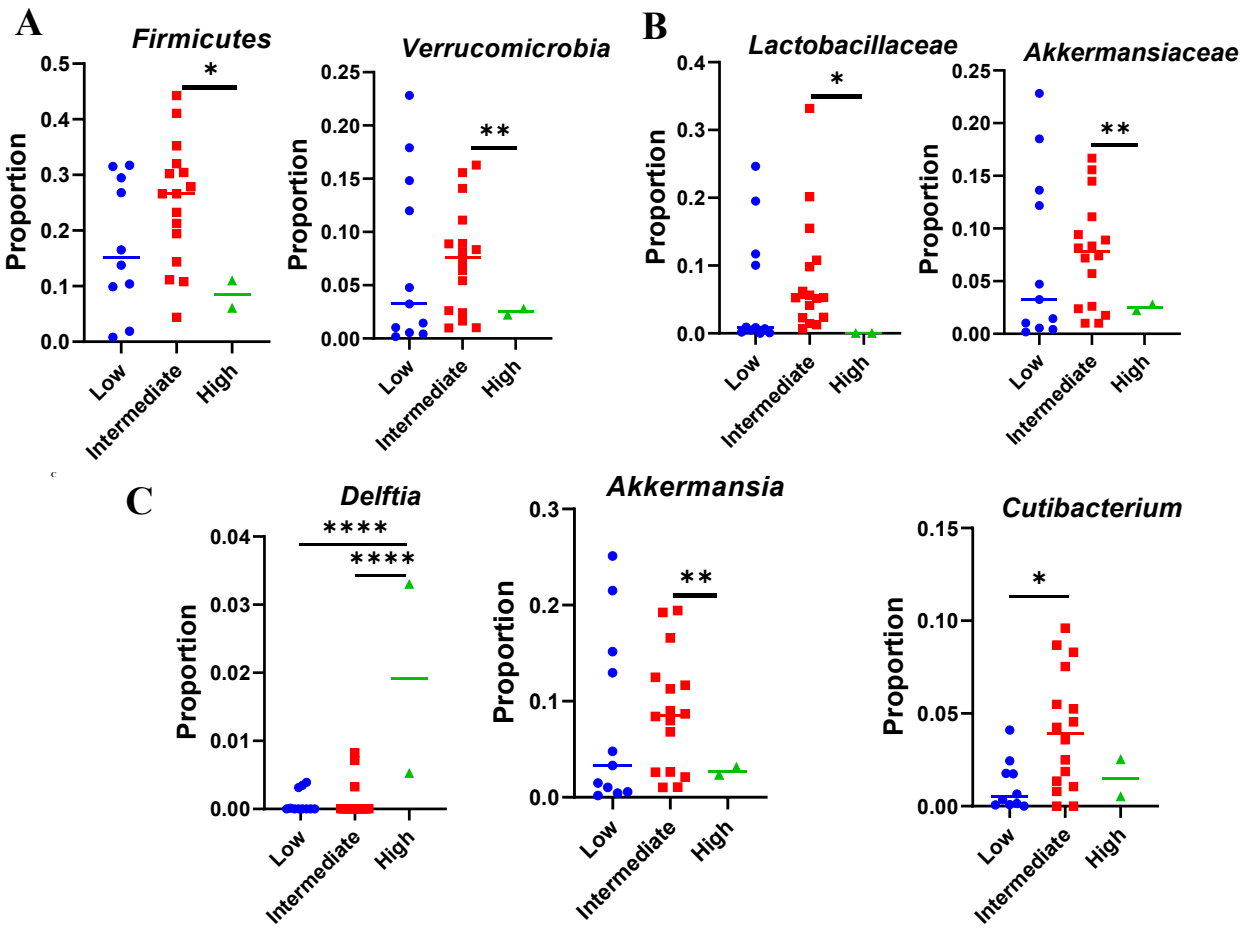


Figure 37: Intratumoral microbiome proportion in distinct TIL groups at Phylum (A), family (B) and Genus (C) levels. *P values obtained from one-way ANOVA or Brown-Forsythe and Welch ANOVA test were provided to indicate significant differences in abundance across the TIL groups. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or **** ($p < 0.0001$). TIL: Tumor infiltrating lymphocytes.*

Chapter Five: Discussion

5.1 Immune Landscape of the TME and its prognostic role

5.1.1 Immunophenotypes in the TME

In this study, four distinct immune phenotypes (IP1–IP4) were identified, each exhibiting unique immune gene expression patterns. IP2 was classified as immunologically inactive, showing consistent downregulation of both immune-suppressive and immune-activating genes, along with the lowest levels of immune cell infiltration. In contrast, IP4 was characterized by an immunologically active TME, with upregulation of most cytotoxic genes analyzed, alongside certain inhibitory molecules including immune check points, and the highest immune cell infiltration However, no differences were observed in PIK3CA mutations between the IPs. Upon incorporation of a validation dataset, these findings were replicated and further demonstrated prognostic significance, with the immune-active phenotype being associated with the best prognosis.

The main finding of this study is the identification of distinct immune phenotypes with unique immune contexture. Similarly, previous research utilizing various methodologies has reported distinct BC immune subgroups based on the immune transcriptome profile of the TME (Tekpli et al. 2019; B. Zhu et al. 2019; Hendrickx et al. 2017). Although BC was initially thought to be a predominantly non-immunogenic tumor, studies have demonstrated that its immune landscape is both dynamic and heterogeneous, with considerable differences across patients. Our findings align with other reports highlighting the spectrum of immune activation within the breast tumor microenvironment, where some BC patients exhibit a T cell-inflamed TME, while others display an immune intermediate, excluded, or desert microenvironment (Hendrickx et al. 2017; Tekpli et al. 2019; Yao, Li, and Wang 2022; Amara et al. 2017). Despite very limited information available about the DEIGs in BC from African women, a recent report on African American women from Carolina Breast Cancer Study also described three immune clusters with gradual immune activation (Hamilton et al. 2022).

Notably, the immune-activated phenotype (IP4) also exhibited immunosuppressive characteristics, with a higher proportion of exhausted T cells and an increased expression of immune inhibitory molecules compared to other IPs. These findings align with the work of Spranger et al. and others, who reported elevated expression of immune inhibitory molecules in T cell-inflamed melanoma phenotypes (Spranger et al. 2013). Similarly, Hendrickx et al. observed that in BC, the PD-1 immunosuppressive signature was more pronounced in the BC-Immune high group, whereas it was less expressed in the BC-Immune low group (Hendrickx et al. 2017). The upregulation of these inhibitory pathways in the immune-activated phenotype may serve as a negative feedback mechanism to regulate the heightened immune response (Spranger et al. 2013; Bedognetti et al. 2016). Although anti-tumor immunity was initiated in these tumors, the response was insufficient to completely eradicate the cancer. For such cases, therapeutic interventions aimed at modulating the immune response could potentially improve patient outcomes and would benefit best from immunotherapy.

In this study, a subset of BCs exhibited an absence of immune responses (IP2) within the TME, characterized by the repression of both immune effector and immune regulatory genes, leading to its designation as an immune-inert phenotype. This subgroup primarily consisted of luminal tumors, particularly luminal A BC, and displayed the lowest Ki-67 proliferation index. In line with our finding, Tekpli et al. found that the cluster with minimal immune infiltration was enriched in ER-positive and luminal cases (Tekpli et al. 2019). Consistent with these findings, lower immune gene expression levels were more frequently observed in BC with ER+ and luminal A subtypes, as identified by both conventional and Sorlie-Perou intrinsic molecular subtyping methods. (Griguolo et al. 2021; Li, Wu, and Han 2023; Goldberg et al. 2021). Additionally, these tumors were associated with low expression of proliferative genes and a lower histological grade contributing to inertness of their TME. (Yersal and Barutca 2014).

Mechanistic studies have shown that T cell-excluded tumors could arise upon disruption of one feature of the cancer-immunity cycle, which extends from antigen recognition by antigen presenting cells, presentation of captured antigen to T cells to homing of activated T cells to the tumor site (Chen and Mellman 2013). Despite the determinants of immune responses being complex, the lower proliferation of such tumors may account in part to their reduced immunogenicity and less immune reaction as described in our study. Higher tumor proliferation might be followed by an enhanced immune response by increasing immunogenic antigens from

apoptotic tumor cells (Bai et al. 2001; J. S. Lee et al. 2001). Even though the role of apoptosis in the induction of $CD8^+$ T cell activation was controversially discussed, emerging studies are proving its essential role in immune stimulation. Nowak and co-authors stated an induced apoptosis in tumor bearing mice associated with a tumor regression. This effect was attributed to an enhanced immune response due to the induction of antigen-specific $CD8^+$ T cell proliferation (A. K. Nowak et al. 2003). These results were further confirmed in BC patients from TCGA and METABRIC cohorts by showing a correlation of high apoptotic tumors with a significantly high infiltration of $CD8^+$, $CD4^+$ memory T cells, M1 macrophages and DC (Murthy et al. 2021). In contrast, in slowly proliferating tumors with less apoptotic cells available antigens capable of priming the adaptive immune response are low resulting in an inert microenvironment.

A notable observation was the clustering of normal breast tissue from healthy donors (used as a control) with the immune-inert phenotype. While some level of immune activity is expected in healthy breast tissue, studies have demonstrated the presence of both innate and adaptive immune cells, which may play a role in immune surveillance. Lymphocytes were the predominant immune cells, with CD8+ T cells being the most abundant among them. These CD8+ T cells primarily exhibit an effector memory phenotype characterized by CD45RO+/CD27− expression (Goff and Danforth 2021; Degnim et al. 2014; Zumwalde et al. 2016).

It has been suggested that mammary microbiota could serve as a source of antigens, potentially driving immune activation. (Parida and Sharma 2019). However, during malignant transformation, tumor cells secrete pro-inflammatory mediators and chemoattractants, leading to enhanced immune cell infiltration. (Gao, Cazares, and Fish 2017). As a result, the immune cell composition of breast tissue gradually increases from normal breast to BC. (Goff and Danforth 2021). Interestingly, in our study, no significant difference was observed in immune activity between IP2 tumors and normal breast tissue, further underscoring the non-immunogenic nature of tumors classified within this phenotype. This finding highlights the immune-cold environment of IP2 tumors, which may have implications for their response to immunotherapy as well chemotherapy.

5.1.2 Prognostic Role of immune subgrouping in BC

There exists increasing evidence that immune-based subtyping in BC has clinical relevance. For example, Hendrickx and co-authors reported an immune phenotype characterised by the highest levels of immune gene expression and immune cell infiltration which correlated with an increased overall survival compared to immune low and intermediate phenotypes (Hendrickx et al. 2017). Similarly, Yao and colleagues identified three immune subsets validated in five BC data sets. The immune-active subtype was consistently associated with a better survival in all data sets (Yao, Li, and Wang 2022). Another study in BC has also reported the prognostic and predictive role of immune clusters. They have identified 3 immune clusters with high, intermediate and low immune gene expression levels. The immune intermediate cluster was demonstrated to have worse prognosis. In addition, the immune high cluster beard a significantly higher number of responders to neoadjuvant chemotherapy (Tekpli et al. 2019). Consistent with these findings, the TCGA validation cohort in our study also revealed improved overall survival for immune-activated phenotypes, both in the entire patient population and specifically among HR-negative cases when stratified by HR status.

5.1.3 PIK3CA mutation and the immune landscape

The immune landscape of tumors has been reported to correlate with their mutational burden (Niknafs et al. 2023). Since tumors generate neoantigens (Efremova et al. 2017), which are directly recognized by immune cells as non-self and induce an immune response, we anticipated a positive correlation between PIK3CA mutations and the immune-active phenotype (IP4). However, our analysis revealed that the two extreme immune phenotypes, IP4 and IP2, exhibited comparable PIK3CA mutation rates, contradicting our initial hypothesis. Our findings align with those of Hendrickx et al. (2017), who reported PIK3CA mutation frequencies of 28.2% and 28.1% in immune-high and immune-low BC phenotypes, respectively, compared to 41.3% in the immune-medium phenotype. While previous studies in BC (Sobral-Leite et al. 2019), colorectal cancer (Ahn et al. 2021), and renal cancer (Li et al. 2021) have reported a correlation between PIK3CA mutations and immune cell infiltration, our finding didn't associate PIK3CA mutation rate with immune phenotypes based on their immune infiltration status. A possible explanation for this is the predominance of PIK3CA mutations in luminal tumor subtypes, which are typically less immunogenic. Notably, 75% of the mutations observed in IP4 occurred within luminal tumors, and since IP2 is predominantly composed of luminal tumors as well, it is

unsurprising to see a high mutation rate in this phenotype. This distribution of mutations may confound the relationship between immune phenotypes and mutation rates. Therefore, we suggest that future studies correlate PIK3CA mutations directly with immune cell infiltration, without subgrouping based on immune phenotypes, to gain a clearer understanding of this association.

5.2 Systemic immunity in BC and immune escape Mechanisms

In this study, we conducted blood immunophenotyping to evaluate the systemic immune response of Ethiopian breast cancer patients by comparing it with healthy donors. Our findings revealed tumor specific immune activation in BC, as indicated by activation of CD4+ and CD8+ T cells (reflected by increased HLA DR expression in BC) as well enhanced effector function of cytotoxic NK cells and CD8+ T cells (evidenced by elevated perforin in BC). Additionally, we correlated systemic immune profiles with various clinicopathologic characteristics. Furthermore, we analyzed immune response dynamics in patients before and after surgical intervention to assess the impact of surgery on immune modulation.

5.2.1 Tumor specific immunity and immune escape Mechanisms

One of the key findings of this study was the expansion of CD8+ T cells BC patients, leading to an inverted CD4/CD8 ratio (i.e., ratio <1) in 50% of cases. Patients with a normal CD4/CD8 ratio exhibited an expansion of CD8+ T cells with a central memory phenotype, whereas those with an inverted ratio showed an increase in effector memory CD8+ T cells. This shift was further associated with higher perforin expression, loss of CD28, and a phenotype indicative of senescence rather than exhaustion, as evidenced by increased CD57 expression with unchanged PD-1 and Tim-3 levels.

An inverted CD4/CD8 ratio has been previously linked to functional immune impairment in infectious disease (G. Bruno et al. 2017) and aging (Turner 2016). It has also been associated with an increased risk of cancer development in people living with HIV irrespective of their $CD4^+$ T cell count (Castilho et al. 2022), increased risk of lung cancer (Sigel et al. 2017) and with reduced cytokine production and cytotoxicity of CD8+ T cells in patients with recurrent respiratory papillomatosis (Holm et al. 2017).

In the context of cancer, an inverted CD4/CD8 ratio has been associated with a CD8+PD-1+ replicative senescence phenotype as well as reduced progression-free survival in chronic lymphocytic leukemia (Nunes et al. 2012). Additionally, a study in gastric cancer have highlighted the predictive value of a high CD4/CD8 ratio in response to neoadjuvant chemotherapy (Skubleny et al. 2023). In breast cancer, a study in TNBC patients observed better response to chemotherapy in patients with higher CD4/CD8 ratio (Li et al. 2022). Similarly, in patients with early BC, lower CD4/CD8 ratio was associated with increased risk of distant recurrence and decreased survival (Campbell 2019).

Beyond alterations in the CD4/CD8 ratio, our study identified signs of immunosenescence in a subset of patients, marked by the accumulation of CD57+ cells, decreased CD28 expression, a reduction in naïve T cells, and an increase in effector memory CD8+ T cell subsets. Immunosenescence is typically associated with aging, characterized by a decline in immune function, a loss of naïve T cells, and an accumulation of terminally differentiated effector and memory cells (Liu et al. 2023). However, young individuals with chronic viral infections as well as cancer patients were also shown to exhibit senescent CD8+ T cells (Zhao, Shao, and Peng 2020).

The overexpression of CD57, along with the downregulation of CD28, marks late-stage differentiation and reduced proliferative capacity in response to cytokines such as IL-2, IL-7, or IL-15. CD57+ cells exhibit shortened telomeres, reduced telomerase activity, and decreased expression of genes involved in cell cycle regulation, and they accumulate with chronic immune activation and aging (Kared et al. 2016). In our cohort, however, the median age of patients exhibiting immune senescence was 40 years, suggesting that these changes are more closely linked to breast cancer itself rather than aging.

Emerging evidence supports the role of tumors in inducing T cell senescence. Ye and colleagues demonstrated that tumor cells from various cancers including breast, melanoma, colon, prostate, ovarian, and head and neck cancer are capable of directly inducing T cell senescence (Ye and Peng 2015). Similarly, Montes and colleagues also demonstrated the induction of senescence in human T cells (CD4 and CD8) up on coculture with various cancer cell lines. They have further demonstrated that tumor-induced senescent cells possess immunosuppressive properties: suppressing proliferation of normal T cells (Montes et al. 2008). Even though senescent CD8+ T cells are capable of producing high amounts of proinflammatory cytokines, they bear defective

immune effector function in caner: have reduced expression of perforin and granzyme B and do not proliferate after TCR stimulation (Zhao, Shao, and Peng 2020) which contrasts with the previously held view in which such cells have high effector role. Given their expansion up on tumor contact and lack of effector function, tumor-driven CD8+ T cell immunosenescence may serve as a critical immune evasion mechanism that suppresses anti-tumor immunity and promotes disease progression in Ethiopian BC patients.

Despite the focus of immunotherapy has mostly been on $CD8^+$ T cells due to their effector functions and direct cytotoxic activity, the importance of $CD4^+$ T cell as helper cells, but also as direct anti-tumor mediator has to be considered (Tay, Richardson, and Toh 2021). Although it was not determined in our BC cohort whether $CD4^+$ T cells are reduced also in their absolute numbers or only in their frequencies within the PBMC as a consequence of the expansion of $CD8^+$ T cells, they also display an activated phenotype, as shown by higher HLA-DR expression. In contrast to $CD8^+$ T cells, activated $CD4^+$ T cells were more shifted towards an exhausted phenotype, as indicated by the high PD1 expression levels. Due to the lack of co-upregulation of other ICPs, these cells were not terminally exhausted (Wu et al. 2019) suggesting a possible responsiveness to reactivation by immunotherapeutic strategies. In line with this assumption, an enhanced frequency of $PD1^+$ $CD4^+$ T cells was reported to be associated with enhanced clinical response to checkpoint inhibitors in metastatic HR^+ BC patients (Yuan et al. 2018).

Enhanced expression of PD1, PD-L1 as well as of CTLA was also found on B cells. Different studies demonstrated that B cells expressing PD1 produce IL-10 upon engagement with PD-L1 (Xiao et al. 2016), whereas $PD\text{-}L1^+$ B cells hinder the cytotoxicity of NK and $CD8^+$ T cells (Takahashi et al. 2020). Furthermore, non-malignant human B cells expressing CTLA-4 have been reported previously (Oyewole-Said et al. 2020), but their role in anti-/pro-tumor immunity remains to be determined. All these reports are reminiscent of regulatory B cells (Bregs) (Catalán et al. 2021). Despite they are most unequivocally identified by functional evaluation or at least by IL-10 production, a good consensus exists on Bregs being $CD24^+CD38^+$. Within our evaluation, we found no differences in the frequencies of translational B cells identified as $CD24^+CD38^+$ cells among the naïve B cells, between BC patients and HC, which argues against an expansion of Bregs but *ex vivo* functional evaluations would be required to confirm this point.

BC patients had also an expansion of (the already highly frequent within the Ethiopian HC) γδ T cells. It is noteworthy that in our staining panel there was only the pan γδ TCR and no functional

markers, with the exception of CD56 and CD16. Thus, a deeper functional evaluation as well as a correlation with the patients´ outcome are required to better understand the role of γδ T cells in Ethiopian BC patients. Indeed, γδ T cells exhibit both pro- and anti-tumor activities (Yijing Zhao, Niu, and Cui 2018) and have been identified as prognostic and in some case predictive marker, both in positive and negative association with the outcome of cancer patients. With respect to BC, tumor infiltrating γδ T cells were found to be of prognostic value in all subtypes, but in particular the HER-2/neu^{+} (Bense et al. 2017). The role of γδ T cells in TNBC seems to be more complex, since improved survival was found for TNBC tumors without PI3K mutation (Boissière-Michot et al. 2021) or when only the Vδ1^{+} γδ T cells were taken into consideration (Wu et al. 2019), but not in other settings (Allaoui et al. 2017). Similarly, expansion of γδ T cells is one of the mechanism associated with enhanced risk of metastasis in BC patients in presence of high levels of cholesterol (Baek et al. 2017). Differences in the frequencies of peripheral γδ T cells between the BC subtypes have also been reported in different cohorts of Chinese patients (Zhang et al. 2020; Song, Wei, and Li 2022b). Interestingly, and contrary to our Ethiopian cohort, the Chinese HC had higher levels of γδ T cells than the BC patients.

In parallel to the expansion of (possible anti-tumor) effector populations, BC patients revealed also an expansion of different suppressive cell populations. Tregs and their highly suppressive subpopulation of CD45RO CCR4^{+} cells had a trend towards expansion in our BC cohort, even if the Treg expansion was not statistically significant. CD45RO+FOXP3hi T cells are an activated and functionally differentiated subset of Tregs derived from naive Treg cells. In Her2-overexpressing BC, a significantly higher proportion of activated Tregs as well as the ratio of activated to resting Tregs was observed in patients lacking a CD8+ T cell response to Her-2, and their accumulation was found to be stage-dependent (Bailur et al. 2015). In vitro studies have also shown that these cells have potent immunosuppressive activity (Watanabe et al. 2019). CCR4 is a chemokine receptor mainly expressed on the most immunosuppressive subsets (CD45RO+FOXP3hi T cells) and is involved in the recruitment of these immunosuppressive populations to the TME (Ketcham, Marshall, and Talay 2018).

Furthermore, differences between BC patients and HC were significant for various MDSC subtypes, with a significant elevation of PMN-MDSC, but a reduction of eMDSC in BC patients. Differences in the expansion of different MDSC subsets in cancer patients has already been reported (Cassetta et al. 2020). For example, a study by (Gonda et al. 2017) found that preoperative BC patients showed MDSC expansion compared to postoperative patients or healthy volunteers. Additionally, MDSC levels were positively correlated with increased IL-6 production and negatively correlated with IFN-γ and IL-12. Their findings also indicated that patients with higher MDSC levels had a poorer prognosis. Importantly, another study in Her-2 positive BC showed that CD8+ T cell responses to Her2 were influenced by circulating MDSC levels. In fact, patients who didn't mount Her2-specific CD8+ T cells were shown to have higher levels of MDSC compared to patients with Her2-specific CD8+ T cells. This was further correlated with survival, with non-metastatic patients with a CD8+ response to Her2 and low levels of MDSC having a 100% survival rate, compared to only 38% in patients with no CD8+ T-cell response to Her2 and high levels of MDSC (Bailur et al. 2015).

5.2.2 Immune Parameters in Relation to Clinicopathologic Characteristic

We have shown earlier that a sub-set of patients in our cohort have exhibited expansion of CD8+ T cells alongside an inverted CD4/CD8 ratio. Further subtype analysis with respect to intrinsic subtypes have revealed that such ratio inversion is significantly more prevalent in luminal tumors than non-luminal tumors. Given that an inverted CD4/CD8 ratio has been previously associated with defective immune response across various conditions, including cancer, our findings support this notion, suggesting that luminal tumors elicit a weaker immune response (Griguolo et al. 2021; Li, Wu, and Han 2023; Goldberg et al. 2021).

In terms of histological type, it has been shown that ILC tumors are predominantly composed of ER+ breast cancer cells and generally exhibit a lower proliferation index (Luveta et al. 2020). However, previous studies have indicated that, when compared to their ER+ IDC counterparts, ILCs display significantly higher immune cell activity (Du et al. 2018; Levine et al. 2018). While we did not perform a separate analysis between ER+ IDC and ILC in this study, it is important to note that all the ILC cases in our cohort were ER+. Our data, in line with previously reported studies, revealed a notable increase in several immune markers in ILC cases, including elevated CD56bright NK cells, effector CD4+ T cells (CD3ζ+ Perforin+), total perforin, and CD57+ cells CD4+ T cells. These findings collectively suggest a heightened immune response in ILC

compared to IDC. Interestingly, we also observed a downregulation of central memory CD4+ T cells, alongside an upregulation of CD57+ and effector cells. This shift could potentially indicate immune senescence in ILC, a phenomenon that warrants further investigation.

5.2.3 Pre- and Post-Operative Immune Parameter

Our study has reported that CD4+ T cells in BC patients exhibit an exhausted phenotype characterized by the expression of immune checkpoint molecules. At the time of diagnosis, these CD4+ T cells also showed a trend toward increased expression of PD-1^{+}CD57^{+} double-positive cells. However, following surgical intervention, there was a significant depletion of these double-positive cells, along with a reduction in effector CD4+ cells. Concurrently, the expression of the costimulatory molecule CD28 was elevated. While immune senescence in CD4+ T cells was not observed when compared to healthy controls, these findings suggest the possible presence of patient-specific immune senescence which was reversed following tumor removal.

BC patients also exhibited significantly elevated levels of CD8+ T cells at diagnosis, leading to an inverted CD4/CD8 ratio in half of the patients, a finding that has been associated with poor clinical outcomes in multiple studies (Li et al. 2022; Bruno et al. 2017). Post-surgery, there was a trend toward a reduction in the proportion of CD8+ T cells; however, the CD4/CD8 ratio remained unchanged. In line with this, the senescent marker CD57 appeared to decline, suggesting a potential reversal of T-cell immune senescence after surgical tumor removal. It is important to note that post-operative blood samples were collected 2-4 weeks after surgery, and only a trend was observed. A larger sample size and a longer follow-up period are necessary to confirm these findings.

Preoperatively, our cohort exhibited tumor-specific expansion of the most suppressive subset of Tregs. In this paired pre- and post-operative analysis, while we did not confirm the depletion of immunosuppressive Tregs, we observed an increase in Tregs with a naive phenotype (CD45RO^{-}CCR4^{-}), which lack immunosuppressive properties. This may indicate a reversal of immune suppression following tumor removal.

In line with our findings, the literature suggests that immune responses can be restored following tumor removal. For instance, research in BC has reported the depletion of the CD117+ granulocyte population upon tumor removal. These cells, rarely detected under homeostatic conditions, have been associated with metastasis promotion (Cattin et al. 2021). Similarly, a

study in colon carcinoma demonstrated the expansion of NK- and NKT-like cells post-surgery, accompanied by increased expression of activating receptors and decreased expression of inhibitory receptors (Krijgsman et al. 2020). In contrast, systemic immune responses in renal cell carcinoma patients remained largely unchanged 30 days after nephrectomy (Wald et al. 2014). It is important to consider that surgery-induced immune alterations may impact our post-operative analyses. While such immune changes typically persist for a few days to weeks, in some cases, they may extend up to six months, potentially introducing bias into these findings. Further studies with extended follow-up are required to fully understand the long-term effects of surgical intervention on immune parameters in BC patients.

5.3 Role of Intratumoral microbiota in the modulation of local immunity

5.3.1 Microbial abundance-immune interactions

Intratumoral microbiota have been implicated in modulating the TME through both immune and non-immune mechanisms. To our knowledge, this study represents the first initiative in Ethiopia to assess the immune landscape of BC in relation to the microbiota, examining both gene expression and cellular components. Our results reveal a statistically significant positive correlation between the relative abundance of *Delftia* and the infiltration levels of TILs, as well as the expression of various immune-related genes. In addition, a strong trend toward a positive correlation between *Corynebacterium* and TIL percentages was observed. Further analysis indicated potential links between the phylum *Actinobacteria* and specific immune-related genes, as well as a relationship between *Cyanobacteria/Chloroplast* and genes involved in antigen processing and presentation highlighting the potential immune activating role of microbiota within the TME.

Previous studies have underscored the crucial role of intratumoral microbiota particularly specific bacterial populations, in shaping anti-tumor immune responses within various tumor microenvironments. For instance: Singh et al. found that in oral squamous cell carcinoma, the relative abundance of certain bacteria within the TME, such as *Fusobacterium*, *Treponema*, *Leptotrichia*, *Corynebacterium*, and *Porphyromonas* affected immune cell infiltration and the expression of genes related to cytokines and transcription factors for T helper cells (Singh et al. 2023). Wang et al further demonstrated that bacteria under Order *Clostridiales* were positively associated with an activated immune microenvironment in TNBC. They demonstrated a correlation between trimethylamine N-oxide (TMAO), the metabolite produced by *Clostridiales*,

with improved efficacy of immunotherapy in the clinical cohort and increased infiltration of CD8+ T cells, M1 macrophages and elevated levels of cytokines in mouse model (Wang et al., 2022).

Conversely, certain microbial populations have been associated with immune suppression. In esophageal squamous cell carcinoma, the relative abundance of *Lactobacillus* was associated with an increased presence of PD-L1+ TAMs. In contrast, *Lactobacillus* has also been correlated with positive immune modulation in cervical and colorectal cancers suggesting tumor specific role of microbiome (Zhang et al. 2023). (J. Shen et al. 2024; Aindelis et al. 2020). In our study, we demonstrated a negative correlation between *Firmicutes* and the expression of a number of immune genes including FURIN, LTK, NTAN1, NCR1, TGF-B and CD274. In addition, Verrucomicrobia presence negatively correlated with CD274, CD68, CXCL8, NTAN1 and TIGIT expression. However, it is important to note that while *Firmicutes* and *Verrucomicrobia* were negatively correlated with immune effector genes, they also showed associations with genes known for their immunosuppressive roles, such as TGF-β and CD274.

5.3.2 Delftia as a predominant immune-modulating microbiota

The primary finding of this study is the identification of *Delftia*, a gram-negative bacillus, as the predominant microbial genus influencing immune transcript expression within the TME. *Delftia*, commonly found in environmental settings such as soil, water, and hospital environments, has primarily been associated with immune-compromised individuals in clinical contexts (Bilgin et al. 2015). In cancer research, *Delftia* has been reported to exhibit differential enrichment in tumor tissues compared to normal tissues, as highlighted in previous studies, including our own work (Jiayan Ma et al. 2020; Desalegn et al. 2023; Khan et al. 2023). Expanding on these findings, this study demonstrates a positive correlation between *Delftia* abundance and various immune-related genes involved in TCR signaling, antigen processing and presentation, effector molecules, and immune modulatory pathways. Furthermore, Delftia was significantly more abundant in high TIL expression group compared to low and intermediate TIL groups. This study provides new evidence of a positive correlation between the specific microbial taxa, Delftia and immune infiltration levels in the breast cancer tumor microenvironment. These findings suggest potential immunomodulatory roles for intratumoral microbiota, which may influence tumor progression and immune responses.

Previous studies have explored the immunomodulatory role of *Delftia* in another context. For instance, *Delftia spp*. have been shown to induce acute pro-inflammatory responses in vitro using human monocytic cell lines. Increased expression of cyclooxygenase-2 (COX-2), tumor necrosis factor (TNF), and myristoylated alanine-rich c-kinase substrate (MARCKS) was observed in *Delftia*-stimulated monocytes (Mcneill et al. 2015). In another study, coculturing *Delftia* with plant cells has been shown to stimulate the expression of defense-related genes such as PR-1 and PR-5, which are involved in salicylic acid (SA) signaling, as well as PDF1.2, which is associated with jasmonic acid/ethylene (JA/ET) signaling pathways. The authors have furthermore demonstrated that the cocultured plants exhibit enhanced resistance when subsequently challenged with bacterial pathogens further building on its immunostimulatory role (Kurokawa et al. 2021).

The role of *Delftia* in cancer, however, remains under explored by previous studies. Mei and colleagues reported higher *Delftia* abundance in the duodenal mucosa of pancreatic cancer patients, accompanied by elevated levels of inflammatory markers such as interleukin-6 (IL-6) and C-reactive protein (CRP). While no direct correlation was established, they speculated that systemic inflammation might be partially attributed to the lipopolysaccharide components of local microbiota (Mei et al. 2018). Conversely, in prostate cancer, *Delftia acidovorans* was negatively associated with regulatory T-cells and downregulation of immune-associated genes such as TGFB2, TLR3, and LPCAT2, which has been shown to regulate macrophage inflammatory gene expression with TLR genes, indicating an immunosuppressive tumor environment (Jiayan Ma et al. 2020).

5.3.3 Other Microbial Phyla and Genera with Immune-Modulating Roles

In this study, the phylum Actinobacteria showed significant positive associations with immune effector molecule-encoding genes, including ITGAX, FAS, and TGFB. ITGAX (CD11c) is predominantly expressed in DCs. CD11c expression in DCs has been correlated with higher expression of MHC molecules and other co-stimulatory molecules and are, or spontaneously become, potent allo stimulatory APC (O'Doherty et al. 1994). Clinically, CD11c positivity was significantly correlated with higher antitumor immune cells and better clinical outcome in TNBC (Lee et al. 2018). Members of Actinobacteria, such as Bifidobacterium, have demonstrated anti-tumor effects by enhancing DC development and maturation, consistent with the positive correlations observed here (Li et al. 2021).

At the family and genus levels, Actinobacteria's correlation with FAS and TGFB expression was particularly notable in *Corynebacteriaceae* and *Corynebacterium*, respectively. *Corynebacterium* also demonstrated a positive correlation with TIL infiltration in Pearson correlation analysis. TILs are established prognostic markers in many cancers, including BC, where higher TIL levels are associated with favorable outcomes and improved responses to chemotherapy and immunotherapy (Nelson et al. 2022). While FAS has been controversially discussed in the context of anti-tumor immunity being implied in both immune activation and tolerance (Tao et al. 2016; Zhu et al. 2017), TGFB is widely recognized as a tumor promoter, contributing to immune suppression and metastasis (Bierie and Moses 2011). The implications of these findings suggest that the observed correlations between *Corynebacterium* and TILs, alongside its influence on immune-related genes such as FAS and TGFB, could indicate a complex role for microbiota in modulating the immune landscape of BC. Specifically, the presence of *Corynebacterium* may enhance anti-tumor immunity through TIL infiltration, while its association with TGFB expression could also point to potential immune suppression within the TME, highlighting the dual role that microbiota might play in shaping tumor progression and response to therapies.

Additionally, our findings indicate that the phylum Cyanobacteria/Chloroplast correlates primarily with genes involved in antigen processing and presentation, such as β2M, TAP1, TAP2, and HLA-I heavy chain. Cyanobacteria-derived metabolites have been shown to induce tumor cell death via caspase activation and oxidative stress (Wijewickrama, Greene, and Cock 2023). The sensing of tumor antigens derived from apoptotic and necrotic cells has been shown to promote DC maturation, resulting in upregulation of genes involved in antigen processing and presentation as seen in this study, potentially leading to tumor-specific CD8+ T-cell responses (Brusa et al. 2008). Supporting this, Shen et al. demonstrated a positive correlation between Cyanobacteria abundance and CD8+ T-cell infiltration levels in the TME of lung adenocarcinoma (S. Shen et al. 2024).

It is important to note that the correlations observed at the phylum level (*Actinobacteria* and *Cyanobacteria*) were based on nominal associations, which were lost after multiple testing correction. While these findings may indicate potential biological interactions, they should be interpreted with caution.

5.3.4 Microbial Diversity-Immune interactions

Our analysis of microbial diversity in relation to individual immune gene expression and TIL levels did not yield statistically significant findings. This contrasts with previous studies that have demonstrated associations between microbial diversity and immune responses in both cancer and non-cancer settings. A diverse gut microbiota in fish has been shown to positively correlate with the expression of genes involved in immune cell development and IL-12 regulation but negatively with the expression of genes related to inflammatory processes, such as macrophage inflammatory protein 1 alpha (MIP-1α) production (Fuess et al. 2021). These finding suggest that microbial diversity may play a role in modulating immune responses by enhancing immune cell development while suppressing excessive inflammation. In HIV, alpha diversity of gut microbiome has been significantly correlated with CD4+ T-cell counts, with a unit increase in the age- and sex-adjusted number of bacterial species associated with an increase of 0.88 CD4+ T-cells/µl (Nowak et al. 2015). In non-small cell lung carcinoma (NSCLC), gut microbiome diversity has been reported to predict response to anti-PD-1 therapy, with patients exhibiting higher microbial diversity demonstrating increased memory CD8+ T-cell subsets and NK cells in response to treatment (Jin et al. 2019). These findings emphasize the potential role of microbial diversity in shaping immune responses and influencing therapeutic outcomes. Similarly, in BC, alpha diversity of the intratumoral microbiome has been linked to an immune-enriched subtype, characterized by high immune cell infiltration and better prognosis (Li et al., 2024). Although these studies highlight the potential of microbial diversity to influence immune responses in various contexts, our study did not reveal significant correlations between microbial diversity and either immune gene expression or immune cell infiltration (TILs). A slight trend was observed with TIL infiltration, suggesting a possible association, but the limited sample size may have restricted our ability to detect statistically robust correlations.

5.4 Limitation of the study

This study enhances our understanding of BC immunology and its correlations with intratumoral microbiota among Ethiopian BC patients, aiding in the stratification of individuals into distinct prognostic groups. However, some limitations should be acknowledged.

A major limitation is the small sample size, which affects the robustness of our survival analysis and may have hindered the detection of significant correlations, particularly when stratified by TIL groups or intrinsic subtypes. The uneven distribution of cases further limited the ability to identify significant differences across subtypes. Additionally, the limited number of immune genes analyzed restricted the comprehensive assessment of the TIME and its role in breast cancer progression. A broader panel of immune genes could provide a more detailed understanding of the immune landscape.

Moreover, due to the limited availability of PBMC, we were unable to perform functional assays to assess the functionality of different immune cells. MDSC levels were determined from frozen PBMC, which may have affected their recovery. Like other cross-sectional patient-based microbiome studies, our findings are also limited by the inability to establish causality. Despite these limitations, the observed trends provide valuable insights that warrant further investigation in larger cohorts with functional validation.

Chapter 6: Conclusion

This study highlights the presence of distinct immune subgroups in Ethiopian breast cancer patients, characterised by unique gene expression and immune cell infiltration patterns, and demonstrates their prognostic relevance in both study and validation cohorts. These findings highlight the potential of immune-based classification to refine risk stratification, guide chemotherapy and immunotherapy selection, and inform personalized treatment strategies for Ethiopian breast cancer patients.

Additionally, this study presents evidence of systemic immune activation specific to breast cancer in Ethiopian patients, accompanied by immune escape mechanisms in peripheral blood immune cells. These mechanisms include CD4+ T cell exhaustion, immune senescence in CD8+ T cells, and the expansion of immunosuppressive populations, such as PNM-MDSC and Tregs. Further functional characterization of these immune populations could provide critical insights into strategies to counteract immune evasion and improve therapeutic outcomes.

Furthermore, the study demonstrates a significant correlation between the intratumoral microbiome and immune response at both genetic and cellular levels, with Delftia emerging as the predominant intratumoral microbiota with this regard. Delftia significantly influences the expression of immune-related genes and correlates with the abundance of TILs, suggesting its potential role in modulating anti-tumor immunity.

All in all, the study provides a comprehensive view of both local (tumor) and systemic immune landscapes and their interaction with the mammary microbiota in Ethiopian breast cancer patients. These findings highlight the complex interplay between tumor biology, host immunity, and microbiota in shaping disease progression. Future studies employing mechanistic approaches and larger sample sizes are warranted to validate these observations, elucidate causal relationships, and identify actionable targets for personalized immunotherapy and microbiota-modulating interventions.

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List of publications

Published articles

1. Yohannes, M., Desalegn, Z., Bauer, M., Stückrath, K., Anberbir, E., Bekuretsion, Y., Assefa, M., Wakuma, T., Worku, Y., Santos, P. S. C., Taylor, L., Adissie, A., Wickenhauser, C., Massa, C., Vetter, M., Kantelhardt, E. J., Seliger, B., & Abebe, T. (2024). Immune landscape of the tumour microenvironment in Ethiopian breast cancer patients. *Breast Cancer Research*, *26*(1). https://doi.org/10.1186/s13058-024-01916-4

2. Yohannes, M., Massa, C., Desalegn, Z., Stückrath, K., Mueller, A., Anberber, E., Bekuretsion, Y., Assefa, M., Santos, P., Addissie, A., Bauer, M., Wickenhauser, C., Taylor, L., Vetter, M., Kantelhardt, E. J., Abebe, T., & Seliger, B. (2024). Blood immune profiling of Ethiopian patients with breast cancer highlights different forms of immune escape. Oncoimmunology, 13(1), 2436227. https://doi.org/10.1080/2162402X.2024.2436227

To be submitted to mBio

3. Role of Intratumoral Microbiota in Immune Modulation in Ethiopian Breast Cancer: The Role of Delftia and Other Microbial Taxa

ANNEXES

Annex I: Patient information sheet

You are kindly invited to participate in this study, which involves patients with breast cancer

a. **Purpose**: The aim of this study is to determine immune profile in breast cancer, its role in prognosis and response to therapy and characterization of microbiota among breast cancer patients using blood, FNA and biopsy from breast tumor and healthy tissue sample from selected hospitals, Addis Ababa, Ethiopia.

b. **Participation**: Participation in this study is voluntary. If you are willing to participate in this study, we will take 10mlof blood before and after surgery is done. In addition, 10 ml blood will also be collected after 4thround and completion of chemotherapy or radiotherapy. The samples you give will be transported in to MLU-Germany for further analysis.

c. **Procedures to be carried on**: the procedure of sample collection is easy and straight forward; blood sample will be collected from vein in the left arm by attending nurse. Left over biopsy (from tumor site) which is to be collected as part of routine diagnostic procedure will also be used. Moreover, healthy adjacent tissue which is 5cm away from tumor site will be collected after breast mastectomy is done.

d. **Risk and discomfort**: There might be a slight risk during sample collection but can be managed easily if they occur. You might also feel some discomfort while your blood is being collected. You may also experience reactions such as thirst, lightheadedness, nausea/vomiting, rapid pulse/palpitations, and fainting. Another possible risk is bruising when blood leaks into tissues and skin around the needle site. If you are encountered some risk during sample collection, necessary medical service will be given according to the routine procedure.

e. **Expected benefits**: there is no direct benefit in being involved in this study. But, the result of this work can contribute in the advancement of knowledge towards the goal of developing convenient tumor marker for breast cancer and effective approaches for therapy.

f. **Confidentiality**: All your personal information collected for the purpose of the present study will be kept confidential by giving study identification number for each questionnaire and special laboratory labeling for the respective specimens . In addition, no one can trace your identity except the principal investigator authorized co-investigators.

g. **Compensation**: No compensation will be provided in participating in this study.

h. **Termination of the study**: Participation in the study is voluntary, and refusal to participate involvesno penalty or loss of benefits to which you are otherwise entitled. The study participants have a right to

- Keep hold information
- Decline to cooperate in the study
- To refuse provision of specimens

I would also like to inform you that this study will be approved by the Department's Ethical and Review Committee and permission to conduct the research was obtained from the respected Hospitals.

For any questions contact

Meron Yohannes

Phone number

+251925907169

Addis Ababa University, Department of medical laboratory Science

OR

The IRB Office of the College of Health Sciences Addis Ababa University by phone 0118961396

Annex II: Patient information sheet (Amharic version)

የጥናቱተሳታፊዎችየመረጃቅጽ

በዚህጥናትላይእንዲሣተፉበአክብሮትተጋብዘዋልይህጥናትከጡትካንሰርየተያየዘችግርያለባቸውተማሚዎችንያካትታል፡፡

ሀ. የጥናቱአላማ፡-

የዚህጥናትአላማየጡትካንሰርአይነቶችንመለየትናከሰውነትየበሽታመከላከልአቅምጋያለውንመስተጋብርንማጥናትይሆናል፡፡
ጥናቱንለማድረግየደምናሙና
ካንሰርካላበትናበካንሰሩአካባቢካለጤነኛየጡትክፍልየጡትቅንጣትናሙናበጥናቱከሚሳተፉየጡትካንሰርታካሚዎችበአዲስአበባ ከሚገኙየጤናድርጅቶችይከናወናል፡፡

ለ. በጥናቱስለመሳተፍ

በዚህጥናትመሳተፍበሙሉፍቃደኝነትላይየተመሰረተነው::በዚህጥናትየሚሳተፉግለሰቦችሁለት/ሦስትጊዜየተለያየመጠንያላቸው የደምናሙናዎች;

ከጡትላይየሚወሰድትንሽ/ቁራጭናሙናይሰጣሉ::ለዚህጥናትአላማመጠኑ10ሚ.ሊየሚሆንየደምናሙናከቀዶጥገናበፊትናበኋላ እንዲሁምበክትትልጊዜ10ሚ.ሊየሚሆንየደምናሙናተሳታፊዎቹበተለያየየጊዜመርሐግብርየሚሰጠውንመድሐኒትለመውሰድበ ሚመጡጊዜየሚሰበሰብይሆናልለጥናቱዓላማየሚሰበሰበውናሙናለተጨማሪየቤተሙከራወደጀረመንየሚወሰድይሆናል፡፡

ሐ. **አጠቃቀም፡-**

በዚህጥናትላይየሚተገበረውየናመናአሰባሰብዘዴቀላልናግልጽሲሆንየሚሰበሰበውምከተሳታፊውክንድበሙያውልምድባላቸውነ ርሶችይሆናል፡፡

ከዚህጋርበተያያዘምለመደበኛውየህክምናአገለግሎትከሚሰበሰበውየተረፈከህመምተኛውቀጭንበሆነመረፌተመጥጦየመወሰድና ሙናእንዲሁምከጡትላይየሚወሰድትንሽ/ቁራጭናሙናየሚወሰድይሆናል፡፡

ከዚህበተጨማሪምበህመምተኛውፈቃደኝነትከጡትካንሰሩ 5cm ርቀትላይካለጤናማየጡትክፍልቅንጣትየጡትናሙናየሚሰበሰብይሆናል፡፡

መ.ሊደርሥየሚችልአደጋ፡

በዚህጥናትበመሳተፍዎምንምአይነትችግርከናመናአሰባሰብጋርበተያያዘአይደርስቦትም፡፡

ነገርግንየደምናሙናበሚሰበሰብበትቅጽበትቀለልያለምቾትየማጣትስሜትእንደማቅለሽለሽማዞርማስታወክፈጣንየደምስርንዝረት ናእራስንመሳትሊገጥምዎትይችላል፡፡

ናሙናውበሚወሰድበትጊዜማንኛውምችግርቢፈጠርበሆስፒታሉየህክምናአገልግሎትበሚሰጡትባለሙያዎችበመደበኘውአገል ግሎትእርዳታይደረግሎታል፡፡

ሠ. ከጥናቱየሚጠበቅጥቅም

በዚህጥናትላይበመሣተፈዎምንምአይነትቀጥተኛጥቅምየለውምነገርግንይህጥናትወደፊትከጡትጋርየተያየዙችግሮችመፍትሔላይ ለሚሠሩሠዎችየራሡንአስተዋፅኦያደርጋል፡፡

ረ.**ሚስጥራዊነት፡-**

ማንኛውምየጥናቱተሳታፊመረጃሚስጥራዊነትበመጀመሪያስለተሳታፊውየሚሞላውመጠይቅልዩበሆነየግልየመለያቁጥርየሚሰየ ምሲሆንየሚሰበሰበውምናሙናልዩበሆነየቤተ-ሙከራቁጥርየሚሰጠውይሆናልል፡፡

የእያንዳንዱንግለሰብመረጃከዋናውተመራማሪናከተፈቀደላቸውአጋርተመራማሪዎችበስተቀርማንምሊያገኝአይችልም፡

ሰ. **መካካሻ፡**

በዚህጥናትላይበመሳተፍዎምንምአየነትከጥናቱጋርበተያያዘማካካሻአይደርግሎትም፡፡

ሰ.ፈቃደኝነትንስለማቋረጥ፡

በዚህመሳተፍሙሉበሙሉበፈቃደኝነትየተመሰረተሲሆንከጥናቱምበማንኛውምጊዜለማቆምቢፈልጉቅጣትየሌለውናጥቅሞንምበ ምንምሁኔታየማይጎድልቦትይሆናል

በዚህምመሰረትየጥናቱተሳታፊዎችየሚከተሉትመብቶችይኖራቸዋል፡

- መረጃየመያዝ, በጥናቱከመሳተፍ/ከመተባበርማቆምናሙናላለመስጠትመከልከል

በተጨማሪምይህጥናትበትምህርትክፍሉናበኮሌጁበሚገኘውየምርምርስነምግባርኮሚቴፈቃድየሚያገኝሲሆንስራውንምለማስጀ መርከጤናድርጅቶችፈቃድየሚጠየቅይሆናል፡፡

ከጥናቱጋርበተያያዘጥያቄቢኖርዎት

የዋናውተመራማሪአድራሻ፤ማሮን

ከጥናቱጋርበተያያዘማንኛውምጥያቄቢኖርዎትበሚከተለውአድራሻጥያቄዎንማቅረብይችላል፡፡

አዲስአበባዩኒቨርሲቲ

ስልክ:+251925907169

ወይምበአዲስአበባዩኒቨርሲቲጤናሳይንስኮሌጅየምርምርሥነ-ምግባርቢሮ

ስልክ: 0118961396

Annex III: Consent form

Serial number:----------

Card number ……

I have been informed that this study is planned to determine breast cancer immunology and characterization of microbiota among breast cancer patients using blood, biopsy (from tumor site and health tissue 5 cm away from the tissue). I am also informed that the information obtained from the card will be transferred to the data extraction form prepared for this study and will be kept confidential by giving unique identification number for each of the filled questionnaire and a specific laboratory labeling for the respective samples. It is therefore with the understanding of the objective of this study that I allow specimens required for the study (blood ,left over biopsy samples), healthy adjacent breast tissue and stool. Nevertheless, I was assured that I have full right to withdraw from this study and that it will never affect my right of getting appropriate treatment.

I ……hereby give my consent to be involved in this study, to give the requested information and allow samples (blood, left over sample (biopsy), healthy adjacent breast tissue and stool which are required for the study.

Signature: …… Date:

_____ _____

Name of investigators signature

_____ _____

Witness Signature

Annex IV: Consent form (Amharic version)

የፈቃደኝነትመጠየቂያቅጽ

ተራቁጥር…….

የካርድቁጥር…….

የጥናቱዓላማምንእንደሆነናዝርዝርሁኔታዎችየተገለጸልኝሲሆንየጥናቱምዋናዓላማስለጡትካንሰርአይነቶችናተያያዝየሰውነትመከ
ላከልጉዳዮችእንዲሁምየጥቃቅንበአይንየማይታዩተህዋስያንከጡትካንሰርጋርያላቸውንተዛምዶሁኔታየሚዳስስትናትመሆኑንተገን
ዝቤያለሁ፡፡ለዚህዐላማምየሚሆንየደም፤

አንዲሁመከመደበኘውህክምናየተረፈ(የጡትቅንጣት/ትንሽናሙናእናበቀጭንመርፌተመጦየመወሰድናሙናእንዲሁምየጡትካን
ሰርካለበትርቆጤነኛየሆነየጡትቅንጣትበተጨማሪምሰገራየሚሰበሰብይሆናል፡

በተጨማሪምየተገኘውመረጃልዩየሆነየመለያቁጥርለእያንዳንዱመጠይቅየሚሰጥሲሆንለያንዳንዱምለተሰበሰበውናሙናየቤተ-
መከራመለያቁጥርየሚሰጠውይሆናል፡፡

እያንዳንዱምመረጃወደመረጃማጠናቀሪያእንደሚገባናሚስጥራዊነቱምየሚጠበቅመሆኑተበረራርቶልኛል፡፡

ስለሆነምየጥናቱዓላማበሚገባስለገባኝየተጠቀሱትናሙናዎች(ጡትቅንጣት/ትንሽናሙናእናበቀጭንመርፌተመጦየመወሰድ
ናሙና፤

እንዲሁምየጡትካንሰርካለበትርቆጤነኛየሆነየጡትቅንጣት)እንዲሰበሰቡመፈቀዴንእገልጻለሁ::አንዲሁምከጥናቱበመንኛውምጊ
ዜየማቆምመብትአንዳለኝናይህምምንምአይነትየጥቅምመጉደልእንደማያደርስብኝተብራርቶልኛል፡፡

እኔ…….በጥናቱ ለመሳተፍያለኝንፈቃደኝነት እንዲሁም
አስፈላጊውንመረጃለመስጠትበተጨማሪምየሚፈለገውንየናሙናአይነት
(ጡትቅንጣት/ትንሽናሙናእናበቀጭንመርፌተመጦየመወሰድ ናሙና፤
እንዲሁምየጡትካንሰርካለበትርቆጤነኛየሆነየጡትቅንጣትበተጨማሪምሰገራ) ለመስጠትፈቃደኛመሆኔንእገልጻለሁ፡፡

ፊርማ ---------- ቀን----------

---------- ፊርማ ----------

የተመራማሪውስም

Annex V: Protocol for flow cytometry

1. Take out cryopreserved PBMC from liquid nitrogen and transfer it in to 10 ml PBS when it thaws up
2. Centrifuge at 350g for 5 min at 22^0C
3. Decant the supernatant and resuspend the cell pellet again with 10ml PBS
4. Transfer 500µl of the cell suspension for cell Dyn analysis while centrifuging the rest of the suspension for 2nd wash
5. Discard the supernatant and resuspend cells at 1-10x10^6 cells/ml in PBS
6. Add 1 µl of Fixable Viabilty stain (FVS700)stock solution for each 1ml of cell suspension and vortex immediately.

   i.e 1:1000 dilution of the stain with the cell suspension

   7. Incubate the mixture for 10-15min. at room temperature or 2-8^0C protected from light. (use aluminum foil)

   8. In the meantime, prepare 10 flow cytometry tubes and add antibodies as give below in table 1.1

   9. When the incubation time is over, wash cells twice with 10ml of PBS.

   10. Resuspend the cell pellet with 1.ml of PBS and transfer 100µl to each tube.

11. Incubate for 15minutes at dark.
12. Wash it 2ml PBS and centrifuge it
13. Wash those cells which are needed for surface staining twice with 2ml of PBS. (tubes 1-5, 11 & 13)
14. Add 300µl of PBS to these tubes and keep them refrigerated until read by flow cytometer.
15. Add 1ml of 1X Fix/Perm buffer to tubes for intracellular staining and vortex.

    N.B The stock is 4x concentrated. Prepare a working solution by diluting 1:4 with diluent buffer immediately before use.

    16. Incubate samples at 2-8^0C for 40-50 min. in dark.

    17. Add 1ml of 1x Perm/Wash working solution, centrifuge and discard the supernatant.

-The stock is 5x concentrated, do a 1:5 dilution with deionize water

- The working solution can be kept at refrigerator up to one week.

18. Add again 2ml of 1x Perm/Wash,centrifuge and discard the supernatant

19. Add antibodies for intracellular staining as shown in the table.

20. Vortex and incubate the samples at 2-8^{0}C for 40-50 min. in dark.

21. Wash the samples twice with 2ml of 1x Perm/Wash

22. Add 300µl of PBS and acquire.

Annex VI: Microbial DNA Isolation Kit (MO BIO)

Sample process

1. Fill the Microtiter deep well 96 plate according to the Table 1. More information about the sample preparation and reagents can be found in the PowerMag Microbial DNA Isolation Kit instruction manual, MO BIO, catalog no. 27200-4.

Row	Row name	Content	Sample/reagent volume
A	Bind	Lysate	450 µl
		100% Ethanol	450 µl
		SwiftMag™ Beads	50 µl
B	Tips		
C	Wash 1	100% Ethanol	1000 µl
D	Wash 2	100% Ethanol	1000 µl
E	Wash 3	100% Ethanol	1000 µl

Table 1. Filling the Microtiter deep well 96 plate

2. Prepare the KingFisher Duo elution strip by adding 100 µl of SwiftMag Elution Buffer into each well.

3. Place a KingFisher Duo 12-tip comb into row B. For more detailed instructions, see the KingFisher Duo user manual.

4. Start the "KF_Duo_MoBio_PowerMag_Microbial_DNA" protocol using arrow keys and START button. You can also run the protocol using a computer. For more details, see the BindIt software user manual.

5. Insert the plate and elution strip into the KingFisher Duo as indicated on the instrument display and press OK to confirm the action.

Note! Make sure that the elution strip is placed in the correct direction into the elution block. Ensure that the perforated end is facing towards the user.

6. The purification protocol will start when the consumables are loaded and OK button is pressed.

7. When the purification process is completed, remove the plate and elution strip. Store the purified DNA accordingly.

Annex VII: Purification of RNA from FFPE sections

1. Using a scalpel, trim excess paraffin off the sample block.

2. Cut sections 5–20 µm thick. If the sample surface has been exposed to air, discard the first 2–3 sections.

3. Immediately place the sections in a 1.5 ml or 2 ml or 2 ml microcentrifuge tube (not supplied), and close the lid.

4. Add 160 µl or 320 µl Deparaffinization Solution, vortex vigorously for 10 s, and centrifuge briefly to bring the sample to the bottom of the tube. Deparaffinization Solution is not supplied with the miRNeasy FFPE Kit and should be ordered separately (cat. no. 19093). If using an alternative deparaffinization method, see Appendix A for further details.

5. Incubate at 56°C for 3 min, then allow to cool at room temperature.

6. Add 150 µl or 240 µl Buffer PKD, and mix by vortexing.

7. Centrifuge for 1 min at 11,000 x g (10,000 rpm).

8. Add 10 µl proteinase K to the lower, clear phase. Mix gently by pipetting up and down.

9. Incubate at 56°C for 15 min, then at 80°C for 15 min. If a heating block without a shaking function is used, briefly mix by vortexing every 3–5 min. If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block has reached 80°C.

10. Transfer the lower, clear phase into a new 2 ml microcentrifuge tube. If processing more than 2 sections, a larger tube may be necessary.

11. Incubate on ice for 3 min. Then centrifuge for 15 min at 20,000 x g (13,500 rpm).

12. Transfer the supernatant to a new microcentrifuge tube (not supplied) taking care not to disturb the pellet. The pellet contains insoluble tissue debris, including crosslinked DNA.

13. Add DNase Booster Buffer equivalent to a tenth of the total sample volume (approximately16 µl or 25 µl) and 10 µl DNase I stock solution. Mix by inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.

Note: DNase I is supplied lyophilized and should be reconstituted Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.

14. Incubate at room temperature for 15 min.

15. Add 320 µl or 500 µl Buffer RBC to adjust binding conditions, and mix the lysate thoroughly.

16. Add 1120 µl or 1750 µl ethanol (100%) to the sample, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 17.

17. Transfer 700 µl of the sample, including any precipitate that may have formed, to an

RNeasy MinElute spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.*

Reuse the collection tube in step 18.

18.Repeat step 17 until the entire sample has passed through the RNeasy MinElute spin column. Reuse the collection tube in step 19.

19. Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.

20. Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane.

Discard the collection tube with the flow-through.

Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

21. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the collection tube with the flow-through.

It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.19 miRNeasy FFPE Handbook 01/2020

22. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14–30 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

Annex VIII: Purification of RNA from fresh frozen tissue

1. Start with cryostat section: 60-100 sections, each with a thickness of up to 10µm in a 1.5 ml-tube.

2. Add 700µl Qiazol Lysis Reagent mix by vortexing or using RNase-free pestle.

3. Place the tube containing the homogenate on the benchtop at room temperature (15–25°C) for 5 min.

4. Add 140 µl chloroform to the tube containing the homogenate and cap it securely.

Shake the tube vigorously for 15 s. Thorough mixing is important for subsequent phase separation.

5. Place the tube containing the homogenate on the benchtop at room temperature for 2–3 min.

6. Centrifuge for 15 min at 12,000 x g at 4°C. After centrifugation, heat the centrifuge up

to room temperature if the same centrifuge will be used for the next centrifugation steps.

After centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous

phase containing RNA; a white interphase; and a lower, red, organic phase.

7. Transfer the upper aqueous phase to a new collection tube (supplied). Add 1.5 volumes

(usually 525 µl) of 100% ethanol and mix thoroughly by pipetting up and down several

times. Do not centrifuge. Continue without delay with step 9.

8. Pipet up to 700 µl of the sample, including any precipitate that may have formed, into an RNeasy Mini spin column in a 2 ml collection tube. Close the lid gently and centrifuge at ≥8000 x g (≥10,000 rpm) for 15 s at room temperature. Discard the flowthrough

9. Repeat step 9 using the remainder of the sample. Discard the flow-through.* Reuse the collection tube in step 11.

11.Pipet 500 µl Buffer RPE onto the RNeasy Mini spin column. Close the lid gently and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the column. Discard the flowthrough. Reuse the collection tube in step 13.

12.Add another 500 µl Buffer RPE to the RNeasy Mini spin column. Close the lid gently and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to dry the RNeasy Mini spin column membrane.

13. Transfer the RNeasy Mini spin column to a new 1.5 ml collection tube (supplied). Pipet 30–50 µl RNase-free water directly onto the RNeasy Mini spin column membrane. Close the lid gently and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.

14. If the expected RNA yield is >30 µg, repeat step 15 with a second volume of 30–50 µl RNase-free water. Elute into the same collection tube.

Annex IX: mRNA expression Assay

1. Remove aliquots of both Reporter CodeSet and Capture ProbeSet reagent from the freezer and thaw at room temperature. Invert several times to mix well and spin down reagent.

2. Create a master mix by adding 70 µL of hybridization buffer to the tube containing the Reporter CodeSet. Do not remove the Reporter CodeSet from this tube. RNase-free water may also be added to this mix if the volume of the individual RNA samples is less than 5 µL and is constant.

3. Label the hybridization tubes. If using strip tubes, ensure they fit in a microfuge or picofuge and cut the strip in half if necessary.

4. Add 8 µL of master mix to each of the 12 tubes. (If water was added to the master mix, increase this volume as necessary). Use a fresh tip for each pipetting step to accurately measure the correct volume.

5. Add up to 5 µl of sample to each tube.

6. If necessary, add RNase-free water to bring the volume of each assay to 13 µL.

7. Invert the Capture ProbeSet tube to mix and spin down the contents. Add 2 µL of Capture ProbeSet to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down and immediately place the tubes in the pre-heated 65°C thermal cycler.

8. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. Ramp reactions down to 4°C and process the following day. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.

9. Load in to MAX/FLEX counter and count read by 555 FOV with digital Analyzer.

Annex X: DNA extraction

1. Carefully peel off the Square Well Mat that covers the PowerMag® Bead Plate and set aside. Add 0.25 grams of soil sample to each well of the PowerMag® Bead Plate.

2. Add 750 µl of PowerMag® Bead Solution / RNase A Solution to each well of the PowerMag® Bead Plate.

3. Check the PowerMag® Lysis Solution before using. If the PowerMag® Lysis Solution has precipitated, heat the solution at 60°C until the precipitate has dissolved. Mix gently.

4. Add 60 µl of PowerMag® Lysis Solution to each well. Secure the Square Well Mat (from step 1) tightly to the PowerMag® Bead Plate.

5. Place each of the PowerMag® Bead Plates (with Square Well Mats securely affixed) between 2 adapter plates (MO BIO Catalog# 11990) and place on the 96 Well Plate Shaker (MO BIO Catalog# 11996). Reference the protocol provided with the adapter plates for proper placement. Shake at speed 20 for 10 minutes.

6. After the first 10 minute cycle, remove the block and rotate it so that the side closest to the machine body is now furthest from the machine. Shake again at speed 20 for 10 more minutes.

7. Centrifuge the PowerMag® Bead Plate at room temperature for 6 minutes at 4500 x g.

8. Carefully and without splashing remove and discard the Square Well Mat and transfer the supernatant to a clean PowerMag® 1 ml Collection Plate.

9. Add 450 µl of PowerMag® IRT Solution to each well and apply Sealing Tape to the PowerMag 1 ml Collection Plate. Vortex horizontally for 5 seconds on the vortex ensuring that the solution is well mixed. Incubate at 4°C for 10 minutes. Centrifuge the PowerMag® 1 ml Collection Plate at room temperature for 6 minutes at 4500 x g. Remove and discard Sealing Tape.

10. Avoiding the pellet, transfer the entire volume (approximately 850 µl) of supernatant to a new PowerMag® 1 ml Collection Plate. For the wells at the center of the plate, it may help to mark a line on the pipet tip to show how far to insert the tip without touching the pellet. Apply Sealing Tape to the PowerMag® 1 ml Collection Plate. Centrifuge again at 4500 x g for 6 minutes to clear any residual IRT pellet that may have carried over.

11. Transfer no more than 850 µl of supernatant to the MO BIO 2 ml Deep Well Plate (DWP) again avoiding any residual pellet.

12. Place the MO BIO 2 ml Deep Well Plate (DWP) containing the supernatant on the epMotion® robotic deck as indicated on the worktable in the epMotion® program.13

13. For each 96 well plate to be processed, place 174 ml of ClearMag® Wash Solution into an Eppendorf 400 ml reservoir placed at the appropriate location on the deck as indicated on the worktable in the epMotion® program.

14. For each 96 well plate to be processed, place 11 ml of ClearMag® Elution Buffer into an Eppendorf 30 ml reservoir placed in an Eppendorf tub holder located at the appropriate location on the deck as indicated on the worktable.

15. For each 96 well plate to be processed, prepare the ClearMag® Binding Solution / ClearMag® Beads by first vortexing the bottle containing the ClearMag® Beads until all beads are resuspended, followed by adding 2 ml of the now resuspended ClearMag® Beads to 85 ml of the ClearMag® Binding Solution in an appropriate mixing vessel (user provided). Vortex well to mix.

16. Transfer the entire volume of ClearMag® Binding Solution/ClearMag® Beads into an Eppedorf 100 ml reservoir placed in an Eppendorf tub holder located at the appropriate location on the deck as indicated on the worktable.

17. Initiate the protocol immediately.

18. Upon completion, cover the wells of the PowerMag® Microplate (MO BIO MTP) with the Elution Sealing Mat provided. DNA is now ready for any downstream application. No further steps are required. We recommend storing DNA frozen (-20°C or -80°C).

Annex XI: PCR amplification and 16S rRNA Gene Sequencing

Amplicon PCR

1 Set up the following reaction of DNA, 2x KAPA HiFi HotStart ReadyMix, and primers:

	Volume
Microbial DNA (5 ng/µl)	2.5 µl
Amplicon PCR Forward Primer 1 µM	5 µl
Amplicon PCR Reverse Primer 1 µM	5 µl
2x KAPA HiFi HotStart ReadyMix	12.5 µl
Total	25 µl

2 Seal plate and perform PCR in a thermal cycler using the following program:

• 95°C for 3 minutes

• 25 cycles of:

— 95°C for 30 seconds

— 55°C for 30 seconds

— 72°C for 30 seconds

• 72°C for 5 minutes

• Hold at 4°C

PCR Clean-Up

1 Centrifuge the Amplicon PCR plate at 1,000 × g at 20°C for 1 minute to collect condensation, carefully remove seal.

2 [Optional - for use with shaker for mixing] Using a multichannel pipette set to 25 µl, transfer the entire Amplicon PCR product from the PCR plate to the MIDI plate. Change tips between samples.

NOTE

3 Vortex the AMPure XP beads for 30 seconds to make sure that the beads are evenly dispersed. Add an appropriate volume of beads to a trough depending on the number of samples processing.

4 Using a multichannel pipette, add 20 µl of AMPure XP beads to each well of the Amplicon PCR plate. Change tips between columns.

5 Gently pipette entire volume up and down 10 times if using a 96-well PCR plate or seal plate and shake at 1800 rpm for 2 minutes if using a MIDI plate.

6 Incubate at room temperature without shaking for 5 minutes.

7 Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.

8 With the Amplicon PCR plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. Change tips between samples.

9 With the Amplicon PCR plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:

a Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.

b Incubate the plate on the magnetic stand for 30 seconds.

c Carefully remove and discard the supernatant.

10 With the Amplicon PCR plate on the magnetic stand, perform a second ethanol wash as follows:

a Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.

b Incubate the plate on the magnetic stand for 30 seconds.

c Carefully remove and discard the supernatant.

d Use a P20 multichannel pipette with fine pipette tips to remove excess ethanol.

11 With the Amplicon PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.

12 Remove the Amplicon PCR plate from the magnetic stand. Using a multichannel pipette, add 52.5 µl of 10 mM Tris pH 8.5 to each well of the Amplicon PCR plate.

13 Gently pipette mix up and down 10 times, changing tips after each column (or seal plate and shake at 1800 rpm for 2 minutes). Make sure that beads are fully resuspended.

14 Incubate at room temperature for 2 minutes.

15 Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.

16 Using a multichannel pipette, carefully transfer 50 µl of the supernatant from the Amplicon PCR plate to a new 96-well PCR plate. Change tips between samples to avoid

cross-contamination.

Index PCR

1 Using a multichannel pipette, transfer 5 µl from each well to a new 96-well plate. The remaining 45 µl is not used in the protocol and can be stored for other uses.

2 Arrange the Index 1 and 2 primers in a rack (i.e. the TruSeq Index Plate Fixture) using the following arrangements as needed:

a Arrange Index 2 primer tubes (white caps, clear solution) vertically, aligned with rows A through H.

b Arrange Index 1 primer tubes (orange caps, yellow solution) horizontally, aligned with columns 1 through 12.

3 Place the 96-well PCR plate with the 5 µl of resuspended PCR product DNA in the TruSeq Index Plate Fixture.

4 Set up the following reaction of DNA, Index 1 and 2 primers, 2x KAPA HiFi HotStart

ReadyMix, and PCR Grade water:

	Volume
DNA	5 µl
Nextera XT Index Primer 1 (N7xx)	5 µl
Nextera XT Index Primer 2 (S5xx)	5 µl
2x KAPA HiFi HotStart ReadyMix	25 µl
PCR Grade water	10 µl
Total	50 µl

5 Gently pipette up and down 10 times to mix.

6 Cover the plate with Microseal 'A'.

7 Centrifuge the plate at 1,000 × g at 20°C for 1 minute.

8 Perform PCR on a thermal cycler using the following program:

• 95°C for 3 minutes

• 8 cycles of:

— 95°C for 30 seconds

— 55°C for 30 seconds

— 72°C for 30 seconds

• 72°C for 5 minutes

• Hold at 4°C

Library Denaturing and MiSeq Sample Loading

Denature DNA

1 Combine the following volumes of pooled final DNA library and freshly diluted 0.2 N NaOH in a microcentrifuge tube:

• 4 nM pooled library (5 µl)

• 0.2 N NaOH (5 µl)

2 Set aside the remaining dilution of 0.2 N NaOH to prepare a PhiX control within the next 12 hours.

3 Vortex briefly to mix the sample solution, and then centrifuge the sample solution at 280 × g at 20°C for 1 minute.

4 Incubate for 5 minutes at room temperature to denature the DNA into single strands.

5 Add the following volume of pre-chilled HT1 to the tube containing denatured DNA:

• Denatured DNA (10 µl)

• Pre-chilled HT1 (990 µl)

6 Place the denatured DNA on ice until you are ready to proceed to final dilution.

Dilute Denatured DNA

1 Dilute the denatured DNA to the desired concentration using the following example:

Final Concentration	2 pM	4 pM	6 pM	8 pM	10 pM
20 pM denatured library	60 µl	120 µl	180 µl	240 µl	300 µl
Pre-chilled HT1	540 µl	480 µl	420 µl	360 µl	300 µl

2 Invert several times to mix and then pulse centrifuge the DNA solution.

3 Place the denatured and diluted DNA on ice.

Denature and Dilution of PhiX Control

. The final library mixture must contain at least 5% PhiX.

1 Combine the following volumes to dilute the PhiX library to 4 nM:

• 10 nM PhiX library (2 µl)

• 10 mM Tris pH 8.5 (3 µl)

2 Combine the following volumes of 4 nM PhiX and 0.2 N NaOH in a microcentrifuge tube:

• 4 nM PhiX library (5 µl)

• 0.2 N NaOH (5 µl)

3 Vortex briefly to mix the 2 nM PhiX library solution.

4 Incubate for 5 minutes at room temperature to denature the PhiX library into single strands.

5 Add the following volumes of pre-chilled HT1 to the tube containing denatured PhiX library to result in a 20 pM PhiX library:

• Denatured PhiX library (10 µl)

• Pre-chilled HT1 (990 µl)

6 Dilute the denatured 20 pM PhiX library to the same loading concentration as the Amplicon library as follows:

Final Concentration	2 pM	4 pM	6 pM	8 pM	10 pM
20 pM denatured library	60 µl	120 µl	180 µl	240 µl	300 µl
Pre-chilled HT1	540 µl	480 µl	420 µl	360 µl	300 µl

7 Invert several times to mix and then pulse centrifuge the DNA solution.

8 Place the denatured and diluted PhiX on ice.

Combine Amplicon Library and PhiX Control

1 Combine the following volumes of denatured PhiX control library and your denatured amplicon library in a microcentrifuge tube:

• Denatured and diluted PhiX control (30 µl)

• Denatured and diluted amplicon library (570 µl)

2 Set the combined sample library and PhiX control aside on ice until you are ready to heat denature the mixture immediately before loading it onto the MiSeq v3 reagent cartridge.

3 Using a heat block, incubate the combined library and PhiX control tube at 96°C for 2 minutes.

4 After the incubation, invert the tube 1–2 times to mix and immediately place in the ice- water bath.

5 Keep the tube in the ice-water bath for 5 minutes.

NOTE

Perform the heat denaturation step immediately before loading the library into the MiSeq reagent cartridge to ensure efficient template loading on the MiSeq flow cell.

Annex XII: PIK3CA Genotyping

In a detection ΔCT cutoff determination experiment, run three or more wild type gDNA samples, and three technical replicates of each sample, with a mutant allele assay(s) and paired gene reference assay. The same amount of gDNA must be used for each sample.

Prepare the PCR mix and the PCR plate as follows:

1. For each sample, calculate the total number of reactions required.

2. Calculate the total volume required each component: volume for 1 reaction × total number of reactions

3. Include 10% excess volume to account for pipetting errors.

Component	Volume for 1 reaction	
	20 µL reaction (96-well plate)	**10 µL reaction (384-well plate)**
TaqMan® Genotyping Master Mix, 2X	10.0 µL	5.0 µL
Prepared gDNA sample†	2.0 – 4.0 µL	1.0 – 2.0 µL‡
(Optional) 50X Exogenous IPC Template DNA	0.4 µL	0.2 µL
(Optional) 10X Exogenous IPC Mix	2.0 µL	1.0 µL
Nuclease-free water	Add water to 18.0 µL	Add water to 9.0 µL
Total volume of super mix	**18.0 µL**	**9.0 µL**
Example: Total volume for 3 technical replicates (includes 10% extra volume for pipetting errors)	**59.4 µL**	**29.7 µL**

4. Label a 1.5-mL microcentrifuge tube, add all components to the labeled tube, cap the tube, then vortex the tube briefly to mix the components.

5. Centrifuge the tube briefly to spin down the contents and eliminate air bubbles.

6. For each set of technical replicates, transfer aliquots of super mix to a microcentrifuge tube, then add the TaqMan® Mutation Detection Assay (mutant allele or gene reference assay) to each tube.

7. Add the appropriate volume of PCR mix to each reaction well of a PCR plate:

PCR plate	Volume of PCR mix per reaction
96-well	20 µL
384-well	10 µL

8. Cover the plate with an optical adhesive film.

9. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles.

In the real-time PCR system software:

1. Select the experiment type: Absolute Quantitation or Quantitation – Standard Curve.

2. For each well that contains a reaction, apply a sample name, assay name, and target or detector name.

3. Enter sample quantity values in the real-time PCR system software:

a. Select Standard as the task for each well of interest.

b. Enter a numeric value. We recommend that the numeric values you enter are relevant to the ng amount of gDNA or copies of DNA input.

4. Set the following thermal-cycling conditions:

• Run mode – Standard

• Sample volume – 10 µL (384-well plates) or 20 µL (96-well plates)

• Thermal-cycling profile – See the table below

Stage	Temp.	Time (mm:ss)	Cycles	Data collection
1	95°C	10:00	1	No
2	92°C	00:15	5	No
	58°C	01:00		No
3	92°C	00:15	40	No
	60°C	01:00		FAM™ or VIC®dye†

5. Load the reaction plate into the real-time PCR instrument, then start the run.

Annex XIII: Data extraction instrument for retrospective study

Retrospective data collection form

Study Code---------- Participant registration/card number ----------	Hospital name ---------- ----------
Contact address(Phone number) _____	Biopsy/block number ----------
Age at diagnosis(in years)--------	Year of diagnosis(G.C) ----------
Permanent residence Addis Ababa(Staying for more than >= 6 months in Addis Ababa) Out of Addis Ababa (staying for <6 months years in Addis Ababa)	If from outside Addis Ababa, the residence 1. Urban 2. Rural
Menopausal status 1. Pre-menopausal 2. Post-menopausal	Tumor clinical stage - 1. Stage I 2. Stage II 3. Stage III 4. Stage IV
Pathological stage(TNM stage) ----------	Histologic grade 1. Well differentiated 2. Moderately 3. Poorly differentiated 4. Missing
Cancer type 1. Ductal 2. Lobular 3. Ductal and lobular 4. Inflammatory 5. Others _____	Hormone receptor status (Immunohistochemistry test) / score 1. ER (positive/negative) 2. PR(positive/negative)
HER2 status((Immunohistochemistry test) 1. Positive 2. Negative	Tumor size in cm _____ Lymph node status 1. Positive 2. Negative no. of LN removed: _____no of LN involved:____

Metastases 1. Yes 2. No	If there was metastases, please specify the specific location ---------- Which examination was done?_____
Type of Rx administered Yes No Round (chemotherapy & radiotherapy 1. Neoadjuvant ☐ ☐ ---------- 2. Surgery ☐ ☐ Date of surgery---------- 3. Chemotherapy ☐ ☐ ---------- Date started_____---------- Type of chemotherapy---------- 4. Radiotherapy ☐ ☐ ---------- Date started----------	
Endpoint measure (specify the date) 1. RecurrenceLocal recurrenceDistant metast☐s ☐ 2. Death ---------- Follow up 3. Date of last contact ---------- 4. Status at last contact ----------	

Name of data collector_____Signature_____

Annex XIV: Questionnaire for the prospective study

Breast cancer Clinical Examination & Surveillance Form

Part one: Identification

1. Chart number/code: _____ Age: _____
2. Phone number _____
3. Permanent Address:_____
4. Duration of stay at residence: _____
5. Place of Birth: _____ Nationality/Ethnic group: _____

6. Occupation:

_____

7. **Marital status**: Single: ☐ Married: ☐ Divorce Widow/Widower:
8. **Religion** ☐
9. Christian: ☐ Muslim: ☐ Other ☐ (specify)

_____

10. Highest education level attained

11. None: ☐ primary: ☐ secondary: ☐ technical school: ☐ university:
12. ☐

Part two: Admission information

13. Health Institution/Centre: _____
14. Reference number: _____Date: ____/ ____ / _____Card number_____
15. Body weight: _____ kgs; Body height: _____cm; BMI _____

Part three: Medical History of patients

No	Patient concerns
1	Have you ever had certain benign breast conditions before? ❒ yes ❒ No If yes, in what age? _____
2	Have you ever had ovarian cancer? ❒ yes ❒ No If yes, in what age? _____
3	Have any of your close blood relatives ever had breast cancer? ❒ yes ❒ No
4	If yes to question no 3, whom ❒ one of your first degree relatives (mother, sister or daughter) ❒ two of your first degree relatives ❒ any of your distantly related relatives (specify) ----------

5	Have you ever had radiation therapy to the chest area as treatment for another cancer? ❒ Yes ❒ No If yes, what age were you? _____
6	Did you note nipple discharge? ❒ yes ❒ No
7	Did you note the presence of palpable breast mass? ❒ yes ❒ No
8	Breast pain? ❒ yes ❒ No
9	Did you note presence of breast skin change or nipple retraction? ❒ yes ❒ No
10	Did you have abnormal screening mammogram? ❒ yes ❒ No
11	Duration of illness (in months)_____
12	Chief compliant

Part four : Other History related to breast cancer development

No	Patient History
11	When did your menstrual periods begin? _____ years
12	Have you ever given birth? ❒ yes ❒ No
13	If yes to question no 12, what age were you when your first child was born? _____
14	How long did you breast-feed your children? _____
15	Do you use contraceptives? ❒ yes ❒ No
16	If yes to question no 15, what type of birth control method do use? ❒ Diaphragm ❒ Birth control pill

	❒ Norplant implant ❒ Depo-Provera (an injected hormonal birth control method) ❒ Hormonal IUDs ❒ Others_____
17	Have you ever worked in area where there is radiation exposure? ❒ yes ❒ No If yes, for how long? _____
18	Do you consume alcohol? ❒ yes ❒ No
19	If yes, how often? ❒ Occasionally ❒ 1 alcoholic drink daily ❒ 2-5 drinks daily
20	Do you smoke? ❒ yes ❒ No
21	If yes, how often? ❒ Occasionally ❒ 1 alcoholic drink daily ❒ 2-5 drinks daily
22	Do you perform physical exercise? ❒ yes ❒ No

Part five: Breast Physical examination findings

	Breast findings	R	L
	Fine nodularity		❒ ❒
	Dense nodularity	❒	❒
	Skin edema	❒	❒

	Nipple/areolar change	❒	❒							
	Tenderness	❒	❒							
	Nipple discharge	❒	❒							
	Mass	❒	❒ which quadrant _____							
	Yes no									
	Symmetry	❒	❒							
	Discrete mass									
	Shape	margins	size		texture		mobility		other	
	❒ round	❒ well-defined	❒<5mm		❒soft		❒fixed		❒_____	
	❒ oval	❒ ill-defined	❒5-9mm		❒ firm		❒mobile		❒_____	
	❒irregular		❒ 1-2cm		❒rubbery					
			❒3-4cm		❒hard					
			❒>4cm							
	Lymph nodes	Axillary			Clavicular					
					Supra				Infra	
		R	L		R	L		R	L	
	WNL	❒	❒		❒	❒		❒	❒	
	Enlarged	❒	❒		❒	❒		❒	❒	
	Fixed	❒	❒							
	Mobile	❒	❒							
	Clinical stage:	❒ I		❒II		❒III		❒IV		
	TNM stage									

Part six : Pathology information (tumor characteristics)

No	Tumor characteristics
33	Tumor type (stage)
34	Estrogen receptor status❒Positive ❒ Negative
35	Progesterone receptor status ❒Positive ❒ Negative
36	Elston histological grading
37	**Histological type** ❒ ductal ❒ lobular ❒ mixed ❒ medulla
38	**Tumor diameter (mm)**
	Tumor type ❒ Ductal ❒Lobular ❒Ductal and lobular /mixed ❒Inflammatory ❒Medulla ❒Others
	Histologic grade ❒Well differentiated ❒Moderately differentiated ❒Poorly differentiated

	❒Missing

Part seven: Clinical Information

39	Liver or lung metastasis ❒ ❒ Spinal metastasis ❒ ❒ Metastasis to other site ❒❒
40	Type of Treatment ❒Neo-adjuvant ❒Radiotherapy ❒ Surgery ❒Chemotherapy
41	Type of chemotherapy administered ----------
42	Round of chemotherapy ❒ 1st Round ❒3rd Round ❒ 2nd Round ❒ 4thRound
43	Clinical outcome ❒ Recurrence ❒ Metastasis ❒ Death ❒ Others
44	Follow up Date of last contact ---------- Status at last contact ----------

Annex XV: Ethical approval and MTA

ADDIS ABABA UNIVERSITY, COLLEGE OF HEALTH SCIENCES (IRB)

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Institutional Review Board

ANNEX 3

Form AAUMF 03-008

IRB's Decision

Meeting No: 01/2018 Date: February 13,2018

Protocol number: 092/17/DMIP

Protocol Title: The genetic land scope of Breast cancer and its interaction with host immunity in Ethiopian Breast cancer patients	
Principal Investigator:	Dr. Tamrat Abebe et al
Institute:	College of Health Sciences, AAU
Elements Reviewed (AAUMF 01-008)	☑ Attached ☐ Not attached
Review of Revised Application ☐ Yes ☐ No	Date of Previous review:
Decision of the meeting:	☑ Approved ☐ Approved with Recommendation ☐ Resubmission ☐ Disapproved

I. Elements approved-
1. Protocol Version No: 02
2. Protocol Version Date: 15/01/2018
3. Informed consent Version No: 02
4. Informed Consent Version Date: 15/01/2018

II. Obligations of the PI-
1. Should comply with the standard international & national scientific and ethical guidelines
2. All amendments and changes made in protocol and consent form needs IRB approval
3. The PI should report SAE within 10 days of the event
4. End of the study, including manuscripts and thesis works should be reported to the IRB
5. The PI should report non-compliance and unanticipated events

III. TO NERC ☒

Institution Review Board (IRB) Approval: Period from: December 16, 2019 to December 15, 2020

Follow up report expected in: 3 Months____ 6 Months_X__ 9 Months _ __One year____

Chairperson, IRB
Dr. Adamu Addissie

Signature
Date: 16/12/2019

Institutional Review Board Office (IRB) Faculty of Medicine ADDIS ABABA UNIVERSITY

Director of Research & Technology Transfer, CHS
Dr. Wondwossen Amogne

Signature

Date 25 Dec 19

ADDIS ABABA UNIVERSITY, COLLEGE OF HEALTH SCIENCES (IRB)

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Institutional Review Board

ANNEX 3

Form AAUMF 03-008

IRB's Decision

Meeting No: 08/2020 Meeting Date: August 26,2020

Protocol number: 082/20/DMIP

Protocol Title: Host immune profile: role in prognosis and prediction of therapy response In Ethiopian breast cancer patients	
Principal Investigator:	Meron Yohannes
Institute:	College of Health Sciences, AAU
Elements Reviewed (AAUMF 01-008)	☑ Attached ☐ Not attached
Review of Revised Application ☐ Yes ☐ No	Date of Previous review:
Decision of the meeting:	☑ Approved ☐ Approved with Recommendation ☐ Resubmission ☐ Disapproved

I. Elements approved-
1. Protocol Version No: 02
2. Protocol Version Date:
3. Informed consent Version No: 02
4. Informed Consent Version Date:

II. Obligations of the PI-
1. Should comply with the standard international & national scientific and ethical guidelines
2. All amendments and changes made in protocol and consent form needs IRB approval
3. The PI should report SAE within 10 days of the event
4. End of the study, including manuscripts and thesis works should be reported to the IRB
5. The PI should report non-compliance and unanticipated events

III. TO NERC ☑

Institution Review Board (IRB) Approval: Period from: December 07, 2020 to December 06, 2021

Follow up report expected in: 3 Months___ 6 Months____ 9 Months _X_One year____

Chairperson, IRB
Dr. Adamu Addisie

Signature _____

Date: 07/12/2020

Institutional Review Board Office (IRB) Faculty of Medicine ADDIS ABABA UNIVERSITY

Director of Research & Technology Transfer, CHS
Dr. Wondwossen Amogne

Signature _____

Date 8/12/2020

College of Health Science Addis Ababa University

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የኢኖቬሽንና ቴክኖሎጂ ሚኒስቴር
The Federal Democratic Republic of Ethiopia
Ministry of Innovation and Technology

MInT
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ቁጥር
Ref.No. 3.ፌ0/193/2018
ቀን
Date ህዳር 23.2018

To: Addis Ababa University College of Health Science
Addis Ababa, Ethiopia

Subject: Letter of Approval

Dear Sir/Madam//Mr./Mrs./Dr,

The National Research Ethics Review Committee (NRERC) has reviewed "**The Genetic Landscape of Breast Cancer and Its Interaction with Host Immunity in Ethiopian Breast Cancer Patients"** project protocol in an expedited manner. We are writing to advise you that NRERC has granted

Full Approval

To the above named project, for a period of **one year (Nov21, 2018- Nov20, 2019)**. All your most recently submitted documents have been approved for use in this study. The study should comply with the standard international and national scientific and ethical guidelines. Any change to the approved protocol or consent material must be reviewed and approved through the amendment process prior to its implementation. In addition, any adverse or unanticipated events should be reported within 24-48 hours to the NRERC. Please ensure that you submit biannual progress report once in six months and annual renewal application 30 days prior to the expiry date.

We, therefore, request you as PI and your esteemed organization to ensure the commencement and conduct of the study accordingly and wish for the successful completion of the project.

With regards

Awol Hussein Mohammed
Research Ethics Directorate
Acting Director

CC.

- HE the Minister
- HE the State Minister (Technology and Research Sector)
- STI Capacity Building, Policy and Research Director General
- NRERC Chairperson
- Dr. Tamrat Abebe (PI)

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From Faciltator to Main Actor

Material Transfer Agreement

This Material Transfer Agreement (MTA) has been prepared for use by Addis Ababa University, Department of Microbiology, Immunology and Parasitology, Ethiopia and Klinik und Poliklinik für Gynäkologie und Institut für Med. Epidemiologie, Biometrie u. Informatik Medizinische Fakultät Martin-Luther-Universität Halle-Wittenberg Universitätsklinikum Halle (Saale), Germany in all transfer of research material (samples, derivatives, and specimens) related to the protocol **"The genetic landscape of breast cancer and its interaction with host immunity in Ethiopian breast cancer patients"**

Provider: Dr. Tamrat Abebe, Addis Ababa University, Department of Microbiology Immunology and Parasitology, Ethiopia

Recipient: Klinik und Poliklinik für Gynäkologie und Institut für Med. Epidemiologie, Biometrie u. Informatik Medizinische Fakultät Martin-Luther-Universität Halle-Wittenberg Universitätsklinikum Halle (Saale)

1. Provider agrees to transfer to recipient's designated (provider) the following research materials /specimen. Formalin-fixed paraffine embedded tumor block, tumor biopsy DNA, RNA, Plasma, serum and PBMC.

   The research material will only be used for research purposes as described in the protocol by recipient's investigator in designated laboratory for the research project described below, under suitable containment conditions. This research material will not be used for commercial purposes such as screening, production or sale for which a commercialization license may be required. Recipient agrees to comply with all National and International guidelines rules and regulations applicable to the Research Project and the handling of the Research Material.

   a) Are the research materials of human origin?

   Yes No [√] [ ]

   b) If yes, are they collected according to the details in the protocol and in adherence to National Research Ethics Review Committee (NRERC) and Addis Ababa University College of Health Sciences (AAU-CHS)Ethics Review Committee recommendations and their approval.

   Yes No [√] [ ]

2. This research material and its derivatives will be used by recipient's investigator solely in connection with the following research project ("Research Project") described with specificity as follows '' **"The genetic landscape of breast cancer and its interaction with host immunity in Ethiopian breast cancer patients"**

3. In all presentations or written publications concerning the research project, recipient will acknowledge provider's contribution of this research material unless requested otherwise.
4. This research material represents a significant contribution on the part of provider and is considered proprietary to provider. Recipient therefore agrees to retain control over this research material and further agrees not to transfer the research material to other people not under her/his direct supervision without advance written approval of provider. The research material will be disposed of as agreed upon per protocol at the end of completion of the project on 2018.
5. The provider does not take any responsibility for loss, damage, wastage or spoilage of the research material during or after shipment to the address provided by the recipient under conditions agreed to in the protocol on shipment of the samples. This research material is provided as a service to the research community. IT IS BEING SUPPLIED TO RECIPIENT WITH NO WARRANTIES, EXPRESS OR IMPLIED, INCLUDING ANY WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. Provider makes no representations that the use of the research material will not infringe any patent or proprietary right of third parties.
6. The recipient shall notify the provider in witting of any intention, improvement, modification discovery or development to the material or the information made by recipient or parties, collaborating with recipient, here in after referred to as "invention". Nothing in this agreement shall, however, be construed as conveying to the provider any rights under any patents or other intellectual property to such invention, other than as explicitly provided herein. At its option the provider shall be entitled to receive sample of any materials derived from the Materials for its own research and evaluation purposes only.
7. The under- signed provider and recipient expressly certify and affirm that the contents of any statements made herein are truthful and accurate.
8. Any additional terms (use an attached page if necessary):
9. The provider maintains, ownership right of the research material and its unmodified derivatives unless stated otherwise.

The provider will retain a copy (aliquot) of every sample sent abroad as much as possible for local research needs.

Material Transfer Agreement

Signature page

For Recipient:

Read and Acknowledged by:

Recipient's Investigator Duly Authorized Signature

Signature/ Stamp

Martin-Luther-Universität
Halle-Wittenberg
Institut für Medizinische Epidemiologie,
Biometrie und Informatik
06097 Halle (Saale)

Date: 15. 1. 18

Mailing Address for Material: Mailing Address for Notices:

For Provider:

Provider's Investigator Duly Authorized Signature

Dr. Tamrat Abebe

Signature/ Stamp

Date: 24-1-18

Mailing Address: Mailing Address for Notices:

Addis Ababa University Addis Ababa University
Department of Microbiology, Immunology and Parasitology
School of Medicine, College of Health
TikurAnbessa Specialized Hospital
Second floor room number 76
P.o.Box 9086, Addis Ababa, Ethiopia

Tel: +251911447227

Fax: +251- 1- 551 30 99Fax: +251- 1- 551 30 99

Annex XVI: CV of the investigator

MERON YOHANNES

(+251) 9 25 90 71 69. meron.yohannes@aau.edu.et, meritijoe@gmail.com

A. EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	Completion Date MM/YYYY	FIELD OF STUDY
Addis Ababa University, Addis Ababa	BS	07/2012	Medical Laboratory Science
Addis Ababa University, Addis Ababa	MS	02/2016	Medical Microbiology
Addis Ababa University	PhD	Active	Medical Microbiology and Immunology

Work Experience/Positions

Positions and Employment

2012-2013 Graduate Assistant, Department of Medical Laboratory, Addis Ababa University

2013-Nov 2015 Assistant Lecturer, Department of Medical Laboratory, Addis Ababa University

Nov 2016- Lecturer, Department of Medical Laboratory, Addis Ababa University

B. Leadership and Professional Training/Courses

- Science 2.0 How to accelerate your research 2017
- Optimizing your project proposal development, monitoring and evaluation-improving health system management 2020
- Using strategic communications as a tool for social change and advocacy 2020
- Unpacking the global launch of COVID-19 Ag-RDT testing 2020

C. Honors, Grants, and Awards

2012 Recognition of excellence from the class of 2012, Addis Ababa University

2012 Female scholarship to pursue MSc, granted by Addis Ababa University

2021 PI of granted research project; "Peripheral immunity as a marker of disease severity in COVID 19 Patients Admitted to COVID 19 treatment canters in Addis Ababa, Ethiopia".

2025 Global scholar in-training award recipient from the American Association for Cancer research.

2018 Jon Shovel travel award recipient to present my Abstract in AACR

D. Professional Memberships

2011- Member, Ethiopian Medical Laboratory Association

2013- Member, Ethiopian Public Health Association

2018- Associate member of American Association of Cancer research

2020- Member of African Society of Laboratory Medicine

E. Contributions to Science

4. Yohannes, M., Desalegn, Z., Bauer, M., Stückrath, K., Anberbir, E., Bekuretsion, Y., Assefa, M., Wakuma, T., Worku, Y., Santos, P. S. C., Taylor, L., Adissie, A., Wickenhauser, C., Massa, C., Vetter, M., Kantelhardt, E. J., Seliger, B., & Abebe, T. (2024). Immune landscape of the tumour microenvironment in Ethiopian breast cancer patients. *Breast Cancer Research*, *26*(1). https://doi.org/10.1186/s13058-024-01916-4

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