

# Advances in tumor microenvironment, immunology and immunotherapy of breast cancer

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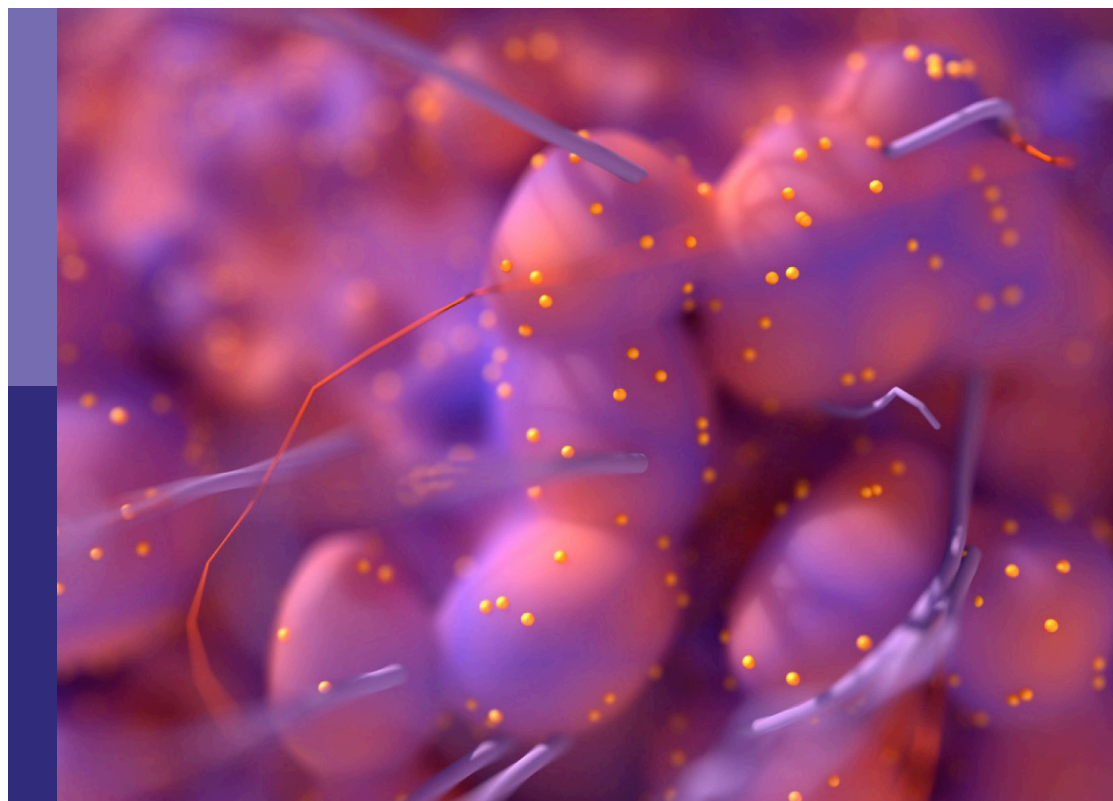
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**Published in**

Frontiers in Oncology



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ISSN 1664-8714  
ISBN 978-2-8325-6892-7  
DOI 10.3389/978-2-8325-6892-7

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# Advances in tumor microenvironment, immunology and immunotherapy of breast cancer

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## Citation

Lin, Y., Zheng, S., Kawaguchi, K., Cui, J., eds. (2025). *Advances in tumor microenvironment, immunology and immunotherapy of breast cancer*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-6892-7

# Table of contents

- 05 **Editorial: Advances in tumor microenvironment, immunology and immunotherapy of breast cancer**  
Yonghong Zhang, Jihong Cui, Shaoquan Zheng, Kosuke Kawaguchi and Ying Lin
- 08 **Hypoxia-inducible factor in breast cancer: role and target for breast cancer treatment**  
Shijiao Zhi, Chen Chen, Hanlin Huang, Zhengfu Zhang, Fancai Zeng and Shujun Zhang
- 28 **The effect of exercise and disease status on mobilization of anti-tumorigenic and pro-tumorigenic immune cells in women with breast cancer**  
Tiia Koivula, Salla Lempiäinen, Joonas Neuvonen, Joonas Norha, Maija Hollmén, Carl Johan Sundberg, Helene Rundqvist, Heikki Minn, Petteri Rinne and Ilkka Heinonen
- 41 **The impact of inhalation versus total intravenous anesthesia on the immune status in patients undergoing breast cancer surgery: a double-blind randomized clinical trial (TeMP)**  
Kristina Kadantseva, Valery Subbotin, Roman Akchulpanov, Levan Berikashvili, Mikhail Yadgarov, Lyudmila Zhukova, Guram Kvetenadze, Oxana Svitich, Polina Kukina, Ivan Kuznetsov, Mariya Shemetova, Anastasiya Smirnova, Petr Polyakov, Andrey Shebankov and Valery Likhvantsev
- 55 **Research on therapeutic clinical trials including immunotherapy in triple-negative breast cancer: a bibliometric analysis**  
Qi Xu, Xiaoyu Feng, Siyuan Qin, Yu Hong, Rui Cui, Jia Liang, Zhuya Xiao and Yuan Li
- 68 **Case report: Triple-negative breast cancer with low tumour-infiltrating lymphocytes infiltration and good prognosis: a case of Tall Cell Carcinoma with Reversed Polarity and review of the literature**  
Shuoying Jiao, Junpeng Chen, Jiumei Shen, Ajing Peng, Ruifu Chen, Baoyong Lai, Chunhua Luo, Yingyi Fan and Xiaohua Pei
- 79 **Resistance mechanisms and prospects of trastuzumab**  
Lizhe Wang, Yu Wang, Yueyang Li, Li Zhou, Jiahui Du, Jin Wang, SiHan Liu, Yongyi Cao, Yuzhi Li, Wenying Yang and Ting Zhu
- 91 **Unveiling the role of TGF- $\beta$  signaling pathway in breast cancer prognosis and immunotherapy**  
Yifan Zheng, Li Li, Wenqian Cai, Lin Li, Rongxin Zhang, Wenbin Huang and Yulun Cao
- 114 **Mature tertiary lymphoid structure associated CD103<sup>+</sup> CD8<sup>+</sup> Trm cells determined improved anti-tumor immune in breast cancer**  
Qin Fang, Shuru Chen, Xiaoyue Chen, Wei Zou, Di Chen, Yukang Huang and Chucheng Wu

- 126 **Breast acinic cell carcinoma with weak progesterone receptor expression: a case report and literature review**  
Caiyun Bai, Xiaodong Xin, Yisen Yang, Fengjiang Qu and Zhimin Fan
- 133 **Case report of concurrent ulcerative colitis and bilateral breast cancer: a literature review of the bidirectional association between autoimmune diseases and breast cancer**  
Xiao Qi, Miao Zhang, Jinhong Zhu and Huaqing Wang



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RECEIVED 12 July 2025  
ACCEPTED 26 August 2025  
PUBLISHED 04 September 2025

## CITATION

Zhang Y, Cui J, Zheng S, Kawaguchi K and  
Lin Y (2025) Editorial: Advances in tumor  
microenvironment, immunology and  
immunotherapy of breast cancer.  
*Front. Oncol.* 15:1664563.  
doi: 10.3389/fonc.2025.1664563

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# Editorial: Advances in tumor microenvironment, immunology and immunotherapy of breast cancer

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## KEYWORDS

tumor microenvironment, immunology, immunotherapy, breast cancer, drug resistance

## Editorial on the Research Topic

### Advances in tumor microenvironment, immunology and immunotherapy of breast cancer

The tumor ecosystems consist of tumor cells embedded within a dynamic tumor microenvironment (TME) comprising immune cells, stromal components, and extracellular factors (1). In breast cancer, the TME exhibits remarkable heterogeneity across molecular subtypes, critically influencing tumor progression, treatment response, and clinical outcomes (2–4). These subtype-specific TME variations have transformed breast cancer management, particularly in immunotherapy and precision oncology approaches. This Research Topic, *Advances in Tumor Microenvironment, Immunology and Immunotherapy of Breast Cancer*, comprises five comprehensive reviews and five original research articles, which collectively explore the interplay between local TME dynamics and systemic mediators in breast cancer progression, therapeutic resistance mechanisms, and the discovery of novel prognostic biomarkers and therapeutic targets.

TME complexity significantly impacts disease characteristics and treatment efficacy. Xu et al. performed a comprehensive bibliometric analysis using Bibliometrix (R) and VOSviewer to map the 27-year evolution of triple-negative breast cancer (TNBC) clinical trials research globally. Their analysis highlights the groundbreaking advances in immunotherapy for TNBC treatment alongside the growing importance of antibody-drug conjugates (ADCs) in targeted therapy approaches. Wang et al. comprehensively reviewed the existing research on the resistance mechanisms of trastuzumab for human epidermal growth factor 2 (HER2+) breast cancer. One aspect in this review particularly emphasizes TME-mediated resistance pathways operating through both cell-autonomous and non-cell-autonomous mechanisms, including impaired antibody-dependent cellular cytotoxicity (ADCC), aberrant secretion of immunosuppressive cytokines, stromal remodeling, and altered immune cell infiltration.

Hypoxia is a significant feature of TME heterogeneity that arises from dysregulated tumor vasculature and heightened metabolic demand. Zhi et al. reviewed the latest research

progress on the role of Hypoxia-inducible factor 1- $\alpha$  (HIF1 $\alpha$ ) in breast cancer progression and therapy resistance across molecular subtypes. Their work highlights HIF1 $\alpha$ -mediated mechanisms including metabolic reprogramming, immune evasion, and treatment resistance, while also evaluating emerging therapeutic strategies targeting the HIF pathway in breast cancer patients.

Notably, rare tumor variants provide unique insights into TME plasticity that directly inform clinical decision-making. While high tumor-infiltrating lymphocytes (TILs) typically correlate with improved survival in TNBC, Jiao et al. reported a rare case of low-TIL TNBC with favorable prognosis, named Tall Cell Carcinoma with Reversed Polarity (TCCRP). Their mechanistic studies implicated that cancer-associated fibroblasts (CAFs) in the interstitium of the tumor contribute to immune evasion in this subtype. These findings suggest fibroblast subpopulation targeting could potentially overcome immunotherapy resistance in TCCRP patients, proposing a novel therapeutic strategy for this unique TNBC variant. Bai et al. reported a rare progesterone receptor (PR)-positive acinic cell carcinoma (AcCC) that exhibited distinct molecular features from conventional TNBC. The PR+/E-cadherin+ phenotype in this rare variant indicates unexpected endocrine responsiveness and targetable pathways, redefining treatment approaches for special breast cancer subtypes.

TME information can provide interpretable prognostic biomarkers. Tertiary lymphoid structures (TLS) serve as critical immunological hubs that orchestrate local antitumor responses and have emerged as robust prognostic biomarkers in cancer. In their comprehensive analysis of 95 treatment-naïve primary breast carcinoma specimens, Fang et al. systematically characterized TLS maturation stages and their associated immune cell composition. The study revealed that peritumoral TLS containing CD103+CD8+ tissue-resident memory T cells (Trm) and activated NK cells were associated with favorable clinical outcomes, with quantitative analyses demonstrating a progressive increase in these effector cell populations correlating with TLS maturation. These findings not only validate TLS as indicators of antitumor immune competence, but also reveal the specific immune cell populations, particularly CD8+ Trm cells and NK cells, that drive their protective effects in breast cancer. Beyond cellular components, non-cellular elements in the TME, like cytokines, chemokines, and other soluble factors, play crucial roles in shaping the tumor immune landscape, thereby significantly influencing anti-tumor immunity and treatment responses. Zheng et al. advanced this understanding by developing TSPRS (TGF- $\beta$  pathway-related prognostic signature), a novel machine learning model that evaluates TGF- $\beta$  activity-associated prognostic signatures. Their work demonstrated significant correlations between TSPRS scores, TME characteristics, and immunotherapy response.

Systemic modulators can profoundly influence cancer immunity beyond the local tumor microenvironment. For systemic physiological modulation, Koivula et al. conducted a clinical trial investigating the immunomodulatory effects of acute exercise in newly diagnosed breast cancer patients. Their results revealed that just 30 minutes of physical activity prior to treatment could significantly alter

circulating immune cell populations, including increased CD8+ T cell and NK cell proportions alongside decreased myeloid-derived suppressor cells (MDSCs). Notably, these exercise-induced changes showed correlation with disease status, suggesting potential applications for prescriptive exercise regimens in cancer therapy. For perioperative interventions, Kadantseva et al. conducted a comparative analysis of inhalation anesthesia (IA) and total intravenous anesthesia (TIVA) in a cohort of 98 breast cancer patients, while no significant differences were observed in the postoperative neutrophil-to-lymphocyte ratio (NLR). However, the IA group exhibited measurable suppression of humoral immunity, which could theoretically impair tumor-specific antibody production and compromise the antitumor activity of immune cells. These findings highlight the underappreciated role of perioperative interventions on sculpting the immunological milieu and determining long-term clinical outcomes in breast cancer management. For autoimmunity-cancer interplay, Qi et al.'s documentation of breast cancer-ulcerative colitis comorbidity provides clinical validation for the bidirectional autoimmunity-cancer interplay, where chronic inflammation may promote carcinogenesis while cancer therapies can trigger autoimmune responses. Collectively, these studies underscore how physiological, pharmacological, and pathological systemic factors collectively shape anticancer immunity and clinical outcomes.

In conclusion, the TME orchestrates breast cancer progression through complex cellular and molecular networks. This Research Topic provides multidimensional insights into TME heterogeneity and delivers new targets and strategies for personalized therapy. The studies featured in this Research Topic collectively advance our understanding of TME immunology, highlighting promising therapeutic targets, resistance mechanisms and novel prognostic tools that may guide clinical decision-making. However, the extraordinary complexity of the TME demands continued investigation using increasingly sophisticated methodologies, including single-cell technologies, spatial transcriptomics, and artificial intelligence approaches. We hope this Research Topic will serve as both a valuable resource and an inspiration for future research aimed at translating TME insights into improved patient outcomes.

## Author contributions

YZ: Writing – original draft. JC: Writing – review & editing. SZ: Writing – original draft. KK: Writing – review & editing. YL: Writing – review & editing.

## Acknowledgments

We would like to express our sincere thanks to all the authors who submitted their studies to the present Research Topic and the reviewers who evaluated the manuscripts. Their efforts help us to communicate current knowledge under this topic to a worldwide audience.



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RECEIVED 15 January 2024

ACCEPTED 26 April 2024

PUBLISHED 10 May 2024

## CITATION

Zhi S, Chen C, Huang H, Zhang Z, Zeng F and  
Zhang S (2024) Hypoxia-inducible factor in  
breast cancer: role and target for breast  
cancer treatment.  
*Front. Immunol.* 15:1370800.  
doi: 10.3389/fimmu.2024.1370800

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# Hypoxia-inducible factor in breast cancer: role and target for breast cancer treatment

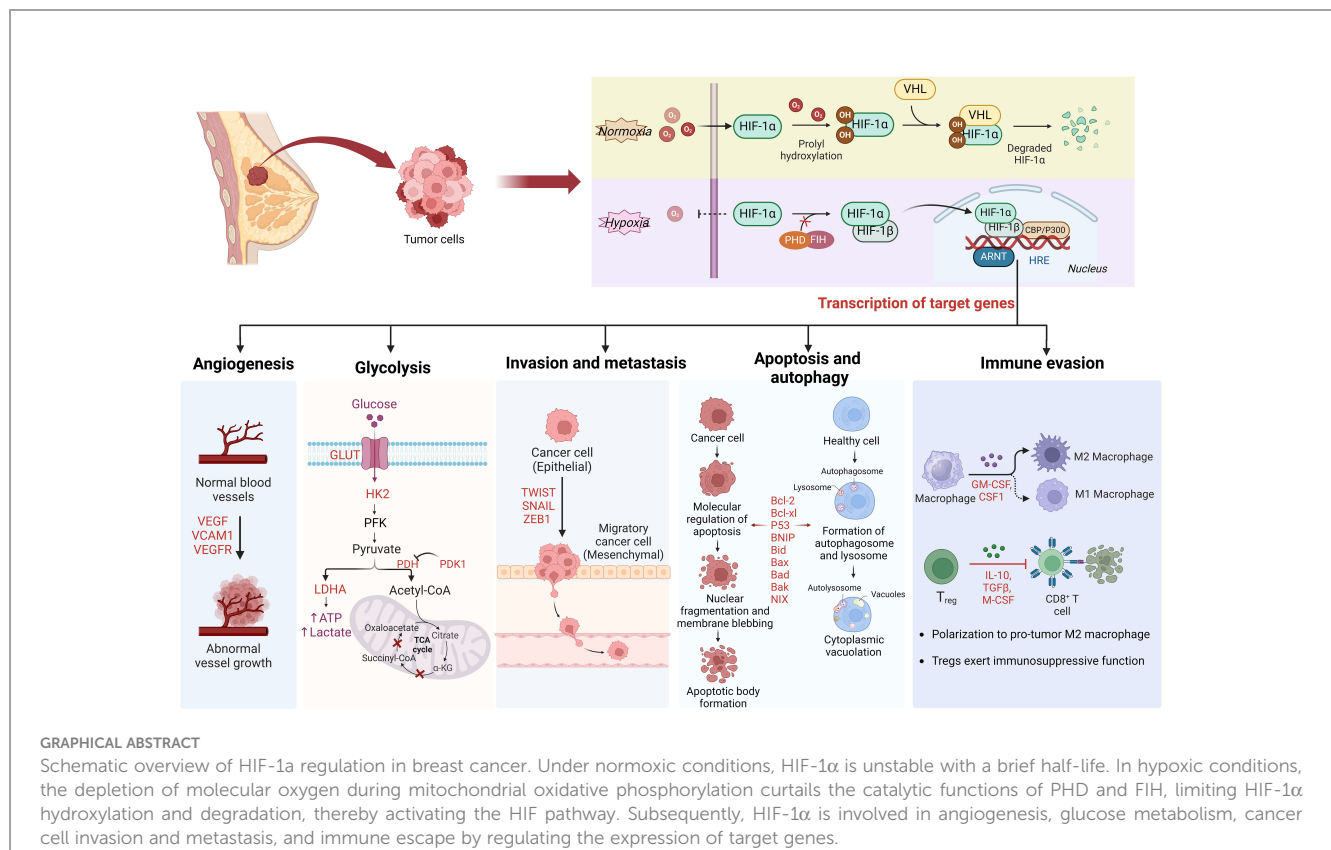
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Globally, breast cancer stands as the most prevalent form of cancer among women. The tumor microenvironment of breast cancer often exhibits hypoxia. Hypoxia-inducible factor 1- $\alpha$ , a transcription factor, is found to be overexpressed and activated in breast cancer, playing a pivotal role in the anoxic microenvironment by mediating a series of reactions. Hypoxia-inducible factor 1- $\alpha$  is involved in regulating downstream pathways and target genes, which are crucial in hypoxic conditions, including glycolysis, angiogenesis, and metastasis. These processes significantly contribute to breast cancer progression by managing cancer-related activities linked to tumor invasion, metastasis, immune evasion, and drug resistance, resulting in poor prognosis for patients. Consequently, there is a significant interest in Hypoxia-inducible factor 1- $\alpha$  as a potential target for cancer therapy. Presently, research on drugs targeting Hypoxia-inducible factor 1- $\alpha$  is predominantly in the preclinical phase, highlighting the need for an in-depth understanding of HIF-1 $\alpha$  and its regulatory pathway. It is anticipated that the future will see the introduction of effective HIF-1 $\alpha$  inhibitors into clinical trials, offering new hope for breast cancer patients. Therefore, this review focuses on the structure and function of HIF-1 $\alpha$ , its role in advancing breast cancer, and strategies to combat HIF-1 $\alpha$ -dependent drug resistance, underlining its therapeutic potential.

## KEYWORDS

breast cancer, hypoxia-inducible factor, angiogenesis, invasion and metastasis, apoptosis and autophagy, drug resistance



## 1 Introduction

Breast cancer (BC) is the most common type of malignancy among women and the second leading cause of cancer-related deaths among women after lung cancer (1–3). The treatment of breast cancer mainly includes surgery, endocrine therapy, chemotherapy, radiotherapy, and targeted therapy, depending on the classification of the tumor, with drug therapy playing an important role. In earlier years, the mortality rate of breast cancer patients has declined due to the reduced risk of the disease, improved treatment methods, and the widespread use of early screening (4). However, the emergence of drug resistance during treatment in recent years has posed a severe challenge to the survival of breast cancer patients (5). Hypoxia, caused by an imbalance between oxygen consumption and supply due to rapid tumor growth, is a common feature of the tumor microenvironment in most solid tumors (4, 6). It promotes tumor growth, metastasis, and treatment resistance by regulating the expression of hypoxia-related genes, ultimately leading to more aggressive and fatal cancers. Hypoxia is associated with enhanced invasive behavior and poorer prognosis and has been identified as a poor indicator of patient outcome (7). The hypoxia-inducible factor (HIF) family, which plays a pivotal role in the cellular response to hypoxic stress, consists of transcription factors that are crucial for managing hypoxic stress at the cellular level (8, 9). Research has consistently demonstrated that Hypoxia-inducible factor 1-alpha (HIF-1 $\alpha$ ) is overexpressed in numerous types of cancer, significantly influencing cancer progression (10–16). HIF-1 $\alpha$  is

responsible for activating genes associated with angiogenesis, cell growth and survival, invasion and metastasis, glucose metabolism, immune system evasion, and resistance to several cancer therapies. Specifically, HIF-1 $\alpha$  is involved in regulating tumor cell metabolism, apoptosis, and autophagy, thereby impacting their survival (8, 17–22). It represents a promising target for anticancer therapy. Therefore, this article provides a detailed description of the structure and function of HIF-1 $\alpha$  as well as its mechanism of action in the development of breast cancer. This review also summarizes the reasons for the emergence of HIF-1 $\alpha$ -dependent drug resistance and strategies to overcome it, providing systematic information for the development of targeted drugs against HIF-1 $\alpha$ .

## 2 Structure and function of HIF-1 $\alpha$

Hypoxia significantly influences numerous pathophysiological conditions in the human body (1). It is a defining characteristic of the solid tumor microenvironment (TME), resulting from rapid tumor growth and inadequate blood supply. An estimated 50%–60% of tumors exhibit anoxic regions (23). Hypoxia is linked to cancer spread and resistance to conventional therapies like chemotherapy and radiotherapy, indicating a grim prognosis for various cancers, including BC, hepatocellular carcinoma (HCC), and cancers of the pancreas, stomach, and colorectum (24). Therefore, targeting hypoxia is seen as a viable strategy in cancer treatment. Cells have evolved sophisticated mechanisms to adapt to anoxic conditions (25).

Within the hypoxic TME, HIF plays a crucial role in tumor adaptation (26). To date, mammals have been found to express three HIF types. HIF is a heterodimer consisting of an oxygen-sensitive  $\alpha$  subunit (HIF-1 $\alpha$ , HIF-2 $\alpha$ , and HIF-3 $\alpha$ ) and an oxygen-insensitive  $\beta$  subunit (HIF-1 $\beta$ ), crucial for regulating gene expression under hypoxic conditions (27–29). While oxygen levels regulate all three HIF  $\alpha$  subunits and they all bind to HIF-1 $\beta$ , research has primarily focused on HIF-1 $\alpha$  and HIF-2 $\alpha$  (29–34). HIF-1 $\alpha$  and HIF-2 $\alpha$  share structural similarities, yet their roles differ across tumor and cell types (29, 35, 36). HIF-3 $\alpha$  has been less studied due to its multiple variants and complex functionality (37, 38). Recent research indicates that specific HIF-1 $\alpha$  subtypes are present in several solid tumors, potentially contributing to tumor progression (1, 39).

HIF-1 consists of the HIF-1 $\alpha$  subunit, composed of 826 amino acids, and the HIF-1 $\beta$  subunit, which has 782 amino acids (Figure 1) (40, 41). HIF-1 $\alpha$  is the most extensively expressed subunit of HIF-1 in mammalian tissues (8). This transcription factor, encoded by the HIF-1 $\alpha$  gene on chromosome 14q2124, responds to hypoxic signals (42, 43). It is part of the basic helix-loop-helix (bHLH)/Period Clock Protein (Per)–Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT)–Single-minded Protein (Sim) (bHLH/PAS) family of transcription factors (44). The bHLH and PAS domains, named after the proteins Per, ARNT, and Sim (40) first identified in *Drosophila*, are essential for DNA binding and the formation of heterodimers between HIF-1 $\alpha$  and HIF-1 $\beta$ , respectively (45). HIF-1 $\alpha$  includes two transactivation domains (TAD): N-TAD and C-TAD, enriched with acidic and hydrophobic amino acids, linked by an inhibitory domain (ID) (42). C-TAD plays a role in HIF-1 $\alpha$  transcription regulation through interaction with the transcriptional coactivator CREB-binding protein (CBP)/p300 in hypoxic conditions, while N-TAD serves as its stable regulator (46, 47). The ID, situated between the two TAD sequences (amino acids 576–785), prevents transcriptional activation by TADs (48). Furthermore, HIF-1 $\alpha$  features an oxygen-dependent degradation domain (ODDD) upstream of the N-TAD region, including two hydroxylation sites, Pro-402 and Pro-564, each bearing a conserved LXXLAP motif (49). This ODDD, located centrally in HIF-1 $\alpha$ , chiefly mediates the protein's oxygen-regulated stability and degradation through the ubiquitin-proteasome pathway (50, 51). HIF-1 $\alpha$  also possesses two nuclear localization signals (NLS), NLSN (N-terminal, 17–33 amino acids) and NLSC (C-terminal, 718–721 amino acids) (45). HIF-1 $\beta$ , also known as ARNT, is expressed constitutively in all cell types and its expression is not influenced by oxygen levels (52). It includes three domains: bHLH, PAS, and C-TAD, but lacks the ODDD and N-TAD domains (45, 53).

Within cells, the regulation of HIF-1 $\alpha$  is stringently dependent on oxygen availability, in contrast to HIF-1 $\beta$ , which is constantly expressed irrespective of oxygen tension (40, 51). Under normoxic conditions, HIF-1 $\alpha$  is unstable with a brief half-life (Figures 1, 2) (40). Pro-402 and Pro-564 in the ODDD and Asn-803 in the C-TAD are hydroxylated by prolyl hydroxylases (PHD) and factor-inhibiting HIF (FIH) using Fe<sup>2+</sup> and 2-oxoglutaric acid as cofactors (40, 51). Hydroxylation at Asn-803 inhibits CBP/p300 binding to HIF-1 $\alpha$ , while hydroxylation at proline residues allows the von

Hippel-Lindau tumor suppressor protein (pVHL), with E3 ubiquitin ligase activity, to recognize hydroxylated HIF-1 $\alpha$  (42, 54). Consequently, HIF-1 $\alpha$  is ubiquitinated and swiftly degraded through the pVHL-mediated ubiquitin-proteasome pathway. The second regulation mechanism involves the hydroxylation of asparaginyl residues by FIH (Figures 1, 2) (55), preventing the association of HIF coactivators (CBP/p300) (55, 56). This asparaginyl hydroxylation occurs in the C-TAD domain at N803 in HIF-1 $\alpha$  and N851 in HIF-2 $\alpha$  (57). Thus, under normoxic conditions, PHD and FIH facilitate HIF degradation through a dual mechanism, suppressing HIF transcriptional activity (58). In hypoxic conditions, the depletion of molecular oxygen during mitochondrial oxidative phosphorylation curtails the catalytic functions of PHD and FIH (Figure 2) (57, 59), limiting HIF-1 $\alpha$  hydroxylation and degradation, thereby activating the HIF pathway (60, 61). The stable HIF-1 $\alpha$  then moves to the nucleus and forms a transcriptionally active heterodimer with HIF-1 $\beta$  (29). The interaction occurs between the heterodimeric complex of HIF-1 $\alpha$ /HIF-1 $\beta$  and CBP/p300, the steroid receptor coactivator-1 family of coactivators, the nuclear redox regulator Ref-1, and the molecular chaperone heat shock protein 90. This interaction facilitates the binding of hypoxia response elements (HRE), leading to increased transcriptional activity of target genes across various signaling pathways and the regulation of cellular adaptive responses to hypoxia (Table 1) (29, 78, 79). However, the degradation of accumulated HIF-1 $\alpha$  happens rapidly upon reoxygenation of hypoxic cells, with the rate of degradation being dependent on the duration of hypoxic stress (80).

## 3 The role of HIF-1 $\alpha$ in breast cancer

### 3.1 Angiogenesis

BC cells necessitate a continual blood supply for oxygen and essential nutrients (81). Initially, during tumor growth, nutrients and oxygen are obtained via diffusion (82). However, as the tumor mass reaches a certain size, diffusion becomes insufficient to sustain growth, prompting the formation of new vasculature to support tumor growth and metastasis (81, 83). Thus, angiogenesis is critical for tumor advancement, proliferation, and metastasis. Tumor blood vessels exhibit high tortuosity, increased vascular permeability, and sluggish blood flow (62) compared to normal vessels, leading to increased local hypoxia, which in turn stabilizes HIF-1 $\alpha$ , fostering tumor invasion and metastasis (84–87).

HIF plays a pivotal role in regulating angiogenesis (Figure 3) (88, 89). BC cells exhibit elevated levels of HIF, stimulating gene expression that facilitates proliferation, metastasis, angiogenesis, and invasion (90, 91). HIF-1 $\alpha$  is notably expressed in precursor lesions and early stages of BC (92). Suppression of the HIF-1 $\alpha$  gene or inhibition of its transcription can hinder tumor cells from secreting vascular endothelial growth factor (VEGF) and impede tumor neovascularization (8, 93, 94).

HIF-1 $\alpha$  shows increased expression particularly in triple-negative BC (TNBC). The nuclear factor kappa B (NF- $\kappa$ B) signaling pathway is activated in TNBC, promoting tumor cell

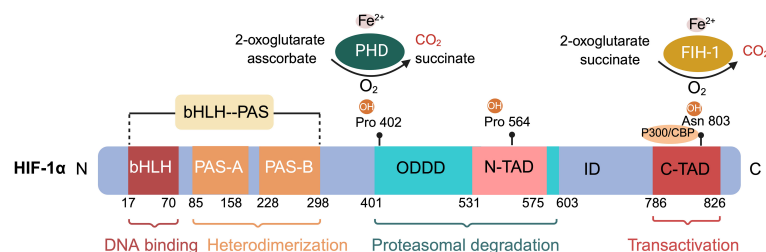


FIGURE 1

Schematic of hypoxia-inducible factor HIF-1 $\alpha$  protein structure and hydroxylation sites at proline and asparagine residues. The basic submotif and the helix-loop-helix domain (bHLH) are located near the N terminus, followed by the Per-ARNT-Sim (PAS) domain. The PAS domain comprises repetitive amino acid sequences PAS-A and PAS-B. The oxygen-dependent degradation domain (ODDD) overlaps with the N-terminal transactivation domain (N-TAD), followed by the C-terminal transactivation domain (C-TAD). Hydroxylation of proline residues within the ODDD and of asparagine residues within the C-TAD of HIF-1 $\alpha$  are highlighted. The non-equilibrium hydroxylation by the prolylhydroxylases (PHD) and the asparagine hydroxylase factor inhibiting HIF-1 including substrates and products is depicted exemplarily for two of the three hydroxylation sites of HIF-1 $\alpha$ .

proliferation, angiogenesis, and evasion of apoptosis (Figure 3) (95). Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) or interleukin (IL)-1 activates inhibitory kappa B kinase (IKKB), leading to NF- $\kappa$ B activation (96). Additionally, under chronic hypoxia in TNBC cell lines, reactive oxygen species (ROS) further activate NF- $\kappa$ B by degrading inhibitor of  $\kappa$ B- $\alpha$  (I $\kappa$ B $\alpha$ ) (97). The NF- $\kappa$ B pathway boosts HIF-1 $\alpha$  mRNA expression by enhancing its transcription (98, 99). TNF- $\alpha$  may elevate HIF-1 $\alpha$  expression by activating NF- $\kappa$ B signaling in TNBC cells and could be influenced by IL-17 (100).

In BC, HIF-1 $\alpha$  primarily induces angiogenesis by regulating the expression of VEGF, hepatocyte growth factor (HGF), vascular cell adhesion molecule 1 (VCAM1), and VEGF receptor (VEGFR) (Figure 3) (62, 101). VEGF, a critical downstream target of the HIF pathway, drives angiogenesis by influencing endothelial cell migration, proliferation, permeability, and survival (102–105).

Under hypoxic conditions, HIF-1 $\alpha$  binds to the VEGF promoter, significantly increasing VEGF mRNA levels in TNBC compared to other BC subtypes (106). HIF-1 $\alpha$  also upregulates breast tumor kinase mRNA and protein expression, which stimulates angiogenesis via hepatocyte growth factor (107). Furthermore, HIF-1 $\alpha$  induces overexpression of C1q binding protein (C1QBP), indirectly stimulating the NF- $\kappa$ B signaling cascade to upregulate VCAM1 expression and promote TNBC angiogenesis (74). Short-term hyperoxia induces ROS formation, leading to increased brain-derived neurotrophic factor expression and VEGFR receptor upregulation through HIF-1 $\alpha$ , promoting angiogenesis (108).

Although HIF-1 $\alpha$  predominates in BC, the HIF-2 $\alpha$  isoform is equally crucial as a key regulator of pathophysiological angiogenesis (109). *RAB11B-AS1*, a long noncoding RNA (lncRNA), enhances VEGFA and angiopoietin-like 4 (ANGPTL4) expression in hypoxic

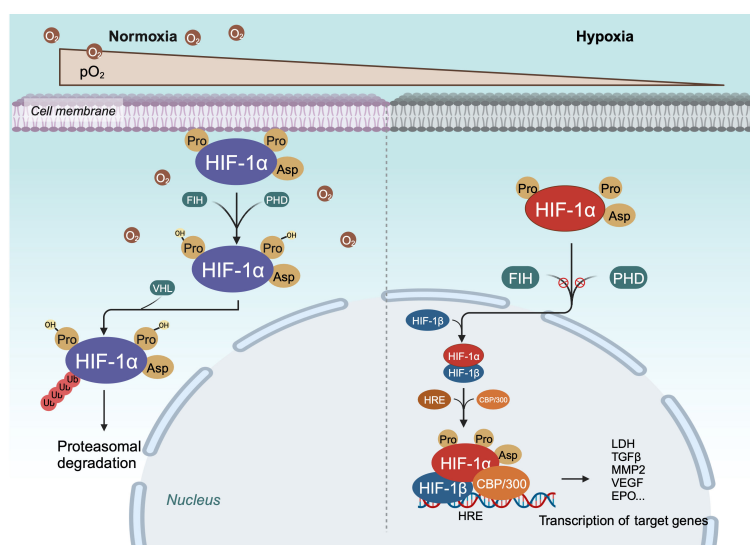


FIGURE 2

Schema of regulation of HIF- $\alpha$  degradation and transcriptional activity. Under normoxic conditions, HIF-1 $\alpha$  is continuously degraded through the key oxygen sensor PHD, which enables HIF-1 $\alpha$  to bind to VHL. Under hypoxic conditions, the hydroxylation of HIF-1 $\alpha$  is inhibited, leading to stabilization of HIF-1 $\alpha$ . Next, HIF-1 $\alpha$  dimerizes with HIF-1 $\beta$  to form a transcriptional activation complex, which binds to HRE and stimulates the transactivation of target genes.



TABLE 1 Target genes of the HIF-1α and their function.

| Target Gene | Expression under hypoxia | Function                                                                | Reference |
|-------------|--------------------------|-------------------------------------------------------------------------|-----------|
| GLUT1       | Up                       | Glycolysis                                                              | (62)      |
| LDHA        | Up                       | Glycolysis                                                              | (62)      |
| PKM2        | Up                       | Glycolysis                                                              | (62)      |
| LOX         | Up                       | Invasion                                                                | (63)      |
| SOD2        | Up                       | Enrichment of braest cancer stem cell                                   | (64)      |
| A2BR        | Up                       | Invasion, and metastasis                                                | (65)      |
| MDR1        | Up                       | Enrichment of braest cancer stem cell                                   | (62)      |
| VEGF        | Up                       | Angiogenesis                                                            | (66)      |
| Bcl-2       | Up                       | Apoptosis                                                               | (67)      |
| GLUT        | Up                       | Glycolysis                                                              | (68)      |
| NANOG       | Up                       | Invasion                                                                | (69)      |
| PDK 1       | Up                       | Glycolysis                                                              | (68)      |
| IL-6        | Up                       | Invasion                                                                | (70)      |
| IL-8        | Up                       | Invasion                                                                | (71)      |
| MMPs        | Up                       | Invasion                                                                | (71)      |
| NF-κB,      | Up                       | Invasion                                                                | (72)      |
| HK2         | Up                       | Glycolysis                                                              | (73)      |
| C1QBP       | Up                       | Angiogenesis                                                            | (74)      |
| P4HA2       | Up                       | Invasion and metastasis.                                                | (75)      |
| SLUG        | Up                       | Plays a key role in the control of epithelial to mesenchymal transition | (76)      |
| TWIST       | Up                       | Plays a key role in the control of epithelial to mesenchymal transition | (77)      |

BC cells in a HIF-2α-dependent manner, promoting tumor angiogenesis and metastasis (110). In conclusion, angiogenesis is a multifaceted process, and HIF-1α significantly contributes to angiogenesis in BC (62, 111).

3.2 Glucose metabolism

Enhanced glucose metabolism plays a vital role in the growth and division of cancer cells, which require significant amounts of biomass and alternate energy sources to offset the diminished oxidative phosphorylation. The activity of HIF is believed to be intimately associated with glucose metabolism. Genes activated by HIF transcription include crucial components of glucose metabolism, such as glucose transporters (GLUT1 and GLUT3), enzymes involved in the glycolytic pathway (hexokinase, phosphofructokinase, aldolase, glyceraldehyde 3-phosphate dehydrogenase,

phosphoglycerate kinase, enolase, pyruvate kinase, and lactate dehydrogenase [LDH]), and pyruvate dehydrogenase (PDH) kinase (PDK) (Figure 4) (28, 55, 112, 113). Du et al. investigated the potential roles and mechanisms of the hypoxic *MIR210HG* axis, finding that *MIR210HG* increases HIF-1α protein levels by directly interacting with the 5'-UTR of HIF1α mRNA. This elevation in HIF-1α protein enhances the expression of enzymes related to glycolysis (pyruvate kinase M2, LDHA) and GLUT1 (114). In hypoxic conditions, HIF-1α facilitates the shift of tumor cells from oxidative to glycolytic metabolism by triggering genes that encode glucose transporters and glycolytic enzymes (115). In particular, the overexpression of HIF-1α in glucose augments glucose absorption in tumor cells through the elevation of glucose transporter levels. The modification of proteins by O-linked β-N-acetylglucosamine (O-GlcNAc) (O-GlcNAcylation) influences glycolysis in BC cells via the HIF-1α/GLUT1 signaling pathway (81, 116). Glucose is processed through the glycolytic pathway in tumor cells, generating substantial amounts of pyruvate (117). Hexokinase 2 (HK2) serves as both the initial and rate-limiting enzyme in the glycolysis pathway (81). CircRNF20 is found to accelerate tumor progression by targeting miR-487a/HIF-1α/HK2 in BC (73). HIF-1α enhances PDK activity and blocks the transformation of pyruvate to acetyl-CoA by inhibiting PDH; thus, reducing the entry of pyruvate into the tricarboxylic acid cycle (55, 118). LDHA converts pyruvate into lactic acid, which is then transported out of the cell by the monocarboxylate transporter (MCT) (62). As cancer cell metabolism shifts to aerobic glycolysis, lactate supplants pyruvate and is expelled into the tumor microenvironment (TME), thereby fostering an immunosuppressive milieu that supports tumor cell proliferation, metastasis, and invasion (119, 120).

Glycolysis can lead to an increase in HIF-1α levels, which in turn raises VEGF expression (121). Aerobic glycolysis stimulates angiogenesis by producing lactate, which acidifies the extracellular environment and enhances VEGF expression (122). Additionally, the end products of glycolysis, lactate and pyruvate, influence VEGF expression through the augmentation of HIF-1α levels (123–125). Hence, it is postulated that the transition to glycolysis precedes angiogenesis. In summary, HIF-1α plays a crucial role in regulating glucose metabolism in BC.

3.3 Invasion and metastasis

Invasion and metastasis involve the spread of cancer cells from the primary tumor site to distant organs, leading to the formation of secondary tumors (81). Early estimates indicate that nearly two-thirds of cancer-related deaths and three-quarters of BC-related deaths result from metastasis (126). EMT, a process in which epithelial cells convert into mesenchymal cells through specific mechanisms, is a key aspect of tumor metastasis (127, 128). Cancer cells that undergo EMT exhibit enhanced invasive capabilities and resistance to apoptosis. Often, EMT is induced by hypoxia, with HIF-1α overexpression linked to various molecules and pathways (Figure 5) (76, 129–132). Research has shown that hypoxia-induced HIF-1α expression leads to the activation of major transcription factors such as TWIST, Snail, Slug, SIP1, STAT3, and ZEB. This activation results in E-cadherin suppression and

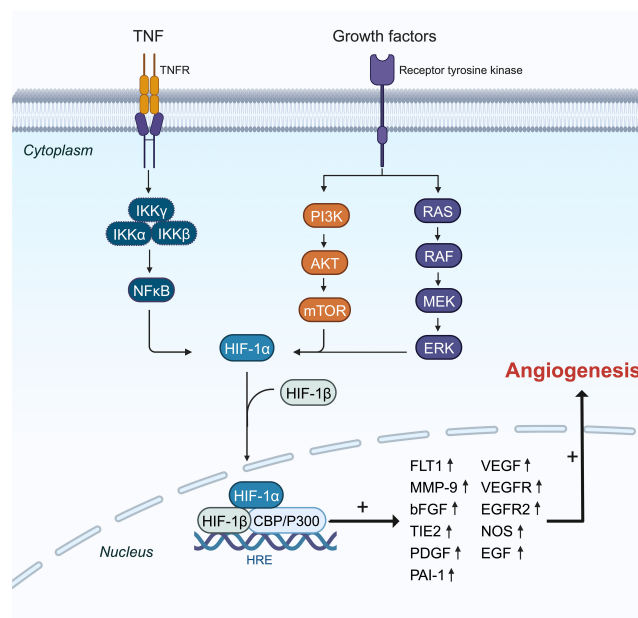


FIGURE 3

The mechanisms and pathways of HIF-1 $\alpha$  overexpression effects on the induction of angiogenesis genes. HIF-1 $\alpha$  translation is amplified by PI3K, RAS, and NF- $\kappa$ B pathways in the cytoplasm, and then it can pass into the nucleus with coactivator (P300) as a transcription factor and enhances the expression of some essential angiogenesis genes like FLT1, MMP9, VEGF, VEGFR, and PAI-1 in the angiogenesis process.

vimentin induction in BC, with HIF-1 $\alpha$  inhibition markedly increasing E-cadherin levels (133). While E-cadherin encourages collective migration of mixed E/M phenotypes by inhibiting TGF- $\beta$ , TGF- $\beta$  activation promotes single-cell migration (134). CSF-1, a regulator of EMT, is influenced by hypoxia. HIF-1 $\alpha$  induces a mixed E/M phenotype via its target gene CSF-1, facilitating

collective migration (135). Additionally, hypoxia has been shown to increase Slug and Snail expression and decrease E-cadherin levels during HIF1-induced EMT through the Notch pathway (136). HIF-1 $\alpha$  activates matrix metalloproteinases 1, 2, 9, and 14, aiding in the breakdown of extracellular matrix components and basement membrane degradation, thereby easing cancer cell migration and

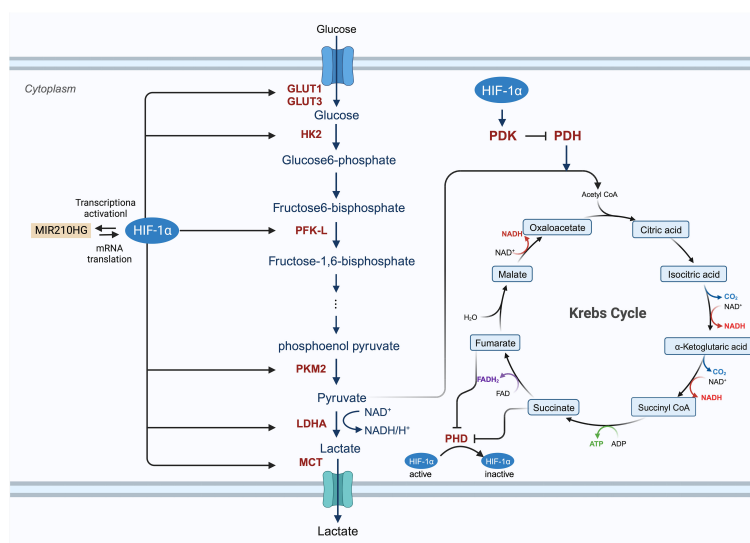


FIGURE 4

The involved procedure of HIF-1 $\alpha$  in cancer glucose metabolism. HIF-1 enhances the expression of glucose transporters GLUT1 and GLUT3 and activates glycolytic enzymes, including hexokinase 2 (HK2), phosphofructokinase (PFK-L), pyruvate kinase isozymes M2 (PKM2) to generate an increasing amount of pyruvate. After this process, pyruvate is largely converted to lactate by lactate dehydrogenase A (LDHA) and removed from the cancer cell by monocarboxylate transporter (MCT). HIF-1 also inhibits the TCA cycle and oxidative phosphorylation process by activating the expression of HIF-1-dependent pyruvate dehydrogenase kinase (PDK), resulting in the decrease of mitochondrial activities and the oxygen consumption in hypoxia.



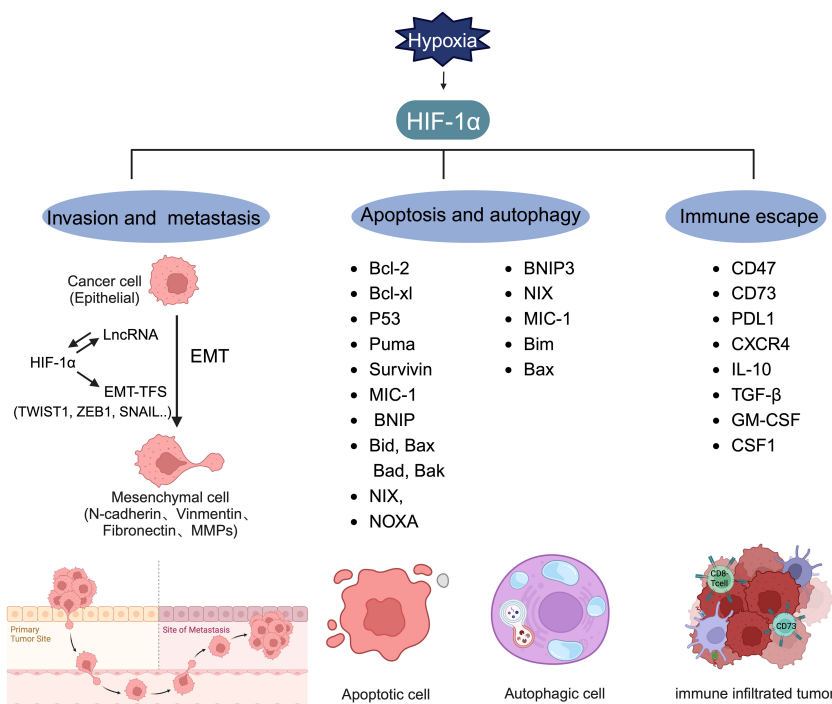


FIGURE 5

Target genes regulated by HIF-1 $\alpha$ . HIF-1 $\alpha$  can regulate the expression level of lncRNA and form a mutual activation pathway with lncRNA, thereby promoting the production of EMT-TFs and promoting the process of tumor EMT. HIF-1 $\alpha$  plays a key role in inducing the transcription of genes involved in invasion, metastasis, apoptosis, autophagy, and immune escape.

spread (75, 113, 130, 137, 138). Chemokine receptors 4 (CXCR4) and 3 (CXCR3), associated with invasion, angiogenesis, metastasis, and prognosis, are upregulated by HIF-1-dependent expression, enhancing cell migration and survival during cycling (139, 140). HIF signaling impacts cell extravasation by modulating genes encoding L1 cell adhesion molecules and ANGPTL4, which reduces endothelial cell adhesion (141). He et al. (2020) showed that hypoxia-induced HIF-1 $\alpha$  regulates BC cell migration and EMT through the MiR3383p/ZEB2 axis (142). Moon et al. identified MRPL52 as a transcriptional target of HIF-1 $\alpha$ , with MRPL52 promoting EMT, migration, and invasion in hypoxic BC cells via the ROS-Notch1-Snail pathway (143).

Recent research indicates that HIF-dependent lncRNAs may contribute to the metastatic phenotype of BC cells. Under hypoxia, lncRNA BCRT1, regulated by HIF-1 $\alpha$  transcriptionally, promotes EMT (144). Liu et al. identified HIF-1 $\alpha$  as a potential transcription factor for lncRNA HLA complex group 18 (HCG18), with a positive correlation between HCG18 and HIF-1 $\alpha$  expression in BC tissue. Knockdown of HIF-1 $\alpha$  reduced HCG18 levels in BC cells, and HIF-1 $\alpha$  binding to specific HREs in the HCG18 promoter stimulates HCG18 expression. *In vivo* assays showed that decreasing HCG18 expression in MDA-MB-231 cells curbed tumor growth and lung metastasis in xenograft mouse models, highlighting HIF1 $\alpha$ 's role as a critical regulator of hypoxia-induced EMT and metastasis (145). Metastasis poses a significant prognostic challenge in BC, and targeting HIF-1 $\alpha$  to inhibit BC metastasis presents a viable strategy.

### 3.4 Apoptosis and autophagy

Apoptosis, a genetically controlled form of cell death, is crucial for normal cellular regulation. Cancer cells, however, often evade apoptosis, contributing to chemotherapy resistance or tumor relapse. This evasion involves a complex interplay of proteins and cytokines. Research indicates that HIF-1 $\alpha$  exhibits a dual role in apoptosis, capable of both inducing and counteracting it (1). HIF-1 $\alpha$  can induce and antagonize apoptosis (Figure 5). The proapoptotic effects of HIF-1 $\alpha$  involve the regulation of genes such as BNIP, Bid, Bax, Bak, Bad, BNIP3, NIX, and NOXA (146), whereas its antiapoptotic effects are seen in the modulation of Bcl-2, Bcl-xL, and myeloid cell leukemia (Mcl-1) expression (1). In MDA-MB-231 cells treated with paclitaxel, a HIF-1 $\alpha$ -dependent alteration in the expression of various pro- and antiapoptotic genes was observed. Under hypoxic conditions, compared to normoxic conditions with paclitaxel, a reduction in proapoptotic gene expression (BAK1, CASP3, CASP8, CASP10, and TNFRSF10A) was noted (147).

HIFs also play a significant role in autophagy, another programmed cell death mechanism (Figure 5). In MCF7 cells subjected to radiation, HIF-1 $\alpha$  induces autophagy by inhibiting the PI3K/AKT/mTOR/p70 pathway and increases the expression of Mcl-1 and BNIP-3 (148). Mcl-1 participates in the neutralization of proapoptotic proteins, inhibiting cytochrome c release from mitochondria (149), while BNIP-3, a mitochondrial protein in the Bcl-2 family, triggers selective mitophagy by releasing Beclin-1 to

initiate autophagy (150, 151). The interplay between autophagy and apoptosis is crucial, especially for inducing cell death in antiapoptotic BC cell lines (152).

### 3.5 HIF-1 $\alpha$ in cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) interact with tumor cells to promote tumor cell growth and metastasis. CAFs, mainly normal interstitial fibroblasts (NFs), are the most abundant cell type in the stroma of breast cancer (153, 154). In breast cancer tumors, only a small fraction of fibroblasts are quiescent; these fibroblasts are responsible for the structural integrity of the extracellular matrix (ECM) and its nutrient supply and contribute to wound healing (155). However, most fibroblasts exhibit an activated phenotype characterized by producing various extracellular matrix components and paracrine mediators (156). Among the many mechanisms involved in transforming NFs to CAFs, local hypoxia has been proven to drive the differentiation of NFs into activated myofibroblasts by triggering the formation of reactive oxygen species (ROS) (157, 158). Additionally, CAF activation is reversible: chronic hypoxia inactivates CAFs, leading to the loss of contractility, reduction of surrounding extracellular matrix remodeling, and ultimately damage CAF-mediated cancer cell invasion (159). Studies have shown that hypoxia-dependent deletion of PHD2 suppresses tumor growth and reduces the metastatic activity of CAFs (160, 161). Hypoxia inhibited prolyl hydroxylase domain protein 2 (PHD2), resulting in the stabilization of HIF-1 $\alpha$ , reduced expression of  $\alpha$ SMA and periostin, and decreased myosin II activity. Treatment with the PHD inhibitor DMOG in an orthotopic breast cancer model significantly reduced spontaneous metastasis to the lungs and liver, which correlated with decreased tumor stiffness and fibroblast activation (159). Another study revealed that the loss of PHD2 is associated with normalization of the vasculature, reduced CAF activation, and decreased intravascular invasion of metastases (161). These findings suggest that blocking PHD2 in CAFs may be a novel strategy for inhibiting prometastatic signals in the breast cancer tumor microenvironment.

In fact, CAFs have been shown to regulate metabolic interdependency between cancer cells and their surrounding microenvironment through the action of HIF (162). HIF-1 $\alpha$  is involved in regulating biventricular metabolic symbiosis between synthetic metabolic cancer cells and catabolic stromal fibroblasts (163). The enhanced glycolytic rate shown by CAFs is partially dependent on HIF signaling (164). Furthermore, the high-energy metabolic byproducts produced by catabolic CAFs are taken up by tumor cells to support their high anabolic demands. The increased metabolic flux in cancer cells generates ROS, which then propagate throughout the tumor microenvironment and through CAFs to promote HIF-dependent metabolic reprogramming (165).

### 3.6 Immune escape

Tumor immune escape, where cancer cells avoid detection and destruction by the host immune system (166), is facilitated under

hypoxic conditions through HIF-1 $\alpha$  overexpression (Figure 5) (167). HIFs increase CD47 immunoglobulin expression and hinder T cell proliferation and activation by attracting myeloid-derived suppressor cells (28). Changes in the expression of CD47, CD73, and PDL1 in TNBC cells treated with chemotherapy agents like carboplatin, doxorubicin, gemcitabine, or paclitaxel enhance cancer cells' ability to evade both innate and adaptive immune responses (168). Increased PDL1 mRNA expression in human TNBC cell lines was linked to elevated HIF-1 $\alpha$  expression due to endoplasmic reticulum oxidoreductase 1- $\alpha$  (ERO1- $\alpha$ ), with ERO1- $\alpha$  knockdown significantly reducing PDL1-mediated T-cell apoptosis, suggesting avenues for therapeutic intervention in hypoxia-mediated immune resistance (169).

Regulatory T cells (Tregs), which suppress immune responses through cytokines and metabolites, play a role in tumor development (170–173). In TNBC, HIF-1 $\alpha$  modulates Tregs' immunosuppressive functions and aggregation by regulating forkhead box P3 (FoxP3) and C-X-C motif CXCR4. HIF-1 $\alpha$  enhances FoxP3 expression by binding to HREs and indirectly increases CXCR4 expression; thus, supporting immunosuppression (174). Moreover, M2 macrophages, known for their immunosuppressive capabilities through IL-10 and TGF- $\beta$  release, are influenced by HIF-1 $\alpha$  (175). This factor drives the polarization of tumor-associated macrophages (TAMs) towards an M2 phenotype, creating an immunosuppressive microenvironment. It has been shown that TAMs in TNBC are more likely to adopt an M2 phenotype compared to other BC subtypes (176, 177). HIF-1 $\alpha$ , present in BRCA1-IRIS overexpressing TNBC cells, secretes high levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), recruiting and polarizing macrophages towards an M2 phenotype; thus, facilitating immune escape (178, 179). HIF-1 $\alpha$ 's ability to polarize TAM to M2 by regulating GM-CSF and macrophage CSF-1 in TNBC underscores its role in promoting immune evasion. HIF can also lead to extracellular acidification by regulating the expression of MCT4, which not only diminishes immune response efficiency but also impacts the efficacy of anticancer drugs (180).

### 3.7 Noncoding RNA regulates HIF-1 $\alpha$

Based on their genomic locations, long noncoding RNAs (lncRNAs) are categorized into five types: antisense, sense, intergenic, intronic, or bidirectional. The location of lncRNAs can be specific to the nucleus, the cytoplasm, or both (25, 181). The expression of lncRNAs is regulated similarly to that of protein-coding RNAs, through mechanisms such as epigenetic modification, gene transcription, and post-transcriptional regulation (182). In tumors, lncRNAs are often abnormally expressed and play roles in the regulation of tumor proliferation, invasion, metastasis, metabolism, angiogenesis, and survival (183, 184). Certain noncoding RNAs can influence tumor-related biological processes by regulating HIF-1 $\alpha$  (Figure 6) (25, 185, 186). For instance, LINC00649 enhances the stability of HIF-1 $\alpha$  mRNA and protein expression by interacting with the nuclear factor 90 (NF90)/NF45 complex (187). MIR210HG specifically impacts triple-negative breast cancer (TNBC) by regulating HIF-1 $\alpha$  at the translation level, thereby increasing HIF-1 $\alpha$  protein expression and influencing the expression of glycolysis genes (114).

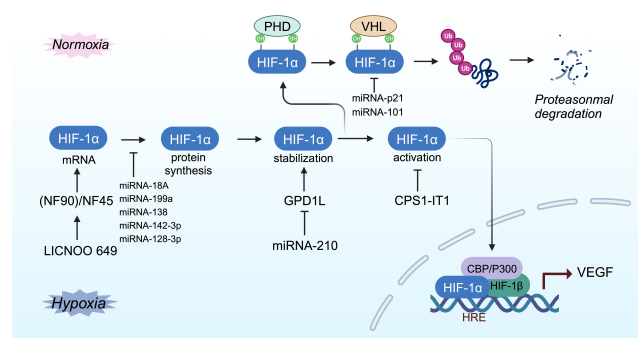


FIGURE 6  
Mechanisms of hypoxia-responsive ncRNA-mediated modulation of HIF-1 $\alpha$  activity.

MicroRNAs (miRNAs) exert a dual regulatory effect on HIF-1 $\alpha$ , both promoting and inhibiting its expression. Saatici et al. identified eight common miRNAs that target HIF-1 $\alpha$ , lysyl oxidase (LOX), and ITGA5, with miR-142-3p and miR-128-3p showing negative correlation with the expression of HIF-1 $\alpha$ , LOX, and ITGA5. Hypoxia can inhibit miR-142-3p, leading to increased HIF-1 $\alpha$  mRNA and protein expression, activating the FAK/Src signaling pathway, and triggering chemotherapy resistance in TNBC (188). However, the regulatory mechanism of miR-142-3p on HIF-1 $\alpha$  remains unclear. Overexpression of miR-101 decreases VHL levels, stabilizing HIF-1 $\alpha$  and inducing VEGFA expression, ultimately enhancing TNBC invasiveness (189). Thus, noncoding RNA regulates HIF-1 $\alpha$  expression in TNBC by maintaining the stability of the HIF-1 $\alpha$  protein and regulating the stability and translation level of HIF-1 $\alpha$  mRNA.

### 3.8 HIF-1 $\alpha$ and drug resistance of breast cancer

In BC treatment options vary by subtype and include surgery, endocrine therapy, chemotherapy, radiotherapy, and targeted therapy, with drug therapy playing a crucial role depending on the tumor classification. However, drug resistance presents a significant challenge in BC treatment (190–192), often due to inherent or acquired resistance over time (193). Hypoxia is a common characteristic of both primary and metastatic BC (194), with HIF-1 $\alpha$  expression in tumor tissues associated with poor prognosis and drug resistance (Figure 7) (132). Research indicates that HIF-1 $\alpha$  may contribute to resistance against conventional therapies via various signaling pathways, including drug efflux, tumor stem cell enrichment, autophagy, and apoptosis (192, 195), necessitating further investigation into HIF-1 $\alpha$ -induced drug resistance mechanisms in BC.

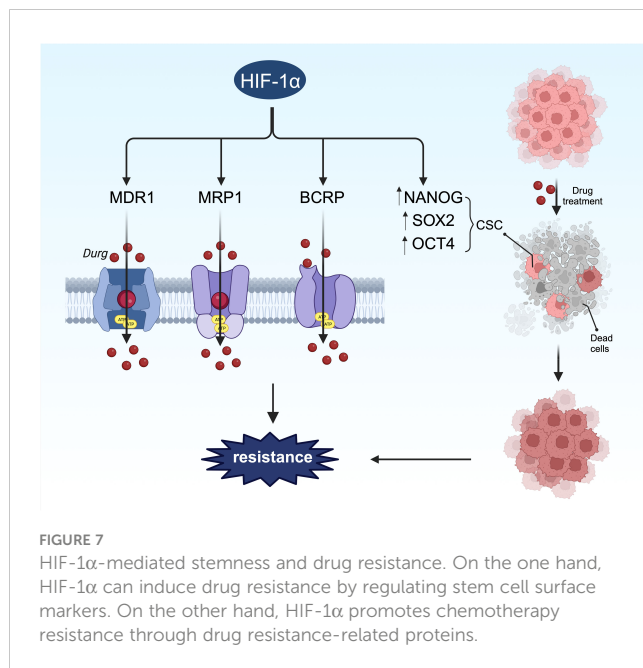
### 3.9 Increased expression of drug outflow pump

Drug efflux transporters such as MDR1, multidrug resistance-associated protein 1 (MRP1), and breast cancer resistance protein

(BCRP) are directly regulated by HIF-1 $\alpha$  (Figure 7), encoded by the ATP-binding cassette (ABC) transporters ABCB1, ABCC1, and ABCG2, respectively. Their promoters contain HREs sensitive to HIF-1 $\alpha$  transcriptional regulation (196). The link between these transporters and drug resistance in BC has been extensively studied (197–199), with all three proteins inducing drug resistance by facilitating drug efflux from tumor cells. Studies have shown that hypoxia-induced MDR1 expression can be significantly reduced by inhibiting HIF-1 expression with antisense oligonucleotides (200). In a separate investigation, 41% of BC tumors exhibited increased levels of MDR1, leading to a threefold higher likelihood of chemotherapy failure (201). Taxanes and anthracyclines represent the primary chemotherapy agents for BC treatment. The upregulation of MDR1, also referred to as P-glycoprotein or Pgp, contributes to resistance against taxanes and anthracyclines (202). The activation of HIF-1 $\alpha$  enhances the resistance of BC cells to these drugs (147, 203, 204). The genes ABCC1 and ABCG2, which encode MRP1 and BCRP, respectively, possess hypoxia response elements (HREs) upstream of their coding sequences. The deletion of these elements can prevent hypoxia-induced activation (196, 205). MRP1 is elevated in cells with activated HIF-1 $\alpha$ , and this effect can be reversed by HIF-1 $\alpha$  siRNA, indicating that ABCC1 is a direct target of HIF-1 $\alpha$ . Another investigation discovered that HIF-1 $\alpha$  binds to the HRE region of the BCRP promoter in LTLTCa cells, with significantly increased binding observed in the presence of CoCl<sub>2</sub> (206). The expression of BCRP correlated with the degree of drug resistance to irinotecan and topotecan (207). Hence, it is proposed that the expression and stability of HIF-1 $\alpha$  can enhance the mRNA and protein levels of MDR1, MRP1, and BCRP, thereby contributing to HIF-1 $\alpha$ -mediated drug resistance.

### 3.10 Upregulation of autophagy

Autophagy has been shown to increase resistance to endocrine and cytotoxic drugs in BC (208–210). HIF-1 $\alpha$  can activate various molecular mechanisms, such as inducing PTP-PEST expression and activating AMPK, which in turn increases BNIP3 expression. This disrupts the interaction between Beclin-1 and Bcl-2 and induces



autophagy, thereby increasing BC cells' resistance to drugs like adriamycin (211, 212). Additionally, endocrine therapies, including tamoxifen and flurvist, have been found to increase autophagy markers in drug-resistant BC cells (213, 214). Thus, HIF-1 $\alpha$  plays a role in inducing BC resistance to both endocrine and cytotoxic drugs by upregulating autophagy.

### 3.11 Inhibition of apoptosis

Apoptosis, leading to cell death, is a crucial outcome of most cancer treatments. Cancer cells, however, have developed mechanisms to evade apoptosis, contributing to resistance to chemotherapy or recurrence of tumors (215). This evasion involves various proteins and cytokines, including the Bcl-2 family, apoptosis inhibitor proteins, and the caspase family, along with cytochrome c and proteases (146). HIF-1 $\alpha$  plays a direct role in the regulation of apoptosis, exhibiting both proapoptotic and antiapoptotic effects (146). The proapoptotic actions of HIF-1 $\alpha$  include the downregulation of proapoptotic members of the Bcl-2 family, such as BNIP3, NIX, and NOXA, while its antiapoptotic effects involve increasing the levels of antiapoptotic proteins like Bcl-2, Bcl-xL, and Mcl-1, and decreasing the levels of proapoptotic proteins such as Bid, Bax, and Bak (216–218). In breast cancer (BC) cells, alterations in apoptotic activity following HIF-1 $\alpha$  activation are associated with increased drug resistance (147, 219, 220), though the precise mechanisms warrant further investigation.

### 3.12 Maintenance of the dryness of cancer cells

Stem cells play a significant role in BC, driving tumor progression and metastasis, and displaying inherent resistance to

chemotherapy and radiotherapy (221, 222). These cells can enhance drug efflux, increase drug metabolism in the tumor, activate survival pathways like Notch and Hedgehog, and dampen apoptosis signaling, all contributing to chemoresistance (223). HIF-1 $\alpha$  directly upregulates genes such as NANOG, SOX2, KLF4, and OCT4, which inhibit differentiation and maintain a stem cell-like phenotype (203, 224). Furthermore, HIF-1 $\alpha$  supports the survival of breast cancer stem cells (BCSC) by inducing ROS-dependent expression of HIF-1 $\alpha$  and HIF-2 $\alpha$ , leading to HIF-mediated expression of IL-6, IL-8, and MDR1. Exposure of MDA-MB-231, SUM-149, and SUM-159 cells to paclitaxel increased the percentage of ALDH+ cells, indicative of stem cell characteristics, by twelvefold *in vitro* and *in vivo* (203). Therefore, HIF-1 $\alpha$  activation promotes the proliferation and enrichment of tumor stem cells, contributing to drug resistance.

### 3.13 Strategies to overcome HIF-1 $\alpha$ -dependent drug resistance

To overcome HIF-1 $\alpha$ -dependent drug resistance, strategies involve directly or indirectly targeting HIF-1 $\alpha$  with inhibitors (225). Direct inhibitors of HIF-1 $\alpha$  target protein-protein/DNA interactions, impacting DNA binding, transcriptional activity of HIF-1 $\alpha$ , heterodimerization with HIF-1 $\beta$ , and interactions with transcriptional coactivators (226–230). Indirect inhibitors aim to downregulate HIF-1 $\alpha$  transcription or translation, reduce HIF-1 $\alpha$  stability, or prevent its degradation, offering potential pathways to mitigate drug resistance in cancer treatment.

### 3.14 Breast cancer therapy targeting HIF-1 $\alpha$

Numerous studies have highlighted the potential of HIF-1 $\alpha$  inhibitors and compounds targeting the HIF-1 $\alpha$  pathway, demonstrating their effectiveness *in vitro* (62, 81)(Table 2). For instance, treatment of MDA-MB-231 cells with the PHD inhibitor molidustat, which stabilizes HIF, resulted in reduced viability, growth, clone formation, cell cycle arrest, and increased chemosensitivity, indicating potential anticancer activity of HIF (249). Among 68 newly synthesized arylformamide compounds, compound 30 m showed the most potent inhibitory activity against HIF-1 $\alpha$  with minimal cytotoxicity, effectively reducing hypoxia-induced HIF-1 $\alpha$  protein accumulation (81, 250). However, clinical trials have failed to show the effect of HIF-1 $\alpha$  inhibitors in BC treatment (81, 251)(Table 3). Preliminary data published in 2013 showed some clinical efficacy of digoxin in patients with stages I–III BC. However, the treatment window of the drug is narrow; serum levels exceeding 1–2 nM produce significant side effects. So far, no follow-up data are available (192, 252). The differential expression of HIF-1 $\alpha$  and HIF-1 $\alpha$  inhibitor monotherapy may be the factors limiting the efficacy of anti-HIF-1 $\alpha$  therapy (1). Yu et al. discussed the lack of clinical effectiveness of HIF-1 $\alpha$  inhibitors (230). First, although both HIF-1 $\alpha$  and HIF-2 $\alpha$  are involved in cancer progression, most inhibitors have targeted only HIF-1 $\alpha$ .

TABLE 2 HIF-1 $\alpha$  inhibitors under investigation in BC.

| Compound                 | Mechanism                                                                                                                                                                                                                                                          | Type of study   | Model                                                        | References |
|--------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|--------------------------------------------------------------|------------|
| KC7F2                    | Decrease HIF-1 $\alpha$ protein accumulation                                                                                                                                                                                                                       | <i>In vitro</i> | MCF-7                                                        | (231)      |
| Quercetin                | Inhibiting HIF-1 $\alpha$ protein accumulation                                                                                                                                                                                                                     | <i>In vitro</i> | SkBr3                                                        | (232)      |
| LXY6006                  | Inhibit HIF-1 $\alpha$ nuclear accumulation                                                                                                                                                                                                                        | <i>In vitro</i> | T47D, MDMBA-231, MX-1                                        | (233)      |
| Aminoflavone             | AF inhibits HIF-1 $\alpha$ expression                                                                                                                                                                                                                              | <i>In vitro</i> | Mouse model (MCF-7)                                          | (234)      |
| 7-Hydroxyneolamellarin A | Inhibit HIF-1 $\alpha$ protein accumulation                                                                                                                                                                                                                        | <i>In vivo</i>  | Mouse model (4T1)                                            | (235)      |
| Methylalpinumisoflavone  | Inhibits HIF-1 activation by blocking the induction of nuclear HIF-1 $\alpha$ protein                                                                                                                                                                              | <i>In vitro</i> | T47D, MDAMB-231                                              | (236)      |
| Cardenolides             | Inhibited HIF-1 transcriptional activity dose-dependently                                                                                                                                                                                                          | <i>In vivo</i>  | MCF-7                                                        | (237)      |
| PX-478                   | Suppresses HIF-1 $\alpha$ levels                                                                                                                                                                                                                                   | <i>In vitro</i> | Mouse model (MCF-7)                                          | (238)      |
| DJ12                     | Decrease HIF-1 $\alpha$ transactivation and DNA binding                                                                                                                                                                                                            | <i>In vitro</i> | MDA-468, ZR-75, MD435                                        | (239)      |
| Isoliquiritigenin        | Isoliquiritigenin inhibits the expression of HIF-1 $\alpha$ by inhibiting the PI3K/Akt and NF- $\kappa$ B signaling pathways, thereby inhibiting the expression of VEGF and the metastasis of TNBC                                                                 | <i>In vitro</i> | MDA-MB-231 cells                                             | (240)      |
| Cardamonin               | Cardamonin inhibits the transcriptional level of HIF-1 $\alpha$ by inhibiting the mTOR/p70S6K pathway, reducing the level of HIF-1 $\alpha$ protein, thereby enhancing mitochondrial oxidative phosphorylation and reducing glucose uptake and lactate production. | <i>In vitro</i> | MDA-MB-231 cells                                             | (241)      |
| Nanoliposomelechinomycin | The activity of HIF-1 can be inhibited by directly inhibiting the transcriptional activity of HIF-1 $\alpha$ and effectively blocking the binding between HIF-1 and HRE.                                                                                           | <i>In vivo</i>  | MDA-MB-231 breast cancer mice and SUM-159 breast cancer mice | (242)      |
| Melittin                 | Melitin inhibits HIF-1 $\alpha$ expression at the transcriptional level mainly by inhibiting NF- $\kappa$ B expression                                                                                                                                             | <i>In vitro</i> | MDA-MB-231 cells                                             | (243)      |
| As4S4 nanoparticles      | As4S4 nanoparticles reduce the transcription level of HIF-1 $\alpha$ by scavenging ROS and inhibit the metastasis of TNBC.                                                                                                                                         | <i>In vivo</i>  | 4T1 breast cancer mice                                       | (244)      |
| Sanguinarine             | Sanguinarine promotes proteasomal degradation of HIF-1 $\alpha$ by inactivating STAT3 under hypoxia and hinders breast cancer growth <i>in vivo</i>                                                                                                                | <i>In vitro</i> | MDA-MB-231 cells                                             | (245)      |
| Ganetespib               | Ganetespib promotes the degradation of HIF-1 $\alpha$ protein, reduces the levels of HIF-1 $\alpha$ protein and target gene proteins and controls angiogenesis, metabolism, invasion and metastasis in TNBC mice.                                                  | <i>In vitro</i> | MDA-MB-231 cells                                             | (246)      |

(Continued)



TABLE 2 Continued

| Compound            | Mechanism                                                                                                                                                                                                                                              | Type of study   | Model            | References |
|---------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|------------------|------------|
| Acridlavine         | Inhibition of TNBC premetastatic niche formation by targeting HIF-1α                                                                                                                                                                                   | <i>In vitro</i> | MDA-MB-232 cells | (247)      |
| Diallyl Trisulfides | DATS inhibits the synthesis of HIF-1α protein by inhibiting the translation level of HIF-1α, thereby reducing the transcriptional activation of downstream target genes L1CAM, snail, slug, VEGF and MMP-2, thereby inhibiting the metastasis of TNBC. | <i>In vitro</i> | MDA-MB-233 cells | (248)      |

TABLE 3 HIF-1α related clinical studies in BC.

| Drug                        | Main Outcomes                                                                                                                                        | Conditions               | Clinical Phase | Study Type     | Status    | NCT Number  |
|-----------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------|----------------|----------------|-----------|-------------|
| Digoxin                     | There was not enough data to analyze HIF-1alpha expression because of the limited tumor samples                                                      | Breast Cancer            | II             | Interventional | Completed | NCT01763931 |
| Bevacizumab, docetaxel      | The rate of serious adverse events is about 18.06% and the rate of other adverse events is 98.61% in total                                           | Breast Cancer            | II             | Interventional | Completed | NCT00559754 |
| Paclitaxel plus bevacizumab | There was no significant difference between HIF-1alpha polymorphism and longer PFS in patients treated with paclitaxel and bevacizumab               | Metastatic Breast Cancer | I              | Observational  | Completed | NCT01935102 |
| Vinorelbine                 | Metronomic dosing of oral vinorelbine in HR+/HER2- MBC as first-line CT after failure of endocrine therapies showed only limited benefit in patients | Metastatic Breast Cancer | II             | Interventional | Completed | NCT03007992 |

Therefore, the role of HIF-2α in drug resistance needs to be understood. Second, patient selection contributes to the success of clinical trials. Accuracy in the measurement of tumor HIF-1α may help to better distinguish responders from non-responders. Finally, although hypoxia varies between and within different BC subtypes, evidence suggests that the hypoxia of the patient's tumor itself is different (253). This heterogeneity may be the main determinant of the overall drug response. Considering the limitations of HIF-1α inhibitors, a combination of HIF-1α inhibitors with chemotherapeutic drugs or other agents may improve outcomes. A preclinical study indicated that digoxin enhances the sensitivity of triple-negative breast cancer cell lines to paclitaxel and gemcitabine *in vivo* (203). Interestingly, molidustat has been found to enhance the cytotoxicity of gemcitabine in MDA-MB-231 cells (249). However, currently, the related combination therapy of BC is in the preclinical stage, and thus, a clear conclusion cannot be drawn. The latest HIF-1α drug delivery system is based on nanocarriers that can improve targeting specificity, overcome solubility problems, reduce drug toxicity, and maintain safe drug concentrations. Furthermore, the mode of drug administration also affects the efficacy of HIF-1α-related drugs and should be investigated in future research (81). In conclusion, the results on BC cell lines show that aspects, such as comparison of HIF-1α and HIF-2α inhibition, double vs. single isomer inhibition, different statuses of hormone receptors, metastasis, and other unexplored issues, should be considered. Thus, we need to understand the role of HIFs in BC before targeting them for clinical application (55).

## 4 Prospect

HIF-1α, an important transcription factor under hypoxic conditions, plays a crucial role in the growth and development of the body as well as various physiological and pathological processes. HIF-1α induces the expression of numerous genes related to angiogenesis, growth and survival, invasion and metastasis, glucose metabolism, epithelial–mesenchymal transition (EMT), immune evasion, and resistance to various cancer treatments. The overexpression of hypoxia-inducible factor-1α (HIF-1α) is also closely related to drug resistance and prognosis in breast cancer patients. Therefore, regulating the activity of HIF-1α may be a breakthrough in treating many diseases. Upregulating the activity of HIF-1α can increase cell survival under hypoxic conditions and enhance angiogenesis in hypoxic tissues. Conversely, HIF-1α inhibitors can block angiogenesis and reduce the survival rate of hypoxic or inflammatory tissues. This article elaborates on the structure and function of HIF-1α, its mechanism of action in developing breast cancer, and drug resistance mechanisms. This review also summarizes strategies to overcome HIF-1α-dependent drug resistance and the current status of targeted HIF-1α therapy for breast cancer.

However, the complex interactions among multiple pathways involving HIF-1α pose greater challenges for its clinical application as an inhibitor. A deeper understanding of the intricate interactions between oxygen tension, the microenvironment, receptor expression, and HIF-1α expression is needed. This not only facilitates the development of new drug combinations but also aids in the



discovery of novel drug targets for breast cancer treatment. Currently, drugs targeting HIF-1 $\alpha$  are mainly focused on preclinical research, and their actual clinical effects, patient tolerance, dosing regimens, and other factors require further evaluation and validation. Additionally, drug delivery and efficacy are limited by factors such as tumor acidosis, hypoxic microenvironments, and elevated interstitial fluid pressure within tumors. Therefore, there is a need to develop more suitable drug carriers and delivery systems to enhance therapeutic outcomes. In conclusion, more comprehensive and in-depth research is required on HIF-1 $\alpha$  and the pathways it mediates. Although current clinical trials have not yet demonstrated satisfactory results for HIF-1 $\alpha$  inhibitors as monotherapy in breast cancer treatment, with continuous and in-depth research on the role of HIF-1 $\alpha$  in cancer development, it is believed that targeted HIF-1 $\alpha$  therapeutics will bring new hope to breast cancer patients in the near future.

## Author contributions

ShiZ: Validation, Writing – original draft. CC: Validation, Writing – review & editing. HH: Visualization, Writing – review & editing. ZZ: Supervision, Writing – review & editing. FZ: Conceptualization, Funding acquisition, Writing – review & editing. ShuZ: Conceptualization, Funding acquisition, Writing – review & editing.

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## Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was supported by the Sichuan Science and Technology Program (2022YFS0623) and the Natural Science Foundation of Southwest Medical University (2022QN079).

## Conflict of interest

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Glossary

|                |                                                        |
|----------------|--------------------------------------------------------|
| BC             | Breast cancer                                          |
| EMT            | Epithelial-mesenchymal transition                      |
| HIF            | Hypoxia-inducible factor                               |
| HIF-1 $\alpha$ | Hypoxia-inducible factor-1 $\alpha$                    |
| HIF-2 $\alpha$ | Hypoxia-inducible factor-2 $\alpha$                    |
| HIF-3 $\alpha$ | Hypoxia-inducible factor-3 $\alpha$                    |
| HIF-1 $\beta$  | Hypoxia-inducible factor-1 $\beta$                     |
| bHLH- PAS      | Basic helix-loop-helix/Per-ARNT-Sim                    |
| TADs           | Transactivation domains                                |
| C-TAD          | C-terminal transactivation domain                      |
| N-TAD          | N-terminal transactivation domain                      |
| CBP/p300       | cAMP response element-binding protein and p300 protein |
| ODDD           | Oxygen-dependent degradation domain                    |
| NLS            | Nuclear localization signals                           |
| PHDs           | Prolyl hydroxylase domain-containing proteins          |
| FIH            | Factor inhibiting HIF-1 $\alpha$                       |
| VHL            | Von Hippel–Lindau protein                              |
| Ub             | Ubiquitin                                              |
| HRE            | Hypoxia response element                               |
| TNBC           | Triple negative breast cancer                          |
| NF- $\kappa$ B | Nuclear factor- $\kappa$ B                             |
| IKK            | Inhibitor of NF- $\kappa$ B kinase                     |
| TNF            | Tumor necrosis factor                                  |
| IL1            | Interleukin 1                                          |
| VEGF           | Vascular endothelial growth factor                     |
| VEGFR          | Vascular endothelial growth factor receptor            |
| HGF            | Hepatocyte growth factor                               |
| VCAM1          | Vascular cell adhesion molecule 1                      |
| BRK            | Breast tumor kinase                                    |
| C1QBP          | Complement C1q binding protein                         |
| BDNF           | Brain-derived neurotrophic factor                      |
| ROS            | Reactive oxygen species                                |
| ANGPTL4        | Angiopoetin-like 4                                     |
| OXPHOS         | Oxidative phosphorylation                              |
| GLUT           | Glucose transporter                                    |
| HK2            | Hexokinase 2                                           |
| PFK1           | 6-phosphofructokinase1                                 |
| ALD            | Aldolase                                               |

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|              |                                                    |
|--------------|----------------------------------------------------|
| ENO          | Enolase                                            |
| GAPD         | Glyceraldehyde-3-phosphate dehydrogenase           |
| PGK          | Phosphoglycerate kinase                            |
| PK           | Pyruvate kinase                                    |
| LDHA         | Lactate Dehydrogenase A                            |
| PKM2         | Pyruvate kinase isozymes M2                        |
| PDK1         | Pyruvate dehydrogenase kinase 1                    |
| PDH          | Pyruvate dehydrogenase kinase                      |
| MCT          | Monocarboxy latetransporter                        |
| TCA          | Tricar boxylic acid                                |
| Twist1       | Twist-related protein 1                            |
| STAT3        | Signal transducer and activator of transcription 3 |
| ZEB          | Zinc finger E-box binding homeobox                 |
| TGF- $\beta$ | Transforming growth factor- $\beta$                |
| CSF-1        | Colony-stimulating factor 1                        |
| MMP          | Matrix metalloproteinase                           |
| CXCR4        | C-X-C chemokine receptor type4                     |
| CXCR3        | C-X-C chemokine receptor type3                     |
| BNIP         | BCL2/adenovirus E1B 19 kDa interacting protein     |
| Bid          | BH3 interacting domain death agonist               |
| BAX          | Bcl-2-associated X protein                         |
| BAK          | BCL2 antagonist/killer                             |
| Bad          | BCL2 associated agonist of cell death              |
| BNIP3        | B cell lymphoma 2 interacting protein 3            |
| NIX          | NIP3-like protein X                                |
| BCL2         | B-cell lymphoma 2                                  |
| Bcl-XL       | B-cell lymphoma 2-extra-large protein              |
| BNIP3L       | BNIP3-like protein                                 |
| PI3K         | Phosphoinositide 3-kinase                          |
| AKT          | Protein kinase B                                   |
| mTOR         | Mechanistic target of rapamycin                    |
| BIM          | BCL2 like 11                                       |
| Tregs        | Regulatory T cells                                 |
| PDL1         | Programmed cell death ligand 1                     |
| ERO1         | Endoplasmic reticulum oxidoreductin 1              |
| FOXp3        | Forkhead box protein 3                             |
| IL10         | Interleukin 10                                     |
| GM-CSF       | Granulocyte-macrophage colony-stimulating factor   |
| NF45         | Nuclear factor 45                                  |
| NF90         | Nuclear factor 90                                  |

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|       |                                                       |
|-------|-------------------------------------------------------|
| LOX   | Lysyl oxidase                                         |
| ITGA5 | Integrin alpha 5                                      |
| MDR1  | Multidrug resistance gene 1                           |
| MRP1  | Recombinant multidrug resistance associated protein 1 |
| BCRP  | Breast cancer resistance protein                      |
| ELP3  | Elongator complex protein 3                           |
| PAK1  | P21-activated protein kinase                          |
| AMPK  | AMP-activated protein kinase                          |
| ATG5  | Autophagy-related gene 5                              |
| CSC   | Cancer stem cell                                      |
| Oct4  | Octamer transcription factor 4                        |
| SOX2  | SRY-box transcription factor 2                        |
| NANOG | Nanog homeobox                                        |
| KLF4  | Kruppel-like transcription factor 4.                  |



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RECEIVED 01 March 2024

ACCEPTED 10 June 2024

PUBLISHED 24 June 2024

## CITATION

Koivula T, Lempiäinen S, Neuvonen J, Norha J, Hollmén M, Sundberg CJ, Rundqvist H, Minn H, Rinne P and Heinonen I (2024) The effect of exercise and disease status on mobilization of anti-tumorigenic and pro-tumorigenic immune cells in women with breast cancer.  
*Front. Immunol.* 15:1394420.  
doi: 10.3389/fimmu.2024.1394420

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# The effect of exercise and disease status on mobilization of anti-tumorigenic and pro-tumorigenic immune cells in women with breast cancer

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**Background:** Mobilization of certain immune cells may improve the ability of the immune system to combat tumor cells, but the effect of acute exercise on mobilizing immune cells has been sparsely investigated in cancer patients. Therefore, we examined how acute exercise influences circulating immune cells in breast cancer patients.

**Methods:** Nineteen newly diagnosed breast cancer patients aged 36–68 performed 30 minutes of moderate-intensity exercise with a cycle ergometer. Blood samples were collected at various time points: at rest, at 15 (E15) and 30 minutes (E30) after onset of the exercise, and at 30 and 60 minutes post-exercise. We analyzed several immune cell subsets using flow cytometry.

**Results:** Acute exercise increased the number of total leukocytes, neutrophils, lymphocytes, monocytes, basophils, total T-cells, CD4<sup>+</sup> T-cells, T helper (Th) 2-cells, Th 17-cells, CD8<sup>+</sup> T-cells, CD4<sup>+</sup>CD8<sup>+</sup> T-cells, CD56<sup>+</sup> natural killer (NK) cells, and CD14<sup>+</sup>CD16<sup>+</sup> monocytes. Many of the changes were transient. Proportions of NK-cells and CD8<sup>+</sup> T-cells increased, while the proportion of myeloid derived suppressor cells (MDSCs) reduced, and proportion of regulatory T-cells remained unchanged by exercise. Several associations were detected between cell mobilizations and disease state. For instance, tumor size correlated negatively with NK cell mobilization at E15, and progesterone receptor positivity correlated negatively with CD8<sup>+</sup> T-cell mobilization.

**Conclusion:** The findings show that the proportions of CD8<sup>+</sup> T-cells and NK cells increased and the proportion of MDSCs proportion decreased in breast cancer

patients after 30-minute exercise, suggesting a change in the profile of circulating immune cells towards more cytotoxic/anti-tumorigenic. The mobilization of some immune cells also appears to be related to the disease state.

#### KEYWORDS

acute exercise, physical activity, breast cancer, white blood cell, immune cell, immunity

## Introduction

Physical exercise has been found to prevent cancer, reduce the side effects of cancer treatments and improve prognosis and patients' quality of life. For example, the likelihood of being diagnosed with breast cancer is 13% lower in people who are most physically active in their leisure time versus the ones who are least (physically) active (1). Moreover, cancer-specific mortality is 31% lower in physically active people post-diagnosis compared to sedentary ones (1). Traditionally, patients were advised to rest during cancer treatments, but due the positive effect of exercise on cancer and treatment related side effects as well as associations with a better prognosis, the view has turned. For example, exercise is the only effective way to reduce treatment related fatigue. Today, exercise is recommended for cancer patients similar to individuals without cancer (2). The current understanding is that many of the health benefits of exercise are attributed to the reduction of cancer risk factors such as obesity, hormonal levels and poor immune surveillance (3).

Tumor immune surveillance is a balance between immune cells that promote tumor progression (pro-tumorigenic cells) and cells that promote tumor rejection (anti-tumorigenic cells) (4). Studies in animal cancer models have shown that exercise shrinks tumors by enhancing the tumor infiltration of anti-tumorigenic cells, thus natural killer (NK) and cytotoxic T cells (5, 6). Furthermore, exercise reduces the accumulation and recruitment of pro-tumorigenic myeloid derived suppressor cells (MDSC) and regulatory T cells to tumors in mice with breast cancer (7, 8). In breast cancer patients, it has been demonstrated that higher CD8<sup>+</sup> T cell count in tumor microenvironment (TME) is associated with a better prognosis and longer overall survival (9, 10). The survival rate of cancer patients has also been linked to the presence of NK cells at TME (11). On the contrary, the high number of infiltrating regulatory T cells and MDSCs is associated with more aggressive breast cancer subtypes and with worse overall survival (12–14).

Acute physical exercise is a powerful stimulus for mobilizing immune cells in healthy individuals, but the phenomenon is less investigated in cancer patients. In healthy individuals, the number of circulating neutrophils, lymphocytes, and monocytes increase in response to different types of exercise (15–18). Previously, we showed that acute 10-minute light-to-moderate-intensity exercise increases the number of total leukocytes, CD8<sup>+</sup> T cells, CD19<sup>+</sup> B cells, CD16<sup>+</sup> NK cells, and CD14<sup>+</sup>CD16<sup>+</sup> monocytes in the

circulation of newly diagnosed breast cancer patients (13) and fairly similar responses were seen in lymphoma patients (19). These studies, however, did not include pro-tumorigenic cells, for example MDSCs or regulatory T cells. Thus, the aim of this study was to investigate immune cell responses to a 30-minute acute exercise session with moderate intensity in breast cancer patients. Specifically, we used flow cytometry to assess responses in circulating leukocytes, neutrophils, lymphocytes (B cells, T cells, NK cells), monocytes, eosinophils, basophils, and MDSCs at multiple time points during and after acute exercise. We wanted to investigate both the number of these cells, but also their proportions, and a profile between anti-tumorigenic and pro-tumorigenic cells. In addition, we aimed to examine whether oncologic disease status affects immune cell mobilization by exercise.

## Materials and methods

This study was conducted at the Turku PET Centre, Turku, Finland between August 2021, and May 2022. The study was approved by the Ethics Committee of the Hospital District of Southwestern Finland (100/1801/2020). Good clinical practice and the Declaration of Helsinki were followed. The study was a part of a larger study and is registered in the international register of clinical trials (Clinicaltrials.gov NCT04990856).

## Participants

Nineteen women with newly diagnosed breast cancer were recruited in this study before the onset of cancer treatments. Participants were recruited from Turku University Hospital. Exclusion criteria were abnormal fatigue, anemia, or physical disability that would hinder the study procedures. All participants provided written informed consent.

## Exercise test

Each participant visited the study laboratory once after study enrollment. Strong physical exertion and alcohol and caffeine consumption were prohibited for at least 24 hours prior the study

day. Participants conducted a 30-minute exercise with a cycle ergometer (Gymstick Vapor 10.0, Gymstick International Oy, Lahti, Finland) with cadence and resistance that they would be able to sustain for 30 minutes. Participants were allowed to change the resistance as many times as they liked during the exercise. However, 70% of age predicted maximal heart rate was calculated using the formula  $(220 - \text{age}) * 0.7$  and participants were asked to reach that heart rate at some point during the exercise. Lactate concentration (Lactate Scout 4, EKF Diagnostics, Barleben, Germany), blood pressure (Apteq AE701f, Rossmax Swiss GmbH, Berneck, Switzerland), and heart rate (Palmsat 2500, Nonin, Plymouth, USA) were measured at the same time points as the blood samples were drawn, and heart rate was also measured continuously during the exercise with Polar H10 heart rate sensor (Polar Electro Oy, Kempele, Finland). Participants' rating of perceived exertion (RPE) was determined with a Borg scale (6–20). Rate pressure product (RPP) was measured by multiplying systolic blood pressure by heart rate. Mean arterial pressure (MAP) was calculated as  $(\text{diastolic blood pressure} * 2 + \text{systolic blood pressure})/3$ . Predicted energy expenditure (EE) during exercise was calculated with the following formula:  $0.744 + 0.0216 * (\text{HRR}) + 0.00699 * (\text{weight}) + 0.00102 * (\text{HRR}) * (\text{weight})$  (20), HRR being heart rate reserve. Participants were allowed to drink water *ad libitum* during and after the exercise.

## Blood sampling and hematology analysis

Prior to starting the exercise bout, an intravenous catheter was placed in the antecubital vein for repeated blood sampling. The first blood sample was drawn after a short period of supine rest before the exercise. The second blood sample was drawn during the exercise, at 15-minute timepoint (E15) and the third at 30-minute timepoint when they were finishing the exercise (E30). Fourth and fifth blood samples were drawn during supine recovery, at 30-minutes post-exercise (P30) and at 60-minutes post-exercise (P60). All blood samples were collected in EDTA-tubes (BD Biosciences, San Jose, USA). We were able to collect all blood samples except P30 and P60 samples from 2 of the participants.

Complete blood counts were determined using whole blood samples at each time point. Total leukocyte count and the number of neutrophils, lymphocytes, monocytes, eosinophils, and basophils were analyzed using Sysmex XN analyzer (Sysmex Corporation, Kobe, Japan).

## Flow cytometry

Flow cytometry was used to determine the number of circulating T cells, B cells, NK cells, monocyte subtypes and MDSCs. Fc Block (BD Biosciences, San Jose, USA) was added to 100  $\mu\text{L}$  whole blood samples. The samples were stained with fluorophore-labeled monoclonal antibodies (BD Biosciences, San Jose, USA). The antibodies used were CD45-FITC (clone HI30), CD3-BV605 (clone UCHT1), CD4-BV421 (clone RPA-T4), CD8-BV786 (clone RPA-T8), CD196-APC (clone 11A9), CD183-PE

(clone 1C6), CD194-PE-Cy7 (clone 1G1), and CD25-APC-Cy7 (clone M-A251) for staining panel 1, CD45-FITC (clone HI30), CD3-BV605 (clone UCHT1), CD19-PE-CY7 (clone HIB19), Anti-HLA-DR-APC-Cy7 (clone L243), CD33-BV711 (clone WM53), CD11b-BV421 (clone ICRF44), CD66b-PE (clone G10F5), and CD14-APC (clone M5E2) for staining panel 2, and CD45-FITC (clone HI30), CD14-APC (clone M5E2), CD16-PE (clone 3G8), CD19-PE-CY7 (clone HIB19), CD56-BV605 (clone NCAM16.2), and CD64-BV421 (clone 10.1) for staining panel 3. Red blood cells were lysed with 1X FACS solution (BD Biosciences, San Jose, USA) and samples were washed with phosphate buffered saline (PBS) (Thermo Fisher Scientific, Waltham, USA). Panel 1 samples were further processed for FoxP3 staining. For that, the samples were incubated with Fixation/Permeabilization working solution (Thermo Fisher Scientific, Waltham, USA) and washed with 1X Permeabilization buffer (Thermo Fisher Scientific, Waltham, USA). Samples were then incubated with FoxP3-antibody (clone 236A/E7) and washed with 1X Permeabilization buffer. 300  $\mu\text{L}$  of PBS was added to each sample and samples were pipetted into a flat bottom 96-well plate. Cell counts were obtained by running 150  $\mu\text{L}$  of each sample with a BD LSR Fortessa™ flow cytometer (BD Biosciences, San Jose, USA). All analyses were performed using FlowJo v10 software (FlowJo, LLC Ashland, Oregon, USA). The gating strategy is presented in Figure 1.

## Biomarker analysis

Characterization of breast cancer biomarkers was determined at Pathology Laboratory of Turku University Hospital as part of the patients' routine diagnostics. Paraffin embedded cancer specimen were stained using Ventana Benchmark Ultra (Roche Diagnostics) for immunohistochemistry (IHC) with ER (SP1), PR (IE2), HER2 (4B5), and Ki-67 (30–9) antibodies (Ventana/Roche Diagnostics). Positive staining at HER2 IHC was always confirmed by *in situ* hybridization according to national and international guidelines (21). The degree of histological differentiation of carcinoma was determined according to Scaff-Bloom-Richardson (22, 23) and staging by the UICC TNM classification (24).

## Plasma volume change

The change in plasma volume (PV) during and after exercise bout was taken into consideration and calculated with following formula:  $\Delta\text{PV} = (\text{Hb}_{\text{pre}} * (1 - \text{Hct}_{\text{post}})) / (\text{Hb}_{\text{post}} * (1 - \text{Hct}_{\text{pre}})) - 1$  (25). White blood cell (WBC) counts were adjusted to reflect the exercise-induced shifts in plasma volume with the following formula:  $\text{WBC}_{\text{corrected}} = \text{WBC}_{\text{uncorrected}} * (1 + \Delta\text{PV})$  (25).

## Statistical methods

After adjusting for plasma volume change, the number of immune cell populations determined by flow cytometry were corrected with the immune cell values obtained from the



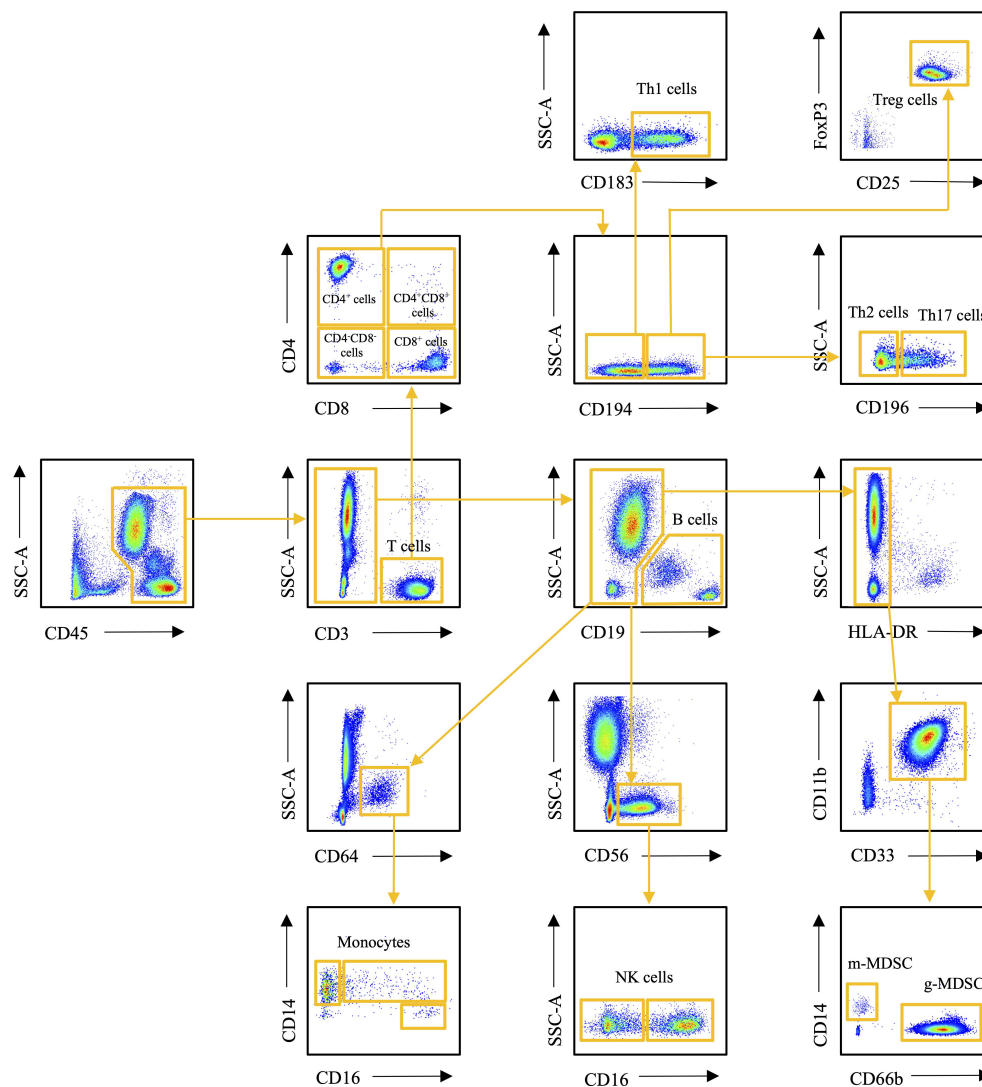


FIGURE 1  
Flow cytometry gating strategy of the immune cells studied.

complete blood count. For the correction the following formula was used:  $WBC_{corrected} = \text{total leukocyte count} \times WBC\% \text{ of } CD45^+ \text{ cells}$ . To determine the effect of acute exercise on immune cells counts and plasma volume, repeated measurement ANOVA was performed. When the main effect (time) was less than  $p < 0.05$ , statistical differences in time points were considered by Tukey-*post-hoc* test. Paired samples t-test was used to compare means of heart rate, blood pressure, and lactate concentration at baseline and during exercise. The associations between immune cell mobilization and disease status and exercise intensity were examined by either Pearson's or Spearman's correlation. Sensitivity analyses were based on the visual inspection of the scatter blots. If we detected a possible outlier, its data was removed for the sensitivity analyses to determine whether the outlier affected the results. Significance was determined at  $p < 0.05$ , and also at  $p < 0.01$  in correlation analyses. All statistical analyses were performed with Graphpad prism 8.0 and SAS 9.4.

## Results

### Participant characteristics

Characteristics of the study participants at baseline and their physiological response to exercise are presented in Table 1. Systolic and diastolic blood pressure, heart rate, and lactate concentration were significantly higher during exercise compared to baseline ( $p < 0.0001$  in all).

### Plasma volume

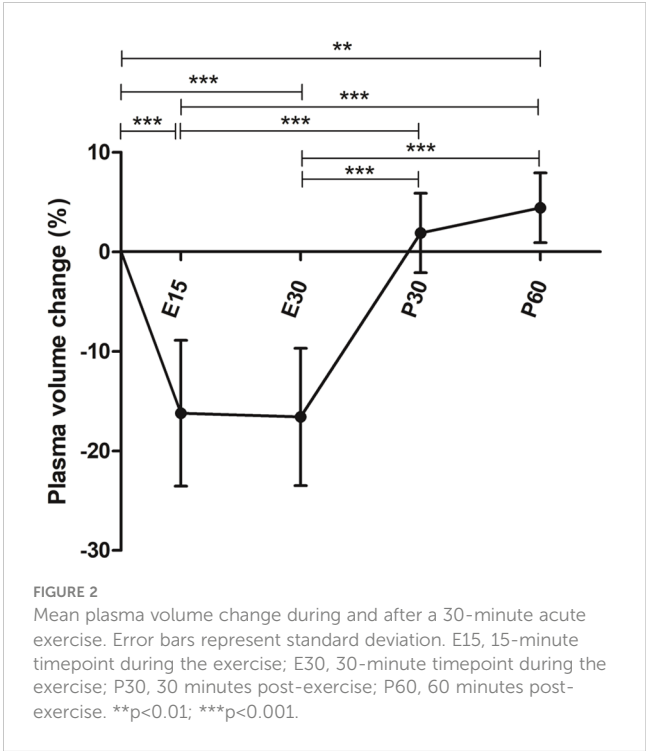
Mean reduction in plasma volume was 16.2% (SD 7.3) at 15-minute timepoint during exercise (E15) ( $p < 0.0001$ ) and 16.6% (SD 6.9) at 30-minute timepoint when they were finishing the exercise (E30) compared to baseline ( $p < 0.0001$ ). The plasma volume was

TABLE 1 Characteristics of the study participants (n=19).

| Resting measurements                         |              |
|----------------------------------------------|--------------|
| Age, years                                   | 56 (8)       |
| Height, cm                                   | 164.3 (4.5)  |
| Weight, kg                                   | 76.9 (14.5)  |
| BMI, kg/m <sup>2</sup>                       | 28.9 (5.4)   |
| Systolic blood pressure, mmHg                | 122 (16)     |
| Diastolic blood pressure, mmHg               | 69 (7)       |
| Heart rate, bpm                              | 69 (10)      |
| Lactate concentration, mmol/l                | 1.2 (0.5)    |
| Disease status                               |              |
| Grade I, %                                   | 16           |
| Grade II, %                                  | 47           |
| Grade III, %                                 | 37           |
| ER+, %                                       | 95           |
| PR+, %                                       | 89           |
| HER2+, %                                     | 37           |
| Tumor size, scale 1–3                        | 2 (1)        |
| Exercising measurements                      |              |
| Heart rate at E15, bpm                       | 128 (21)***  |
| Heart rate at E30, bpm                       | 138 (22)***  |
| Heart rate % of maximal heart rate at E15, % | 78 (13)      |
| Heart rate % of maximal heart rate at E30, % | 84 (14)      |
| Systolic blood pressure at E15, mmHg         | 165 (33)***  |
| Systolic blood pressure at E30, mmHg         | 165 (28)***  |
| Diastolic blood pressure at E15, mmHg        | 109 (19)***  |
| Diastolic blood pressure at E30, mmHg        | 114 (22)***  |
| Lactate concentration at E15, mmol/l         | 3.6 (1.3)*** |
| Lactate concentration at E30, mmol/l         | 3.8 (2.0)*** |
| RPE at E15, Borg 6–20                        | 12 (1)       |
| RPE at E30, Borg 6–20                        | 14 (2)       |
| Ergometer’s workload, distance, km           | 15.9 (1.6)   |

Data presented as mean (SD). Significant p-values; \*\*\*<0.001 as compared to rest. BMI, body mass index; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; RPE, rating of perceived exertion; E15, 15-minute timepoint during the exercise; E30, 30-minute timepoint during the exercise.

2.2% (SD 4.0) above baseline at 30-minutes post-exercise (P30) (p=0.2286) and 4.9% (SD 3.5) above baseline at 60-minutes post-exercise (P60) (p=0.0009) (Figure 2). Therefore, absolute number of white blood cells were adjusted for plasma volume change.



### Effect of acute exercise on different leukocyte subgroups

Acute exercise increased the number of total leukocytes by 22% (p<0.0001), neutrophils by 17% (p=0.0380), lymphocytes by 36% (p<0.0001), monocytes by 15% (p=0.0009), and basophils by 55% (p=0.0042) at E15 (Figures 3A–E). Increases in neutrophils and monocytes were observed only at E15 but the number of total leukocytes, lymphocytes, and basophils were also elevated at E30 compared to baseline (p=0.0234, p=0.0006, p=0.0070, respectively). Furthermore, the number of basophils was still elevated at P30 (p=0.0279). The number of eosinophils did not change (Figure 3F).

Total number of T cells increased by 27% at E15 compared to baseline (p=0.0002) and remained elevated at E30 (p=0.0242) before returning to baseline after the exercise (Figure 4A). The number of total CD4<sup>+</sup> T helper (Th) cells increased only at E15 by 19% (p=0.0025) (Figure 4B). Further analysis of CD4<sup>+</sup> cells showed that the number of Th1 cells increased 27% at E15 but it was not significant (Figure 4C). Th2 and Th17 cells increased by 24% (p=0.0013) and by 23% (p=0.0060) at E15, respectively (Figures 4D, E). There was no change in regulatory T cells (Figure 4F). The number of CD8<sup>+</sup> cytotoxic T cells increased by 36% at E15 (p=0.0014), remained elevated at E30 (p=0.0139), and decreased to baseline after the exercise (Figure 4G). CD4<sup>+</sup>CD8<sup>+</sup> double positive T cells did not change significantly, but the number of CD4<sup>+</sup>CD8<sup>−</sup> double negative T cells increased by 43% at E15 (p=0.0397) and then returned to baseline (Figures 4H, I).

The number of CD19<sup>+</sup> B cells decreased by 18% at P30 (p=0.0084) and 17% at P60 (p=0.0084) compared to E15, but

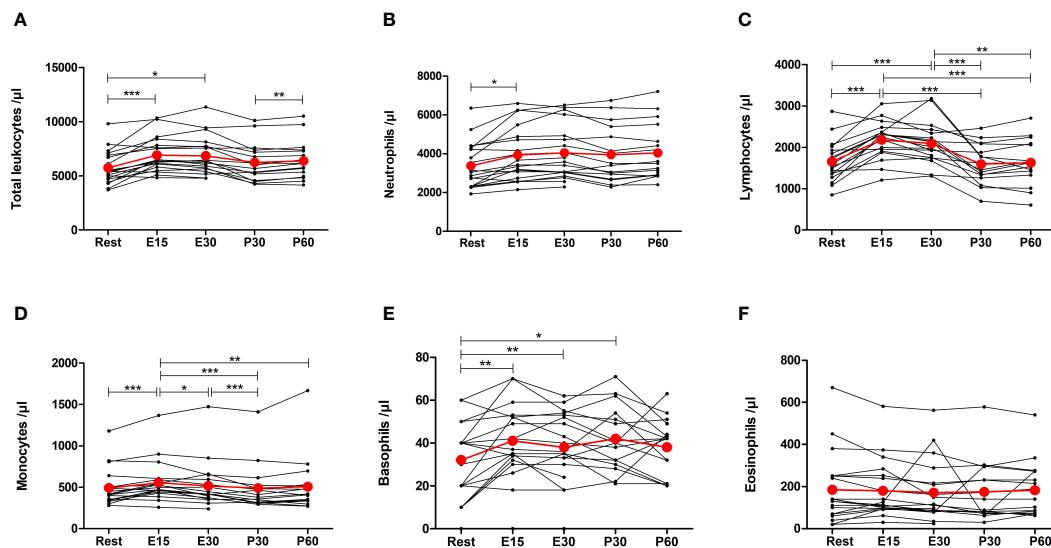


FIGURE 3

Exercise-induced changes in (A) total leukocytes, (B) neutrophils, (C) lymphocytes, (D) monocytes, (E) basophils, and (F) eosinophils. The number of total leukocytes, neutrophils, lymphocytes, monocytes, and basophils increased during exercise. The number of eosinophils did not change. Red lines represent the mean. E15, 15-minute timepoint during the exercise; E30, 30-minute timepoint during the exercise; P30, 30 minutes post-exercise; P60, 60 minutes post-exercise. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

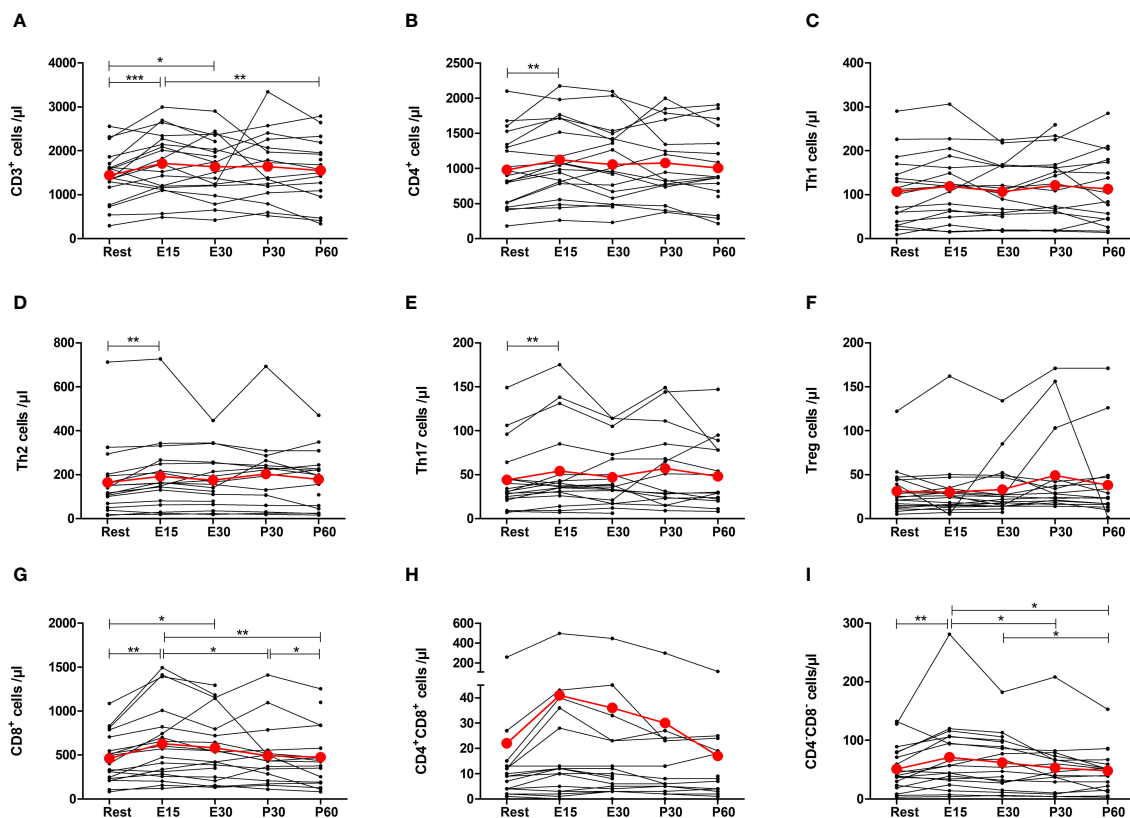


FIGURE 4

Exercise-induced changes in (A) total T cells, (B) CD4<sup>+</sup> helper T cells, (C) Th1 cells, (D) Th2 cells, (E) Th17 cells, (F) regulatory T cells, (G) CD8<sup>+</sup> cytotoxic T cells, (H) CD4<sup>+</sup>CD8<sup>+</sup> T cells, and (I) CD4<sup>+</sup>CD8<sup>-</sup> T cells. The number of total T cells, CD4<sup>+</sup> T cells, Th2 cells, Th17 cells, CD8<sup>+</sup> T cells, and CD4<sup>+</sup>CD8<sup>+</sup> T cells increased during exercise. The number of Th1 cells, regulatory T cells, and CD4<sup>+</sup>CD8<sup>-</sup> T cells did not change. Red lines represent the mean. E15, 15-minute timepoint during the exercise; E30, 30-minute timepoint during the exercise; P30, 30 minutes post-exercise; P60, 60 minutes post-exercise. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

there was no significant change from baseline (Figure 5A). The number of total NK cells, CD16<sup>+</sup> NK cells, and CD16<sup>-</sup> NK cells increased by 154% ( $p=0.0001$ ), 202% ( $p=0.0001$ ) and 60% ( $p=0.0096$ ) at E15, respectively, and remained elevated at E30 before returning to baseline (Figures 5B–D). Further, there was a 72% increase in the number of CD14<sup>+</sup>CD16<sup>+</sup> monocytes at E15 compared to baseline ( $p=0.0178$ ), but the number of CD14<sup>+</sup>CD16<sup>-</sup> monocytes or CD14<sup>+</sup>CD16<sup>+</sup> monocytes did not change (Figures 5E–G). The number of total MDSCs, g-MDSCs, or m-MDSCs did not change (Figures 5H–J).

In addition to absolute cell counts, immune cell proportions of total leukocytes were analyzed (Table 2). The proportion of neutrophils decreased at E15 ( $p=0.0227$ ) and then increased at P30 and P60 compared to baseline ( $p=0.0015$ ,  $p=0.0027$ , respectively). On the contrary, the proportion of lymphocytes increased at E15 ( $p=0.0003$ ) and decreased at P30 and P60 compared to baseline ( $p=0.0242$ ,  $p=0.0440$ , respectively). Monocyte proportion was below baseline at all time-points ( $p<0.05$  in all) and eosinophil proportion was decreased only at E15 compared to baseline ( $p=0.0200$ ). The proportion of Th2 cells increased at P30 compared to baseline ( $p=0.0375$ ). The proportion of CD19<sup>+</sup> B cells decreased at E15 compared to baseline ( $p=0.0397$ ). The proportions of total NK cells and CD16<sup>+</sup> NK cells increased at E15 ( $p=0.0006$ ,  $p=0.0001$ , respectively) and at E30 ( $p=0.0031$ ,  $p=0.0005$ , respectively) and decreased at P30 compared to

baseline ( $p=0.0373$ ,  $p=0.0347$ , respectively). Further, the proportions of total MDSCs and g-MDSCs decreased at E15 ( $p=0.0003$ ,  $p=0.0003$ , respectively) and at E30 ( $p=0.0014$ ,  $p=0.0015$ , respectively) compared to baseline. The proportions of other immune cells of total leukocytes remained unchanged in response to exercise (Table 2).

The proportions of different T cell subsets of total T cells and different helper T cells of total helper T cells were also examined (Table 3), and we found that the proportion of CD4<sup>+</sup> T cells of total T cells decreased at E15 and E30 compared to baseline ( $p=0.0070$ ,  $p=0.0181$ , respectively) whereas the proportion of CD8<sup>+</sup> T cells of total T cells increased at E15 and E30 compared to baseline ( $p=0.0089$ ,  $p=0.0348$ , respectively). The proportions of different helper T cells did not change (Table 3).

CD8<sup>+</sup>/Treg and CD56<sup>+</sup> NK cell/Treg ratios were also analyzed. CD8<sup>+</sup>/Treg ratio increased from 27 at baseline to 48 at E15 and NK cell/Treg ratio from 21 at baseline to 49 at E15 but the changes were not significant.

We also conducted correlation analyses between immune cell mobilization and exercise intensity and disease state that are found in Supplementary Material. Several associations were detected between cell mobilizations and disease state. For instance, tumor size correlated negatively with NK cell mobilization at E15, and progesterone receptor positivity correlated negatively with CD8<sup>+</sup> T-cell mobilization.

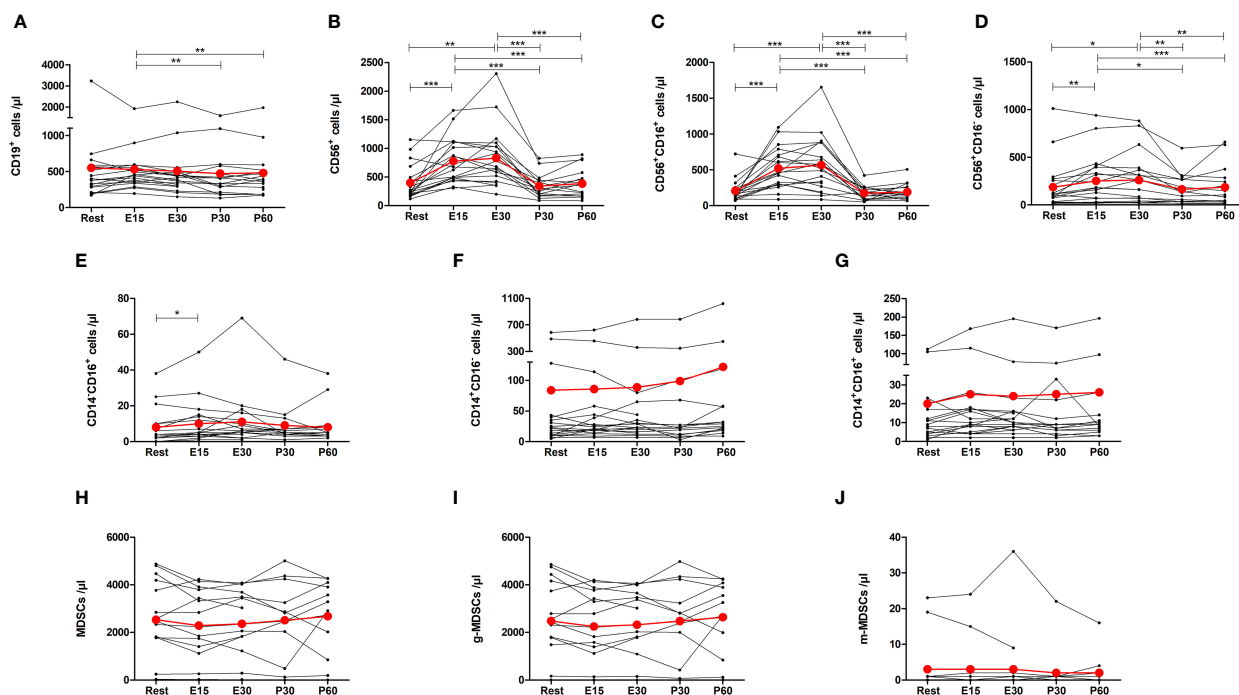


FIGURE 5

Exercise-induced changes in (A) CD19<sup>+</sup> B cells, (B) total NK cells, (C) CD16<sup>+</sup> NK cells, (D) CD16<sup>-</sup> NK cells, (E) CD14<sup>+</sup>CD16<sup>+</sup> monocytes, (F) CD14<sup>+</sup>CD16<sup>-</sup> monocytes, (G) CD14<sup>+</sup>CD16<sup>+</sup> monocytes, (H) total myeloid derived suppressor cells (MDSCs), (I) granulocytic MDSCs, and (J) monocytic MDSCs. The number of total NK cells, CD16<sup>+</sup> NK cells, CD16<sup>-</sup> NK cells, and CD14<sup>+</sup>CD16<sup>+</sup> monocytes increased during exercise. The number of B cells decreased after exercise when compared to E15. The number of CD14<sup>+</sup>CD16<sup>-</sup> monocytes, CD14<sup>+</sup>CD16<sup>+</sup> monocytes, or MDSCs did not change. Red lines represent the mean. E15, 15-minute timepoint during the exercise; E30, 30-minute timepoint during the exercise; P30, 30 minutes post-exercise; P60, 60 minutes post-exercise. \* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$ .

TABLE 2 Exercise-induced changes in immune cell proportions.

|                                     | Rest        | E15            | E30           | P30          | P60          |
|-------------------------------------|-------------|----------------|---------------|--------------|--------------|
| % of total leukocytes               |             |                |               |              |              |
| Neutrophils                         | 58.0 (7.6)  | 55.8 (8.5)*    | 58.1 (7.9)    | 61.6 (8.6)** | 62.1 (8.6)** |
| Lymphocytes                         | 29.7 (7.7)  | 32.8 (8.3)***  | 31.6 (7.7)    | 27.7 (8.1)*  | 27.2 (8.3)*  |
| Monocytes                           | 8.5 (2.7)   | 8.0 (2.4)*     | 7.4 (2.2)**   | 7.5 (2.3)*** | 7.6 (2.7)*** |
| Eosinophils                         | 3.2 (2.9)   | 2.7 (2.2)*     | 2.6 (2.3)     | 2.8 (2.5)    | 3.0 (2.4)    |
| Basophils                           | 0.6 (0.3)   | 0.6 (0.3)      | 0.6 (0.3)     | 0.7 (0.3)    | 0.6 (0.2)    |
| CD3 <sup>+</sup>                    | 27.0 (11.4) | 27.6 (12.0)    | 26.0 (11.0)   | 29.7 (12.5)  | 27.2 (12.4)  |
| CD4 <sup>+</sup>                    | 17.1 (8.0)  | 16.7 (8.4)     | 15.9 (8.2)    | 18.3 (8.7)   | 16.9 (8.7)   |
| Th1                                 | 1.8 (1.3)   | 1.7 (1.1)      | 1.6 (1.0)     | 1.8 (1.3)    | 1.7 (1.3)    |
| Th2                                 | 2.7 (1.8)   | 2.7 (1.8)      | 2.6 (1.5)     | 3.3 (2.2)*   | 3.0 (1.9)    |
| Th17                                | 0.8 (0.5)   | 0.8 (0.6)      | 0.7 (0.5)     | 0.9 (0.7)    | 0.8 (0.6)    |
| Treg                                | 0.6 (0.5)   | 0.5 (0.5)      | 0.5 (0.5)     | 0.8 (0.9)    | 0.6 (0.7)    |
| CD8 <sup>+</sup>                    | 8.7 (6.5)   | 9.5 (7.4)      | 8.8 (6.4)     | 10.1 (6.0)   | 8.8 (6.3)    |
| CD4 <sup>+</sup> CD8 <sup>+</sup>   | 0.5 (1.6)   | 0.6 (1.9)      | 0.6 (1.8)     | 0.6 (1.8)    | 0.3 (0.7)    |
| CD4 <sup>+</sup> CD8 <sup>-</sup>   | 0.9 (0.6)   | 1.0 (0.7)      | 0.9 (0.6)     | 1.0 (0.6)    | 0.8 (0.5)    |
| CD19 <sup>+</sup>                   | 8.8 (6.6)   | 7.7 (3.8)*     | 7.4 (5.1)     | 7.3 (4.7)    | 7.3 (4.7)    |
| CD56 <sup>+</sup>                   | 6.9 (5.1)   | 11.2 (4.7)***  | 11.7 (5.3)**  | 5.0 (2.5)*   | 5.8 (3.7)    |
| CD56 <sup>+</sup> CD16 <sup>+</sup> | 3.5 (1.8)   | 7.5 (3.7)***   | 7.9 (4.4)***  | 2.6 (1.0)*   | 2.9 (1.4)    |
| CD56 <sup>+</sup> CD16 <sup>-</sup> | 3.4 (4.7)   | 3.6 (3.8)      | 3.7 (3.7)     | 2.4 (2.2)    | 2.8 (3.2)    |
| CD14 <sup>+</sup> CD16 <sup>-</sup> | 1.3 (2.3)   | 1.1 (1.8)      | 1.0 (1.8)     | 1.1 (2.2)    | 1.4 (2.6)    |
| CD14 <sup>+</sup> CD16 <sup>+</sup> | 0.3 (0.4)   | 0.3 (0.4)      | 0.3 (0.4)     | 0.3 (0.5)    | 0.3 (0.5)    |
| CD14 <sup>+</sup> CD16 <sup>+</sup> | 0.1 (0.2)   | 0.1 (0.1)      | 0.1 (0.2)     | 0.1 (0.1)    | 0.1 (0.1)    |
| MDSC                                | 44.6 (26.5) | 32.9 (20.8)*** | 34.8 (20.7)** | 40.8 (27.9)  | 41.1 (25.6)  |
| gMDSC                               | 43.8 (26.7) | 32.3 (20.9)*** | 34.3 (20.9)** | 40.3 (27.9)  | 40.6 (25.6)  |
| mMDSC                               | 0.1 (0.1)   | 0.0 (0.1)      | 0.1 (0.2)     | 0.0 (0.1)    | 0.0 (0.1)    |

Data presented as mean (SD).  
p-values; \*<0.05, \*\*<0.01, \*\*\*<0.001 as compared to rest.  
E15, 15-minute timepoint during the exercise; E30, 30-minute timepoint during the exercise; P30, 30 minutes post-exercise; P60, 60 minutes post-exercise.

## Discussion

In this study, the effect of 30-minute acute exercise on circulating immune cell populations was examined in newly diagnosed breast cancer patients. The absolute number of total leukocytes, neutrophils, lymphocytes, monocytes, basophils, total T cells, CD4<sup>+</sup> T cells, Th2 cells, Th17 cells, CD8<sup>+</sup> T cells, CD4<sup>+</sup>CD8<sup>-</sup> T cells, NK cells, and CD14<sup>+</sup>CD16<sup>+</sup> monocytes increased by exercise. The number of eosinophils, regulatory T cells, CD4<sup>+</sup>CD8<sup>+</sup> T cells, B cells, MDSCs, CD14<sup>+</sup>CD16<sup>+</sup>, or CD14<sup>+</sup>CD16<sup>-</sup> monocytes did not change during or after exercise compared to baseline. Furthermore, the proportions of total lymphocytes and NK cells of total leukocytes increased during exercise, while the proportions of neutrophils, monocytes, eosinophils, B cells, and MDSCs decreased. Overall, the profile of immune cells changed towards more cytotoxic/anti-tumorigenic by acute exercise.

The mobilization of immune cells seems to occur rapidly after the start of the exercise. However, the extent to which different immune cell subtypes are mobilized varies considerably (26). Here, both the absolute cell count and proportion of lymphocytes increased during exercise. In healthy individuals, NK cells are most sensitive to exercise stimuli due to their high number of adrenergic receptors (26–28). In this study, the increase in NK cells was also markedly greater than the increase of any other cell type. CD16<sup>+</sup> NK cells increased by 202% at E30 compared to resting state, whereas the maximal increase in total lymphocytes was only 36%. The proportion of total NK cells and CD16<sup>+</sup> NK cells of total leukocytes also increased during exercise. The increase in CD16<sup>-</sup> NK cell count was lower than in CD16<sup>+</sup> NK cells, only 60%, and the proportion of CD16<sup>-</sup> NK cell did not change, which is in line with our previous studies with lymphoma and breast cancer patients (19, 29). Unexpectedly, no correlation between change in heart rate and NK cells was found in this study,



TABLE 3 Exercise-induced changes in T cell proportions.

|                                         | Rest        | E15           | E30          | P30         | P60         |
|-----------------------------------------|-------------|---------------|--------------|-------------|-------------|
| % of total T cells (CD3 <sup>+</sup> )  |             |               |              |             |             |
| CD4 <sup>+</sup>                        | 64.5 (14.5) | 61.6 (16.5)** | 61.5 (16.2)* | 63.7 (11.1) | 65.3 (13.4) |
| CD8 <sup>+</sup>                        | 30.9 (14.5) | 33.2 (15.8)** | 33.2 (15.7)* | 31.8 (11.4) | 30.1 (13.2) |
| CD4 <sup>+</sup> CD8 <sup>+</sup>       | 2.6 (8.2)   | 3.4 (10.6)    | 3.24(10.6)   | 2.8 (9.6)   | 2.6 (8.3)   |
| CD4 <sup>+</sup> CD8 <sup>-</sup>       | 3.3 (1.7)   | 3.7 (2.5)     | 3.7 (2.4)    | 3.2 (1.9)   | 3.1 (1.7)   |
| % of helper T cells (CD4 <sup>+</sup> ) |             |               |              |             |             |
| Th1                                     | 11.8 (8.1)  | 11.5 (7.5)    | 11.4 (7.5)   | 11.2 (7.6)  | 12.0 (8.5)  |
| Th2                                     | 17.7 (14.5) | 17.9 (14.4)   | 17.9 (13.1)  | 18.7 (16.4) | 18.3 (15.5) |
| Th17                                    | 4.6 (2.9)   | 4.9 (3.5)     | 5.0 (3.7)    | 5.0 (3.5)   | 5.2 (4.4)   |
| Treg                                    | 3.4 (2.3)   | 2.8 (2.1)     | 3.5 (2.8)    | 4.5 (4.4)   | 3.8 (3.5)   |

Data presented as mean (SD).

p-values; \* $<0.05$ , \*\* $<0.01$  as compared to rest.

E15, 15-minute timepoint during the exercise; E30, 30-minute timepoint during the exercise; P30, 30 minutes post-exercise; P60, 60 minutes post-exercise.

although NK cell mobilization is known to be depend on exercise intensity via adrenergic signaling (26). There was, however, positive correlation between NK cell mobilization and change in lactate concentration. NK cell mobilization has also been studied in breast cancer survivors, where a significant increase was observed after acute exercise of 10x 3 minute intervals at 60% of VO<sub>2</sub>max (30). Further, in a recently published study, Schenk et al. (31) found that NK cell counts increase after acute exercise in prostate cancer patients. The settings of that study were similar to the current study; it was conducted before the start of cancer treatments and the intensity of the exercise (30-min at 75% of VO<sub>2</sub>max) was comparable as the heart rate percentage of maximal heart rate was 78 at E15 and 84 at E30 in the current study (32). The prognostic value of NK cells has been studied in many types of cancer including breast cancer (33). To date, the prognostic value of tumor-infiltrating NK cells is more evident, however, there are handful of studies also reporting that peripheral NK cell count independently predicts better survival in cancer patients (34–36).

Based on previous studies conducted in healthy individuals (26, 37), it was hypothesized that the increase in CD8<sup>+</sup> T cells would be more pronounced than the increase in CD4<sup>+</sup> T cells subsets. However, the 36% increase in CD8<sup>+</sup> T cell count during exercise was only slightly greater than those in CD4<sup>+</sup> T cells (19%), Th1 cells (27%), Th2 cells (24%), and Th17 cells (23%). The proportion of CD8<sup>+</sup> T cells of total T cells, however, increased and the proportion of CD4<sup>+</sup> T cells decreased during the exercise. Double negative CD4<sup>+</sup>CD8<sup>-</sup> T cells also increased significantly by 43% during exercise. Some CD4<sup>+</sup>CD8<sup>-</sup> T cells are  $\gamma\delta$  T cells which based on studies conducted with healthy individuals, have high response potential to acute exercise (26). Furthermore, absolute CD19<sup>+</sup> B cell count did not change, and it has been previously described that B cells respond to adrenergic signaling to a lesser extent than NK cells and T cells (26), which is likely to explain these results. Based on previous studies in healthy individuals, it appears that lymphocytes with cytotoxic potential, hence NK cells, CD8<sup>+</sup> T cells, and  $\gamma\delta$  T cells, are mobilized more than other lymphocytes during acute

stress (38). In this study, the same pattern was observed in breast cancer patients. Comprehensive clinical significance of the mobilization of cytotoxic immune cells is yet to be investigated, but there is great evidence from preclinical studies that immune cell mobilization enhances tumor killing. Studies in animal models have found that epinephrine and IL-6 mediated mobilization of NK cells during exercise decreases tumor size (5), and that administration of lactate reduces tumor growth in cytotoxic T cell-dependent manner (6). It is not yet known whether exercise produces similar responses in cancer patients, but this study shows that cytotoxic immune cells are increased by exercise and that the mobilization of lymphocytes correlates with lactate concentration.

To the best of our knowledge, the effect of acute exercise on MDSCs has not been studied in humans before, including cancer patients. MDSCs are immunosuppressive cells that proliferate under chronic diseases such as cancer. In fact, cancer cells attract MDSCs to the tumor site where they enhance a pro-tumor microenvironment (39). In the present study, we found no change in the absolute number of total MDSCs, granulocytic MDSCs, or monocytic MDSCs after exercise. However, the proportions of total MDSCs and g-MDSCs decreased during exercise compared to baseline. Because MDSCs are pro-tumorigenic, it can be considered that a decrease in their proportion and on the other hand increase in cytotoxic T and NK cells changes the blood profile toward more cytotoxic and is thus beneficial for cancer patients. It is not known, however, if these changes in circulating blood correlate with changes in tumor microenvironment during and after exercise. Another pro-tumorigenic cell type is regulatory T cells, which inhibits the activity of effector T cells (40). Here we did not see any change in their number or proportion during or after exercise, which can again be a positive sign for cancer surveillance. Previously, regulatory T cell counts have been studied in patients with chronic lymphocytic leukemia (41). Those authors saw no change immediately after 45–60 minutes of cycling/running exercise, but a decrease was found 1 hour afterwards. In breast cancer survivors, the proportion of regulatory T cells is decreased 24 h after half marathon (42). A

study conducted in mice finds that CD8<sup>+</sup> T cell/regulatory T cell ratio doubled in breast cancer tumors after 8 weeks of training due to decreased number of regulatory T cells recruited to the tumor site (8). Further, another study with breast cancer mouse model finds that accumulation of MDSCs in tumor is reduced after 30 days of daily exercise when compared to sedentary control group (7). Such results are promising, but further investigation is needed to see whether these changes occur also in humans.

Granulocytes and monocytes have high functional diversity in tumor sites; some subtypes have pro-tumoral and some anti-tumoral effects (43–45). Therefore, it is difficult to predict whether their mobilization during exercise is beneficial to cancer patients. In the present study, the increase in absolute neutrophil count was significant only at E15 compared to baseline, which was unexpected since prolonged increase in neutrophils after exercise is commonly observed in healthy individuals (46, 47). The basophil count increased at E15 and was still elevated 30 minutes post-exercise. However, the proportion of basophils did not change. Oppositely, the absolute count of eosinophils did not change, but the proportion of eosinophils decreased at E15 compared to baseline. Exercise-induced changes in basophils and eosinophils have been studied relatively little in healthy individuals with inconsistent results (27, 47–49), and to the best of our knowledge, this is the first study to examine basophil and eosinophil responses to acute exercise in cancer patients. In breast cancer survivors, neutrophil, basophil, and eosinophil count clustered as granulocytes has been studied in response to half marathon, after which the granulocyte count increases but then decreases to baseline 24 h after exertion (42). However, it is well recognized that the intensity and duration of exercise affects the magnitude of immune cell mobilization (50–52), and thus a half marathon is very different stimulus compared to the 30-minute cycle ergometer exercise used in this study. Moreover, we found that total monocytes and non-classical (CD14<sup>+</sup>CD16<sup>+</sup>) monocytes increased at E15, but there was no change in classical or intermediate monocytes. The mobilization of non-classical monocytes, but not of classical or intermediate monocytes, have been linked to adrenergic activation and it has been suggested that they mobilize more compared to other monocyte subpopulations also in healthy individuals (26, 38). However, the number of intermediate (CD14<sup>+</sup>CD16<sup>+</sup>) and classical (CD14<sup>+</sup>CD16<sup>+</sup>) monocytes have been observed to increase after a 45-minute exercise bout in breast cancer survivors (53), and in our previous study, the number of intermediate monocytes, but not classical or non-classical monocytes was increased in breast cancer patients immediately after 10 minutes of exercise (29). Thus, the data are conflicting.

Based on the results from animal cancer models (5, 6), the immune cell mobilization due to exercise is believed to act as a key regulator in the positive effects of exercise on cancer. Thus, it is an encouraging non-pharmacologic strategy in cancer prevention and treatment with little to no adverse effects (1). In preclinical studies, exercise has also been shown to increase the effect of combined radiotherapy and immunotherapy in reducing tumor volume when compared to sedentary controls (7). Previous studies in healthy individuals combined with our results in breast cancer patients show that the mobilization of immune cells occurs fast and transiently after starting the exercise (54). In this study, the

maximal increases in immune cell counts were all observed at E15, except for NK cells, which peaked at E30. For many subpopulations, the number decreased back to baseline at the end of the exercise. In fact, it is suggested that the positive effects of immune cell modulation by exercise occurs during and soon after exercise and with regular exercise, the effect accumulates (55). Although the phenomenon is well studied in healthy individuals, exercise-induced mobilization may not be exactly similar in people having chronic diseases, such as cancer. Breast cancer type and disease stage may contribute to the magnitude of this phenomenon and therefore we also analyzed associations between well-established biomarkers and immune cell mobilization. We found that CD8<sup>+</sup> T cell mobilization at E15 correlated negatively with ER positivity and that total T cell and CD8<sup>+</sup> T cell mobilization correlated negatively with PR positivity both at E15 and at E30. Interestingly, similar significant correlation between ER positivity and CD8<sup>+</sup> T cells were found in our recent study where breast cancer patients did a 10-minute acute cycling exercise with light-to-moderate exercise intensity (29). The explanation for these observations is however unknown, but it may appear that cancer status indeed affects these responses. However, we also acknowledge that some correlations can be just random findings. In fact, when significance level of the p-value was set to 0.01, only the correlation between PR positivity and CD8<sup>+</sup> T cell mobilization remained significant. No difference have been observed in the number of circulating T cells between different breast cancer subtypes, but HER2-positive and triple negative breast cancers typically have higher level of lymphocytes infiltrated in the tumor than hormone receptor positive cancers (56). This area warrants further studies to explore whether cancer status affects exercise-induced immune cell mobilization.

The study has certain strengths and limitations. The biggest strength of our study was the timing of the consecutive blood sampling, especially the two samples during the exercise. If blood samples would have been drawn only pre- and immediately post-exercise, a lot of information would have been lost as many of the changes were transient. Indeed, the majority of the significant changes were observed half-way during the exercise. Second, we analyzed complete blood counts and calculated plasma volume change during the exercise. Therefore, we were able to correct the effect of plasma volume change on immune cell concentrations. However, it is known that intensity of exercise has an effect on immune cell mobilization, but we did not control the intensity in a standardized manner that could enable the same relative exercise intensity for each patient. This could have led to variance in responses to exercise. Controlling this would have required maximal fitness testing, which would have been time-consuming and stressful. This study was conducted in a short window between cancer diagnosis and treatments making it also challenging to schedule the maximal fitness tests and perform the study protocol. However, all patients reached the intended 70% of maximal heart rate goal, and the patients classified the rate of exertion during the exercise bout as moderate on a Borg scale with very small standard deviations. As a strength, the exercise itself represented a real-world situation as the subjects were allowed to exercise as they would exercise in their daily living. Further, a correlation analysis was performed to examine the influence of

exercise intensity as well as disease status on immune cell mobilization in these participants. Given that our correlation values were not particularly strong, and p-values were mostly just below 0.05, we acknowledge that we must be precautionary with the interpretations of these associations. These analyses are explorative in nature and findings must be repeated in other studies.

## Conclusions

The present study aimed to examine changes in circulating immune cell counts in breast cancer patients conducting acute cycling exercise before onset of a curative oncologic treatment. We found that 30-min acute exercise is sufficient to mobilize total leukocytes, neutrophils, lymphocytes, monocytes, basophils, total T cells, CD4<sup>+</sup> T cells, Th 2 cells, Th 17 cells, CD8<sup>+</sup> T cells, CD4<sup>+</sup>CD8<sup>+</sup> T cells, NK cells, and CD14<sup>+</sup>CD16<sup>+</sup> monocytes. Additionally, our findings suggest that the profile of immune cells change towards more cytotoxic/anti-tumorigenic as the proportions of CD8<sup>+</sup> T cells and NK cells increased and the proportion of MDSCs proportion decreased in breast cancer patients with 30-minute exercise. Further studies are however needed to examine the clinical significance of this exercise-induced immune cell mobilization and they also should focus on the function of pro-tumor and anti-tumor immune cells in the context of exercise and cancer and whether cancer status affects exercise-induced immune cell mobilization.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The studies involving humans were approved by Ethics Committee of the Hospital District of Southwestern Finland. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

TK: Data curation, Formal analysis, Investigation, Writing – original draft. SL: Investigation, Writing – review & editing. JNe: Investigation, Writing – review & editing. JNo: Investigation, Writing

– review & editing. MH: Conceptualization, Writing – review & editing. CS: Writing – review & editing. HR: Conceptualization, Methodology, Writing – review & editing. HM: Conceptualization, Writing – review & editing. PR: Conceptualization, Methodology, Writing – review & editing. IH: Conceptualization, Funding acquisition, Writing – review & editing.

## Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The study was financially supported by Academy of Finland and Hospital District of South-West Finland.

## Acknowledgments

The authors thank the personnel of Turku PET Centre for their help in this project and the participants for volunteering their time to this study.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1394420/full#supplementary-material>.

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RECEIVED 16 March 2024

ACCEPTED 16 July 2024

PUBLISHED 26 July 2024

## CITATION

Kadantseva K, Subbotin V, Akchulpanov R, Berikashvili L, Yadgarov M, Zhukova L, Kvetenadze G, Svitich O, Kukina P, Kuznetsov I, Shemetova M, Smirnova A, Polyakov P, Shebankov A and Likhvantsev V (2024) The impact of inhalation versus total intravenous anesthesia on the immune status in patients undergoing breast cancer surgery: a double-blind randomized clinical trial (TeMP). *Front. Oncol.* 14:1401910. doi: 10.3389/fonc.2024.1401910

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# The impact of inhalation versus total intravenous anesthesia on the immune status in patients undergoing breast cancer surgery: a double-blind randomized clinical trial (TeMP)

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**Background:** Breast cancer (BC) mortality primarily stems from metastases rather than the primary tumor itself. Perioperative stress, encompassing both surgical and anesthetic factors, profoundly impacts the immune system, leading to alterations in neuroendocrine pathways and immune functions, potentially facilitating tumor progression and metastasis. Understanding the immunomodulatory effects of different anesthesia techniques is crucial for optimizing perioperative care in patients with BC. The neutrophil-to-lymphocyte ratio (NLR) serves as one of the key indicators of perioperative immune response.

**Objective:** To compare the effects of inhalation anesthesia (IA) and total intravenous anesthesia (TIVA) on perioperative immune response in BC surgery patients.

**Methods:** In this randomized, double-blind clinical trial, BC surgery patients were randomized to receive either TIVA with propofol or IA with sevoflurane. The primary endpoint was NLR assessment. Secondary immune parameters measured included natural killer cells, various T cell subsets, B cells, the immuno-regulatory index [T-helpers (CD3+CD4+)/cytotoxic T-cells (CD3+CD8+)], matrix metalloproteinases (MMP-9), complement components, and immunoglobulins, preoperatively and at 1 and 24 hours postoperatively.

**Results:** The study included 98 patients (IA: 48, TIVA: 50). The baseline characteristics exhibited remarkable similarity across the groups. No significant difference in absolute NLR values was found between IA and TIVA groups at any time point (1 hour:  $p = 0.519$ , 24 hours:  $p = 0.333$ ). Decreased IgA and IgM levels post-surgery suggested potential negative impacts of IA on humoral immunity compared to TIVA. CRP levels increased more by 24 hours ( $p = 0.044$ ) in IA compared to TIVA. No significant differences were observed in natural killer cells, T cell subsets, B cells, MMP-9 levels or complement components between groups. Significant differences in the immuno-regulatory index between the TIVA and IA groups at one hour postoperatively ( $p = 0.033$ ) were not maintained at 24 hours.

**Conclusion:** While there were no notable differences in NLR among the types of anesthesia, the observed disparities in immunoglobulin content and C-reactive protein levels between groups suggest that we cannot dismiss the potential immunosuppressive effects of inhalational anesthesia in breast cancer surgeries. Further investigation needed to clarify the impact of various anesthesia methods on immune function and their implications for long-term cancer outcomes.

#### KEYWORDS

anesthesia, cancer, immunity, propofol, sevoflurane

## 1 Introduction

The leading cause of mortality in breast cancer (BC) is metastases, not the primary tumor, leading to 30–40% mortality despite the application of surgery, radiation, and chemotherapy (1). Mastectomy and breast resection are primary treatment methods for BC. It is well-established that perioperative stress, associated with neuroendocrine and immune dysfunctions, plays a crucial role in enhancing the survival of circulating tumor cells and minimal residual disease (2). Various clinical studies have demonstrated that anesthesia also contributes to perioperative stress and may influence cancer recurrence and survival (3, 4). It is hypothesized that surgical interventions in oncological patients may induce suppression of cellular immunity, potentially contributing to adverse long-term outcomes. Multiple studies have shown that using inhaled anesthetics during cancer surgery could lead to worse survival outcomes than using the intravenous anesthetic propofol (5–8).

In a 2023 meta-analysis of 14,036 patients, findings indicated total intravenous anesthesia (TIVA) significantly enhances post-surgical oncological prognosis compared to inhalation anesthesia (IA). This was evidenced by improved overall survival, increased recurrence-free survival, and diminished post-operative pathological manifestations among cancer surgery patients (9). This analysis underscores TIVA's efficacy in fostering more favorable long-term health outcomes in oncological patient care (9). The observed disparities in clinical outcomes may primarily result from the specific effects exerted by anesthetic agents on immune cell populations, notably natural killer (NK) cells,

cytotoxic T lymphocytes (CTLs), and T-helper (Th) cells. Furthermore, the influence of anesthesia on the neutrophil-to-lymphocyte ratio (NLR) elucidates the intricate relationship between anesthesia and immune modulation during surgical interventions (10–12). Oncological processes involve alterations in immune system function at both local and systemic levels, as evidenced by blood parameter changes (13). The neutrophil-to-lymphocyte ratio serves as a marker for assessing inflammation levels, prognosticating cancer and other etiologies, particularly in preoperative preparation (14).

NLR is a simple marker in peripheral blood and is used to assess inflammatory response and physiological stress during the perioperative period (15). Anesthetic technique may influence NLR, thereby modulating the inflammatory response and surgical outcomes (16). Research spanning a variety of cancer types and stages has demonstrated the prognostic importance of the NLR, revealing that higher NLR levels correlate with diminished survival rates (17).

The study aimed to evaluate the relationship between NLR and anesthetic types in patients undergoing BC surgery.

## 2 Materials and methods

### 2.1 Trial design

We conducted a prospective, randomized, double-blind superiority clinical trial. The protocol was approved by the

institutional ethics committee (# 2/2021), registered at <https://clinicaltrials.gov> (NCT04800393). The study was started on the 29th of March 2022. The randomization was conducted before the surgery, following the signing of informed consent by patients who presumably met the eligibility criteria. This process employed block randomization with variable block sizes ranging from 20 to 40 patients to ensure statistical balance and allocation concealment across study arms. Researchers, in collaboration with a biostatistician, prepared a series of numbered, opaque, and sealed envelopes, each indicating one of the anesthesia methods used (TIVA or IA). Patients were randomly allocated to TIVA or IA groups before induction of anesthesia. In compliance with the double-blind study protocol, patients and outcome assessors were blinded regarding group assignment. The protocols were implemented to maintain the confidentiality of allocation envelopes, preventing unauthorized disclosure of group assignments, thereby upholding the integrity and validity of the study's findings. The investigators planned and designed this study in accordance with the recommendations of the Declaration of Helsinki (18). The protocol was drawn up in accordance with the recommendations of SPIRIT 2013 (19). The manuscript adheres to the Consolidated Standards of Reporting Trials (CONSORT) guidelines (20). The CONSORT checklist is provided in the [Supplementary Table S1](#).

## 2.2 Study population

During the study period from March 2022 to September 2023, an assessment of surgeries for BC was conducted. All patients scheduled for this type of surgical intervention were evaluated according to the existing inclusion and exclusion criteria. If a patient met eligibility criteria, a bedside evaluation and written informed consent were obtained. The study enrolled participants who voluntarily provided informed consent, within the age range of 45 to 74 years, diagnosed with primary operable BC at stages IA-IIA (T1–2, N0, M0) through cytological verification, without prior chemotherapy. In the study, two post-randomization exclusion criteria were applied: withdrawal of informed consent (refusal to continue participation in the study) and incomplete surgical intervention (non-resected lesions verified in the early postoperative period, 2 weeks post-surgery). Patients with a history of other oncological diseases in other locations, history of substance abuse, or autoimmune diseases were not included in the study. All eligibility criteria are presented in [Supplementary Table S2](#).

## 2.3 Outcomes

The primary endpoint was NLR.

The secondary endpoints were matrix metalloproteinase 9 (MMP-9), immuno-regulatory index [T-helpers (CD3+CD4+)/cytotoxic T-cells (CD3+CD8+)], NK cells (CD3-CD16+) in the blood, C-reactive protein (CRP).

Other endpoints included: T cells of blood (CD3+), T helpers of blood (CD3+CD4+), Cytotoxic T cells of blood (CD3+CD8+), B cells of blood (CD19+CD3-), T cells (CD3+) + B cells (CD19+

CD3-) + NK cells (CD3-CD16+) of blood, IgA, IgM, IgG, complement component C3, complement component C4.

All endpoints were assessed at three time points: before induction of anesthesia, 1 hour after and 24 hours after completion of surgery.

## 2.4 Anesthesia

The enrolled patients were not premedicated. Intraoperative monitoring included electrocardiography (ECG), pulse oximetry, and non-invasive arterial blood pressure measurement. Induction of anesthesia in both groups was performed using propofol at a dosage of 1.5–2.2 mg/kg, fentanyl at 3–5 µg/kg, and a muscle relaxant (either rocuronium bromide or cisatracurium). Muscle relaxation was maintained until neuromuscular blockade reached a Train-Of-Four (TOF) count of 10–0%. Tracheal intubation was carried out with a tube of the appropriate size.

After induction and tracheal intubation, patients from both study groups underwent mechanical ventilation in a pressure control ventilation–volume guaranteed mode using General Electric Avance CS2 or General Electric Medical Systems, USA. Ventilation parameters included an oxygen fraction (FiO<sub>2</sub>) of 35–40%, tidal volume (TV) of 6–8 ml/kg, positive end-expiratory pressure (PEEP) of 5 cm H<sub>2</sub>O, an inhalation to exhalation ratio (I:E) of 1:2, and a respiratory rate sufficient to maintain normocapnia (35–45 mm Hg).

Within the TIVA group, propofol was administered for anesthetic maintenance with a dosage of 0.1–0.2 mg/kg/min based on the Schnider model. Conversely, the IA group received sevoflurane for maintaining anesthesia levels. Specifically, sevoflurane was administered at an end-tidal concentration of approximately 1 MAC. The gas mixture used consisted of 35–40% oxygen and air with no nitrous oxide (N<sub>2</sub>O) administered. The flow rates were adjusted to maintain optimal oxygenation and ventilation parameters throughout the procedure. The mean blood pressure was maintained above 60 mmHg. Fentanyl was dosed individually by the anesthesiologist.

From the start of skin suture application, patients were transitioned to assisted ventilation in the pressure support ventilation-pro mode with the following parameters: flow trigger at 0.2 L/min, support pressure necessary to achieve TV of 6–8 ml/kg with a maximum airway pressure not exceeding 35 cm H<sub>2</sub>O, PEEP of 5–8 cm H<sub>2</sub>O, and maintaining normocapnia. Extubation of patients was conducted upon reaching TOF 0.95 and higher.

Transfer from the operating room to the postoperative recovery unit or the ward was based on the severity and extent of the surgical intervention, hemodynamic stability, restoration of spontaneous breathing, and absence of the need for oxygen support. For transfer from the operating room to the ward, the patient, post-extubation, had to score 9 or more points on the modified Aldrete recovery scale.

## 2.5 Blood samples

Venous blood samples were collected before the induction of anesthesia, one hour after surgery, and 24 hours following the

completion of the surgery. Each blood collection was performed into three tubes: for immunological research with a separating gel or coagulation activator (to obtain serum); for flow cytometry with K2-EDTA (2-substituted potassium salt of ethylenediaminetetraacetic acid); for general blood test with K2-EDTA – 10 ml.

After each blood collection, the tubes with the biomaterial were immediately transported to the clinical immunology laboratory for further determination of the parameters under study.

Multiparametric analysis helps determine the phenotype of cell populations. Flow cytometry analysis was conducted to evaluate various immune cell populations using specific CD markers with a BD FACSCanto II flow cytometer (BD Biosciences, USA). Detection on this flow cytometer occurred through two types of signals: light scattering (forward and side scatter) and fluorescence emission. Data analysis for identifying T cells (CD3+), T-helper cells (CD3+CD4+), cytotoxic T cells (CD3+CD8+), B cells (CD19+CD3-), and NK cells (CD3-CD16+) involved gating procedures that restrict the analysis to signals from cell populations that meet specific morphological and expression (fluorescence) profiles. Fluorescence plots were then generated within the selected gates. Detailed gating strategies and representative flow cytometry plots from several patients are provided in the [Supplementary Figures S1, S2](#) to enhance transparency and validate our data (21).

The concentration of serum proteins CRP, IgA, IgM, IgG, C3, C4 was measured by nephelometry using a “BN ProSpec” laser nephelometer (22). The concentration of MMP-9 in serum was determined by enzyme immunoassay using the Human MMP-9 Quantikine ELISA Kit strictly in accordance with the instructions for the kit (23).

## 2.6 Data collection

For each study participant, a case report form was completed based on comprehensive examination results. This form documented all data in alignment with the preoperative period, including demographic information (age, gender, height, weight), history of comorbidities, and initial laboratory findings; intraoperative period, detailing anesthesia type, laboratory parameters, and intraoperative monitoring data; and postoperative period, noting complications during hospitalization, laboratory parameters. Morphological data were collected, encompassing cancer staging based on various classifications, including TNM staging, molecular subtype, and HER2 status. Subsequently, these data were entered into an electronic database for further analysis.

## 2.7 Statistical analysis

The sample size for this study was determined based on an evaluation of NLR in patients undergoing BC surgery with TIVA or IA, as reported in two prior studies (15, 24). We calculated that a cohort of 130 patients would provide the study with 80% power to detect an effect size of 0.78. This calculation assumes a standard deviation of 1.5 and a two-sided alpha of 0.05. Additionally, it

accounts for an anticipated 10% loss due to loss of follow-up and withdrawal of consent.

Due to difficulties in the supply and procurement of certain reagents, the steering committee stopped recruitment on September 4, 2023, after 98 patients had undergone randomization. This decision was made without knowledge of the interim analysis results. We assessed the sample power (superiority) for NLR with a margin ( $\delta$ ) = 1 (25).

All analyses were conducted using IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY, USA: IBM Corp. Continuous variables with normal and skewed distributions were expressed as mean  $\pm$  Standard Deviation (SD) and median with Interquartile Range (IQR), respectively. Normality of data was evaluated using the Shapiro-Wilk test and histogram analysis.

Dichotomous variables were analyzed using a two-tailed Chi-square test or Fisher's exact test, with the Fisher-Freeman-Halton extension applied when necessary. The Mann-Whitney U test compared nonparametric continuous variables between independent groups. For paired samples, analysis involved the Friedman test with Dunn's *post-hoc* test or the Wilcoxon signed-rank test.

All tests were two-sided, with a significance threshold set at  $p < 0.05$ .

A logistic regression model with backward stepwise selection (Wald) was used for sensitivity analysis, aiming to identify predictors of the primary outcome and control for baseline imbalances. Variables with a univariate  $p$ -value  $< 0.10$  were included in the model, with anesthesia method being a mandatory inclusion. Collinearity and overfitting risks were assessed using stepwise regression and Spearman correlation tests. In multivariate analyses, variables were reported as odds ratios (ORs) with 95% CIs.

We conducted two types of analyses – intention-to-treat and per-protocol (for the primary endpoint) due to the inadvertent inclusion of 3 patients who did not meet the inclusion criteria (stage 0).

## 3 Results

### 3.1 Patients

A total of 324 patients were assessed for eligibility at the A. Loginov Moscow Clinical Scientific Center from March 29, 2022, to September 4, 2023. Of these, 278 met the inclusion criteria; however, 180 were excluded due to autoimmune diseases (158 patients) or a history of cancer at another location (22 patients). Consequently, 98 patients were randomized: 48 to the IA group and 50 to the TIVA group. The primary outcome analysis included 97 patients, with one patient excluded due to missing data from a blood draw error (Figure 1).

The baseline patient characteristics, oncological characteristics, type of surgery, and duration of anesthesia were similar between study groups (Tables 1, 2). The median age was 62 years (IQR, 55–68). The majority of patients underwent radical mastectomy (69 patients, 70%). The ICU stay did not exceed 24 hours, and the

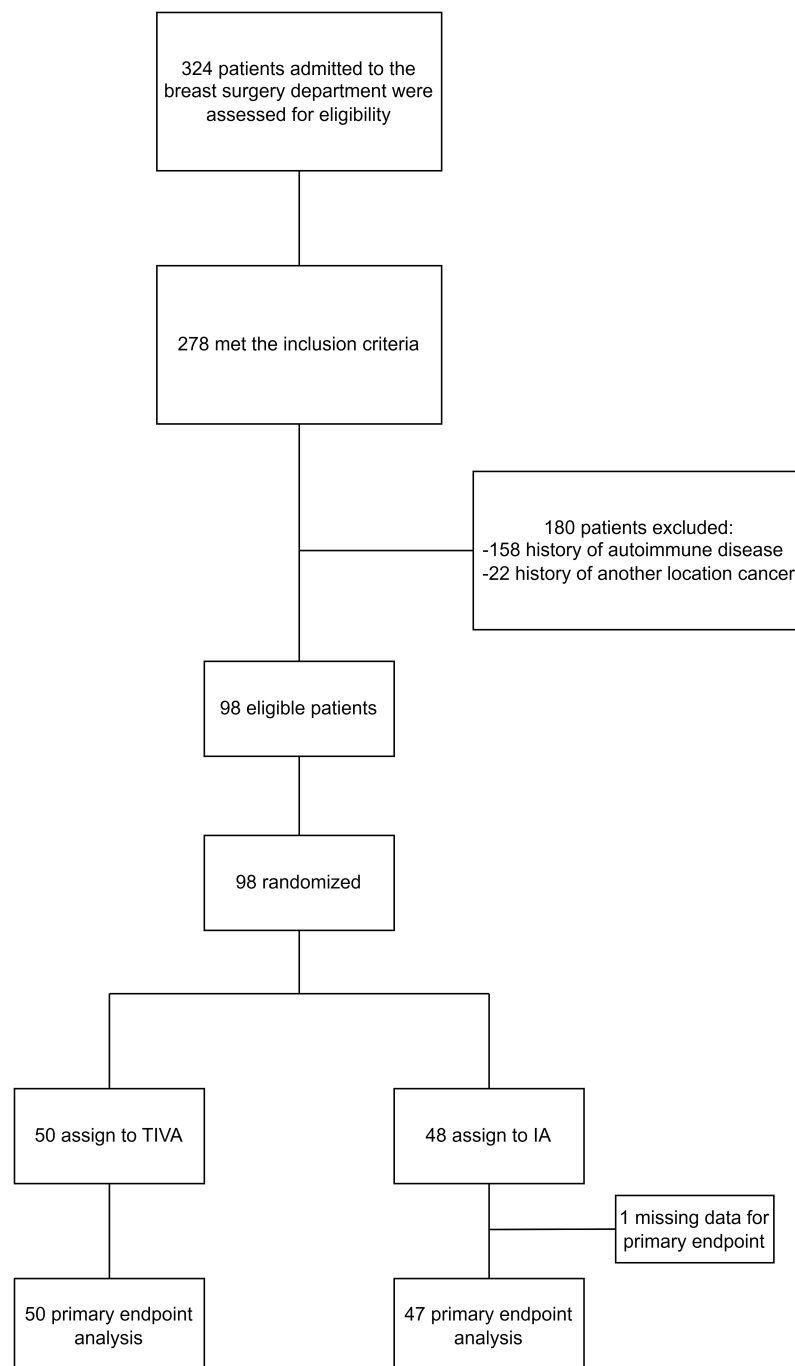


FIGURE 1  
Flow chart of the study.

length of stay in the hospital was 3 days in the IA group (IQR, 3–5) compared to 4 days in the TIVA group (IQR, 3–5),  $p = 0.131$ .

### 3.2 Primary outcome

The absolute NLR did not significantly differ between the IA and TIVA groups either pre-operatively (1.7, IQR 1.24–2.43 in the

IA group versus 1.65, IQR 1.37–2.17 in the TIVA group,  $p = 0.767$ ), at 1 hour post-operatively (4.54, IQR 2.40–9.00 versus 4.42, IQR 2.50–6.67,  $p = 0.519$ ), and at 24 hours post-operatively (3.25, IQR 2.47–4.73 versus 3.00, IQR 2.39–3.64,  $p = 0.333$ ), as indicated in Table 3. No differences between groups were noted in the percentage of change from baseline to 1 hour ( $p = 0.294$ ) and to 24 hours ( $p = 0.636$ , Figure 2). Similar results were observed when assessing the relative NLR (Table 3).



TABLE 1 Patients' baseline characteristics.

| Characteristics                       |                 | IA,<br>N = 48         | TIVA,<br>N = 50         | p-value            |
|---------------------------------------|-----------------|-----------------------|-------------------------|--------------------|
| Age, years; N, Me (IQR)               |                 | 48, 62.5<br>(56–68)   | 50, 61<br>(54–68)       | 0.545 <sup>1</sup> |
| BMI, kg/m <sup>2</sup> ; N, Me (IQR)  |                 | 46, 29<br>(23.8–32.0) | 50, 27.6<br>(23.4–31.2) | 0.687 <sup>1</sup> |
| Comorbidities, No. (%)                |                 |                       |                         |                    |
| COVID-19 history                      |                 | 29, 60%               | 27, 54%                 | 0.521 <sup>2</sup> |
| Uncontrolled diabetes                 |                 | 3, 6.3%               | 0, 0%                   | 0.114 <sup>3</sup> |
| Chronic obstructive pulmonary disease |                 | 1, 2.1%               | 1, 2.0%                 | 0.999 <sup>3</sup> |
| Cerebrovascular diseases              |                 | 1, 2.1%               | 0, 0%                   | 0.490 <sup>3</sup> |
| Peripheral artery diseases            |                 | 1, 2.1%               | 0, 0%                   | 0.490 <sup>3</sup> |
| Diabetes                              |                 | 5, 10.4%              | 2, 4.0%                 | 0.264 <sup>3</sup> |
| Arterial hypertension                 |                 | 30, 63%               | 28, 56%                 | 0.513 <sup>2</sup> |
| Chronic kidney disease                |                 | 0, 0%                 | 1, 2.0%                 | 0.999 <sup>3</sup> |
| Coronary artery disease               |                 | 3, 6.3%               | 4, 8.0%                 | 0.999 <sup>3</sup> |
| Atrial fibrillation                   |                 | 2, 4.2%               | 0, 0%                   | 0.237 <sup>3</sup> |
| Arrhythmias                           |                 | 3, 6.3%               | 1, 2.0%                 | 0.357 <sup>3</sup> |
| Heart failure                         |                 | 6, 12.5%              | 2, 4.0%                 | 0.155 <sup>3</sup> |
| Liver failure                         |                 | 0, 0%                 | 0, 0%                   | NA                 |
| Dementia                              |                 | 0, 0%                 | 0, 0%                   | NA                 |
| Rheumatologic disease                 |                 | 0, 0%                 | 0, 0%                   | NA                 |
| Peptic ulcer disease                  |                 | 0, 0%                 | 1, 2.0%                 | 0.999 <sup>3</sup> |
| Hemiplegia                            |                 | 0, 0%                 | 0, 0%                   | NA                 |
| Leukemia                              |                 | 0, 0%                 | 0, 0%                   | NA                 |
| Lymphoma                              |                 | 0, 0%                 | 0, 0%                   | NA                 |
| HIV/Hepatitis                         |                 | 0, 0%                 | 0, 0%                   | NA                 |
| Morphometric characteristics          |                 |                       |                         |                    |
| Tumor site (right)                    |                 | 23, 48%               | 20, 40%                 | 0.430 <sup>2</sup> |
| TNM classification                    | T <sub>is</sub> | 1, 2.2%               | 2, 4.0%                 | 0.579 <sup>4</sup> |
|                                       | T1              | 34, 71%               | 31, 62%                 |                    |
|                                       | T2              | 13, 27%               | 17, 34%                 |                    |
|                                       | N0              | 48, 100%              | 50, 100%                | NA                 |
|                                       | M0              | 48, 100%              | 50, 100%                | NA                 |
| Tumor cell differentiation (G)        | G1              | 8, 17%                | 10, 20%                 | 0.945 <sup>2</sup> |
|                                       | G2              | 25, 54%               | 26, 52%                 |                    |
|                                       | G3              | 13, 28%               | 14, 28%                 |                    |
| Stage                                 | 0               | 1, 2.2%               | 2, 4.0%                 | 0.204 <sup>3</sup> |
|                                       | IA              | 34, 71%               | 28, 56%                 |                    |
|                                       | IIA             | 13, 27%               | 20, 40%                 |                    |

(Continued)

TABLE 1 Continued

| Characteristics              |                 | IA,<br>N = 48 | TIVA,<br>N = 50 | p-value            |
|------------------------------|-----------------|---------------|-----------------|--------------------|
| Morphometric characteristics |                 |               |                 |                    |
| Molecular Subtypes           | No data         | 2, 4.2%       | 1, 2.0%         | 0.598 <sup>4</sup> |
|                              | Luminal A       | 21, 44%       | 17, 34%         |                    |
|                              | Luminal B       | 23, 48%       | 28, 56%         |                    |
|                              | Triple negative | 2, 4.2%       | 4, 8.0%         | 0.999 <sup>3</sup> |
| Her2neu+                     |                 | 2, 4.2%       | 2, 4.0%         |                    |

<sup>1</sup>Mann-Whitney test, <sup>2</sup>chi-square test, <sup>3</sup>Fisher's exact test, <sup>4</sup>Fisher-Freeman-Halton extension. NA, not applicable; IA, inhalation anesthesia; TIVA, total intravenous anesthesia; Me, median value; IQR, interquartile range.

TABLE 2 Surgical and anesthesia characteristics.

| Characteristics                         |                   | IA, N = 48                                                        | TIVA,<br>N = 50   | p-value            |
|-----------------------------------------|-------------------|-------------------------------------------------------------------|-------------------|--------------------|
| Surgery type                            | Partial resection | 18, 37.5%                                                         | 11, 22.0%         | 0.093 <sup>2</sup> |
|                                         | Mastectomy        | 30, 62.5%                                                         | 39, 78.0%         |                    |
| Anesthesia time, min.                   |                   | 87.5 (75.0–102.5)                                                 | 90.0 (70.0–105.0) | 0.839 <sup>1</sup> |
| Surgery time, min.                      |                   | 70.0 (60.0–90.0)                                                  | 70.0 (60.0–90.0)  | 0.937 <sup>1</sup> |
| Time of anesthetic administration, min. |                   | 85 (75–100)                                                       | 90 (70–105)       | 0.908 <sup>1</sup> |
| Intraoperative Fentanyl (mg)            |                   | 0.3 (0.3–0.4)                                                     | 0.4 (0.3–0.5)     | 0.534 <sup>1</sup> |
| Intraoperative propofol (mg)            |                   | 1.5–2.2 mg/kg-induction of anesthesia<br>No - maintain anesthesia | 800 (600–1000)    | NA                 |
| Intraoperative sevoflurane (MAC)        |                   | 0.9 (0.8–1.0)                                                     | No                | NA                 |

<sup>1</sup>Mann-Whitney test, <sup>2</sup>chi-square test. NA, not applicable; IA, inhalation anesthesia; TIVA, total intravenous anesthesia.

### 3.3 Secondary outcomes

Patients in the IA group exhibited significantly lower CRP levels preoperatively ( $p = 0.023$ ) and 1 hour post-operatively ( $p = 0.038$ ). However, the increase in CRP from baseline to 24 hours post-operatively was more marked in the IA group ( $p = 0.044$ ), as detailed in [Table 3](#). Levels of MMP-9 and NK cells were comparable in both groups at all assessment time points ([Table 3](#)). The immunoregulatory index (CD4+/CD8+ ratio) was comparable in both groups at all assessment time points, however, the change from baseline to 1 hour post-operatively was statistically different ( $p = 0.033$ ), as detailed in [Table 3](#).

**TABLE 3** Serum biomarkers in patients receiving total intravenous anesthesia vs. patients receiving inhalation anesthesia.

| Laboratory parameters                          |                                    | IA, N = 48<br>N, Me (IQR)      | TIVA, N = 50<br>N, Me (IQR)   | p-value <sup>1</sup> |
|------------------------------------------------|------------------------------------|--------------------------------|-------------------------------|----------------------|
| Absolute NLR (abs. neutrophil/abs. lymphocyte) | Preoperative                       | 47, 1.7 (1.24–2.43)            | 50, 1.65 (1.37–2.17)          | 0.767                |
|                                                | 1 h. postoperative                 | 47, 4.54 (2.4–9)               | 49, 4.42 (2.5–6.67)           | 0.519                |
|                                                | % of change from baseline to 1 h.  | 47, 246.9 (36.4–343.6)         | 50, 135.7 (34.2–261.1)        | 0.294                |
|                                                | 24 h. postoperative                | 45, 3.25 (2.47–4.73)           | 49, 3 (2.39–3.64)             | 0.333                |
|                                                | % of change from baseline to 24 h. | 47, 79.3 (19.6–182.5)          | 50, 64.4 (18.5–138.8)         | 0.636                |
| Relative NLR (rel. neutrophil/rel. lymphocyte) | Preoperative                       | 47, 1.69 (1.26–2.39)           | 50, 1.64 (1.37–2.18)          | 0.885                |
|                                                | 1 h. postoperative                 | 47, 4.5 (2.41–8.5)             | 49, 5.02 (2.77–6.89)          | 0.950                |
|                                                | 24 h. postoperative                | 45, 3.25 (2.5–4.64)            | 49, 3 (2.37–3.65)             | 0.303                |
| CRP, mg/l                                      | Preoperative                       | <b>48, 0.7 (0.3–2.18)</b>      | <b>50, 1.49 (0.8–3.9)</b>     | <b>0.023</b>         |
|                                                | 1 h. postoperative                 | <b>48, 0.77 (0.32–2.25)</b>    | <b>50, 1.45 (0.8–3.9)</b>     | <b>0.038</b>         |
|                                                | % of change from baseline to 1 h.  | 48, -3.19 (-11.18–1.4)         | 50, -0.44 (-6.16–5.26)        | 0.112                |
|                                                | 24 h. postoperative                | 48, 5.84 (2.49–10.7)           | 50, 5.54 (2.87–11.4)          | 0.848                |
|                                                | % of change from baseline to 24 h. | <b>48, 466 (195–1009)</b>      | <b>50, 261 (74–631)</b>       | <b>0.044</b>         |
| IgA, g/l                                       | Preoperative                       | <b>48, 2.33 (1.61–3.04)</b>    | <b>50, 1.09 (0.47–2.25)</b>   | <b>&lt;0.001</b>     |
|                                                | 1 h. postoperative                 | <b>48, 2.27 (1.56–2.98)</b>    | <b>50, 1.42 (0.83–2.42)</b>   | <b>&lt;0.001</b>     |
|                                                | % of change from baseline to 1 h.  | <b>48, -3.01 (-9.25–2.62)</b>  | <b>50, 6.22 (-8–81)</b>       | <b>0.011</b>         |
|                                                | 24 h. postoperative                | <b>48, 2.23 (1.69–2.75)</b>    | <b>50, 1.11 (0.47–1.95)</b>   | <b>&lt;0.001</b>     |
|                                                | % of change from baseline to 24 h. | 48, -5.64 (-15.2–2.99)         | 50, -5.84 (-26.32–6.46)       | 0.629                |
| IgM, g/l                                       | Preoperative                       | 48, 0.82 (0.51–1.22)           | 50, 0.74 (0.34–1.06)          | 0.058                |
|                                                | 1 h. postoperative                 | 48, 0.84 (0.61–1.16)           | 50, 0.72 (0.39–1.2)           | 0.207                |
|                                                | % of change from baseline to 1 h.  | <b>48, -4.55 (-13.06–0.81)</b> | <b>50, 9.3 (-11.91–68.25)</b> | <b>0.031</b>         |
|                                                | 24 h. postoperative                | <b>48, 0.81 (0.59–1.16)</b>    | <b>50, 0.57 (0.36–0.87)</b>   | <b>0.005</b>         |
|                                                | % of change from baseline to 24 h. | 48, -3.4 (-15.31–10.53)        | 50, -9.63 (-35.29–17.3)       | 0.298                |
| IgG, g/l                                       | Preoperative                       | <b>48, 10.1 (8.25–12.7)</b>    | <b>50, 5.76 (2.66–10.6)</b>   | <b>&lt;0.001</b>     |
|                                                | 1 h. postoperative                 | <b>48, 10.05 (8.21–11.7)</b>   | <b>50, 7.98 (3.97–10.2)</b>   | <b>&lt;0.001</b>     |
|                                                | % of change from baseline to 1 h.  | <b>48, -4.77 (-11.73–1.17)</b> | <b>50, -4.3 (-7.19–80.5)</b>  | <b>0.013</b>         |
|                                                | 24 h. postoperative                | <b>48, 9.76 (8.3–11.2)</b>     | <b>50, 5.64 (2.13–9.91)</b>   | <b>&lt;0.001</b>     |
|                                                | % of change from baseline to 24 h. | 48, -7.85 (-15.48–0.81)        | 50, -8.53 (-30.56–2.4)        | 0.589                |
| C3, g/l                                        | Preoperative                       | 48, 1.25 (1.08–1.36)           | 50, 1.3 (1.11–1.49)           | 0.161                |
|                                                | 1 h. postoperative                 | 48, 1.2 (1.03–1.35)            | 50, 1.34 (1.13–1.47)          | 0.078                |
|                                                | 24 h. postoperative                | 48, 1.23 (1.03–1.39)           | 50, 1.31 (1.17–1.48)          | 0.086                |
| C4, g/l                                        | Preoperative                       | 48, 0.31 (0.25–0.35)           | 50, 0.31 (0.24–0.35)          | 0.752                |

(Continued)

TABLE 3 Continued

| Laboratory parameters                            |                                       | IA, N = 48<br>N, Me (IQR)      | TIVA, N = 50<br>N, Me (IQR)     | p-value <sup>1</sup>    |
|--------------------------------------------------|---------------------------------------|--------------------------------|---------------------------------|-------------------------|
|                                                  | 1 h. postoperative                    | 48, 0.3 (0.23–0.33)            | 50, 0.31 (0.23–0.35)            | 0.709                   |
|                                                  | 24 h. postoperative                   | 48, 0.3 (0.26–0.34)            | 50, 0.31 (0.24–0.39)            | 0.621                   |
| MMP-9, ng/ml                                     | Preoperative                          | 29, 19.7 (13.7–20.0)           | 11, 17.4 (13.4–19.3)            | 0.254                   |
|                                                  | 24 h. postoperative                   | 29, 20.0 (15.9–20.0)           | 11, 17.4 (14.7–20.0)            | 0.492                   |
| Absolute neutrophil count,<br>10 <sup>9</sup> /L | Preoperative                          | 47, 3.7 (3.1–4.6)              | 50, 3.3 (2.5–4)                 | 0.086<br>0.866<br>0.217 |
|                                                  | 1 h. postoperative                    | 47, 5.6 (4–7)                  | 49, 5.4 (4.2–7.2)               |                         |
|                                                  | 24 h. postoperative                   | 45, 6.8 (5.3–9.1)              | 49, 5.8 (4.6–8.6)               |                         |
| Absolute lymphocyte count,<br>10 <sup>9</sup> /L | Preoperative                          | 47, 2.06 (1.7–2.6)             | 50, 2.1 (1.5–2.35)              | 0.237                   |
|                                                  | 1 h. postoperative                    | 47, 1.2 (0.7–1.7)              | 50, 1.3 (1–1.6)                 | 0.558                   |
|                                                  | 24 h. postoperative                   | 45, 2 (1.8–2.4)                | 49, 2 (1.6–2.5)                 | 0.604                   |
| Relative neutrophil count, %                     | Preoperative                          | 47, 57.4 (48.4–63.9)           | 50, 55.45 (51.9–62)             | 0.865                   |
|                                                  | 1 h. postoperative                    | 47, 77.9 (62.7–87.4)           | 49, 78.5 (67.2–83.3)            | 0.789                   |
|                                                  | 24 h. postoperative                   | 45, 69.5 (64.4–74.6)           | 49, 67.6 (63.5–71.8)            | 0.440                   |
| Relative lymphocyte count, %                     | Preoperative                          | 47, 33.3 (26.5–39)             | 50, 33.75 (28.4–38.1)           | 0.894                   |
|                                                  | 1 h. postoperative                    | 47, 17.2 (10.2–26)             | 50, 16.4 (11.9–25.5)            | 0.928                   |
|                                                  | 24 h. postoperative                   | 45, 21.5 (15.8–25.6)           | 49, 23 (19.6–26.8)              | 0.269                   |
| T cells (CD3+), %                                | Preoperative                          | 47, 70.4 (64.9–78.9)           | 50, 70.75 (62.2–78)             | 0.829                   |
|                                                  | 1 h. postoperative                    | 47, 67.5 (54.1–74.9)           | 50, 65.95 (54.9–72.3)           | 0.549                   |
|                                                  | 24 h. postoperative                   | 47, 73.8 (65.5–81.2)           | 50, 71.9 (67.2–77.3)            | 0.654                   |
| T helper cell (CD3+CD4+), %                      | Preoperative                          | 47, 61.5 (53.9–66.8)           | 50, 59.75 (53.1–70.8)           | 0.798                   |
|                                                  | 1 h. postoperative                    | 47, 61.1 (53.7–68.2)           | 50, 56.8 (43.7–69.3)            | 0.292                   |
|                                                  | 24 h. postoperative                   | 47, 65.7 (56.7–71.5)           | 50, 63.4 (52.6–72.2)            | 0.518                   |
| Cytotoxic T cell (CD3+CD8+), %                   | Preoperative                          | 47, 32 (25.3–38.6)             | 50, 32.65 (22.4–39.4)           | 0.940                   |
|                                                  | 1 h. postoperative                    | 47, 31 (26–37.6)               | 50, 36 (23–45.1)                | 0.392                   |
|                                                  | 24 h. postoperative                   | 47, 27 (22.7–36.2)             | 50, 30.15 (21.7–38.4)           | 0.433                   |
| Immunoregulatory index<br>(CD4+/CD8+ ratio)      | Preoperative                          | 46, 1.85 (1.51–2.46)           | 50, 1.66 (1.34–3.16)            | 0.872                   |
|                                                  | 1 h. postoperative                    | 47, 1.95 (1.47–2.52)           | 50, 1.54 (0.97–2.84)            | 0.203                   |
|                                                  | % of change from baseline to<br>1 h.  | <b>46, 1.58 (-16.00–18.40)</b> | <b>50, -8.15 (-35.00–10.16)</b> | <b>0.033</b>            |
|                                                  | 24 h. postoperative                   | 45, 2.57 (1.68–3.02)           | 49, 2.13 (1.37–3.01)            | 0.394                   |
|                                                  | % of change from baseline to<br>24 h. | 46, 9.08 (-5.78–33.18)         | 50, 13.39 (-10.35–34.59)        | 0.924                   |
| B cells, %                                       | Preoperative                          | 47, 8.9 (7–11.5)               | 50, 9.65 (6.2–13.1)             | 0.762                   |
|                                                  | 1 h. postoperative                    | 47, 10.7 (8.1–14.3)            | 50, 10.8 (7.7–16.8)             | 0.991                   |
|                                                  | 24 h. postoperative                   | 47, 12.7 (8.5–15.7)            | 49, 12.3 (9.1–16.8)             | 0.484                   |
| NK cells, %                                      | Preoperative                          | 47, 16.2 (11.8–23.4)           | 50, 14.9 (8.7–22.3)             | 0.191                   |
|                                                  | 1 h. postoperative                    | 47, 16 (10.3–27.9)             | 50, 17.5 (10.7–25)              | 0.988                   |
|                                                  | 24 h. postoperative                   | 47, 11.3 (8–16.6)              | 48, 8.95 (6.45–14.15)           | 0.189                   |

(Continued)

TABLE 3 Continued

| Laboratory parameters                                       |                     | IA, N = 48<br>N, Me (IQR)   | TIVA, N = 50<br>N, Me (IQR) | p-value <sup>1</sup> |
|-------------------------------------------------------------|---------------------|-----------------------------|-----------------------------|----------------------|
| T cells (CD3+), B cells (CD19+CD3), NK cells (CD3-CD16+), % | Preoperative        | <b>46, 98.5 (96.6–99.8)</b> | <b>50, 97.2 (95.4–98.6)</b> | <b>0.021</b>         |
|                                                             | 1 h. postoperative  | 47, 98.3 (95.5–99.9)        | 50, 97.25 (93.8–99.2)       | 0.151                |
|                                                             | 24 h. postoperative | 45, 98.4 (96.8–99.4)        | 48, 97.4 (94.6–98.95)       | 0.105                |

<sup>1</sup>Mann-Whitney test. IA, inhalation anesthesia; TIVA, total intravenous anesthesia; Me, median value; IQR, interquartile range. Statistically significant differences are marked with bold font.

3.4 Other outcomes

IgA and IgG levels were significantly higher in the IA group both before surgery ( $p < 0.001$ ) and at 1 hour and 24 hours post-operatively ( $p < 0.001$ ), compared to TIVA group. The IgM level was significantly higher in the IA group at 24 hours after surgery ( $p = 0.005$ ). Notably, in the IA group, all immunoglobulin levels generally decreased 1 hour post-operatively compared to baseline, whereas in the TIVA group, remained unchanged or increased (IgA:  $p = 0.11$ , IgM:  $p = 0.031$ , IgG:  $p = 0.013$ ), Table 3, Figure 2. Significant differences were also observed in the level of T cells (CD3+)+B cells (CD19+CD3-)+NK cells (CD3-CD16+) pre-operatively ( $p = 0.021$ ). Levels of C3, C4, absolute and relative neutrophils and lymphocytes, as well as T (CD3+), B, T helper cells (CD3+ CD4+), CTLs (CD3+CD8+) were comparable in both groups at all assessment time points (Table 3).

3.5 Trends in serum biomarkers levels

Absolute and relative NLR, absolute and relative neutrophil count, and B cells significantly increased at 1 hour and 24 hours from baseline in both groups ( $p$  ranging from  $<0.001$  to  $0.01$ , Table 4). IgM and IgG levels decreased at 24 hours post-operatively in the IA group. Levels of C3 and C4 also decreased in the IA group at 1 hour post-operatively. T cell (CD3+) levels decreased in both groups at 1 hour post-operatively, while the immunoregulatory index increased at 24 hours in the IA group. All trends in serum biomarker levels are presented in Table 4.

3.6 Sensitivity analysis for the primary outcome

For the regression analysis, we categorized patients based on their absolute NLR level at 24 hours ( $\geq 3$  [median value] and others) and included the following predictors in the analysis: type of anesthesia, baseline CRP, baseline IgA, IgM, IgG, and T cells (CD3+)+B cells (CD19+CD3-)+NK cells (CD3-CD16+), and type of surgery. The results of the univariate and multivariate analyses for the association of baseline variables with the primary outcome confirmed the lack of significant effect for the type of anesthesia (univariate OR: 1.2, 95% CI 0.5–2.7,  $p = 0.660$ ; adjusted OR: 1.6, 95% CI 0.6–4.2,  $p = 0.382$ ).

According to the per-protocol analysis, the intention-to-treat result was confirmed, and no significant differences were found

between IA and TIVA at all time points ( $p = 0.982$  at baseline,  $p = 0.715$  at 1 hour, and  $p = 0.429$  at 24 hours postoperatively).

3.7 Power assessment

The sample power for the primary endpoint (superiority for NLR at 24 hours) for values of 3.25 in the IA group vs. 3 in the TIVA group with a standard deviation of 2, a 5% alpha level, and 97 patients was 83.7%.

4 Discussion

4.1 Key findings

The study findings reveal a consistent suppression of cellular immunity in both groups, evidenced by decreased lymphocyte levels and increased neutrophil counts postoperatively, both relative and absolute. This led to a uniform elevation of the NLR across all examined time points. The result indicates that the observed changes are attributable to the presence of perioperative stress in patients undergoing BC surgery, rather than to the specific type of anesthetic agent used.

The most significant finding of this study was the suppression of humoral immunity in the IA group, reflected by decreased IgA and IgM levels, whereas these markers were elevated in the TIVA group. These observations highlight the potential immunosuppressive consequences of IA, which could have implications for postoperative recovery and long-term outcomes in oncological settings (26, 27). The postoperative decrease in IgA and IgM levels may impair the body's specific antibody production, undermining resistance to infectious diseases and potentially impacting long-term oncological outcomes, while simultaneously compromising the production of tumor-specific antibodies essential for opsonization and enhancement of phagocytosis by macrophages and dendritic cells, critical for the effective functioning of the complement system and support of CTLs' anti-tumor activity (28).

Significant changes in CRP levels from baseline to 24 hours postoperatively in both groups underscore a pronounced inflammatory response, reflecting perioperative stress. However, a more substantial increase was observed in the IA group compared to the TIVA group. This difference may be attributed to the anti-inflammatory properties of propofol and a shift in immune balance towards a cytotoxic anti-tumor response (29, 30). Elevated CRP levels contribute to cancer progression by serving as a marker of

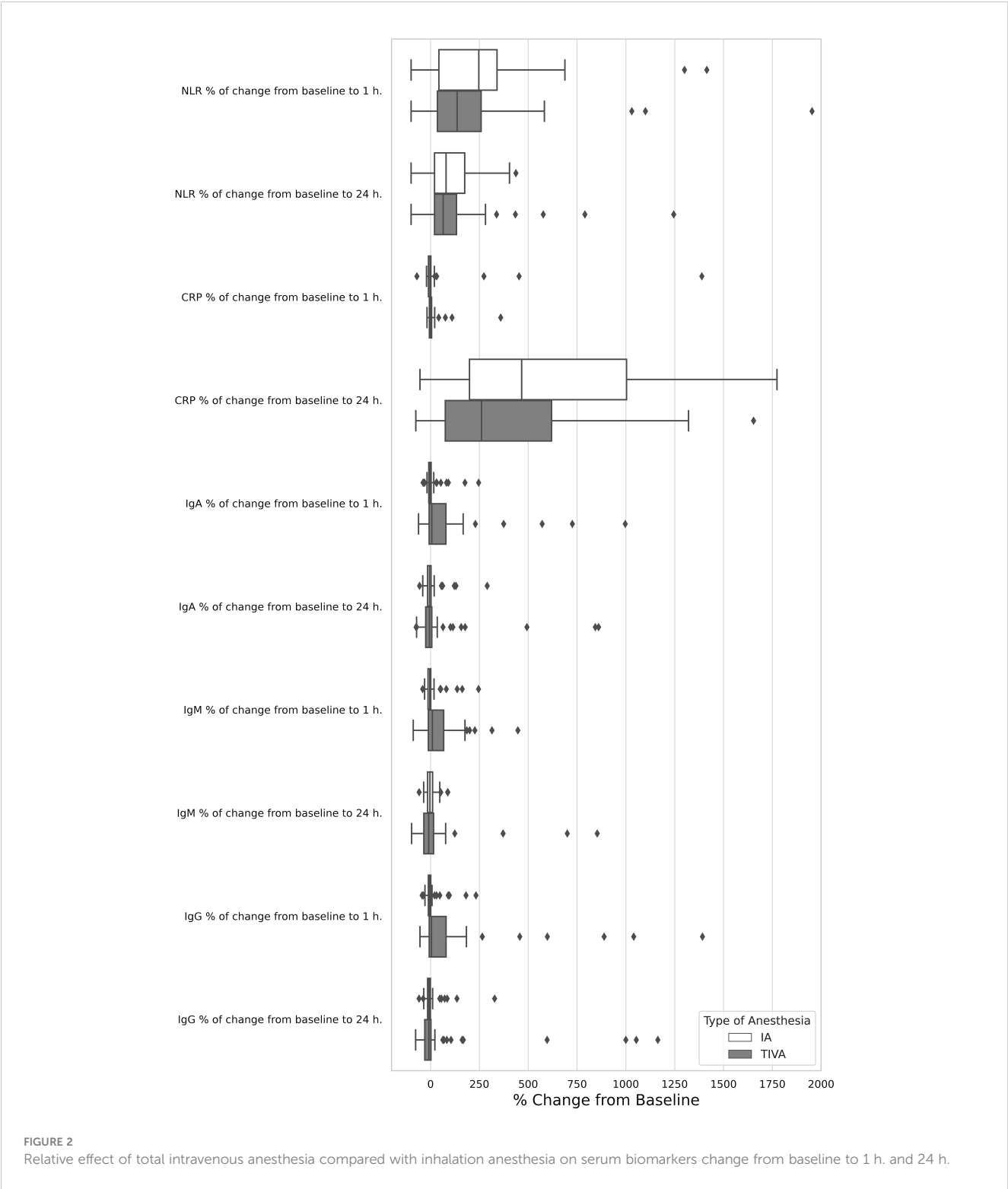


FIGURE 2  
Relative effect of total intravenous anesthesia compared with inhalation anesthesia on serum biomarkers change from baseline to 1 h. and 24 h.

disease advancement and enhancing inflammation, which supports tumor growth by aiding DNA damage, angiogenesis, and metastasis (31, 32). Additionally, CRP's role in pathogen opsonization and complement system activation underscores its involvement in creating a tumor-friendly microenvironment (31–33).

Regarding MMP-9, one of the primary markers indicating adverse progression of BC (34), and cellular immunity parameters, no significant intergroup differences were identified. Moreover, the intragroup dynamics, demonstrating a decrease in T cells (CD3+), NK cells, and an increase in B cells, were



TABLE 4 Trends in serum biomarker levels in patients receiving total intravenous anesthesia vs. patients receiving inhalation anesthesia.

| Laboratory parameters                                       | IA <sup>1</sup> | TIVA <sup>1</sup> | IA                                       |                                           | TIVA                                     |                                           |
|-------------------------------------------------------------|-----------------|-------------------|------------------------------------------|-------------------------------------------|------------------------------------------|-------------------------------------------|
|                                                             |                 |                   | Trend from baseline to 1 h. <sup>2</sup> | Trend from baseline to 24 h. <sup>2</sup> | Trend from baseline to 1 h. <sup>2</sup> | Trend from baseline to 24 h. <sup>2</sup> |
| Absolute NLR                                                | <0.001          | <0.001            | ↑<0.001                                  | ↑<0.001                                   | ↑<0.001                                  | ↑<0.001                                   |
| Relative NLR                                                | <0.001          | <0.001            | ↑<0.001                                  | ↑<0.001                                   | ↑<0.001                                  | ↑<0.001                                   |
| CRP, mg/l                                                   | <0.001          | <0.001            | 0.248                                    | ↑<0.001                                   | 0.999                                    | ↑<0.001                                   |
| IgA, g/l                                                    | 0.006           | 0.002             | 0.109                                    | ↑0.006                                    | 0.401                                    | 0.154                                     |
| IgM, g/l                                                    | 0.015           | 0.094             | 0.065                                    | ↓0.028                                    | NA                                       | NA                                        |
| IgG, g/l                                                    | <0.001          | 0.001             | ↓0.049                                   | ↓<0.001                                   | 0.999                                    | ↓0.010                                    |
| C3, g/l                                                     | 0.001           | 0.083             | ↓0.001                                   | 0.074                                     | NA                                       | NA                                        |
| C4, g/l                                                     | 0.014           | 0.103             | ↓0.013                                   | 0.459                                     | NA                                       | NA                                        |
| MMP-9, ng/ml                                                | NA              | NA                | NA                                       | 0.163 <sup>3</sup>                        | NA                                       | 0.374 <sup>3</sup>                        |
| Absolute neutrophil count, 10 <sup>9</sup> /L               | <0.001          | <0.001            | ↑<0.001                                  | ↑<0.001                                   | ↑<0.001                                  | ↑<0.001                                   |
| Absolute lymphocyte count, 10 <sup>9</sup> /L               | <0.001          | <0.001            | ↓<0.001                                  | ↓<0.001                                   | ↓<0.001                                  | ↓<0.001                                   |
| Relative neutrophil count, %                                | <0.001          | <0.001            | ↑<0.001                                  | ↑<0.001                                   | ↑<0.001                                  | ↑<0.001                                   |
| Relative lymphocyte count, %                                | <0.001          | <0.001            | ↓<0.001                                  | ↓<0.001                                   | ↓<0.001                                  | ↓<0.001                                   |
| T cells (CD3+), %                                           | 0.001           | <0.001            | ↓0.07                                    | 0.540                                     | ↓0.028                                   | 0.485                                     |
| T helper cell (CD3+CD4+), %                                 | 0.020           | 0.005             | 0.999                                    | 0.07                                      | 0.121                                    | 0.750                                     |
| Cytotoxic T cell (CD3+CD8+), %                              | 0.016           | 0.004             | 0.999                                    | ↓0.016                                    | 0.297                                    | 0.297                                     |
| Immunoregulatory index (CD4+/CD8+ ratio)                    | 0.034           | 0.003             | 0.999                                    | ↑0.037                                    | 0.258                                    | 0.258                                     |
| B cells, %                                                  | <0.001          | <0.001            | ↑<0.001                                  | ↑<0.001                                   | ↑0.010                                   | ↑<0.001                                   |
| NK cells, %                                                 | 0.001           | <0.001            | 0.999                                    | ↓0.016                                    | 0.662                                    | ↓0.018                                    |
| T cells (CD3+), B cells (CD19+CD3), NK cells (CD3-CD16+), % | 0.805           | 0.905             | NA                                       | NA                                        | NA                                       | NA                                        |

<sup>1</sup>Friedman test, <sup>2</sup>Dunn's post-hoc test; <sup>3</sup>Wilcoxon signed-rank test; NA, not applicable; IA, inhalation anesthesia; TIVA, total intravenous anesthesia. The symbols ↑ and ↓ represent an increase and a decrease in the parameter over time, respectively.

comparable across both groups, further confirming the impact of perioperative stress on cellular immunity indicators, regardless of the anesthetic type.

4.2 Comparison with previous studies

According to previous studies, exceeding a NLR threshold of 3.3 indicated a decrease in survival rates among patients with BC. (35, 36). Although this parameter exceeded the threshold value in both groups at one hour postoperatively, it returned to lower levels within twenty-four hours. Despite its potential, the study found no significant variance in NLR values between groups, which did not substantiate the hypothesis that IA affects the immune response in BC surgery more than intravenous anesthesia.

The results of this study, demonstrating a more pronounced increase in CRP levels 24 hours postoperatively in the IA group compared to the TIVA group, align with findings from the study in

non-cardiac surgery with oncological patients (37). However, the study involving gastric cancer patients has shown no significant differences in CRP levels between the volatile anesthesia and TIVA groups at any postoperative time point (38). This necessitates further investigation to understand the varying impacts of anesthetic techniques on inflammatory responses in patients with cancer.

To the best of our knowledge, in this study, for the first time, suppression of humoral immunity was demonstrated through the impact of IA on the levels of IgA and IgM in patients with BC.

Our results diverge from prior findings that indicated an impact of specific anesthetic techniques, such as sevoflurane-remifentanyl and propofol-remifentanyl combinations, on cellular immunity in BC surgeries. Contrary to the suppression of NK cell cytotoxicity and other cellular immune responses observed with these specific anesthetic regimens in the referenced study, our data revealed no significant alterations in cellular immune function (24). In a 2020 study, Efremov et al. assessed the effects of IA compared to TIVA on

cell-mediated immunity during kidney cancer surgeries, producing findings consistent with our research. It was demonstrated that there were no significant differences in NK cell counts, total T lymphocytes, or T lymphocyte subpopulations between the IA and TIVA groups (39). Our research aligns with previous findings, demonstrating that both TIVA and IA exhibit negligible effects on cellular immune responses. This is evidenced by comparable perioperative levels of circulating natural killer cells, helper T cells (Th1, Th17), and cytotoxic T lymphocytes, thus underscoring the limited impact of anesthetic choice on cellular immunity in the context of oncological surgeries (12, 40).

Contrasting with our study, Likhvantsev et al. (2022) documented post-surgical decreases in MMP-9 for TIVA recipients versus IA ( $p = 0.030$ ) (41).

### 4.3 Limitations

Despite the study being conducted as a prospective, double-blind trial with unequivocally interpretable laboratory data, it nevertheless had several limitations. First, our study was conducted in line with routine clinical practice, thereby incorporating propofol for induction in the IA group. However, while this approach may have influenced the actual magnitude of the IA effect, it simultaneously enhanced the clinical relevance of the findings. Secondly, cell count measurements might not fully reflect function or actual differences in cytotoxic activity. A significant limitation of the study was the baseline imbalance between groups in levels of CRP, IgA, and IgG, however, the sample power for the primary endpoint exceeded 80%. An additional limitation of our study was the inability to perform analyses for interleukin 6 and phagocytosis, due to supply restrictions for the necessary reagents. Furthermore, there was an imbalance in the number of patients between the IA and TIVA groups assessed for MMP-9, attributed to the relatively large size of the varying block randomization.

### 4.4 Future directions

Further research is necessary to understand IA's impact on immune function, including cytokine profiles, specific antibodies, and lymphocyte subpopulations. However, our findings prompt reconsideration of IA's use in oncologic patient surgery, with a preference for TIVA due to propofol's positive effect on anti-tumor immunity.

## 5 Conclusion

In conclusion, although there were no notable differences in NLR among the types of anesthesia, our study demonstrated that inhalational anesthesia significantly affected humoral immunity

post-surgery, while cellular immunity remained largely unaltered. This selective suppression could potentially compromise the patient's ability to eradicate residual cancer cells and increase the risk of postoperative complications and tumor recurrence. These findings suggest a more cautious approach to the use of IA, particularly in breast cancer surgeries, where maintaining immune function is crucial for long-term disease management and patient survival.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The studies involving humans were approved by A.S. Loginov Independent ethics committee (# 2/2021). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

KK: Data curation, Methodology, Writing – original draft, Writing – review & editing. VS: Writing – original draft, Writing – review & editing, Supervision, Validation. RA: Data curation, Writing – original draft, Writing – review & editing. LB: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. MY: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. LZ: Supervision, Writing – original draft, Writing – review & editing. GK: Supervision, Writing – original draft, Writing – review & editing. OS: Writing – original draft, Writing – review & editing. PK: Writing – original draft, Writing – review & editing. IK: Writing – original draft, Writing – review & editing. MS: Writing – original draft, Writing – review & editing. ASm: Data curation, Writing – original draft, Writing – review & editing. PP: Writing – original draft, Writing – review & editing. ASH: Data curation, Writing – original draft, Writing – review & editing. VL: Conceptualization, Methodology, Project administration, Writing – original draft, Writing – review & editing.

## Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. The study was funded by a grant from the Russian Science Foundation, No. 23–25–00219, <https://rscf.ru/project/23-25-00219/>.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2024.1401910/full#supplementary-material>

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## OPEN ACCESS

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RECEIVED 26 April 2024

ACCEPTED 03 September 2024

PUBLISHED 14 October 2024

## CITATION

Xu Q, Feng X, Qin S, Hong Y, Cui R, Liang J,  
Xiao Z and Li Y (2024) Research on  
therapeutic clinical trials including  
immunotherapy in triple-negative breast  
cancer: a bibliometric analysis.  
*Front. Oncol.* 14:1423924.  
doi: 10.3389/fonc.2024.1423924

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# Research on therapeutic clinical trials including immunotherapy in triple-negative breast cancer: a bibliometric analysis

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**Background:** Breast cancer, particularly triple-negative (TNBC), is a leading malignancy with aggressive traits and high metastasis rates. Clinical trial is an important tool for optimizing therapeutic strategies in the evaluation of the safety and efficacy for TNBC. Our bibliometric study of TNBC clinical trials aims to assess therapeutic strategies, identify trends, and explore advancements in treatment. We focus on mapping knowledge development, including key research entities and topics, and analyzing research trends and emerging methods. This analysis intends to inform future research, especially in personalized and precision medicine for TNBC.

**Methods:** We selected publications on clinical trials for the treatment of TNBC from 1997 to 2024 in the Web of Science Core Collection (WoSCC). After an initial screening, we downloaded key data including titles, publication years, authors, countries, institutional affiliations, journals, keywords, and abstracts, and saved them in BibTex format. We then conducted a bibliometric analysis using Bibliometrix in R and VOSviewer to illustrate the prospects, highlights, and trends of TNBC treatment options. Furthermore, to emphasize the hot topics in TNBC treatment strategies, we performed a bibliometric analysis of immunotherapy using the same approach.

**Results:** 1907 publications were included, most of which were from China, Italy, and the United States. The number of annual publications has increased dramatically since 2010. The focus of TNBC clinical trial research has shifted from understanding the biology, such as breast cancer subtyping and genotyping, to novel therapeutic approaches. The major advancement in clinical trials is the switch from late-stage palliative treatment to early preoperative neoadjuvant therapy, as more TNBC cases are discovered at an early stage. Immunotherapy is also highlighted with additional alternatives for advanced or metastasized TNBC, such as targeted inhibitors with unusual mutation rates and antibody drug conjugates (ADC).



**Conclusions:** This investigation made it apparent how immunotherapy has recently made major advancements in TNBC treatment plans and how ADCs, or targeted therapies, are currently popular for TNBC. By identifying significant papers, comprehending trending topics, and collaborating across multiple disciplines, this study may accelerate research on TNBC therapy options.

#### KEYWORDS

**bibliometric analysis, TNBC, clinical trial, neoadjuvant therapy, antibody drug conjugates, immunotherapy**

## Introduction

Nearly fifteen percent of breast cancer is triple negative breast cancer (TNBC). Compared to other subtypes, early-stage TNBC is linked to a higher risk of recurrence, and advanced stage illness has a miserable outcome with a median survival of about 18 months (mos) (1). Recent studies have shown that a certain percentage of HER2 low-expressing tumors also exist in TNBC, but for the majority of patients with TNBC do not express HER2 and also lack estrogen and progesterone receptor, thus rendering them ineffective for hormonal or anti-TNBC therapy (2). Patients with TNBC have a high recurrence rate within 3 years of diagnosis and a high mortality rate within 5 years compared to other subtypes (3). Chemotherapy is the primary strategy for systematic therapy in both the neoadjuvant and metastatic situation because there has been a dearth of specific therapies due to the lack of receptor expression (4). Nevertheless, systemic chemotherapy generates adverse effects, and the majority of patients ultimately develop resistance (5). Therefore, there is an urgent need for cutting-edge therapeutic options for TNBC that extensively improve outcomes (6).

An interdisciplinary focus entitled bibliometrics providing researchers with statistical and mathematical tools for an in-depth and dispassionate assessment of a particular field of study (7). The availability of several databases (Web of Science, Scopus, Direct Science, etc.) and software applications that facilitate the study of substantial quantities of data (VOSviewer, Leximancer, HistCite, etc.) is the reason for the popularity of bibliometric analysis (8). Thus, it has been used in various areas such as cardiovascular diseases (9), Alzheimer's disease (10), Diabetes (11) and so on, becoming progressively more significant in evaluating hotspots and developing clinical trial procedures about TNBC treatment. Bibliometric analysis helps decipher and map cumulative scientific knowledge and evolutionary nuances in mature fields by making sense of large amounts of unstructured data in a rigorous manner (12).

Bibliometric analysis offers a new method of learning and research, allowing researchers to quickly grasp an overview and to discern the frontiers. Although some articles have employed

bibliometric analysis tools, they are typically used to analyze the overall situation of TNBC research (13, 14). There has not yet been a bibliometric analysis specifically focused on clinical trials related to TNBC treatment. This study investigated the research output, the distribution of interdisciplinary research, publication sources, nations and regions with active research, institutions, and researchers, helping researchers to select appropriate publications, journals and collaborators. We also employed thematic map analysis, co-citation analysis, and keyword co-occurrence analysis to establish the conceptual framework of clinical trials focused on treating triple-negative breast cancer (TNBC). This offered us the opportunity to evaluate research frontiers, hot themes, and foundations with the goal of enlightening the current state of the field, directing future investigations, and stimulating improvements in the study of TNBC therapies. This study not only summarizes the current state of knowledge but also provides insights into future research directions, particularly in the realms of personalized and precision medicine. Furthermore, we have conducted a more in-depth analysis of the hot topic of immunotherapy and highlighted the potential of antibody-drug conjugates (ADC) as emerging treatment methods. At the same time, compared to existing research, our work offers a more macro perspective, taking into account the geographical distribution and collaboration patterns of global research, rather than just focusing on the quantity or quality of research output.

## Materials and methods

### Data sources & search strategy

Web of Science Core Collection (WoSCC) covers journals from all disciplines and global regions, and includes only journals that demonstrate a high level of editorial rigor and best practices, as well as providing detailed metadata and citation links for each article, and containing a large number of development access articles, which can provide a wide range of bibliographic resources for bibliometric analyses, and thus be a powerful tool for bibliometric analyses. All of the material utilized in this study originates from the

Science Citation Index-Expanded database from the WoSCC, and were downloaded on February 9, 2024. The search phrases were “(TS = [clinical trial]) AND TS=(triple-negative breast cancer) AND TS=(treatment)” and the keywords were “clinical trial,” “treatment,” and “triple-negative breast cancer” within WoSCC (Figure 1). The requirements to be included for the review articles and research about the first retrieval were limited to those published in English during 1997 and 2024. This enabled the discovery of 1907 pertinent works regarding the retravel. Each study’s critical data, containing the title, year of publication, authors, country, institutional affiliation, journal, keywords, and abstract, has been gathered in BibTex format via WoSCC and imported into RStudio for further analysis.

## Data analysis

The study gathered the metadata from databases concerning the included documents, which included active countries/regions, institutions, and researchers. The primary analysis pipelines employed R’s 3.6.3, version 3.6.3, using biblioshiny from the bibliometrix package (version 3.1.4, <http://www.bibliometrix.org>) (15). Bib format raw files typically were loaded; metadata (sources, writers, and citations) was subsequently calculated and plotted; this was followed by a more in-depth examination of clustering, conceptual structure, intellectual structure, and social structure. Furthermore, bibliometric analysis, which includes co-authorship analysis, keyword co-occurrence analysis, citation and co-citation analysis, and bibliographic coupling, was conducted employing VOSviewer software (version 1.6.17).

The outcomes of related functions executed by different software applications were compared in order to generate more plausible and reputable conclusions. The biblioshiny app and period co-occurrence analysis were used to generate thematic maps. The research horizons in the field of clinical trials concerning treatment for TNBC have been investigated through the use of thematic maps.

## Statistical analysis

The Spearman rank correlation coefficients were employed to assess correlations. The R software ggpubr package (version 0.4.0)

developed the plots. R (version 3.6.3) was used for the statistical analysis, and all reported p-values were two-sided.

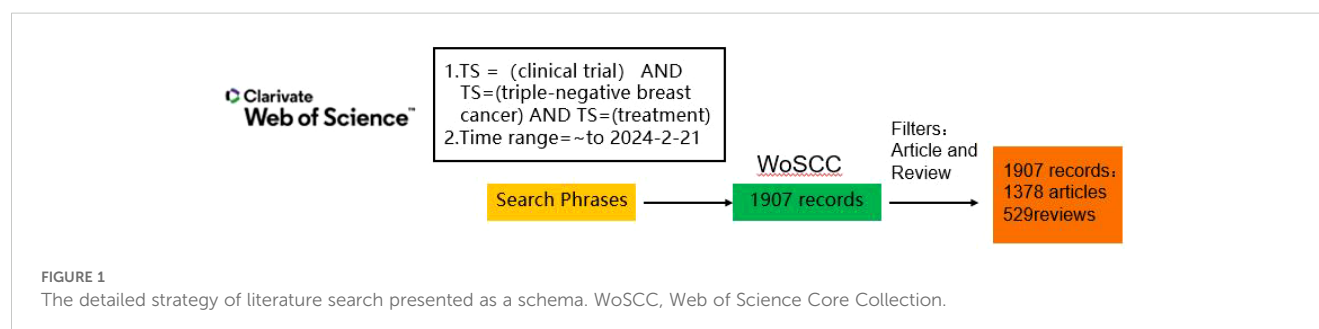
## Results

### General landscape of included documents on clinical trial about treatment in TNBC

From the WoSCC database, a total of 1907 documents were gathered, all of which were unique (Figure 1B). WoSCC offers an extensive overview of the literature; for instance, it provides citation data, which is why files developed by WoSCC were selected as the data source for analysis. Reports from 1997 to 2010 were dispersed (Figure 2A). Figure 2A shows how the annual output of articles and reviews developed dramatically after 2010, while the total research output was not substantial before that time. 2020 marked the peak of all research creation, after which it steadily declined. A substantial association between publication year and output was identified using a polynomial model fit (Coef: 0.81, 0.79, and 0.71 for articles, reviews, and total literature, respectively) (Figure 2A).

Table 1 shows that the United States (n=675) had the highest output, followed by China (n=240), Italy (n=171), Germany (n=85), and Japan (n=74), depending on the nation or location of corresponding authors. Multi-country publications (MCPs) represented 181 (26.8%) of the 675 papers from the United States. China and Italy had MCP rates of 13.8% and 31.6%, respectively, indicating that China had a lower MCP rate over Italy (Figure 2B, Table 1). The restricted cooperation between these countries or the institutions in each nation is remarkable. According to Table 2, the UNIV TEXAS MD ANDERSON CANC CTR (n = 340) and Fudan University (n = 77) were the two most significant affiliations between the United States and China. The University of Milan in Italy, UNIV TEXAS MD ANDERSON CANC CTR in the United States, and Fudan University in China are displayed as representative points of collaboration both within and between these nations on a collaboration map (Figure 2C).

448 journals published the literature that was used in this investigation. The top 20 journals featuring the most documents on this keyword in cancer are listed in Table 3. With 75 included documents, the journal Cancers achieved second in terms of published significant publications, while the Journal of Clinical Oncology ranked third with 63 included articles (Figure 3A,



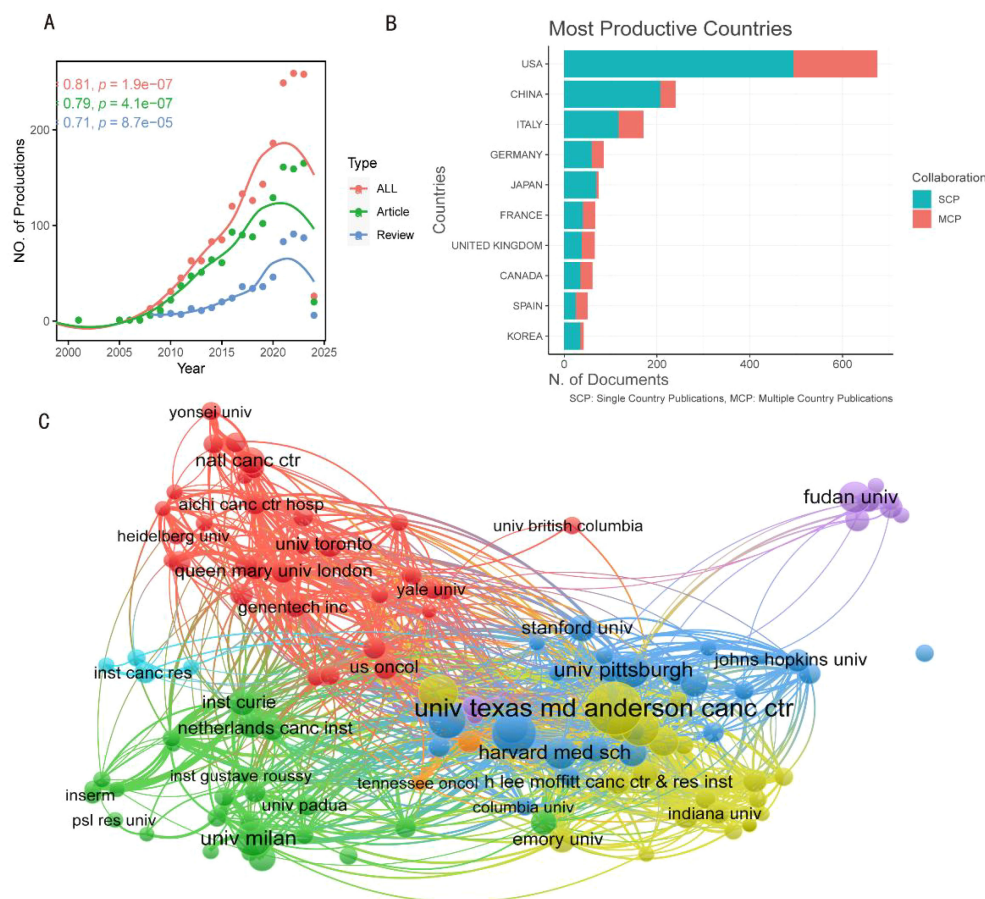


FIGURE 2

The general landscape of documents on therapeutic clinical trials about TNBC research: output, countries and affiliations. (A) The annual outputs of research on therapeutic clinical trials about TNBC from 1997 to 2024. (B) The number of documents published in different country and collaborative situation of corresponding authors. SCP, single country publications, MCP, multiple country publication. (C) Vos viewer-curated selective framework of scientific collaborations between affiliations from various countries. The number of publications is represented by the size of the circles, whereas different colors correspond to different clusters. The research centers' strength of link is indicated by the thickness of the lines.

Table 3). With 139 articles published, Breast Cancer Research and Treatment was the most productive journal. With 38 publications published in total, containing 34 articles and 4 reviews, RuGo HS was the most productive author (Figure 3B). The top 20 most relevant authors' works over time are shown in Figure 3C. Additionally, there are more published articles that expand the circle. There are more citations the darker the color (Figure 3C).

## Current highlights in clinical trial about treatment in TNBC

The keywords that show up in these publications have been selected to reflect contemporary subjects that will improve scholars' comprehension of scientific findings. These publications frequently include "clinical trial," "treatment," and "triple-negative breast cancer," in addition to the terms "survivor" and "neoadjuvant chemotherapy (Figures 4A, B)." This indicates that paclitaxel, docetaxel, and other comparable substances are the most consistently prescribed treatments for triple-negative breast

cancer. Simultaneously, there is an increasing trend in the adjuvant treatment of breast cancer utilizing monoclonal antibodies like pembrolizumab, demonstrating the significance of immunotherapy in the battle against breast cancer. Phase II trials account for the majority of the double-blind, open clinical trials for triple-negative breast cancer. These findings indicate that immunotherapy is equally important as chemotherapy in the management of breast cancer.

The 25 most cited publications worldwide are listed in Table 4. Goldhirsch A 2011 Annoncol had 2,728 citations, followed by Cortazar 2014 Lancet with 2,265 citations (Table 4). Goldhirsch a 2011 Annoncol focused on the subtype strategy of breast cancer treatment, and determined that, according to the classification of subtypes, breast cancer was suitable for endotherapy chemotherapy in along with pembrolizumab immunotherapy (16); Cortazar 2014 Lancet focuses on the neoadjuvant therapy of breast cancer (17). With all factors considered, these noteworthy studies offer a brief overview of the subject and widen experts' viewpoints to reveal the critical role that immunotherapy plays in the treatment of breast cancer.

TABLE 1 Most relevant countries by corresponding authors.

| Country        | Articles | <sup>†</sup> SCP | MCP | Freq  | MCP_Ratio |
|----------------|----------|------------------|-----|-------|-----------|
| USA            | 675      | 494              | 181 | 0.354 | 0.268     |
| CHINA          | 240      | 207              | 33  | 0.126 | 0.138     |
| ITALY          | 171      | 117              | 54  | 0.09  | 0.316     |
| GERMANY        | 85       | 59               | 26  | 0.045 | 0.306     |
| JAPAN          | 74       | 69               | 5   | 0.039 | 0.068     |
| FRANCE         | 67       | 40               | 27  | 0.035 | 0.403     |
| UNITED KINGDOM | 66       | 38               | 28  | 0.035 | 0.424     |
| CANADA         | 61       | 35               | 26  | 0.032 | 0.426     |
| SPAIN          | 50       | 25               | 25  | 0.026 | 0.5       |
| KOREA          | 42       | 35               | 7   | 0.022 | 0.167     |
| AUSTRALIA      | 37       | 21               | 16  | 0.019 | 0.432     |
| INDIA          | 33       | 28               | 5   | 0.017 | 0.152     |
| BELGIUM        | 31       | 13               | 18  | 0.016 | 0.581     |
| NETHERLANDS    | 28       | 17               | 11  | 0.015 | 0.393     |
| SWEDEN         | 19       | 15               | 4   | 0.01  | 0.211     |
| AUSTRIA        | 17       | 10               | 7   | 0.009 | 0.412     |
| BRAZIL         | 16       | 9                | 7   | 0.008 | 0.438     |
| IRELAND        | 16       | 10               | 6   | 0.008 | 0.375     |
| GREECE         | 15       | 8                | 7   | 0.008 | 0.467     |
| POLAND         | 15       | 12               | 3   | 0.008 | 0.2       |

<sup>†</sup>SCP, Single Country Publications; MCP, Multiple Country Publications.

Trending topics of clinical trial about treatment in TNBC

Hot topics of interest are presented in current publications for researchers; nevertheless, current techniques are not enough to create breakthroughs. Through keyword mutations, researchers can better grasp changes in relevant research hot topics to help obtain information more effectively. A glimpse of the evolution of keywords in recent years can be seen in [Figures 5A, B](#). The image shows how a significant section of the page is devoted to numerous forms of breast cancer. Various breast cancer subtypes and metastatic tumors maintained to be hot topics from 1997 to 2014, despite negligible breakthroughs in associated therapeutic studies. Clinical trials associated with breast cancer rose after 2015, nevertheless from 2015 to 2018, genotypes and subtypes of the disease remained prominent. After 2019, the main focus of research shifted to therapeutic approaches, particularly studies concerning gene alterations associated with breast cancer, with a particular emphasis on chemotherapeutic drugs and antibody drug conjugates. Furthermore, after 2021, Pi3K inhibitors and ADC medications, sacituzumab govitecan, are becoming increasingly well-liked and could eventually replace immune profiles as the

TABLE 2 Most relevant affiliations.

| Affiliation                     | Articles |
|---------------------------------|----------|
| UNIV TEXAS MD ANDERSON CANC CTR | 340      |
| FUDAN UNIV                      | 163      |
| DANA FARBER CANC INST           | 117      |
| NETHERLANDS CANC INST           | 109      |
| UNIV PITTSBURGH                 | 91       |
| UNIV N CAROLINA                 | 90       |
| MEM SLOAN KETTERING CANC CTR    | 86       |
| INST CURIE                      | 80       |
| MAYO CLIN                       | 78       |
| UNIV CALIF SAN FRANCISCO        | 78       |
| SUN YAT SEN UNIV                | 77       |
| UNIV MILAN                      | 73       |
| HARVARD MED SCH                 | 72       |
| OHIO STATE UNIV                 | 69       |
| STANFORD UNIV                   | 65       |
| JOHNS HOPKINS UNIV              | 64       |
| MED UNIV VIENNA                 | 61       |
| UNIV LIBRE BRUXELLES            | 61       |
| UNIV WASHINGTON                 | 61       |
| WASHINGTON UNIV                 | 60       |
| UNIV COLORADO                   | 59       |
| CHINA MED UNIV                  | 57       |
| EUROPEAN INST ONCOL             | 56       |
| BAYLOR COLL MED                 | 55       |
| NATL CANC CTR                   | 52       |

preferred option for treating TNBC ([Figures 5A, B](#)). As demonstrated in the [Figure 6A](#), topic mapping, on the other hand, makes it possible to visualize four distinct categories of themes. Using keywords, the concept map distributes topics into four quadrants according to their centrality and density. The two main areas of keywords are research on the nature and classification of diseases as well as investigation on the effectiveness and methods of therapy. Basic subjects are represented by the bottom-right quadrant. The bottom-right quadrant displays research on triple-negative breast cancer survival rates and treatment approaches. Topics that are advancing or declining are shown in the bottom-left quadrant. Included are studies on the nature and subtypes of breast cancer, revealing a possible decline in research on the subject. It is remarkable that the “movement theme” (located in the top-right quadrant) is recognized for having a high density and centrality. Neoadjuvant therapy is a major topic in the “movement theme” further discussed in the literature. Niche topics in the top-left



**TABLE 3** The top 20 most relevant journals in fields of therapeutic clinical trials on TNBC between 1997 and 2024.

| Sources                                  | Articles | ↑JCR | IF   |
|------------------------------------------|----------|------|------|
| BREAST CANCER RESEARCH AND TREATMENT     | 139      | Q1   | 3.8  |
| CANCERS                                  | 75       | Q1   | 5.2  |
| JOURNAL OF CLINICAL ONCOLOGY             | 63       | Q1   | 45.3 |
| CLINICAL BREAST CANCER                   | 60       | Q2   | 3.1  |
| CLINICAL CANCER RESEARCH                 | 50       | Q1   | 11.5 |
| FRONTIERS IN ONCOLOGY                    | 45       | Q2   | 4.7  |
| ANNALS OF ONCOLOGY                       | 41       | Q1   | 50.5 |
| BREAST                                   | 37       | Q1   | 3.1  |
| BREAST CANCER RESEARCH                   | 35       | Q1   | 7.4  |
| ONCOLOGIST                               | 29       | Q1   | 5.8  |
| PLOS ONE                                 | 26       | Q1   | 3.7  |
| EUROPEAN JOURNAL OF CANCER               | 25       | Q1   | 8.4  |
| THERAPEUTIC ADVANCES IN MEDICAL ONCOLOGY | 25       | Q1   | 4.9  |
| CANCER RESEARCH                          | 24       | Q1   | 11.2 |
| NPJ BREAST CANCER                        | 24       | Q1   | 5.9  |
| BMC CANCER                               | 22       | Q2   | 3.8  |
| MOLECULAR CANCER THERAPEUTICS            | 20       | Q1   | 5.7  |
| BREAST CARE                              | 17       | Q1   | 2.1  |
| SCIENTIFIC REPORTS                       | 17       | Q1   | 4.6  |
| CANCER TREATMENT REVIEWS                 | 16       | Q1   | 11.8 |

↑JCR and IF were curated from Web of Science of 2024.  
IF, Influence factor; JCR, Journal Citation Reports™.

quadrant have limited importance in the field, although they have high density due to insignificant external links (low centrality). There are numerous topics on the development, heterogeneity, and susceptibility of cancer (Figure 6A).

The comprehensive findings of the VOSviewer software-assisted keyword co-occurrence analysis are presented in an organized fashion (Figure 6B). The most commonly searched keywords from 2016 to 2021 are displayed in light to dark purple hues. The majority of studies concerned general treatments for breast cancer, with chemotherapy and disease survival being previously identified primary themes. In addition, research on “immunotherapies,” like the PD-L1 monoclonal antibody pembrolizumab, has recently shifted from general breast cancer treatment to more specific therapies that target distinct subtypes of breast cancer, involving pembrolizumab for TNBC (Figure 6B). Despite the fact that immunotherapy is still quite popular right now, ADCs and other targeted treatments like sacituzumab govitecan can be noticed in the Figure 6B. The confidence of the results is demonstrated by the fact that these findings generally fall in line with those derived from literature-based methods (Figure 6B). In summary, clinical trial results indicate the critical

role that immunotherapy plays in TNBC, making it a popular area of study today. These findings additionally pointed out the trend toward ADC or targeted treatments, offering fresh approaches to the management of TNBC.

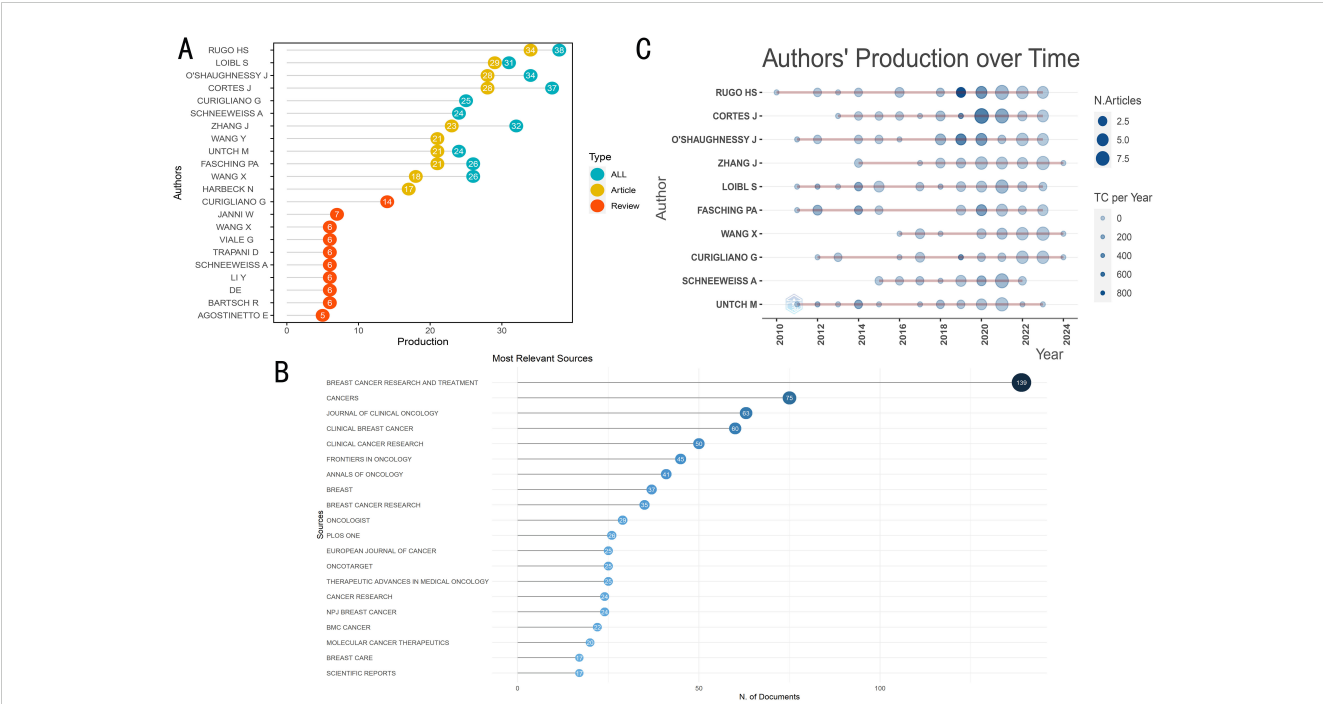
## Overview of immunotherapy in TNBC

The role of immunotherapy in that leadership of TNBC is growing. We employed a similar analysis procedure using the fixed keywords “TNBC” and “immunotherapy” in order to carry out a more thorough investigation of TNBC immunotherapy (Supplementary Figure S1A). After 2015, this field saw quickly development, as we saw when we acquired documents from 2018 (Supplementary Figure S1B). With 65 papers total—55 articles and 10 reviews—the most prolific author, Li Y, has drawn attention to significant trends in the field and prompted further study (Supplementary Figure S1C). These articles frequently include the query words “PD-L1,” “chemotherapy,” “pembrolizumab,” and “t-cells,” in addition to the terms “immunotherapy” and “Triple-negative Breast cancer” (Supplementary Figures S1D, E). In the meantime, the combination of atezolizumab and albumin-bound paclitaxel was investigated in the most widely cited article by Schmid P in 2018 in N ENGL J MED. The results indicated that atezolizumab plus albumin-bound paclitaxel raised progression-free survival in the intention-to-treat population and the PD-L1-positive subgroup of patients with metastatic triple-negative breast cancer (18). Pembrolizumab monotherapy was investigated as a second-line or later therapy for previously treated mTNBC patients in the second most cited publication by Adams S in 2019 in ANN ONCOL, demonstrating its efficacy (19) (Table S1). Overall, improvement and assessment of immunotherapy methods that target PD-L1 are the main topics of both articles. The most cited articles’ keywords support TNBC immunotherapy’s trending challenges. A tree diagram can show particular frequencies for each keyword. Conceptual maps emphasize important research hotspots, like photodynamic therapy, and combination neoadjuvant chemotherapy and immunotherapy (Supplementary Figures S1F, G).

## Discussion

Bibliometric analyses are being used more and more to evaluate developments and trends in different fields of research. As for TNBC treatment, immunotherapy-such as pembrolizumab, a monoclonal antibody targeting PD-L1-is currently the hot challenge. Nevertheless, indications of ADC and other targeted therapies are already starting to appear. However, there is no study on bibliometric analysis of clinical trials regarding TNBC treatment studies. Based on available data, since 2010, there have been a significant number of clinical trial studies on TNBC treatment, with a significant upsurge in studies on TNBC immunotherapy after 2015. Research on general therapies for breast cancer was concentrated after 2010, when TNBC treatment was likely to center around chemotherapy, neoadjuvant therapy, and other





**FIGURE 3**  
The general landscape of documents on therapeutic clinical trials about TNBC research: journals and authors. **(A)** The number of documents published in the most relevant journals. **(B)** Articles and reviews produced by highly relevant authors. **(C)** The production of top ten corresponding author over time. TC: total citations.

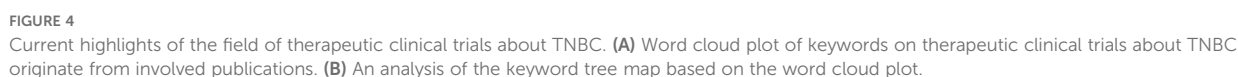
**TABLE 4** Most globally cited documents.

| Paper                               | DOI                              | Type    | TC   | TC per Year | Normalized TC | ↑JCR | IF    |
|-------------------------------------|----------------------------------|---------|------|-------------|---------------|------|-------|
| GOLDHIRSCH A, 2011, ANN ONCOL       | 10.1093/annonc/mdr304            | Article | 2728 | 194.86      | 18.47         | Q1   | 50.5  |
| CORTAZAR P, 2014, LANCET            | 10.1016/S0140-6736(13)62422-8    | Review  | 2265 | 205.91      | 24.08         | Q1   | 168.9 |
| ADAMS S, 2019, ANN ONCOL            | 10.1093/annonc/mdy517            | Article | 2195 | 365.83      | 27.85         | Q1   | 50.5  |
| ADAMS S, 2019, ANN ONCOL-a          | 10.1093/annonc/mdy518            | Article | 2195 | 365.83      | 27.85         | Q1   | 50.5  |
| VON MINCKWITZ G, 2012, J CLIN ONCOL | 10.1200/JCO.2011.38.8595         | Article | 1760 | 135.38      | 18.31         | Q1   | 45.3  |
| NANDA R, 2016, J CLIN ONCOL         | 10.1200/JCO.2015.64.8931         | Article | 1449 | 161.00      | 23.05         | Q1   | 45.3  |
| SCHMID P, 2020, N ENGL J MED        | 10.1056/NEJMoa1910549            | Article | 1283 | 256.60      | 29.68         | Q1   | 158.5 |
| MASUDA N, 2017, N ENGL J MED        | 10.1056/NEJMoa1612645            | Article | 1017 | 127.13      | 17.97         | Q1   | 158.5 |
| GELMON KA, 2011, LANCET ONCOL       | 10.1016/S1470-2045(11)70214-5    | Article | 922  | 65.86       | 6.24          | Q1   | 51.1  |
| CORTES J, 2020, LANCET              | 10.1016/S0140-6736(20)32531-9    | Article | 831  | 166.20      | 19.22         | Q1   | 168.9 |
| SILVER DP, 2010, J CLIN ONCOL       | 10.1200/JCO.2009.22.4725         | Article | 732  | 48.80       | 8.51          | Q1   | 45.3  |
| SCHMID P, 2020, LANCET ONCOL        | 10.1016/S1470-2045(19)30689-8    | Article | 725  | 145.00      | 16.77         | Q1   | 51.1  |
| SIKOV WM, 2015, J CLIN ONCOL        | 10.1200/JCO.2014.57.0572         | Article | 663  | 66.30       | 11.54         | Q1   | 45.3  |
| O'SHAUGHNESSY J, 2011, N ENGL J MED | 10.1056/NEJMoa1011418            | Article | 652  | 46.57       | 4.41          | Q1   | 158.5 |
| KIM C, 2018, CELL                   | 10.1016/j.cell.2018.03.041       | Article | 586  | 83.71       | 9.74          | Q1   | 64.5  |
| HUDIS CA, 2011, ONCOLOGIST          | 10.1634/theoncologist.2011-S1-01 | Review  | 581  | 41.50       | 3.93          | Q1   | 5.8   |

(Continued)

| Paper                       | DOI                           | Type    | TC  | TC per Year | Normalized TC | ↑JCR | IF    |
|-----------------------------|-------------------------------|---------|-----|-------------|---------------|------|-------|
| TUTT A, 2018, NAT MED       | 10.1038/s41591-018-0009-7     | Article | 567 | 81.00       | 9.42          | Q1   | 82.9  |
| DENKERT C, 2017, LANCET     | 10.1016/S0140-6736(16)32454-0 | Review  | 547 | 68.38       | 9.67          | Q1   | 168.9 |
| MITTENDORF EA, 2020, LANCET | 10.1016/S0140-6736(20)31953-X | Article | 541 | 108.20      | 12.52         | Q1   | 168.9 |
| VOORWERK L, 2019, NAT MED   | 10.1038/s41591-019-0432-4     | Article | 521 | 86.83       | 6.61          | Q1   | 82    |

IF, Influence factor; JCR, Journal Citation Reports™; TC, Total Citations; Normalized TC, calculated by dividing the actual count of citing items by the expected citation rate for documents with the same year of publication



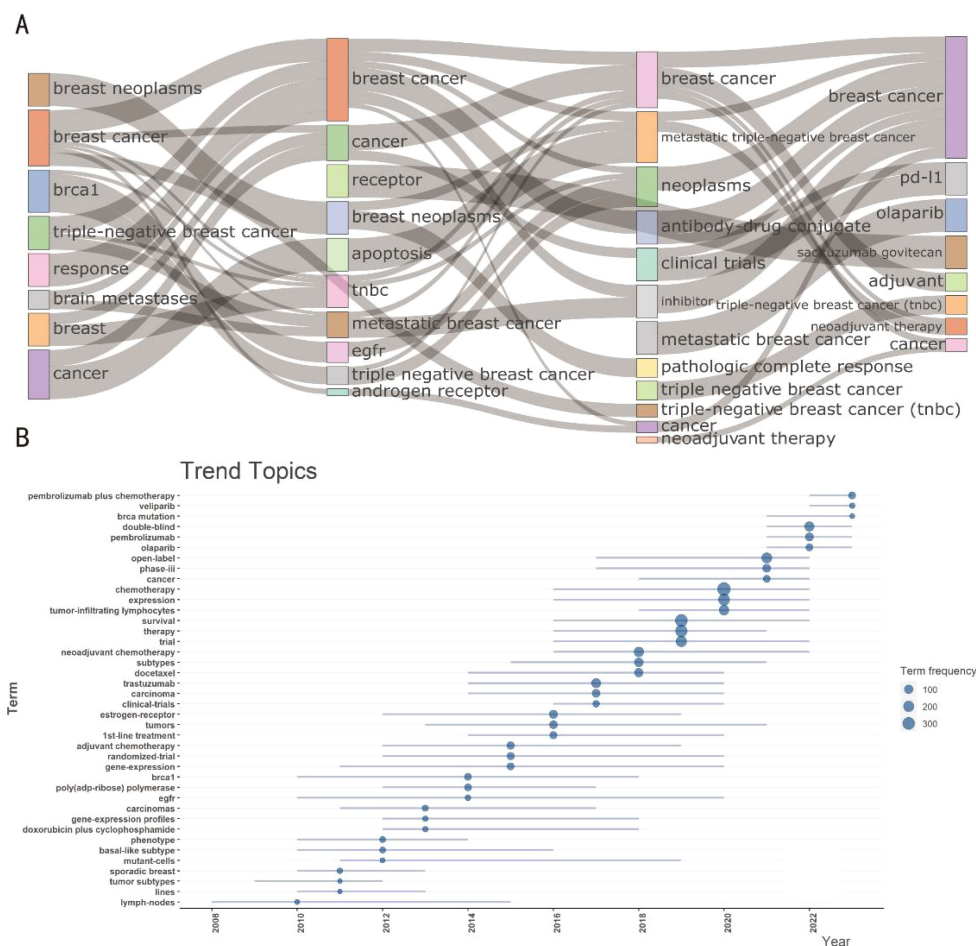


FIGURE 5

Trending topics of therapeutic clinical trials about TNBC. **(A)** Evolution of keywords across time eras. The thematic Evolution function in bibliometrix in R was used to obtain nodes and edges. The subjects of research are closely related. There were several subjects that span multiple academic disciplines and had a lot in common with one another. Concurrently, research themes changed over time and gave rise to new terminology, denoted by curving lines. **(B)** Trend topics evolution during 1997 to 2024. The size of the dot represented its frequency, while the horizon line represented its period in years.

methods. Furthermore, after 2020, the use of pi3K inhibitors and sacituzumab govitecan medications is starting to become obvious, indicating potential directions for TNBC treatment. However, it is also clear that performing clinical studies costs some time. The majority of pertinent journals have impact factors (IFs) that are relatively large. China comes in second to the United States in terms of research findings about TNBC immunotherapy and treatment in clinical trials. China and the United States are also actively working together on research. Another country with a high rate of research output is Italy, most likely as a result of its highly significant affiliation with the University of Milan.

It is noteworthy that the papers of Goldhirsch A., 2011, in *Annals of Oncology* and Cortazar, 2014, in *The Lancet* have received the greatest citations in clinical trials on TNBC therapy possibilities. In 2011, Goldhirsch A concentrated on subtype-specific methods to breast cancer treatment, using subtyping to figure out if the cancer is amenable to immunotherapy with pembrolizumab and chemotherapy, endocrine therapy, or chemotherapy. Cortazar, in 2014, in *The Lancet*, focuses on

neoadjuvant therapy for breast cancer. Both articles are closely related to immunotherapy for TNBC. The two most cited investigations on the topic of immunotherapy for TNBC are by Adams S., 2019, in *Annals of Oncology*, and Schmid P., 2018, in *The New England Journal of Medicine*. In the former, atezolizumab and nab-paclitaxel are combined, and it is shown that this prolongs progression-free survival in patients with metastatic triple-negative breast cancer who have intention-to-treat as well as in the subgroup of patients who have PD-L1 positive. The latter investigated pembrolizumab monotherapy as a follow-up or second-line treatment for patients with mTNBC who had already received treatment. Studies performed in both directions emphasize the role immunotherapy plays in TNBC, with pembrolizumab serving as the main PD-L1 monoclonal antibody medication.

Immunotherapy for TNBC is growing more and more popular, according to two levels of analysis: the topic maps of the literature studies and the keyword co-occurrence analysis in the VOSviewer software. Chemotherapy and neoadjuvant therapy, the typical treatment for breast cancer, were the topic of previous study. But

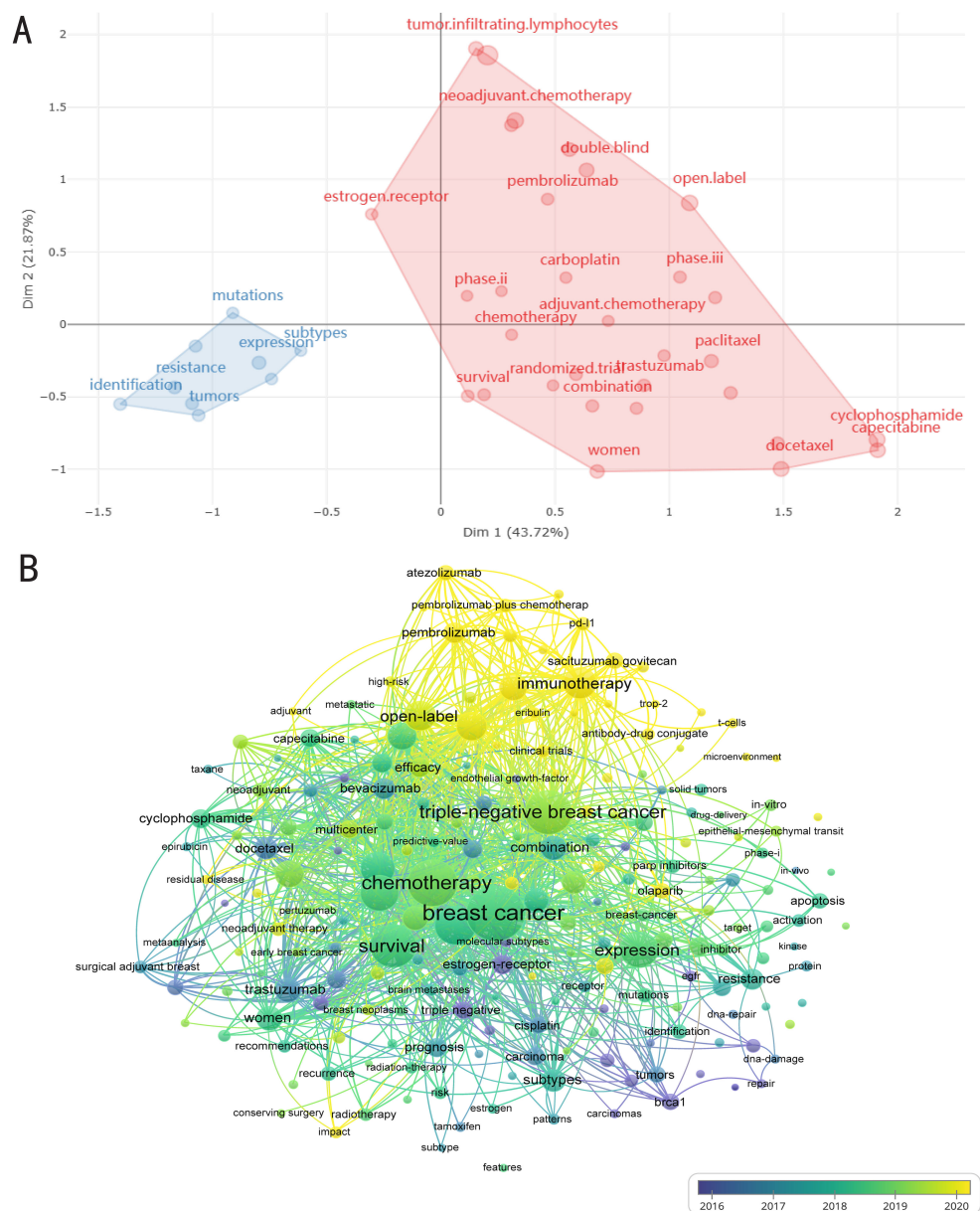


FIGURE 6

Trending topics of therapeutic clinical trials about TNBC. (A) Conceptual structure map of keywords. MCA, Multiple correspondence analysis. Dim: Dimension. Two colors represent two clusters. (B) the VOSviewer software's examination of keyword co-occurrence. Trending topics from 2016 to 2020 are shown with lighter color for later years and darker purple for earlier years. The nodes' sizes indicate how frequently they occur. The nodes' appearance in the same publication is shown by the curves joining them. The likelihood of the two keywords occurring together increases with the distance between two nodes.

these days, immunotherapies like “pembrolizumab” and “PD-L1 monoclonal antibody”—targeted medicines for various subtypes of breast cancer—have acquired popularity as a means of treating TNBC, and their significance cannot be ignored. Though immunotherapy is now quite popular, alternative targeted medicines, like ADC medications and PI3K inhibitors, are starting to gain traction and could end up becoming the standard for treating TNBC in the future.

Due to its heterogeneity and acquired resistance, single-agent therapy has not produced encouraging outcomes in clinical studies battling invasive TNBC, although demonstrating promising results

in cell line and preclinical models. Presently, about eighty percent of clinical studies are looking into novel combination therapy-based therapies for TNBC (20).

Chemotherapy or neoadjuvant chemotherapy alone is not the best option for treating TNBC. Chemotherapy is not sufficient to treat TNBC; more sophisticated targeted therapies are required to enhance the prognosis of particular patient subgroups (21). TNBC is a heterogeneous tumor with higher genetic instability, frequent copy number changes, and complex structural rearrangements, and these features make TNBC more suitable for immunotherapy. Currently, immune checkpoint inhibitors combined with



chemotherapy, PARP inhibitors, cancer vaccines, or NK cell treatment are the main targets of immunotherapy research for TNBC (22). PD-L1 is an attractive target for therapy because of its high mutation activity and 20% elevation in TNBC patients (23). However, the discrepancy between immunohistochemical PD-L1 assay performance and inter-reader reproducibility has also raised concerns. Related studies have shown that high tumor-infiltrating lymphocytes (TILs) are also associated with response to PD-1/PD-L1 inhibitors in breast cancer patients. Thus, the combined analysis of PD-L1 and TILs could serve as a more comprehensive immunooncology biomarker (24). Further published studies may soon allow for a more thorough bibliometric analysis on the application of PD-L1 in immunotherapy for TNBC.

Targeted therapy has become increasingly important in TNBC, providing new options for TNBC treatment through the discovery of new therapeutic targets. Targeted therapy for TNBC is mainly based on different subtypes of gene expression profile and related molecular pathways. TNBC can currently be further categorized into subtypes, with different treatment strategies chosen according to the subtype. Basal-like TNBC (BL-TNBC) can be subdivided into two subgroups, the BL1 and BL2 subgroup. BL1-TNBC is similar to that seen in BRCA1 or BRCA2 mutation carriers (25). Tumors in this group tend to have a high genomic instability, high mutational burden, and deficiencies in homologous recombination repair (HRR). They may also express certain markers such as cytokeratins 5/6 (CK5/6) and epidermal growth factor receptor (EGFR), and lack the expression of HER2 (26). Tumors in the BL2 group may not exhibit the same level of genomic instability or HRR deficiency. They might still express basal cytokeratins and EGFR but could have different responses to treatment and outcomes compared to the BL1 group. Thus, targeting DNA repair defects can be an effective therapeutic strategy for TNBC of the BL1 subgroup characterized by BACRness or BRCA mutations, and platinum and PARP inhibitors have been shown to have significant efficacy in the treatment of such TNBC (27). Moreover, mesenchymal-like TNBC are represented by enriched genes involved in the biological regulation of EMT and CSC, and thus, therapeutic strategies such as c-MET-targeted therapy, immune checkpoint blockade, and so on, are effective. For HER2-enriched TNBC, EGFR-targeted therapy and PI3K/AKT/mTOR pathway-targeted therapy are effective (28). As a result, subtype-based therapeutic strategies are being investigated. Relevant studies have shown that AR targeted therapy is typically used to treat the LAR subtype; PD-1/PD-L1 inhibitors are usually prescribed to treat the IM subtype; PARP inhibitors are typically used to treat the BL1 subtype; mTOR inhibitors are typically used to treat the BL2 subtype; and PI3K inhibitors are typically used to treat the M/MSL subtype (29, 30). Relevant clinical trials have demonstrated that AKT inhibitors such as capivasertib and ipatasertib play a significant role in the PI3K/AKT/mTOR signaling pathway, both occupying important positions in the treatment of metastatic triple-negative breast cancer (TNBC) (31, 32). However, the therapeutic effects of the related treatments in different TNBC subtypes are still under investigation, and targeted therapies are no longer limited to specific subtypes. The percentage of various therapies can be inferred from the frequency with which these terms occur.

Nonetheless, TNBC treatment remains challenging particularly because other breast tumors do not express similar therapeutic targets. Another problem with TNBC is its heterogeneity; different subtypes of triple-negative cancers respond variably to the therapies that are now available (33).

Although immunotherapy has been a well-liked addition to TNBC's expanding toolkit, only a small percentage of patients have shown any real improvement. Novel immune checkpoint targets, intratumoral injections that directly modify the tumor microenvironment, and small molecule inhibitors are among the latest strategies being researched in an effort to elicit immune responses in refractory cancers. Given the risk for irreversible autoimmune damage and the lack of predictive accuracy of early PD-L1 expression, it is vital to identify patients who will benefit most from treatment as the use of immunotherapy to treat early-stage TNBC increases (1). Antibody-drug conjugates, or ADCs, have shown potential as a practical and effective class of cancer treatments during recent trials. Trastuzumab deruxtecan was the first ADC medication authorized for the treatment of TNBC, while sacituzumab govitecan came next. Even though Trastuzumab deruxtecan works by targeting binding to the receptor HER2, it also has an important role in TNBC. On the one hand, TNBC usually do not express HER2, but in some cases do express low to moderate levels of HER2. HER2 status is based on the results of HER2 testing, called an immunohistochemistry (IHC) test or fluorescence *in situ* hybridization (FISH) test. HER2-positive is defined as IHC 3+ or FISH-positive. Therefore, certain TNBCs may have low or moderate expression of HER2 (IHC 1+ or 2+). Moreover, therapeutics may have a bystander effect, whereby the cytotoxicity released kills tumor cells that do not express ADC-targeted antigens cells, thus making them an effective means of treating TNBC (34). The FDA has approved the most recent class of antibody-drug coupling payloads, which is represented by these medications taken together. However, as studies continue to progress, it has been found that only a fraction of TNBC patients respond to ADC therapy and often develop resistance. Therefore, studies on combination therapy are becoming more and more essential (35).

Our study also has certain limitations. On the one hand, the limitations of our study are reflected in potential biases within the analysis process, including sample selection bias, the limitations of the data collection time frame, and possible flaws in the analytical methods (36). On the other hand, although our study captures the shifts in research hotspots within the field of TNBC treatment, it does not provide specific details about related studies. Different from traditional review, bibliometric analysis cannot offer systematic details; researchers still need to read specific documents. Future perspectives need professional instinct and accumulated experience, due to lower weight among all the papers which may be overshadowed by highly influential publications.

Our study employs a methodological approach that swiftly amalgamates the insights derived from literature analysis. Our synthesis reveals that the cutting-edge advancements in TNBC clinical trials are predominantly centered around antibody-drug conjugates (ADCs) and targeted therapies. This trend underscores a blurring of the traditional lines that define breast cancer subtypes,



suggesting that certain individuals with triple-negative breast cancer may derive benefits from therapies involving HER2-targeting ADCs (37). Significantly, the potential for groundbreaking progress in this domain is increasingly linked to the emergence of novel technological advancements. By conducting a bibliometric analysis keyed to the utilization of ADCs in TNBC, we can deduce that the advent of innovative ADC technologies may precipitate a transformative shift in the field. Notably, the prospect of ADCs incorporating radioactive isotopes holds the promise of enhancing the therapeutic impact of radiation treatments for patients with advanced metastatic triple-negative disease (38). Moreover, the advent of therapies that target less common molecular markers has effectively surmounted the constraints imposed by conventional subtyping, heralding an era where classification based on specific molecular targets is poised to become a pivotal direction in the evolution of cancer treatment strategies (39).

## Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding authors.

## Author contributions

QX: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. XF: Data curation, Formal analysis, Methodology, Writing – review & editing. SQ: Data curation, Writing – review & editing. YH: Data curation, Writing – review & editing. RC: Data curation, Writing – review & editing. JL: Data curation, Writing – review & editing. ZX: Data curation, Methodology, Supervision,

Writing – review & editing. YL: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Supervision, Writing – review & editing.

## Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work is supported by National Natural Science Foundation of China under grant 81902369.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2024.1423924/full#supplementary-material>

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RECEIVED 19 July 2024

ACCEPTED 29 October 2024

PUBLISHED 18 November 2024

## CITATION

Jiao S, Chen J, Shen J, Peng A, Chen R, Lai B, Luo C, Fan Y and Pei X (2024) Case report: Triple-negative breast cancer with low tumour-infiltrating lymphocytes infiltration and good prognosis: a case of Tall Cell Carcinoma with Reversed Polarity and review of the literature. *Front. Oncol.* 14:1455893. doi: 10.3389/fonc.2024.1455893

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# Case report: Triple-negative breast cancer with low tumour-infiltrating lymphocytes infiltration and good prognosis: a case of Tall Cell Carcinoma with Reversed Polarity and review of the literature

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**Background:** Triple-negative breast cancer (TNBC) is a great challenge for clinical management due to the high rate of metastatic recurrence and lack of recognised therapeutic targets, while tumour-infiltrating lymphocytes (TILs) infiltration in TNBC has been discovery provides an opportunity for immunotherapy, and studies have suggested that the level of TILs infiltration is positively correlated with TNBC survival. We found a rare case of TNBC with low TILs infiltration but good prognosis, named Tall Cell Carcinoma with Reversed Polarity (TCCRP), and we investigated it from the perspective of TILs infiltration in order to improve the clinical data of TCCRP.

**Case presentation:** a 54-year-old woman with a left breast mass, breast ultrasound showed BI-RADS IVb, puncture diagnosis of invasive ductal carcinoma, postoperative pathology showed papillary structure, cubic and high columnar arrangement of cells, nuclei far away from the base, interstitial fibroplasia, diagnosed as TCCRP. Immunohistochemistry revealed that the tumours were triple-negative breast cancer (negative for ER, PR, and HER-2), with a low Ki-67 proliferation index. TILs were < 10%, with a small infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, positive expression of SMA and FAP, and IDH2, PIK3CA gene mutation. The patient underwent postoperative chemotherapy, 11 months follow-up, no recurrence and metastasis.

**Conclusion:** TCCRP is a rare TNBC with inert biological behaviours and good prognosis. We found low infiltration of TILs in the pathological tissue of this case,

which may be a characteristic of TCCRP, and the presence of Cancer-Associated Fibroblasts (CAF) in the interstitium of the tumour in this case may have suppressed the anti-tumour immunity to some extent, and further studies on the immune characteristics of the tumour microenvironment (TME) in TCCRP are needed.

#### KEYWORDS

triple-negative breast cancer, Tall Cell Carcinoma with Reversed Polarity, tumour-infiltrating lymphocytes, cancer-associated fibroblasts, case report

## 1 Introduction

Breast cancer is currently the second most prevalent cancer in the world, accounting for 11.6% of all cancers globally, and the fourth leading cause of cancer deaths worldwide (1). Triple-negative breast cancer (TNBC), a subgroup of breast cancer defined by the lack of expression of oestrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2, accounts for 15 to 20% (2). Clinical management of TNBC is a great challenge due to the high incidence of visceral metastases and the lack of recognised therapeutic targets (3). In recent years, immunotherapy has emerged as a promising treatment modality for TNBC (4). The tumour microenvironment (TME) is immunologically complex and involves the interaction of multiple immune cells. Among them, tumour-infiltrating lymphocytes (TILs) have become a hotspot of interest in clinical studies on the immune aspects of TNBC (5), and although breast cancer is not considered an immunothermal tumour, a large infiltration of TILs can be found in TNBC (6). TILs include T cells, B cells and natural killer cells (NK), and in T cells, CD8<sup>+</sup> cytotoxic T cells (CTL) are essential for tumour destruction, CD4<sup>+</sup> T helper 1 (Th1) cells secrete cytokine mediators that help CTL improve toxicity. The level of TILs has an important predictive value in TNBC, and a study published in the *Lancet* noted that an increase in TILs was associated with prolonged overall survival in TNBC patients (7). Interestingly, we identified a case of a rare TNBC infiltrated by low TILs but with a good prognosis, called Tall Cell Carcinoma with Reversed Polarity (TCCRP). We first examined the expression of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells and tried to analyse this case from the perspective of TILs infiltration to further improve the clinical data of this rare disease and provide ideas for treatment.

## 2 Case presentation

### 2.1 Case characteristic

In July 2023, a 54-year-old woman presented with a 10-year history of a left breast mass. The patient had discovered a left breast mass 10 years earlier without systematic treatment, and recently went to the hospital for examination after realising that the mass

had increased in size compared to the previous one. Physical examination revealed well-developed and symmetrical breasts without dimpling or skin changes resembling an orange peel. No bleeding or discharge was observed upon bilateral nipple compression. A mass was palpable just below the left areola, measuring 3.0cm×2.0cm, hard texture, unclear borders, poor mobility, no enlarged lymph nodes were detected in the axilla and clavicle bilaterally. There was no family history of breast cancer in the patient. Ultrasound showed a solid hypoechoic mass (BI-RADS: IVb) (Figure 1A). Mammogram showed benign changes in the glands of both breasts (BI-RADS:2) (Figure 1B), and no mass was seen. Puncture biopsy was performed, and the cancer cells were arranged in solid, papillary or irregular glandular structures (Figures 2A, B), and invasive ductal carcinoma was diagnosed, left mastectomy with sentinel lymph node biopsy was done, and the intraoperative pathology reported no metastasis of cancer macromolecules in lymph nodes (0/7).

The excised specimen was fixed in 10% formalin buffer and paraffin embedded. Sections were stained with haematoxylin, eosin and immunohistochemistry. Microscopically, the tumour tissue was seen to have a lobe-like structure, with papillary, sieve-like and glandular duct-like structures within the lobe, with cells arranged in a cubic and high columnar pattern, with nuclei distant from the base, slightly enlarged, ovoid, with thickened nuclear membranes and occasional nuclear grooves, with reddish-stained cytoplasm, interstitial fibroplasia, and foamy cells were seen in the foci (Figures 3A–E).

Immunohistochemistry showed that TTF-1 and TG were negative, and GATA-3 was positive, indicating that the breast origin was not from the thyroid gland. P63 and Calponin were negative, indicating that the myoepithelium was absent and invasive, and the diagnosis of invasive ductal carcinoma was supported by the positivity of P120, E-cad, and CK8, and the negativity of ER, PR, and HER-2, which was TNBC. Calretinin positivity could support the diagnosis, CK5/6, CD56 positivity, EGFR, EMA, CD117, GCDFP-15, AR, Syn, CgA were all negative, KI67 was 10%, and the value-added index was low. For TILs and subpopulation detection, TILs were < 10% (Figure 4A), and the tumour interstitium was infiltrated by a small number of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes (Figures 4B, C). We compared it with common TNBC (Figures 4D–F). Detection of



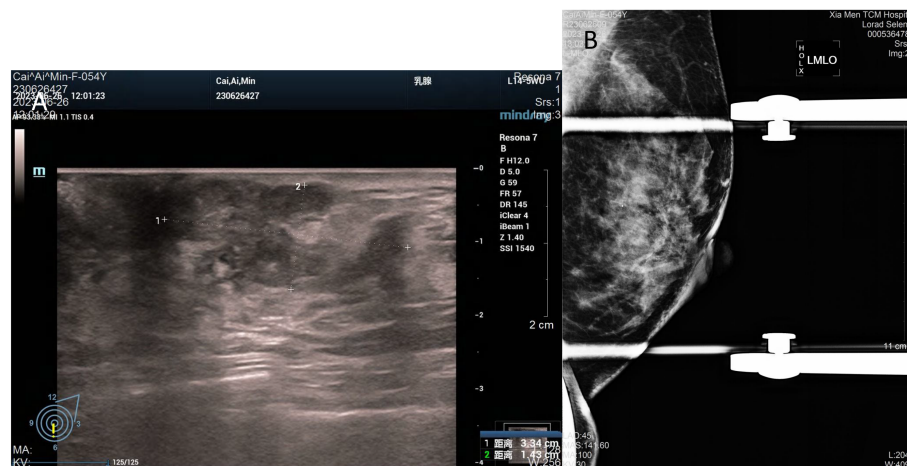


FIGURE 1

Imaging of the tumour. (A) Ultrasound shows an irregular, solid hypoechoic mass measuring 33mmx14mm with angular margins next to the nipple at 6 o'clock in the left breast. (B) Mammogram shows benign changes in both breasts, no mass was seen.

Cancer-Associated Fibroblasts (CAF) markers showed positivity for SMA and FAP (Figures 5A, B), and Sanger sequencing detected mutations in IDH2 (R172G) and PIK3CA (H1047R) genes (Figures 6A, B). Clinical staging was pT<sub>2</sub>N<sub>0</sub>M<sub>0</sub>, stage IIA, diagnosed according to the Chinese Society of Clinical Oncology (CSCO) Breast Cancer Guidelines 2023 (8).

The patient has received 4 cycles of post-operative chemotherapy and has been followed for 15 months and is currently disease-free.

## 2.2 Literature review

### 2.2.1 Literature search results

A bibliographic search of PubMed was performed using the following keywords: “Tall cell carcinoma with reversed polarity of the breast(TCCRP)” OR “Breast Tumor Resembling the Tall Cell Variant of Papillary Thyroid Carcinoma (BTPTC)” OR “Tall Cell Variant of Papillary Breast Carcinoma (TCVPBC)” OR “Solid

Papillary Carcinoma with Reverse Polarity (SPCRP)” OR “Solid Papillary Breast Carcinomas Resembling the Tall Cell Variant of Papillary Thyroid Neoplasms (BPTC)”. A total of 106 papers were retrieved. We screened 66 remaining studies for eligible title and abstract after removing duplicates ( $n = 40$ ), and 40 were excluded due to ineligibility or irrelevance (Figure 7). The abstract and title were read, and 22 articles were eliminated. After determining eligibility, 18 articles were excluded because 7 were “review articles”, 1 was “letter”, 3 were “incomplete case information”, and 7 were “not full text”. As a result, we finally included 26 studies. For each paper included, we recorded authors, demographics, clinical characteristics, treatment, and follow-up.

### 2.2.2 Characteristics of included studies

A total of 26 relevant papers involving 96 patients were published, and we summarised the clinical data of the patients (Table 1). All reported patients were female, age range 40-85 years with a mean of 61.6 years, tumour size 0.6-5 cm with a mean of

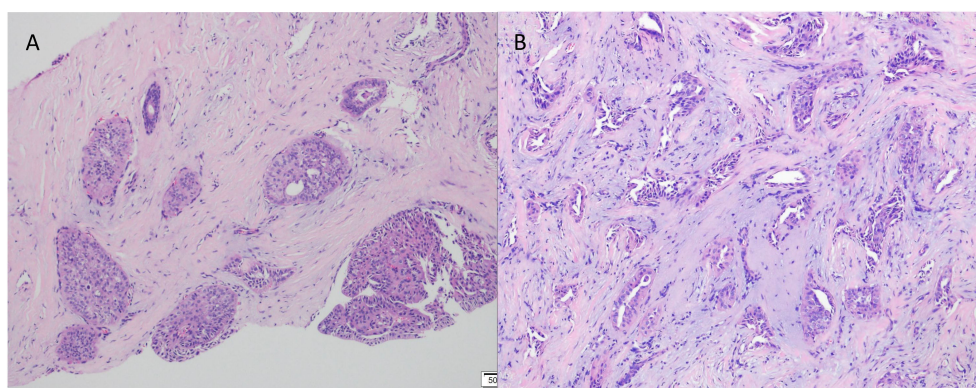


FIGURE 2

Pathological examination by puncture using haematoxylin and eosin (HE) staining. (A) Cancer cells were arranged in solid, papillary structures (x100). (B) Cancer cells arranged in an irregular adenoidal structure with infiltrative growth, tumour cells were columnar to cuboidal with mild nuclear morphology and acidophilic cytoplasm. Interstitial fibrous tissue hyperplasia and mucoid changes (x200).



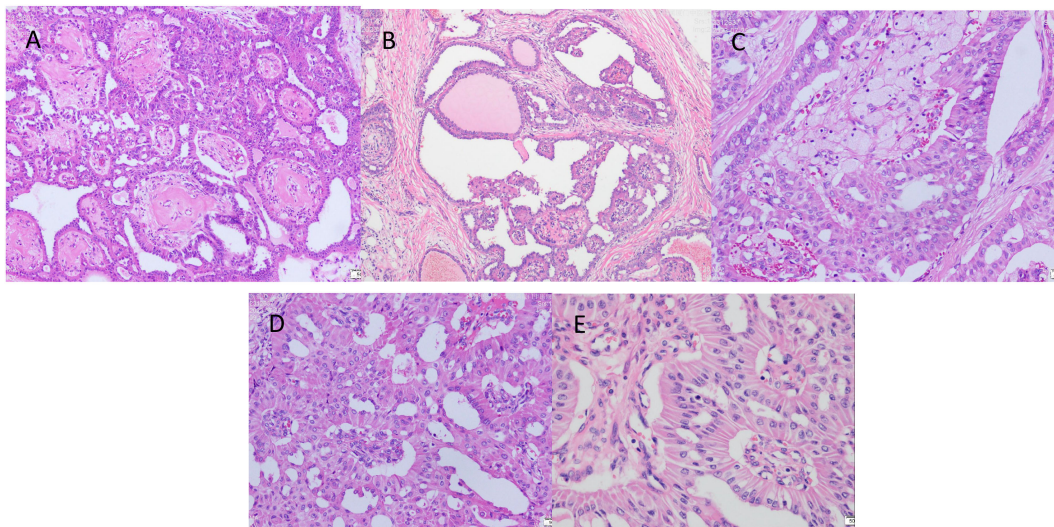


FIGURE 3

The postoperative pathological examination with haematoxylin and eosin (HE) staining. **(A)** Cancer cells arranged in a papillary structure (x100). **(B)** Cancer cells are arranged in a glandular structure, some of which are cystically dilated, similar to thyroid follicles, with individual glandular lumens containing gelatinous, homogeneous, strongly eosinophilic material, and interstitial fibrous connective tissue (x100). **(C)** Foam cell aggregates are seen in the interstitium of cancerous tissue (x200). **(D)** The surface of the papilla is covered with cancer cells in a cubic or high columnar shape, the nucleus is far from the base, the cytoplasm is reddish stained, and the interstitium is seen as a fibrovascular axis (x200). **(E)** Cancer cells are arranged in high columnar shape with mild nuclear morphology, eosinophilic granular cytoplasm, and nuclei are located away from the base at the luminal margin (x400).

1.9 cm, 61.5% of patients reported imaging data including Ultrasound, Mammogram and MRI, 44.8% of patients belonged to TNBC, and among the patients who underwent genetic testing so far, there were 59.4% had IDH2 mutations and 33.3% had PIK3CA mutations. Local excision was the mainstay of treatment, and

metastasis was found in 4 cases during follow-up, 1 case had intramammary lymph node metastasis at the time of consultation, 1 case had axillary lymph node metastasis at the time of consultation, 1 case had axillary lymph node metastasis at the time of consultation and bone metastasis 32 months later, and 1

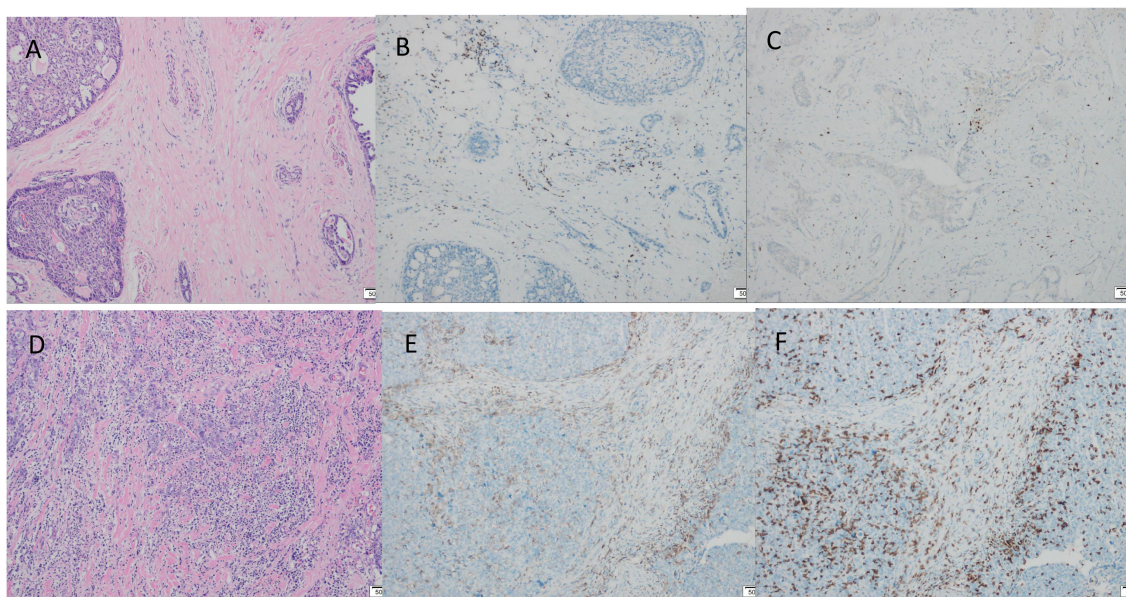
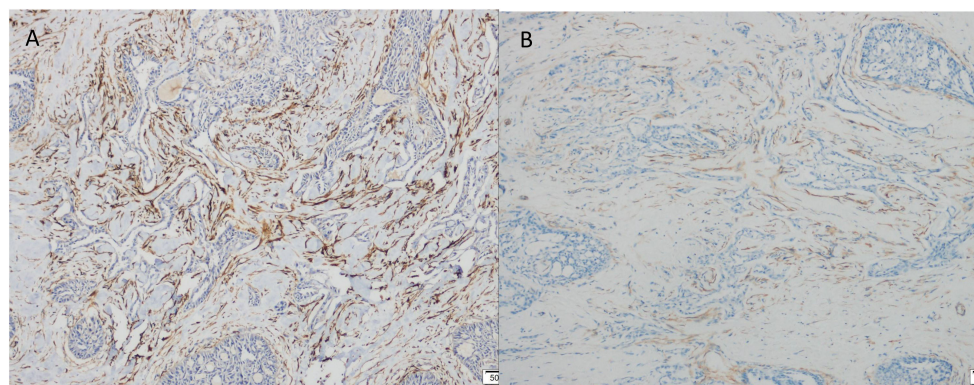


FIGURE 4

Tumour TILs infiltration assessment, TILs were stained by haematoxylin and eosin (HE) staining, CD4<sup>+</sup> T lymphocytes, CD8<sup>+</sup> T lymphocytes were stained by immunohistochemical (IHC) staining. **(A)** TILs < 10% (x100). **(B)** A small number of interstitial CD4<sup>+</sup> T lymphocytes infiltrated (x100). **(C)** Small amount of interstitial CD8<sup>+</sup> T lymphocyte infiltration (x100). **(D-F)** Common triple-negative breast cancer control. **(D)** TILs > 40% (x100). **(E)** CD4<sup>+</sup> positive expression (x100). **(F)** CD8<sup>+</sup> positive expression (x100).



**FIGURE 5**  
Immunohistochemical (IHC) staining. **(A)** FAP positive expression (x100). **(B)** SMA positive expression (x100).

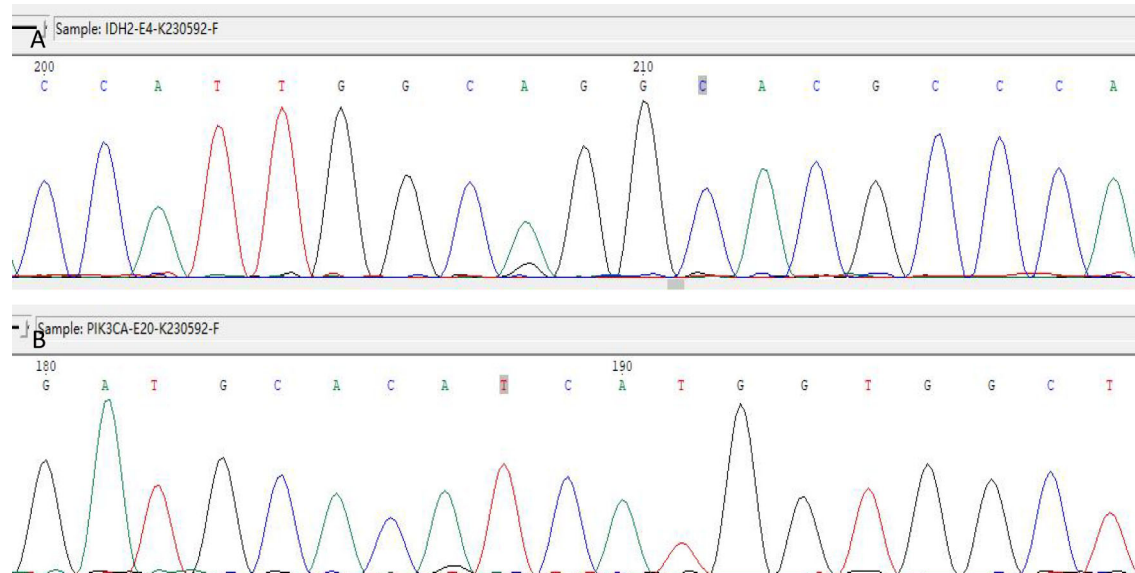
case had local recurrence and axillary lymph node metastasis 5 years after surgical excision. No fatal patients have been reported, and the follow-up time was 0-132 months, with a mean of 25.1 months.

### 3 Discussion

TCCRP is a rare subtype of breast cancer which was first identified by Eusebi in 2003 (9) and was initially named Breast Tumour Resembling the Tall Cell Variant of Papillary Thyroid Cancer (BTPTC) because of the similarity of the pathologic features to the high cell variant of Papillary Thyroid Carcinoma (BTPTC), an important morphological feature of this tumour is the proximity of the cell nuclei to the luminal surface of the gland, known as polarity flip. In 2019, the WHO Blue Book classification of breast

tumours (5th edition) included this rare tumour (35) and named Tall Cell Carcinoma with Reversed Polarity (TCCRP), which was recognised as an independent subgroup included in rare and salivary gland-type tumours.

In this paper, we study and summarise the relevant TCCRP literature in recent years: patients are mainly middle-aged and elderly women, usually consulting for a palpable breast lump or screening mammogram, Ultrasound and Mammogram are predominantly used in the imaging data, but the description of BI-RADS grading is missing in many cases, and TNBC has the highest percentage in TCCRP, and a few patients have weak positivity of the hormone receptors were weakly positive, and the remaining patients lacked HER-2 testing for definitive molecular staging. In immunohistochemistry, breast origin markers GCDFP-15 and GATA-3 were frequently strongly positively expressed,



**FIGURE 6**  
IDH2 R172 and PIK3CA H1047 mutations detected by Sanger sequencing. **(A)** IDH2 gene detected the c.514A > G (p.R172G) mutation. **(B)** The PIK3CA gene was detected with the c.3140A > G (p.H1047R) mutation.



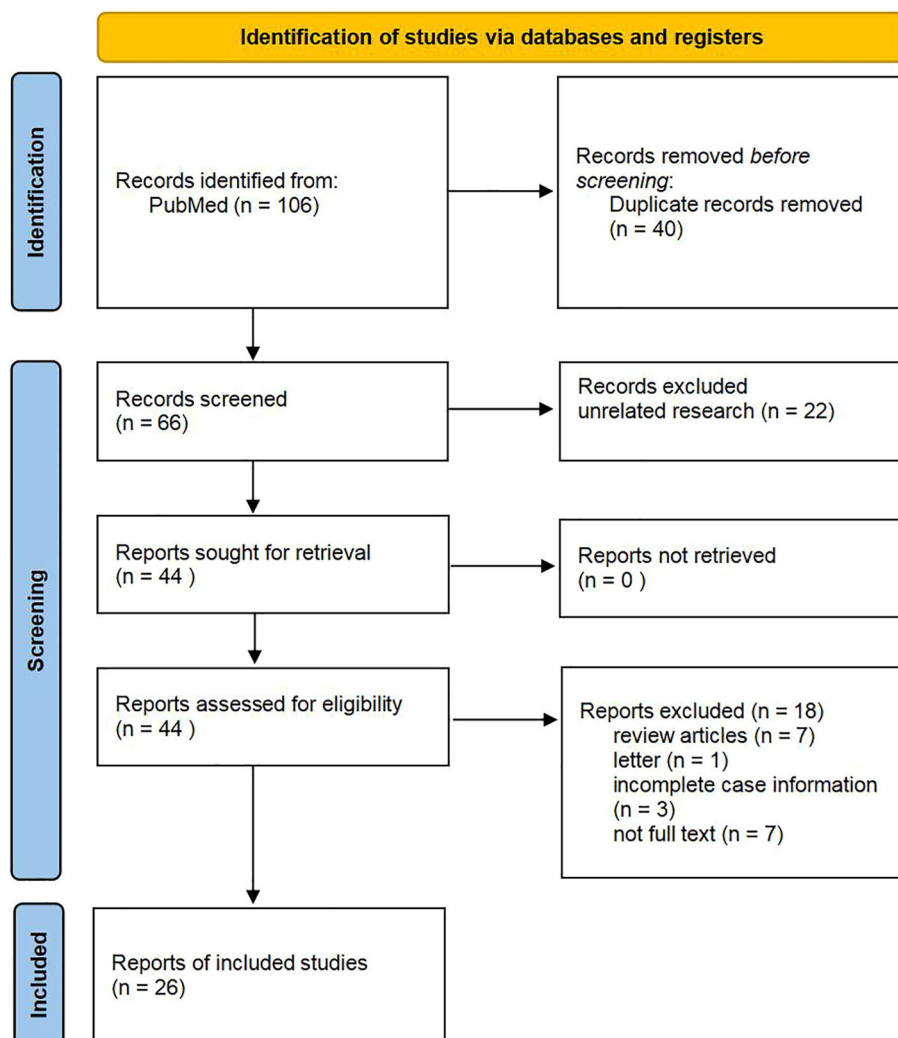


FIGURE 7  
Flow diagram of study selection.

thyroid papillary carcinoma markers TTF-1 and TG were negative, the majority of TCCRP had a Ki67 proliferative index of less than 10% indicating a better prognosis, CK5/6 was usually positive, and p63 was usually negative suggesting a certain degree of aggressiveness. In terms of diagnosis, early studies have shown that TCCRP does not have molecular genetic alterations typical in papillary thyroid carcinoma, such as BRAF and RET gene mutations, Chiang et al. (15) found that mutations in the IDH2 and PIK3CA genes are characteristic mutations in TCCRP, and Alsadoun et al. (20) suggested that Calretinin is also a useful TCCRP marker, as Calretinin expression is rare in low-grade breast cancers, whereas high-grade breast cancers with poorer prognosis are usually positive for Calretinin. It is now generally accepted that TCCRP has an inert biological behaviour and a good prognosis, and in terms of treatment, some scholars support the absence of active clinical management, while others have proposed to consider the use of IDH2 inhibitors.

The clinical, pathologic, and immunohistochemical features of our TCCRP case were consistent with previous reports of a 54-year-

old woman with a painless breast mass that presented clinically and had been present for 10 years, similar to the history reported by Tosi (11). However, the Mammogram in this patient suggested benign, and it was considered that the diagnosis was missed due to the extremely dense type of the patient's gland and the low sensitivity of the Mammogram for the mass, and Foschini (17) also suggested that the lesions were mostly interpreted as benign on Mammogram examination or Ultrasound because of the regularity of their margins. The diagnosis of TCCRP on the basis of puncture pathology is difficult as it shows columnar to cuboidal tumour cells, with nuclei seen in very few areas away from the base. Postoperative pathology had typical features of TCCRP, with papillary, glandular-like structure of cancer cells, some of which were cystically dilated, similar to thyroid follicles, and the surface of the papillae was covered with cancer cells in a cuboidal or high columnar shape, with nuclei far away from the base. The interstitium of the cancerous tissue was slender fibrous connective tissue with fibrovascular axons, and foam cell aggregates were seen. Immunohistochemistry was triple-negative, KI67 was 10% with a

TABLE 1 Summary of TCCRP case report clinical information.

| Literature                | Cases | Age<br>(The average) | Tumour<br>size (cm) | Tumour<br>site<br>(cases) | Lymph<br>node<br>metastasis              | Imaging                  | TNBC | IDH2<br>mutation | PIK3CA<br>mutation | Treatment                                                               | Follow-up<br>(average month)                                                     |
|---------------------------|-------|----------------------|---------------------|---------------------------|------------------------------------------|--------------------------|------|------------------|--------------------|-------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| Eusebi et al.2003 (9)     | 5     | 56-74 (63)           | 0.8-2.0 (1.4)       | L (2)<br>R (2)<br>NA (1)  | UK                                       | UK                       | 0    | ND               | ND                 | Wide excision (3);<br>quadrantectomy (1);UK (1)                         | 26-108 (54),NED (4)<br>UK (1)                                                    |
| Cameselle et al.2006 (10) | 1     | 64                   | 4.1                 | R                         | axillary<br>lymph node                   | UK                       | 0    | ND               | ND                 | Mastectomy+ALND+C/<br>XRT/HT                                            | 32, bone metastases                                                              |
| Tosi et al.2007 (11)      | 4     | 45-80 (58)           | 2-5 (3)             | R (4)                     | intramammary<br>lymph node (1)<br>No (3) | UK                       | 0    | ND               | ND                 | Quadrantectomy (4)                                                      | 3-10 (6.5)NED                                                                    |
| Chang et al.2009 (12)     | 1     | 66                   | 1.1                 | L                         | No                                       | Ultrasound               | 0    | ND               | ND                 | quadrantectomy and<br>SLN excision                                      | 12, NED                                                                          |
| Masood et al.2012 (13)    | 1     | 57                   | 3.7                 | L                         | No                                       | Ultrasound,<br>Mammogram | 0    | ND               | ND                 | mastec-<br>tomy                                                         | UK                                                                               |
| Colella et al.2015 (14)   | 1     | 79                   | 3                   | R                         | No                                       | Ultrasound               | 1    | ND               | ND                 | mastectomy with ALND                                                    | 18                                                                               |
| Chiang et al.2016 (15)    | 13    | 51-79 (65)           | 0.6-1.8 (1.1)       | L (8)<br>R (5)            | No                                       | UK                       | 6    | 10               | 8                  | S (1)<br>S+XRT (2)<br>S+C (1)<br>S+XRT+C (1)<br>NA (8)                  | 12-77 (34)<br>NED (7)<br>UK (6)                                                  |
| Bhargava et al.2017 (16)  | 3     | 48-65 (63)           | 0.9-1.7 (1.3)       | L (2)<br>R (1)            | UK                                       | MRI (1)                  | UK   | 2                | 2                  | S (3)                                                                   | 0-19 (13)<br>NED (2)<br>UK (1)                                                   |
| Foschini et al.2017 (17)  | 13    | 48-85 (62.6)         | 0.6-2.5 (1.5)       | L (7)<br>R (6)            | axillary lymph<br>node (2)<br>No (11)    | Ultrasound,<br>Mammogram | 10   | ND               | ND                 | wide excision (11)<br>wide excision+ALND (1)<br>wide excision+XRT+C (1) | 24-132 (71)<br>Recurrence and lymph<br>node metastasis (1)<br>NED (10)<br>UK (2) |
| Pitino et al.2017 (18)    | 1     | 65                   | 0.7                 | R                         | No                                       | Ultrasound,<br>Mammogram | 0    | ND               | ND                 | quadrantectomy<br>+SLN excision                                         | 34,NED                                                                           |
| Lozada et al.2018 (19)    | 6     | 58-85 (60)           | 0.6-2.1 (1.2)       | UK                        | No                                       | UK                       | 6    | 6                | 4                  | UK                                                                      | UK                                                                               |
| Alsadoun et al.2018 (20)  | 9     | 52-75 (66)           | 0.9-4 (1.7)         | L (5)<br>R (4)            | UK                                       | UK                       | 4    | 6                | ND                 | quadrantectomy (8)<br>quadrantectomy+SLN (1)                            | 53<br>NED (1)<br>UK (8)                                                          |
| Gai et al.2018 (21)       | 1     | 55                   | UK                  | R                         | No                                       | Mammogram                | 1    | ND               | ND                 | simple mastectomy+SLNB                                                  | UK                                                                               |
| Zhong et al.2019 (22)     | 9     | 63-79 (70)           | 0.7-1.8 (1.3)       | R (5)<br>L (4)            | No                                       | UK                       | 4    | 7                | 6                  | UK                                                                      | 16-64 (32)<br>NED (9)                                                            |

(Continued)

TABLE 1 Continued

| Literature                 | Cases | Age<br>(The average) | Tumour size (cm) | Tumour site (cases) | Lymph node metastasis | Imaging                    | TNBC | IDH2 mutation | PIK3CA mutation | Treatment                                                                | Follow-up (average month) |
|----------------------------|-------|----------------------|------------------|---------------------|-----------------------|----------------------------|------|---------------|-----------------|--------------------------------------------------------------------------|---------------------------|
| Ding et al.2019 (23)       | 1     | 70                   | 1.6              | L                   | No                    | Mammogram                  | 1    | 1             | 1               | quadrantectomy                                                           | 9,NED                     |
| Haefliger et al.2020 (24)  | 1     | 60                   | 0.8              | R                   | No                    | Mammogram                  | 1    | 1             | 1               | quadrantectomy +SLN excision                                             | 8,NED                     |
| Pareja et al.2020 (25)     | 14    | 46-85 (63)           | 0.6-2.6 (1.3)    | UK                  | No                    | UK                         | UK   | 14            | 7               | quadrantectomy (2)<br>quadrantectomy+XRT (3)<br>mastectomy (1)<br>UK (8) | UK                        |
| Jassim et al.2021 (26)     | 1     | 40                   | 5.5              | R                   | No                    | Mammogram                  | 0    | 1             | ND              | mastectomy+ALND                                                          | 6,NED                     |
| Zhang et al.2021 (27)      | 1     | 45                   | 1                | L                   | No                    | Ultrasound, Mammogram      | 1    | 1             | 1               | wide local excision+SLNB                                                 | UK                        |
| Trihia et al.2021 (28)     | 1     | 71                   | 0.9              | R                   | No                    | Mammogram                  | 1    | ND            | ND              | Quadrantectomy+SLNB                                                      | UK                        |
| Wei et al.2021 (29)        | 2     | 70-72 (71)           | 1.6-2.2 (1.9)    | R (2)               | No                    | Ultrasound                 | 2    | 2             | 2               | mastectomy (1)<br>mastectomy+SLNB (1)                                    | 6-33 (19.5),NED           |
| Cui et al.2021 (30)        | 1     | 62                   | 2                | L                   | No                    | Ultrasound                 | 1    | 1             | ND              | mastectomy                                                               | 6,NED                     |
| Sasaki et al.2022 (31)     | 3     | 44-71 (57)           | 0.4-1.4 (0.9)    | L (2)<br>R (1)      | No                    | UK                         | 1    | 3             | ND              | Wide excision+SLNB (3)                                                   | 8-19 (14),NED             |
| Lee et al.2023 (32)        | 1     | 64                   | 1.6              | R                   | No                    | Ultrasound, Mammogram, MRI | 1    | ND            | ND              | Quadrantectomy+SLNB                                                      | 12,NED                    |
| Elghobashy et al.2023 (33) | 1     | 41                   | 2.2              | L                   | No                    | Ultrasound, Mammogram      | 1    | 1             | ND              | Quadrantectomy +SLNBX+XRT                                                | 19,NED                    |
| Lei et al.2024 (34)        | 1     | 65                   | 2                | L                   | No                    | Ultrasound, Mammogram, MRI | 1    | 1             | ND              | Quadrantectomy                                                           | 48,NED                    |

ALND, axillary lymph node dissection; C, chemotherapy; HT, endocrine therapy; L, left; ND, not done; NED, disease-free survival; R, right; SLN, sentinel lymph node; SLNB, sentinel lymph node biopsy; UK, unknown; XRT, radiation therapy.



good prognosis, Calretinin was positive, and genetic testing suggested IDH2 and PIK3CA mutations. The current follow-up period is 15 months and the patient will be followed for a long time in the future. In addition, there are no studies on TILs infiltration in the TME of TCCRP, and we compared the present case with a case of common TNBC according to the international guidelines on TILs detection published by the International Immunology-Oncology Biomarker Working Group in 2014 (36), with immunohistochemical detection of CD4<sup>+</sup> T cell and CD8<sup>+</sup> T cell expression to first explore the characteristics of TCCRP TILs infiltration.

Breast cancer used to be regarded as a low-immunogenic tumour, but in recent years, studies have revealed the presence of TILs infiltration in the stroma of TNBC, which is attributed to the high degree of genetic instability and mutational load leading to the production of a large number of neoantigens (37), which are capable of strong immune responses and strong immunoediting effects. And the higher the degree of TILs infiltration the better the prognosis, clinical studies have shown that high levels of specific phenotypic TILs (CD4<sup>+</sup>, CD8<sup>+</sup>) can positively predict the long-term prognosis of TNBC (38). It has been suggested that immune-rich TNBC may be under immune surveillance, which consistently eliminates many immunogenic clones and thus reduces clonal heterogeneity and achieves a balanced state of immune surveillance and clonal heterogeneity, while TNBC with low TILs have a poor prognosis and may represent the onset of immune escape and tumour evolution toward greater clonal heterogeneity and genomic diversity (39). Our case of TCCRP was a low-immunogenic tumour with low levels of TILs but good prognosis, a specific TNBC subtype. The reason may be that TCCRP has a more stable genome with low somatic copy number as well as clonal heterogeneity, and there was no significant neoantigen production, which resulted in the absence of a strong immune response. In addition, we observed a large amount of fibrous connective tissue in the TCCRP mesenchyme, and the fibrotic mesenchyme was able to inhibit anti-tumour immune responses and allow the tumour to undergo immune escape. Fibroblasts are the main cells of the tumour mesenchyme that can be induced by cancer cells to become Cancer-Associated Fibroblasts (CAF), and these fibroblasts usually express cytoskeletal proteins ( $\alpha$ -SMA) or fibroblast activation proteins (FAP), and it is now proposed that CAFs can establish an immune-suppressing by interacting with the TME components and thus establishing an immune-suppressive microenvironment (40). CAF-enriched tumours exhibit an immunocold tumour microenvironment, and transcriptomics, flow cytometry, and quantitative histopathology analyses have demonstrated a negative correlation between CAF density and the anti-tumour phenotype of CD8<sup>+</sup> T cells (41), which is consistent with the characteristics of the TCCRP in this case. In addition, CAF was able to promote the occurrence of M2 polarisation of tumour-associated macrophages, as well as a significant increase in the proportion of other immunosuppressive cells (regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC)) in TME, resulting in an immunosuppressive microenvironment (42). Four main subpopulations of CAF have been identified, which are categorised as CAF-S1 to CAF-S4 based on the expression of

differentially activated markers, TNBC are generally enriched in CAF-S1 or CAF-S4, and FAP is expressed only in the CAF-S1 subpopulation (43). In this case, FAP was positive for SMA, which belongs to the CAF-S1 subpopulation, and CAF-S1 has an immunosuppressive function, and targeting of CAF may also help to allow more immune infiltration and restore immunosuppression. It has been shown that tumours in CAF receptor Endo180 knockout mice exhibit increased CD8<sup>+</sup> T cell infiltration and enhanced sensitivity to immune checkpoint blockade (ICB) (41). FAP can also be used as a therapeutic target, for example FAPI-04 can be used to target breast cancers with high levels of activated fibroblasts (44). We hypothesised that targeting fibroblast subpopulations to improve clinical response to immunotherapy may be an effective treatment modality for TCCRP. It is also important to note that our findings are preliminary, as the 15-month follow-up is shorter than the mean of 25.1 months, and longer follow-up is needed to validate our findings. We have limitations in studying the immune characteristics of TME in our TCCRP case, CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells in TILs contain multiple cell subpopulations, which need to be differentiated on the basis of surface markers and secreted cytokines, and each subpopulation plays a different role in the immune response, for example, Th1 cells promote tumour killing, Treg suppress the immune response, Th1 cell markers include CD28, CD44, secrete IFN- $\gamma$  and the Treg cell marker is FOXP3. In addition, the expression of other immune cells in the TME has an association with breast cancer prognosis. For example, there was a significant independent positive correlation between higher mature Tumour-Infiltrating Dendritic Cells (DCs) and longer recurrence-free survival (RFS) (45). Infiltration of tumour-associated macrophages (TAM) is usually associated with poor clinicopathological features, and a retrospective study had reported a poor prognostic role for M2-polarised TAM in breast cancer patients (46). There was also a study that preliminarily showed that increased natural killer (NK) cell expression was associated with improved RFS (47). Overall, in addition to TILs expression, the infiltration of other immune cells in the TME is also an important study to assess the prognosis of the tumour, and we initially only examined the infiltration of TILs, which has some limitations and does not represent the overall immune infiltration of the TCCRP. Subsequently, flow cytometry and single cell sequencing techniques can be used to further investigate the immune profile of TME in TCCRP, as well as the crosstalk between CAF subpopulations and immune cells to better understand the immune profile of TCCRP.

## 4 Conclusion

TCCRP is a rare type of TNBC with inert biological behaviour and good prognosis, the typical pathological feature is the papillary morphology of the nuclei away from the base. This case has the feature of low TILs infiltration, which may be a characteristic of TCCRP, the presence of CAF in the interstitium of the tumour in this case may have suppressed anti-tumour immunity to some extent, and further studies are needed regarding the immune characteristics of the TME in TCCRP.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## Ethics statement

The studies involving humans were approved by Research Ethics Committee of the Third Affiliated Hospital of Beijing University of Chinese Medicine. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## Author contributions

SJ: Writing – original draft. JC: Writing – original draft. JS: Writing – original draft. AP: Writing – original draft. RC: Writing – original draft. BL: Writing – review & editing. CL: Writing – review & editing. YF: Writing – review & editing. XP: Writing – review & editing.

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## Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This study was supported by National Natural Science Foundation of China (8237153504), Beijing University of Chinese Medicine top innovative talent training funding project (90010961020119).

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## OPEN ACCESS

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RECEIVED 21 February 2024

ACCEPTED 11 November 2024

PUBLISHED 25 November 2024

## CITATION

Wang L, Wang Y, Li Y, Zhou L, Du J,  
Wang J, Liu S, Cao Y, Li Y, Yang W and  
Zhu T (2024) Resistance mechanisms  
and prospects of trastuzumab.  
*Front. Oncol.* 14:1389390.  
doi: 10.3389/fonc.2024.1389390

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# Resistance mechanisms and prospects of trastuzumab

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Breast cancer that overexpresses Human Epidermal Growth Factor Receptor 2 (HER2+) due to gene amplification or overexpression constitutes 15-20% of all breast cancer cases. Trastuzumab, the first FDA-approved monoclonal antibody targeting HER2, serves as the standard first-line treatment for HER2-positive advanced breast cancer, as recommended by multiple clinical guidelines. Currently, accumulated clinical evidence reveals a considerable degree of variability in the response of HER2+ breast cancer to trastuzumab treatment. Specifically, over 50% of patients either do not respond to or develop resistance against trastuzumab. The specific mechanisms of resistance to trastuzumab are currently unclear. This paper aims to review the existing research on the resistance mechanisms of trastuzumab, based on its target, from aspects such as genetic loci, molecular structure, signaling pathways, and the tumor microenvironment and to outline current research progress and new strategies.

## KEYWORDS

trastuzumab, HER2, breast cancer, tumor microenvironment, resistance

## 1 Introduction

The prevalence of breast cancer (BC) among women has risen to become the leading cause of cancer incidence globally, with a considerable associated mortality rate. Among the molecular subtypes, HER2-positive breast cancer accounts for 15-20% of cases and is often associated with high invasiveness, metastatic potential, and treatment resistance, serving as a predictor of poor prognosis (1). Since its approval by the FDA in 1998, trastuzumab has emerged as a groundbreaking anti-HER2 monoclonal antibody. By binding to the extracellular domain (ECD) of HER2, trastuzumab effectively blocks intracellular HER2 signaling pathways, leading to cell cycle arrest and antibody-dependent cellular cytotoxicity. These mechanisms have collectively contributed to significant improvements in the prognosis of HER2-positive patients (2). However, a substantial body of clinical and research evidence indicates that resistance to trastuzumab is inevitable. The mechanisms of trastuzumab resistance remain unclear, with prevailing hypotheses including HER2 mutations or isoform formation, activation of alternative signaling



pathways, epitope masking, and changes in the tumor microenvironment, among others. These challenges have led to the development of new treatment strategies, such as antibody-drug conjugates and dual antibody therapies. This article aims to provide a comprehensive overview of the resistance mechanisms of trastuzumab through its interaction with HER2 (3, 4).

## 2 HER family

The members of the HER family, HER1 (EGFR, ERBB1), HER2, HER3 (ERBB3), and HER4 (ERBB4), play a central role in regulating cell proliferation and differentiation in embryonic and human tissues (5). They are critical in the upregulation, mutation, and hyperactivation of human cancers due to their structural similarities and specificity (6). These receptors use multiple activation mechanisms to identify growth factors through diverse recognition methods. They are crucial in regulating cancer phenotypes by interacting with proximal signal transduction components, thereby serving as primary targets for drugs designed to treat tumor cells (7).

## 3 HER2

The HER2 gene, also known as Neu, ErbB2, or CD340, was initially identified in human breast cancer cells (8). It belongs to the receptor tyrosine kinase (RTK) family, specifically the epidermal growth factor receptor (EGFR) subfamily. HER2 is a tyrosine kinase receptor membrane glycoprotein encoded by the ErbB gene, located on the long arm of chromosome 17 in band 2 region 1 (17q21), and is classified as an oncogene (9). The protein encoded by this gene undergoes glycosylation, has a molecular weight of 185 kDa, and is anchored to the cell membrane. This transmembrane protein consists of three distinct domains: the extracellular domain (ECD), the transmembrane domain (TM), and the intracellular domain (ICD). These domains work together to form the complete structure of the transmembrane protein (10).

## 4 HER Receptor Activation

The activation of HER family members primarily depends on autocrine or paracrine pathways (7).

ErbB receptors feature an extracellular region of about 630 amino acids, structured into four unique domains: I/L1, II/CR1, III/L2, and IV/CR2. These domains are sequentially organized, alternating in pairs. The receptors also include a single transmembrane domain and a cytoplasmic tyrosine kinase (9). Each structural domain includes specific sub-domains and amino acid residues, which can be classified into ligand-binding and dimerization domains. Except for HER3, all receptors contain a cytoplasmic tyrosine kinase region. Except for HER2, all receptors bind specific ligands through their extracellular domains. Upon dimerization, HER2 exhibits strong kinase activity and preferential binding. The dimer features leucine-rich residues in extracellular

sub-domains I and III and cysteine residues in sub-domains II and IV, primarily connected by disulfide bonds (10, 11).

Tyrosine kinase phosphorylation is triggered through heterodimerization, leading to the activation of several signaling pathways, notably Ras/MAPK, PI3K/AKT/mTOR, PLC $\gamma$ /PKC, and JAK2/STAT. These pathways play essential roles in key biological processes, including cell growth, programmed cell death, differentiation, blood vessel formation, and cellular invasiveness (12, 13).

## 5 Mechanisms of trastuzumab resistance

### 5.1 Gene overexpression or deletion

After anti-HER2 treatment, the overexpression or deletion of associated genes can result in mutations in the target itself, causing decreased or lost HER2 expression, which in turn diminishes drug efficacy. Research on resistance to trastuzumab has identified mutations in genes such as ATM, CDH1, GNAS, MLH1, RB1, SMARCB1, SMO, TP53, and VHL (14). The p53 signaling pathway is activated by DNA damage, emphasizing its vital role in cellular stress response mechanisms (15). These mutations may contribute to the development of resistance to therapy (16).

For example, ATM and RB 1 are mainly associated with cell cycle regulation and cellular senescence, and have significant associations with drug resistance in trastuzumab. Studies have shown that ATM (ataxia telangiectasia mutated) plays a key role in the DNA damage response, and that cells lacking ATM show resistance to apoptosis induced by low levels of DNA double-strand breaks, which may lead to early senescence (17). Moreover, RB 1 (retinoblastoma protein), as an important tumor suppressor, regulates the cell transition from G1 to S phase, and its loss of function is closely related to the development of various cancers. In HER 2-positive breast cancers, trastuzumab resistance is often associated with dysregulation of cell cycle regulation, especially as inactivation of RB 1 may lead to cellular resistance to therapy (18).

The interaction between ATM and RB1 may significantly influence the regulation of cell proliferation and apoptosis. ATM modulates cell proliferation and the apoptotic response by controlling the levels of the ARF (alternative reading frame) tumor suppressor protein. In contrast, RB1 regulates cell cycle progression by interacting with E2F transcription factors, thereby impacting cell growth and treatment response. Consequently, the dysregulation of ATM and RB1 might be a crucial factor underlying the resistance of HER2-positive breast cancer cells to trastuzumab. The role of ATM and RB1 in cell cycle control and cellular senescence, as well as their connection to trastuzumab resistance, offers important insights into the mechanisms underlying therapeutic resistance in breast cancer (19).

### 5.2 HER2 density or structural changes

#### 5.2.1 HER2 mutations or isoform formation

Due to the widespread presence of mutations, splicing, and post-translational modifications in tumor cells, HER2 inevitably produces



isoforms and various subtypes. Current research indicates the existence of HER2 splicing variants lacking exon 16 (HER2Δ16), and the co-expression of HER2 subtypes has a unique impact on the phenotype of breast tumors. The activation of oncogenes, with a primary mechanism closely tied to epithelial-mesenchymal transition (EMT), is integral to this process (20). The formation of stable disulfide bonds at the molecular level eventually activates downstream signaling pathways, potentially causing a delayed response and reduced efficacy of trastuzumab (21).

Under certain conditions, proteolytic cleavage can result in the formation of specific mutant forms of the epidermal growth factor receptor. For example, soluble EGFR (sEGFR) can be produced either by selective splicing of receptor mRNA or by site-specific proteolytic cleavage of the receptor, releasing the extracellular domain. Similar to EGFR, matrix metalloproteinase (MMP) and ADAM (A Disintegrin And Metalloprotease domain) cleavage of HER2-ECD produces a truncated form of HER2, known as p95HER2—a 95 kDa truncated fragment of the HER2/neu protein. The absence of the extracellular domain in p95HER2 leads to resistance to trastuzumab, and p95HER2 can form homodimers, bypassing the binding site of trastuzumab or diminishing its effect, thereby activating multiple downstream pathways (Figure 1A). This variant is prevalent in cancer patients, accounting for 22.4%, and has been closely associated with breast cancer metastasis and recurrence (22, 23).

Moreover, the truncated form of the extracellular domain of the antigen might represent another possible resistance mechanism, although this has not yet been clinically confirmed for trastuzumab deruxtecan. This concept has, however, been previously demonstrated with trastuzumab; tumors expressing the complete receptor responded well to trastuzumab, whereas those expressing the truncated p95HER2 form showed resistance (24).

### 5.2.3 HER2 epitope masking

Trastuzumab resistance primarily arises from antibody binding obstruction, which is facilitated by MUC4. MUC4 covers the

trastuzumab binding site on HER2, leading to trastuzumab resistance upon prolonged exposure (Figure 1B) (25). TNF $\alpha$ -induced expression of MUC4 leads to trastuzumab resistance, impairing Antibody-Dependent Cellular Cytotoxicity (ADCC). CD44, a transmembrane hyaluronic acid receptor overexpressed in JIMT-1 cells, is proven to interact with polymerized hyaluronic acid.

Although HER2-targeted antibodies have been effective for treating HER2-positive cancers, a considerable number of patients do not respond despite sustained antigen expression. Overexpression of extracellular matrix (ECM) components, such as MUC4 or CD44/hyaluronic acid, creates a physical barrier that masks the HER2 antigen, hindering its recognition (Figure 2). Multichannel targeted delivery approaches, like the synergistic action of KLA-R16, survivin siRNA, and Herceptin, can help mitigate resistance in recurrent breast cancer driven by CD44 overexpression (26).

In preclinical models, HER2-redirected CAR T cells have shown considerable potential in eliminating HER2-positive trastuzumab-sensitive tumor cell lines, such as SKBR3 and BT474. Moreover, clinical trials have demonstrated that these CAR T cells can effectively treat trastuzumab-resistant patients. However, evidence of their anti-tumor efficacy in trastuzumab-resistant tumors is still lacking (27). ECM components contribute to trastuzumab resistance by sterically hindering antigen recognition, which could also impact the effectiveness of emerging antibody-drug conjugates (ADCs). Therefore, the clinical application of trastuzumab-derived HER2-specific CAR T cells may provide a promising option for treating trastuzumab-resistant tumors (28).

### 5.2.4 HER2 shedding

Recent studies suggest that dysregulation in the shedding of extracellular domains (ECD) from transmembrane receptors may contribute to tumor progression and resistance. Specifically, the shedding of the HER2 receptor results in the release of soluble HER2-ECD, which retains the trastuzumab recognition epitope while leaving behind the oncogenic, membrane-associated p95HER2

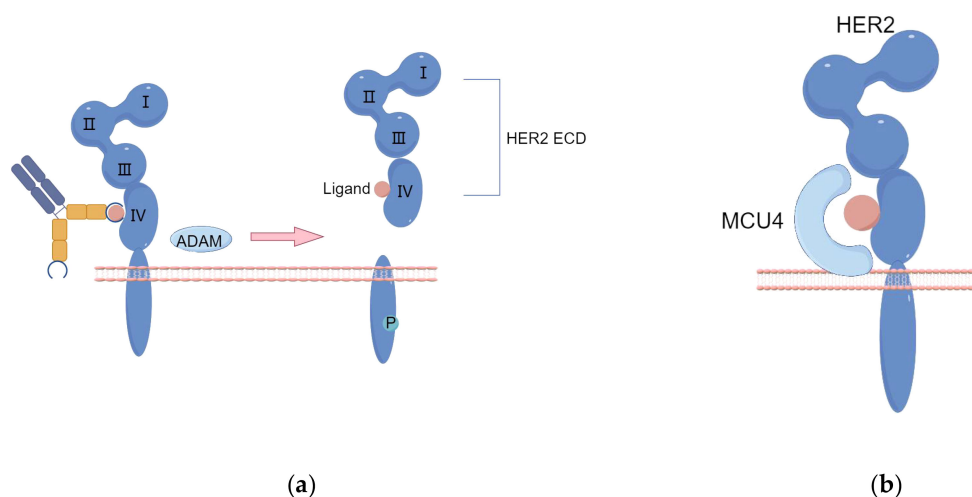


FIGURE 1

(A) The proteolytic cleavage of HER2-ECD by ADAM enzymes results in the production of a truncated form of HER2 protein, referred to as p95HER2. (B) Enhanced activation of the Signal Transducer and Activator of STAT3 pathway leads to the upregulation of MUC1/MUC4 expression, subsequently contributing to the development of resistance against trastuzumab. (By Figdraw).

fragment. The presence of p95HER2 plays a significant role in trastuzumab resistance (29).

Studies have found that upregulating DPAGT1 persistently promotes HER2 shedding, leading to resistance to trastuzumab (30).

### 5.2.5 Heterodimer formation

Recent research indicates that increased IGF2 protein expression is observed in trastuzumab-resistant breast cancer

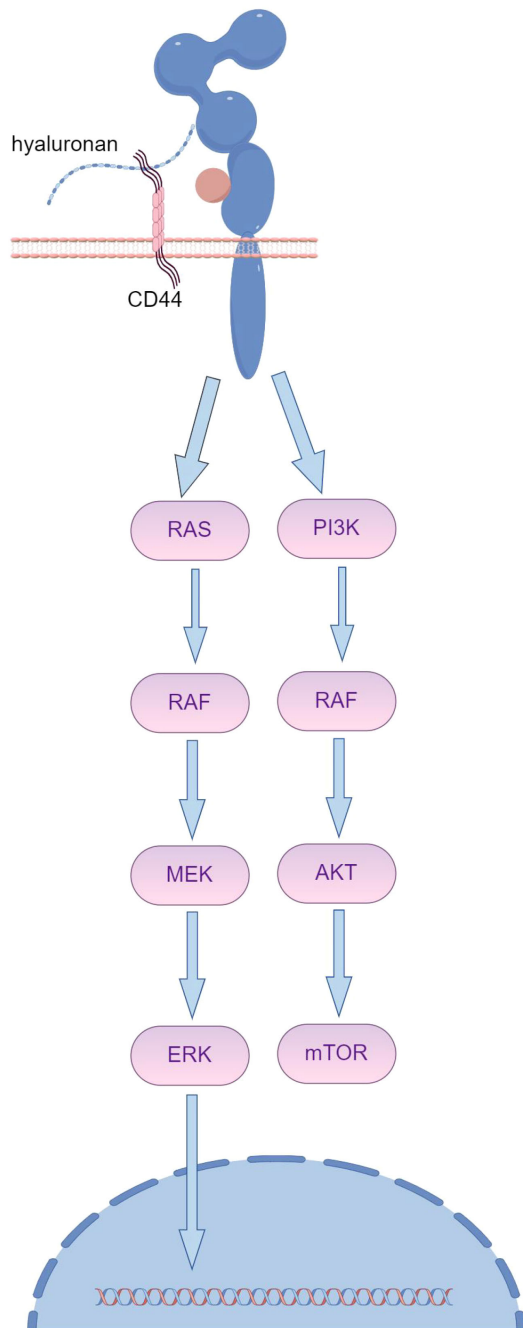
strains, which may be linked to the formation of IGF1R/HER2 heterodimers (Figure 3). It has been demonstrated that miR-98-5p can bind to the 3'UTR of IGF2 mRNA (31). Additionally, a significant correlation has been identified between the downregulation of miR-98-5p, the upregulation of IGF2, and trastuzumab responsiveness. These factors may thus serve as predictive markers for trastuzumab sensitivity and as potential targets to combat trastuzumab resistance.

AXL, a member of the TAM (TYRO3, AXL, MER) receptor tyrosine kinase family, is associated with epithelial-mesenchymal transition. The formation of HER2 heterodimers has been linked to trastuzumab resistance (32). AXL activates the PI3K/AKT and MAPK pathways through binding with its ligand, Growth Arrest-Specific 6 (GAS6), extracellular domain-mediated dimerization, or through signaling cascade formation with other transmembrane receptors, ultimately leading to resistance phenotypes. Combined targeting of AXL and anti-HER2 therapies could be a potential strategy to overcome trastuzumab resistance (33).

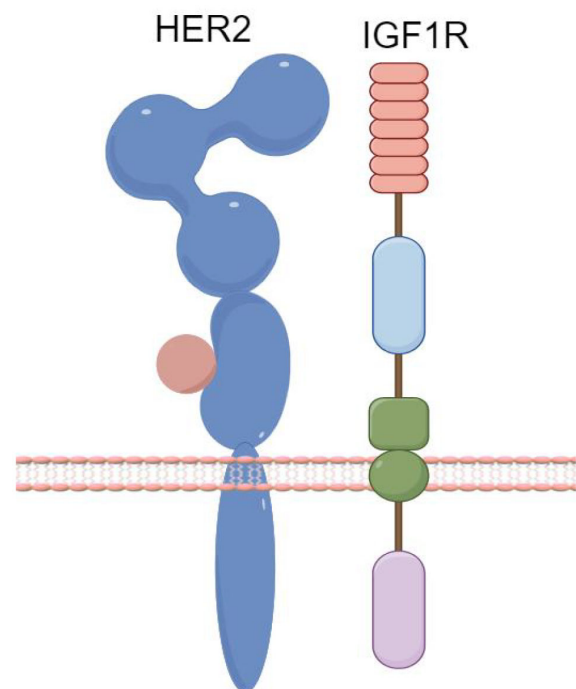
## 6 Activation of the downstream signaling pathway of HER2

### 6.1 Constitutive activation of the PI3K/AKT pathway

Recent research highlights that the primary downstream signaling pathways of HER2 are RAS/RAF/MEK/ERK and PI3K/AKT, with mutations in the PI3K/AKT pathway accounting for a significant portion of resistance cases (Figure 4) (34). Specifically,

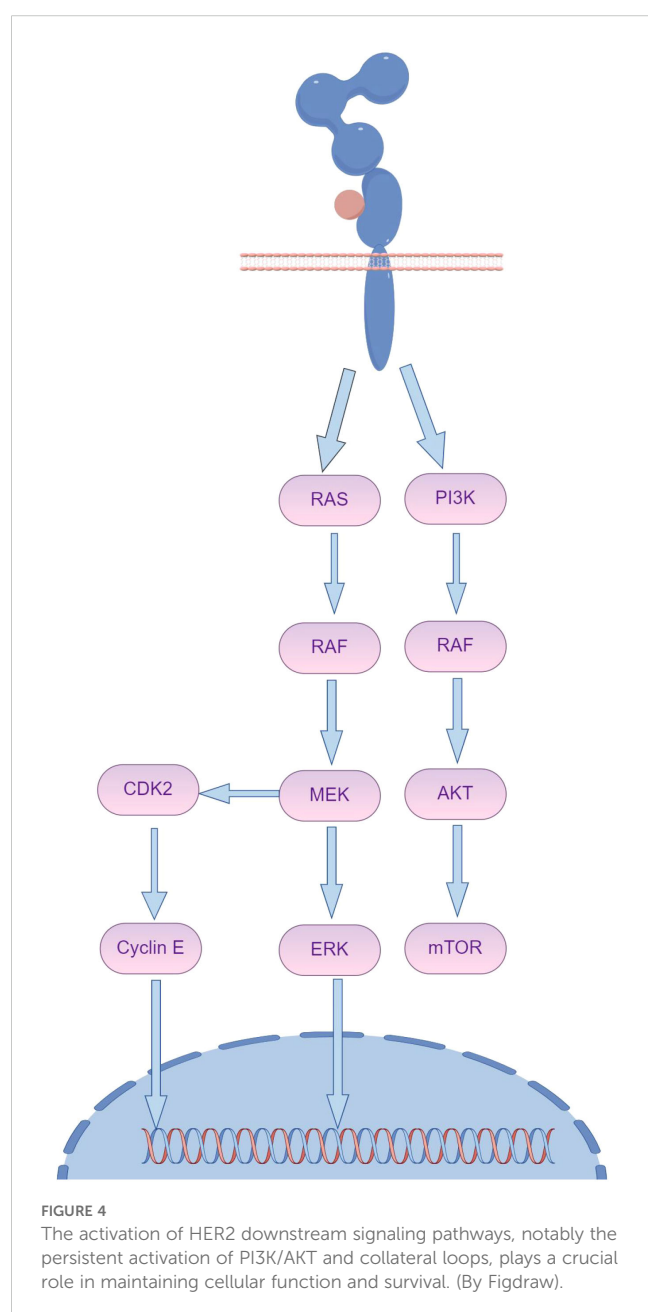


**FIGURE 2**  
The overexpression of ECM components, such as MUC4 or CD44/hyaluronic acid, creates a spatial barrier that shields the HER2 antigen from recognition, subsequently leading to resistance to trastuzumab. (By Figdraw).



**FIGURE 3**  
Formation of IGF1R/HER2 Heterodimers. (By Figdraw).

mutations in PI3K prevent the inhibition of AKT even when the complex is disrupted, allowing downstream signaling to negate trastuzumab's inhibitory effects on HER2 (35). The use of multiple targeted therapies has shown effectiveness in addressing this resistance. The HER family is essential for tumor cell proliferation, regulated by various ligands. Upregulation of integrins has been closely associated with trastuzumab resistance, particularly due to the activation of the PI3K pathway. Targeting both HER2 and integrins can partially mitigate trastuzumab resistance driven by PI3K activation. Recombinant protein-drug conjugates, RP-HI and RPDC-HI, have been developed to target these interactions, blocking the HER family-integrin interactions and subsequently inhibiting downstream signaling, thus alleviating trastuzumab resistance (36).



Activation of certain signaling pathways may contribute significantly to resistance in malignant cells against antibody-drug conjugates (ADCs). One such pathway is PI3K/AKT/mTOR, which plays a key role in cell survival, growth, and metabolic processes (Figure 5). Activation of this pathway has been linked to decreased ADC sensitivity, which reduces the efficacy of the cytotoxic payloads carried by ADCs and prolongs cell survival (31, 37). This phenomenon has been observed in patients carrying PIK3CA mutations or PTEN deletions during trastuzumab treatment. Specifically, PTEN downregulation has been linked to trastuzumab treatment failure, suggesting that PTEN deficiency or PIK3CA hyperactivation reduces trastuzumab sensitivity by activating the PI3K/AKT pathway. These findings highlight the importance of considering inhibitors targeting these signaling pathways in future therapeutic approaches to improve the effectiveness of trastuzumab and related drugs and overcome resistance issues.

The cyclin-dependent kinase inhibitor protein p27Kip1 is a critical factor in trastuzumab responsiveness, and its downregulation has been shown to promote trastuzumab resistance. Additionally, activation of the cyclin D1-CDK4/6 (cyclin-dependent kinase 4/6) pathway has been found to limit the antitumor activity of HER2 inhibitors *in vivo*. The combined use of CDK4/6 inhibitors with anti-HER2 and endocrine therapies has shown promising potential in treatment strategies, providing a potential avenue for overcoming trastuzumab resistance (37).

## 6.2 PTEN loss

The loss of PTEN leads to the continuous activation of the PI3K/AKT signaling pathway. In HER2-positive cancers, decreased PTEN expression or PTEN polymorphism hinders trastuzumab-mediated growth inhibition (38). PTEN levels vary between HER2-negative and HER2-positive cancers (39).

## 7 Tumor microenvironment

### 7.1 Immune-mediated resistance

#### 7.1.1 ADCC escape

ADCC, standing for Antibody-Dependent Cellular Cytotoxicity, is an immune response capable of lysing target cells. The immune system possesses a crucial defense mechanism, a nonspecific cytotoxic reaction that involves a range of immune cells, namely natural killer cells, macrophages, and monocytes (40).

#### 7.1.2 ADCC regulation

Human Leukocyte Antigen G (HLA-G) is a non-classical MHC I molecule. In the tumor microenvironment, trastuzumab induces abnormal production of TGF- $\beta$  and IFN- $\gamma$ , which subsequently enhances the HLA-G/KIR2DL4 signaling pathway, where HLA-G is the sole ligand for KIR2DL4 (41). In breast cancer cells that exhibit high HLA-G expression, binding to KIR2DL4 on natural killer (NK) cells suppresses their anti-tumor activity, thereby reducing the therapeutic efficacy of trastuzumab. Experimental studies suggest

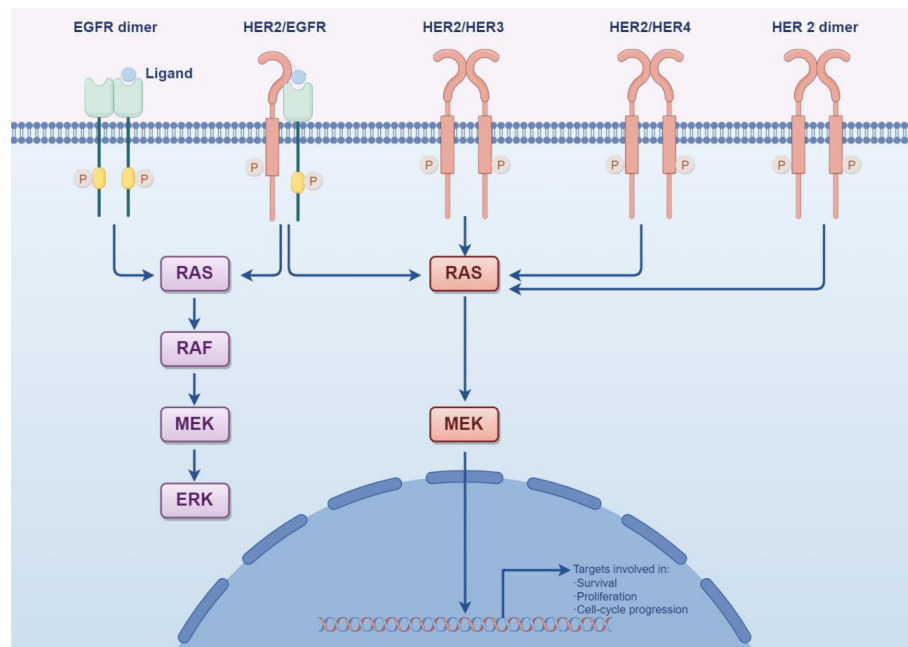


FIGURE 5

HER2 can create a “homodimer” as well as form “heterodimers” with HER1, HER3, and HER4. These various dimers significantly activate the PI3K/Akt and MAPK signaling pathways within cells, thereby controlling processes such as tumor cell proliferation, differentiation, migration, and apoptosis. This activation ultimately reduces the effectiveness of trastuzumab and contributes to drug resistance over time. (By Figdraw).

that disrupting the HLA-G/KIR2DL4 pathway can enhance the immune response of NK cells against breast cancer cells, thereby improving trastuzumab’s anti-tumor effect (42).

Given the significant role of antibody-dependent cellular cytotoxicity (ADCC) in trastuzumab’s immune response against cancer, a more strategic combination therapy approach could be beneficial for HER2-positive cancer (43). However, IFN- $\gamma$  also induces PD-L1 expression in HER2-overexpressing breast cancer cells. Altogether, these findings highlight the potential of combining HLA-G and PD-L1/PD-1 targeted therapies as a strategy to address trastuzumab resistance (44, 45).

## 7.2 Adaptive immunity

Recent studies have shown that during treatment with trastuzumab, it can induce human peripheral blood mononuclear cells (PBMCs) or natural killer cells (NK cells) to secrete interferon-gamma (IFN- $\gamma$ ) (46). This secretion of interferon-gamma (IFN- $\gamma$ ) can prompt cancer cells to increase the expression levels of Major Histocompatibility Complex-I (MHC-I) and CD86. Ultimately, this upregulated expression of MHC-I and CD86 can enhance T-cell-mediated anti-tumor immune responses (47).

The Fc gamma receptor (Fc $\gamma$ R) plays a crucial role in the anti-tumor action of trastuzumab. Traditionally, Fc $\gamma$ R was thought to be expressed only in cells of the hematopoietic system, but recent studies have shown its presence in non-leukocyte cells, such as fibroblasts. In breast cancer, high infiltration of CD16+ fibroblasts correlates with poor prognosis and trastuzumab resistance (48). The Rho family guanine nucleotide exchange factor VAV2 is essential

for the function of CD16 in fibroblasts, and targeting VAV2 can potentially reverse trastuzumab resistance. Recent research involving HER2-positive breast cancer patients and experimental models also suggests that the gut microbiome may act as an exogenous tumor factor contributing to cancer progression and treatment resistance (49).

Cancer-associated fibroblasts (CAFs) constitute a significant population of cells within the tumor microenvironment (TME) across various cancer types, breast cancer included (50). Research indicates that immunosuppressive cells resident in the TME, specifically CAFs and cancer-associated macrophages, play a role in immune evasion by tumor cells (51). In the context of cancer, CAFs have been shown to facilitate the migration of monocytes, leading to their transition towards the M2 phenotype through the secretion of factors such as chitinase 3-like 1 (Chi3L1). Nevertheless, the precise mechanism by which CAFs contribute to tumor resistance against trastuzumab-mediated ADCC remains elusive and warrants further investigation (52, 53).

## 8 The association between angiogenesis and trastuzumab resistance

The relationship between trastuzumab resistance and angiogenesis remains unclear; however, multiple studies suggest that, in contrast to trastuzumab-sensitive SKBR3 cells, the culture supernatant from trastuzumab-resistant SKBR3 cells (referred to as SKBR3-TR) significantly enhances endothelial cell elongation,

providing valuable insights. Alpha-B-crystallin, found in vascular endothelial cells, plays an important role in tumor angiogenesis by regulating vascular structure (54). Interestingly, Alpha-B-crystallin (aB-crystallin) is highly upregulated in SKBR3-TR cells. Silencing aB-crystallin significantly reduces tube formation induced by SKBR3-TR and simultaneously inhibits the activation of the mechanistic target of rapamycin (mTOR) in endothelial cells (Figure 6). Moreover, rapamycin, an mTOR inhibitor, successfully reverses the tube formation promoted by SKBR3-TR. Overall, aB-crystallin enhances the ability of SKBR3-TR cells to activate mTOR in endothelial cells, thus facilitating angiogenesis (55).

The mTOR inhibitor rapamycin affects the formation of tube-like structures induced by SKBR3-TR cells (56). Research shows that treating human aortic endothelial cells (HAECs) with SKBR3-TR cell culture medium increases the amount of phosphorylated mTOR (p-mTOR) in HAECs. However, adding rapamycin to HAECs co-cultured with SKBR3-TR cells reduces p-mTOR levels. Additionally, HAECs stimulated with culture medium treated with SKBR3-TR cells form tube-like structures, and adding rapamycin further inhibits this formation. The results indicate that tube-like structure formation induced by SKBR3-TR cells is related to mTOR activation, and the mTOR inhibitor rapamycin can reverse the promotion of tube-like structure formation induced by SKBR3-TR cells, offering potential in addressing trastuzumab resistance (12, 57).

## 9 Tumor-associated regulatory factors

### 9.1 Transcription factors, redox environment

Unlike typical RNA molecular structures, circular RNA (circRNA) lacks 3' or 5' ends, and its unique spatial configuration is closely associated with the specific progression of diseases. Studies have shown a strong correlation between circRNA and resistance in breast cancer (58). circCDYL2 promotes trastuzumab resistance by maintaining HER2 downstream signal transduction. Specifically, circCDYL2 stabilizes GRB7 forming a circCDYL2-GRB7-FAK complex, and maintaining the activity of downstream signaling molecules AKT and ERK1/2. Studies have shown that HER2+ breast cancer patients with high expression of circCDYL2 have poorer outcomes and shorter disease-free survival (DFS) and overall survival (OS) after trastuzumab treatment. Therefore, to some extent, circRNA could be a potential research direction for reducing trastuzumab resistance (59, 60).

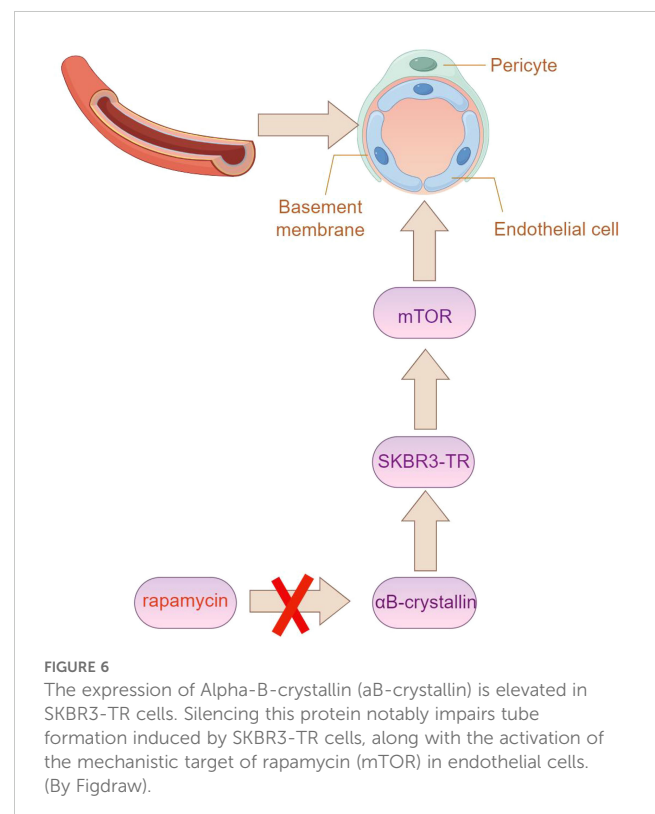
### 9.2 Autophagy evasion

Autophagy is a multistep process that allows cells to maintain homeostasis under stress by degrading dysfunctional or unwanted intracellular components. Growing evidence suggests that autophagy plays a crucial role in tumorigenesis, including processes such as tumor formation, growth, invasion, metastasis, dormancy, and resistance to therapy. In a three-dimensional *in vitro* model,

chronic trastuzumab exposure was found to increase autophagy and sustain cell survival, leading to trastuzumab resistance (58). In breast cancer, autophagy has emerged as a novel resistance mechanism for anti-HER2 therapies. Studies suggest that autophagy not only aids in cellular stress responses but may also help tumor cells evade therapeutic effects by promoting survival. For instance, trastuzumab-resistant cells displayed increased autophagic activity, which correlated with their ability to resist the drug. Therefore, targeting autophagy may present new opportunities for overcoming trastuzumab resistance.

Targeting autophagy-related protein 5 (ATG5), a protein involved in autophagosome precursor formation, has been shown to suppress autophagy and reverse trastuzumab resistance in HER2-positive breast cancer. ATG5 plays a key role in autophagy, and loss of its function could reduce cellular tolerance to chemotherapeutic agents. Although trastuzumab is initially effective in treating HER2-positive breast cancer, resistance eventually develops in many patients (51, 59). By targeting ATG5, autophagy can be effectively inhibited, thus enhancing trastuzumab's efficacy and overcoming drug resistance. This finding highlights the importance of autophagy in drug resistance mechanisms and offers new insights for treating HER2-positive breast cancer.

Autophagy inhibitors and bioactive molecules can enhance the efficacy of breast cancer treatments. Autophagy inhibitors prevent cancer cells from evading therapy by blocking autophagy. Chloroquine, a commonly used autophagy inhibitor, can inhibit autophagy and sensitize breast cancer cells to chemotherapy. Other inhibitors, such as hydroxychloroquine and bafilomycin A1 (BafA1), also suppress autophagy. Bioactive molecules, including inhibitors of AMPK (AMP-activated protein kinase) and mTOR





(mechanistic target of rapamycin), can interfere with autophagy-regulating pathways in breast cancer cells, thereby enhancing chemotherapy effectiveness (57, 60).

In addition, some miRNAs and lncRNAs have been found to regulate autophagy in breast cancer cells. By modulating the expression of these molecules, the efficacy of anti-cancer treatments can be improved (60). In summary, using autophagy inhibitors and bioactive molecules to interfere with the autophagy process in breast cancer cells can improve the anti-cancer effects of drugs. This provides new insights into developing more effective strategies for treating breast cancer (61).

## 10 Impaired lysosomal function

Antibody-drug conjugates (ADCs) are internalized by cells through binding to specific target molecules, followed by receptor-mediated endocytosis, ultimately reaching the lysosome (62). Within the acidic environment of lysosomes, ADCs undergo chemical or enzymatic cleavage, releasing cytotoxic drugs that then exert their lethal effects on cancer cells. However, the efficacy of ADCs can be compromised due to various factors, leading to reduced effectiveness. One key mechanism of resistance involves the transport of cytotoxic drugs from the lysosomal lumen to the cytosol. This mechanism is particularly relevant for ADCs with non-cleavable linkers, which may depend on specialized transporters to facilitate drug transfer from lysosomes to the cytosol. Abnormalities in the expression levels or function of these transporters can impede drug release, thereby contributing to increased ADC resistance (63).

Studies have investigated the mechanism of T-DM1 resistance by isolating three distinct resistant HER2-positive clones. Compared to the parental clones, these resistant clones maintained similar HER2 expression levels, with no changes in internalization and trafficking pathways. However, one clone showed elevated lysosomal pH levels and disrupted proteolytic activity, leading to the accumulation of T-DM1. This lysosomal dysfunction impaired T-DM1 processing, ultimately reducing its antitumor efficacy. Understanding this functional defect not only sheds light on the various mechanisms of ADC resistance but also provides potential strategies for overcoming such resistance (16).

## 11 Cell migration

The formation of heterodimers results in the recruitment of downstream signaling molecules, which inevitably influences cell movement through this recruitment process. Cell movement, a complex and multi-step process, involves actin remodeling, focal adhesion (FA) turnover, and other regulatory factors, requiring dynamic regulation of FA assembly and disassembly (43). Proteins such as c-Src and focal adhesion kinase (FAK) play key roles in regulating FA formation and turnover. Studies have shown that Heregulin (HRG) triggers a precise sequence of events via the HER2/HER3 signaling pathway, altering cell morphology and promoting migration in HER2-positive breast cancer cells.

Specifically, HRG induces actin cytoskeleton reorganization and FA complex formation in BT-474 breast cancer cells, facilitating actin nucleation.

This signaling cascade is activated by HER2/HER3, leading to the involvement of kinases and scaffolding proteins like c-Src, FAK, and paxillin. Phosphorylation of paxillin recruits PAK1 kinase, which further phosphorylates cortactin. Simultaneously, paxillin interacts with N-WASP, co-regulating the Arp2/3 complex, resulting in local actin reorganization. These changes ultimately enhance cell migration. HRG enhances cell migration by modifying cell morphology and rearranging the actin cytoskeleton in HER2-positive breast cancer cells, involving numerous kinases and scaffolding proteins, including c-Src, FAK, paxillin, PAK1, cortactin, and N-WASP. These findings are crucial in understanding the molecular mechanisms underlying migration in HER2-positive breast cancer cells and drug resistance, providing a foundation for developing novel and more specific therapeutic strategies to disrupt the progression and metastasis of HER2-positive breast cancer (46).

## 12 Discussion

Breast cancer that overexpresses Human Epidermal Growth Factor Receptor 2 (HER2+) due to gene amplification or overexpression constitutes 15-20% of all breast cancer cases. The specific mechanisms of resistance to trastuzumab are currently unclear. This paper aims to review the existing research on the resistance mechanisms of trastuzumab, based on its target, from aspects such as genetic loci, molecular structure, signaling pathways, and the tumor microenvironment and to outline current research progress and new strategies.

The activation of HER family members primarily depends on autocrine or paracrine pathways. ErbB receptors possess an extracellular segment of approximately 630 amino acids, organized into four distinct structural domains: I/L1, II/CR1, III/L2, and IV/CR2. These domains are arranged in tandem, alternating with double-domain units. Additionally, the receptors contain a single transmembrane region and a cytoplasmic tyrosine kinase. Each structural domain includes specific sub-domains and amino acid residues, which can be classified into ligand-binding and dimerization domains. Except for HER3, all receptors contain a cytoplasmic tyrosine kinase region. Except for HER2, all receptors bind specific ligands through their extracellular domains. Upon dimerization, HER2 exhibits strong kinase activity and preferential binding. The dimer features leucine-rich residues in extracellular sub-domains I and III and cysteine residues in sub-domains II and IV, primarily connected by disulfide bonds.

Heterodimerization triggers the phosphorylation of tyrosine kinases, subsequently leading to the activation of several signaling pathways, including Ras/MAPK, PI3K/AKT/mTOR, PLC $\gamma$ /PKC, and JAK2/STAT. These pathways regulate essential cellular processes, such as proliferation, apoptosis, differentiation, angiogenesis, and invasiveness.

Following anti-HER2 treatment, gene overexpression or deletion can result in mutations within the target itself, causing

reduced or absent HER2 expression, which diminishes drug sensitivity. Studies on trastuzumab resistance have identified mutations in genes such as ATM, CDH1, GNAS, MLH1, RB1, SMARCB1, SMO, TP53, and VHL. Notably, TP53 mutations are the most frequently observed, particularly in circulating cell-free DNA (cfDNA) from patients with HER2-positive metastatic breast cancer. Additionally, breast cancer is the most common malignancy (25.5%) among women harboring pathogenic TP53 mutations. Importantly, the p53 signaling pathway plays a crucial role in the cellular response to DNA damage, highlighting its significance in the stress response. These genetic alterations can contribute to therapeutic resistance.

Given the widespread occurrence of mutations, splicing, and post-translational modifications in tumor cells, HER2 inevitably produces isoforms and various subtypes. Current research has shown the presence of HER2 splicing variants that lack exon 16 (HER2Δ16), and the co-expression of HER2 subtypes exerts a unique influence on breast tumor phenotypes. Oncogene activation, which is predominantly linked to epithelial-mesenchymal transition (EMT), is integral to this process. The formation of stable disulfide bonds at the molecular level ultimately activates downstream pathways, potentially leading to delayed responses and decreased efficacy of trastuzumab.

Moreover, variability in tumor antigen expression can influence the effectiveness of trastuzumab-based drug conjugates. Trastuzumab deruxtecan (T-DXd), an antibody-drug conjugate (ADC), was evaluated in the KRISTINE trial, a phase II study involving T-DM1 combined with pertuzumab in neoadjuvant therapy. HER2 heterogeneity was defined as HER2-negative regions detected by fluorescence *in situ* hybridization (FISH) in 10% of cases, or ERBB2 gene amplification detected in more than 5% but fewer than 50% of tumor cells. None of the ten patients exhibiting HER2 heterogeneity reached the primary endpoint of a pathologic complete response (pCR) of 0%. The study results indicated that patients with higher HER2 heterogeneity had poorer progression-free survival (PFS) and overall survival (OS) compared to those with lower heterogeneity.

Under specific conditions, proteolytic cleavage may give rise to certain mutant forms of the epidermal growth factor receptor. For instance, soluble EGFR (sEGFR) can be generated either by selective mRNA splicing of the receptor or by site-specific proteolytic cleavage, which releases the extracellular domain. Similarly, cleavage of HER2-ECD by matrix metalloproteinases (MMPs) or ADAM (A Disintegrin And Metalloprotease domain) produces a truncated version of HER2, known as p95HER2—a 95 kDa truncated form of the HER2/neu protein. The absence of the extracellular domain in p95HER2 results in trastuzumab resistance, as p95HER2 forms homodimers that bypass trastuzumab binding or reduce its efficacy, thus activating various downstream signaling pathways. This variant is found in approximately 22.4% of cancer patients and is closely associated with breast cancer metastasis and recurrence.

Additionally, the truncated version of the antigen's extracellular domain could represent another resistance mechanism for trastuzumab deruxtecan that has yet to be clinically demonstrated. This phenomenon has previously been observed

with trastuzumab: tumors expressing the full-length receptor respond well, whereas those expressing truncated p95HER2 are resistant.

The resistance rate to trastuzumab in clinical settings is increasingly significant. Although the mechanisms of resistance are not fully understood, they can be investigated through genetic loci, molecular structure, signaling pathways, and the tumor microenvironment to develop targeted interventions.

As the primary target of trastuzumab, anti-HER2 treatment often leads to overexpression or absence of related genes, primarily manifested as mutations in ATM, CDH1, GNAS, MLH1, RB1, SMARCB1, SMO, TP53, and VHL. Among these, TP53 mutations are the most common (25.5%), with major variants being p.Ser241Phe and c.376-2dup. Combining HER2-targeted drugs with anti-mutp53 therapy may synergize in treating HER2-positive breast cancer patients. Moreover, mutations such as those in ATM and RB1 cannot be disregarded. Targeting trastuzumab resistance using small molecule tyrosine kinase inhibitors (TKIs) alone or in combination with monoclonal antibodies or ADCs has shown some effectiveness; however, the mechanisms of resistance due to gene overexpression remain unclear and require further investigation.

HER2 expression is influenced by its mutations, density, and isoforms. The main variant, currently HER2Δ16, lacks exon 16, resulting in crosstalk through epithelial-mesenchymal transition and the formation of intermolecular disulfide bonds, which delays response and reduces trastuzumab efficacy. Proteolytic cleavage of HER2-ECD, resulting in the truncated form p95HER2, and the formation of intermolecular homodimers can also contribute to trastuzumab resistance.

HER2 lacks specific antibodies and forms dimers with other ligands through amino acid residues, exhibiting strong kinase activity and binding preference. The exposure sufficiency of HER2 dimers after their formation affects trastuzumab target efficiency. Antibody binding impediment is a primary mechanism of trastuzumab resistance, mainly involving mucin 4 (MUC4) and CD44. Multi-channel targeted delivery, such as KLA-R16, survivin siRNA, and trastuzumab, can alleviate recurrent breast cancer resistance caused by spatial barriers. Additionally, trastuzumab-derived HER2-specific CAR T cells specifically target resistant cells with spatial barriers, showing promising prospects for further research. Elevated levels of IGF2 protein can lead to the formation of IGF1R/HER2 heterodimers. Moreover, AXL, through binding with its ligand growth arrest-specific protein 6 (GAS6) and extracellular domain-mediated dimerization, or by forming signal cascades with other transmembrane receptors to activate PI3K/AKT and MAPK pathways, can also contribute to trastuzumab resistance. Drug conjugation strategies, such as targeting AXL alongside anti-HER2 drugs, may represent a potential treatment approach for trastuzumab resistance.

Activation of the downstream signaling pathways of HER2 is a key issue in trastuzumab resistance, involving constitutive activation or crosstalk of signaling pathways, and various regulatory factors such as the loss of phosphatase and tensin homolog (PTEN), leading to constitutive activation of the PI3K/AKT pathway. Various small molecular proteins that induce

changes in cell conformation also affect key downstream signaling pathways, such as the overactivation of the HER2-SHCBP1-PLK1 axis by SHC linker protein 1 (SHC1), representing a novel mechanism of cancer resistance to trastuzumab. Activation of the cyclin D1-CDK4/6 (cyclin-dependent kinase 4/6) axis also plays a significant role in trastuzumab resistance, and CDK4/6 inhibitors combined with anti-HER2 and endocrine therapy have demonstrated certain therapeutic benefits.

At the submicroscopic level, the tumor microenvironment plays a significant role in the interactions between receptors and small molecular proteins, primarily involving regulation, immune escape, and functional impairment of antibody-dependent cellular cytotoxicity (ADCC), with multiple immune cells influencing cytotoxic responses that are closely linked to resistance. Trastuzumab-resistant tumor cells exhibit high proliferation of endothelial cells, with alpha-B-crystallin in vascular endothelial cells regulating vascular morphology. Additionally, alpha-B-crystallin enhances the ability of SKBR3-TR cells to activate mTOR in endothelial cells, promoting angiogenesis and contributing to trastuzumab resistance. Experiments have demonstrated that mTOR inhibitors can inhibit this process, offering a potential solution to trastuzumab resistance.

Tumor-associated regulatory factors exert regulatory effects on downstream HER2 signaling, with possible mechanisms involving competitive inhibition, prevention of degradation of key downstream proteins, and inhibition of ubiquitin-mediated degradation. For instance, the truncated peptide segment ( $\beta$ -TrCP-343aa) encoded by circ- $\beta$ -TrCP competitively binds with NRF2, preventing NRF2 protein degradation mediated by SCF  $\beta$ -TrCP, and the protective effect of  $\beta$ -TrCP-343aa on NRF2 protein requires GSK3 activity. Subsequently, elevated transcription of NRF2 upregulates a series of antioxidant genes, leading to trastuzumab resistance, and provides a potential therapeutic target to overcome trastuzumab resistance. Additionally, evasion of autophagy and cell migration are emerging areas in the study of trastuzumab resistance, involving signal transduction of various kinases and scaffolding proteins, providing a broad research field and potential for developing novel, more specific treatment strategies to intervene in the progression and metastasis of HER2-positive breast cancer.

Moreover, variability in tumor antigen expression can influence the effectiveness of trastuzumab-based drug conjugates. Trastuzumab deruxtecan (T-DXd) is an antibody-drug conjugate (ADC) consisting of a humanized anti-HER2 monoclonal antibody covalently linked to the topoisomerase I inhibitor DXd. The high drug-to-antibody ratio (8:1) ensures that, after T-DXd internalizes and its adaptors are cleaved, high concentrations of DXd are delivered to the target tumor cells. The membrane permeability of DXd allows it to diffuse through the cell membrane, exerting antitumor activity against surrounding tumor cells, regardless of HER2 expression. The ADC structure is complex, and resistance can develop at various stages, including antigen expression, recognition, internalization, degradation, and drug release. Antigen-associated resistance mechanisms include reduced HER2/NECTIN4 levels, tumor heterogeneity, and truncation of the antigen extracellular domain. Studies suggest that reduced HER2

levels may lead to refractory disease, while tumor heterogeneity affects ADC efficacy, leading to worse progression-free survival (PFS) and overall survival (OS). The KRISTINE and ZEPHIR trials demonstrated that patients with higher tumor heterogeneity had poor treatment outcomes. Antigen truncation, such as p95HER2, can also bypass trastuzumab binding, leading to drug resistance.

In addition to antigen resistance, tumor cells may also develop resistance to the drug payload. Upregulation of drug efflux transporters ABCC2 and ABCG2 has been found to reduce intracellular drug accumulation, resulting in resistance. Furthermore, binding chemistry, drug-to-antibody ratio (DAR), and binding sites affect ADC efficacy, and optimizing DAR and binding methods is crucial to balancing efficacy and tolerance.

Lysosomal dysfunction and activation of signaling pathways are also mechanisms of drug resistance. Impaired lysosomal acidification can limit drug release, while upregulation of the PI3K/AKT/mTOR and Wnt/ $\beta$ -catenin pathways is also associated with resistance.

The mechanisms of resistance to trastuzumab require further exploration to develop effective solutions.

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## Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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RECEIVED 29 August 2024

ACCEPTED 04 November 2024

PUBLISHED 27 November 2024

## CITATION

Zheng Y, Li L, Cai W, Li L, Zhang R, Huang W and Cao Y (2024) Unveiling the role of TGF- $\beta$  signaling pathway in breast cancer prognosis and immunotherapy.  
*Front. Oncol.* 14:1488137.  
doi: 10.3389/fonc.2024.1488137

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# Unveiling the role of TGF- $\beta$ signaling pathway in breast cancer prognosis and immunotherapy

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**Introduction:** The TGF- $\beta$  signaling pathway (TSP) is pivotal in tumor progression. Nonetheless, the connection between genes associated with the TSP and the clinical outcomes of breast cancer, as well as their impact on the tumor microenvironment and immunotherapeutic responses, remains elusive.

**Methods:** Breast cancer transcriptomic and single-cell sequencing data were obtained from the The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO) databases. We identified 54 genes associated with the TSP from the Molecular Signatures Database (MSigDB) and analyzed both data types to evaluate TSP activity. Using weighted gene co-expression network analysis (WGCNA), we identified modules linked to TSP activity. To assess patient risk, we used 101 machine learning algorithms to develop an optimal TGF- $\beta$  pathway-related prognostic signature (TSPRS). We then examined immune activity and response to immune checkpoint inhibitors and chemotherapy in these groups. Finally, we validated ZMAT3 expression levels clinically and confirmed its relevance in breast cancer using CCK-8 and migration assays.

**Results:** At the single-cell level, TSP activity was most notable in endothelial cells, with higher activity in normal tissues compared to tumors. TSPRS was developed. This signature's accuracy was confirmed through internal and external validations. A nomogram incorporating the TSPRS was created to improve prediction accuracy. Further studies showed that breast cancer patients categorized as low-risk by the TSPRS had higher immune phenotype scores and more immune cell infiltration, leading to better prognosis and enhanced immunotherapy response. Additionally, a strong link was found between the TSPRS risk score and the effectiveness of anti-tumor agents. Silencing the ZMAT3 gene in the TSPRS significantly reduced the proliferation and invasiveness of breast cancer cells.

**Discussion:** Our study developed a TSPRS, which emerges as a potent predictive instrument for the prognosis of breast cancer, offering novel perspectives on the immunotherapeutic approach to the disease.

## KEYWORDS

breast cancer, TGF- $\beta$ , prognostic signature, tumor microenvironment, immunotherapy

## Introduction

Breast cancer, with its high incidence and mortality rates among women globally, stands as a leading cause of cancer-related deaths. According to the latest data from the International Agency for Research on Cancer in 2020, breast cancer has become the most prevalent cancer worldwide, posing a significant threat to women's health (1). The complexity of breast cancer is further underscored by its classification into various subtypes such as luminal A, luminal B, HER2-positive, and triple-negative breast cancer (TNBC), each with distinct biological behaviors and treatment responses. Among these, TNBC is particularly challenging due to its high malignancy, lack of relevant targets, poor prognosis, and limited treatment options.

Although conventional treatments such as surgical resection, radiotherapy, chemotherapy, targeted therapy, and endocrine therapy have improved outcomes, they are not always successful, leading to a persistent search for more effective strategies. In this context, immunotherapy has emerged as a groundbreaking approach that has shown remarkable success in treating melanoma and lung cancer (2, 3). This success has paved the way for exploring the potential benefits of immunotherapy in breast cancer, traditionally considered to have “low immunogenicity”.

The pursuit of effective immunotherapy for breast cancer has concentrated on vaccines, Chimeric Antigen Receptor T cell (CAR-T) therapy, and immune checkpoint inhibitors (ICIs). ICIs, in particular, have become a focal point of clinical research. While monotherapy with ICIs has not been ideally successful, there is a growing interest in combination therapies. Despite the low immunogenicity attributed to breast cancer, its high heterogeneity presents both challenges and opportunities. TNBC, for example, is characterized by stronger immune infiltration and higher genomic instability compared to other subtypes (4), suggesting a better response to immunotherapy in certain patient populations. Meanwhile, hormone receptor-positive breast cancers, once deemed “cold” tumors, are now recognized to exhibit varying levels of immune activity (5, 6), indicating that targeted therapies could be beneficial for a subset of these patients.

The role of TGF- $\beta$ , a multifunctional cytokine, in cancer progression illustrates the complexity of the tumor microenvironment (TME) in breast cancer. TGF- $\beta$  can act as both a tumor suppressor and promoter, depending on the stage of the disease and the cellular context (7–9). Its dual role in the immune response further complicates the landscape, as it can inhibit anti-tumor immunity while promoting immune tolerance and tumor escape. Understanding the activity of the TGF- $\beta$  signaling pathway (TSP) in the breast cancer TME is thus crucial for advancing immunotherapy strategies.

In our study, we sought to dissect this complexity by analyzing breast cancer transcriptome and single-cell sequencing data from the TCGA and GEO databases. We identified 54 TSP genes from

MSigDB and assessed the activity of the TSP in breast cancer at both the single-cell and bulk transcriptome levels. Using weighted gene co-expression network analysis (WGCNA), we analyzed the module most related to the TSP activity score. Furthermore, we employed a combination of 101 machine learning algorithms to construct a prognostic model capable of predicting patient outcomes, delineating the TME landscape, and estimating the response to ICIs and sensitivity to anti-tumor drugs.

## Materials and methods

### Data collection

We sourced a gene expression matrix from 1,081 breast cancer specimens and 99 non-cancerous tissues adjacent to tumors from The Cancer Genome Atlas (TCGA, <https://portal.gdc.cancer.gov/>) repository. This investigation concentrated on 1,029 breast cancer instances that had comprehensive survival data and a post-diagnosis duration exceeding 30 days. Additionally, we retrieved the GSE20711 cohort, comprising 88 breast cancer cases with exhaustive clinical profiles, from the Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>) archive. Furthermore, our study included an analysis of single-cell RNA-sequencing data from five primary breast cancer samples, accessed from the GSE180286 series within the GEO repository. A compilation of 54 genes implicated in the TSP was procured from the Molecular Signatures Database (MSigDB, [www.gsea-msigdb.org/gsea/msigdb](http://www.gsea-msigdb.org/gsea/msigdb)). Figure 1 illustrates the flowchart of the data analysis process.

### Processing of single-cell RNAseq data

The “Seurat” package (10) was employed for the analysis of single-cell sequencing data. Initial quality control (QC) involved filtering out cells with mitochondrial gene content exceeding 20% and selecting genes present in a minimum of three cells with expression levels ranging from 200 to 7,500. The next step was to identify a subset of 2,000 highly variable genes for further examination. To correct for batch effects across the various samples, we utilized the “Harmony” algorithm. For the construction of cellular clusters, functions “FindClusters” and “FindNeighbors” within “Seurat” were applied, and the resulting clusters were visualized using the t-distributed Stochastic Neighbor Embedding (t-SNE) technique. Cell type identification and refinement were performed using the “SingleR” tool, guided by known marker genes associated with various cell types.

The activity of specific gene sets within each cell was quantified using the single-sample Gene Set Enrichment Analysis (ssGSEA) method. When comparing differentially expressed genes (DEGs) between two cohorts, the “FindMarkers” utility in “Seurat” was our method of choice. We identified differentially expressed genes with a log fold-change threshold of 0.25 and a minimum percentage of 0.25 while maintaining default settings for other parameters. Additionally, the “CellChat” R package (11) was implemented to explore cellular interactions.

**Abbreviations:** BRCA, Breast Cancer; DEGs, Differentially Expressed Genes; OS, Overall Survival; ssGSEA, single sample Gene Set Enrichment Analysis; TCIA, The Cancer Immunome Atlas; TMB, Tumor Mutational Burden.

## Weighted gene co-expression network analysis

To conduct the WGCNA analysis on the TCGA-BRCA bulk RNA-seq data, we utilized the “WGCNA” R package (12). Initially, we determined an optimal soft threshold  $\beta$  that adhered to the requirements for constructing a scale-free network. Subsequently, we converted the weighted adjacency matrix into a topological overlap matrix (TOM) and calculated the dissimilarity (dissTOM). For gene clustering and module identification, we employed the dynamic tree-cutting method. Ultimately, we pinpointed the module exhibiting the strongest correlation with the TSP activity scores for further investigation.

## Construction of prognostic signature

We conducted differential analysis between normal and tumor samples in the TCGA bulk RNA-seq data using the ‘DEseq2’ R package, with criteria of  $|\log FC| > 0.5$  and  $p.adjust < 0.05$ . Subsequently, we identified the intersection between the DEGs at the bulk RNA-seq level and the genes belonging to the TSP-related module identified through WGCNA. These genes were denoted as TGF- $\beta$  signaling pathway-related genes (TSPRG). To develop a robust prognostic signature characterized by high predictive accuracy, we followed the subsequent steps:

1. To ensure a balanced distribution of clinical characteristics, we randomly partitioned the TCGA-BRCA dataset into a training set and an internal validation set, maintaining a ratio of 6:4. Additionally, we utilized the GSE20711 dataset as an external validation set.
2. In the TCGA-BRCA training dataset, we conducted univariate Cox regression analysis to identify TSPRG with potential prognostic significance. Subsequently, we employed ten machine learning algorithms, namely CoxBoost, Ridge, Lasso, Random Survival Forest (RSF), Stepwise Cox, Elastic Net (Enet), survival support vector machine (survival-SVM), Generalized Boost Regression Modeling (GBM), Supervised Principal Components (SuperPC), and Partial Least Squares Regression for Cox (plsRcox). To perform variable selection and construct models, we generated 101 combinations of these ten algorithms within the TCGA-BRCA training dataset, employing a tenfold cross-validation framework.
3. We assessed the performance of all constructed models in both the TCGA internal validation set and the GSE20711 dataset. For each model, we computed the concordance index (C-index) across the training, internal validation, and external validation sets. Subsequently, we ranked the models based on their mean C-index to determine their predictive performance. We selected algorithms that demonstrated both robust performance and clinical translational significance. Consequently, we developed a final signature, termed the TGF- $\beta$  signaling pathway-

related signature (TSPRS), which can effectively predict overall survival in BRCA patients.

## Survival analysis and predictive nomogram construction

The TCGA training set, internal validation set, and GSE20711 set were divided into high-risk and low-risk groups based on the median TSPRS risk score. We conducted Kaplan-Meier (KM) curve analysis using the “survminer” R package to assess whether there was a significant difference in overall survival (OS), progression-free survival (PFS), and disease-free survival (DFS) between the high-risk and low-risk groups. Additionally, we performed receiver operating characteristic (ROC) curve analysis using the “timeROC” package to evaluate the sensitivity and specificity of the TSPRS in predicting OS in BRCA patients.

Furthermore, we examined the correlation between the TSPRS and various clinical characteristics, including age, T, M, N, and stage. Univariate and multivariate Cox regression analyses were conducted on the TCGA-BRCA datasets to determine whether the TSPRS served as an independent prognostic factor for predicting survival in BRCA patients.

To enhance the prognostic accuracy and predictive capability of our model, we developed a nomogram that incorporated TSPRS and clinical characteristics to quantify the expected survival of BRCA patients. Finally, we evaluated the precision discrimination and accuracy of the nomogram using ROC curves, the C-index, and calibration curves.

## Tumor mutation burden analyses

We acquired Tumor Mutation Burden (TMB) files containing somatic mutation data from TCGA. We estimated and visualized the differences in TMB levels between the two risk subgroups using the “maftools” R packages. The correlation between risk scores and TMB scores was assessed and depicted using the “limma”, “ggpubr”, “ggplot2”, and “ggExtra” R packages.

KM analysis was employed to examine the survival differences between groups with varying TMB levels as well as between different risk status subgroups. The “Survival” and “survminer” R packages facilitated this analysis.

## Association of TSPRS with tumor microenvironment and response to immunotherapy

To elucidate the relationship between the TSPRS and immune cell infiltration within the BRCA TME, our study utilized computational methodologies including CIBERSORT, ESTIMATE, and ssGSEA. These algorithms were instrumental in quantifying the degree of immune cell infiltration and the activity of immune-related functions.

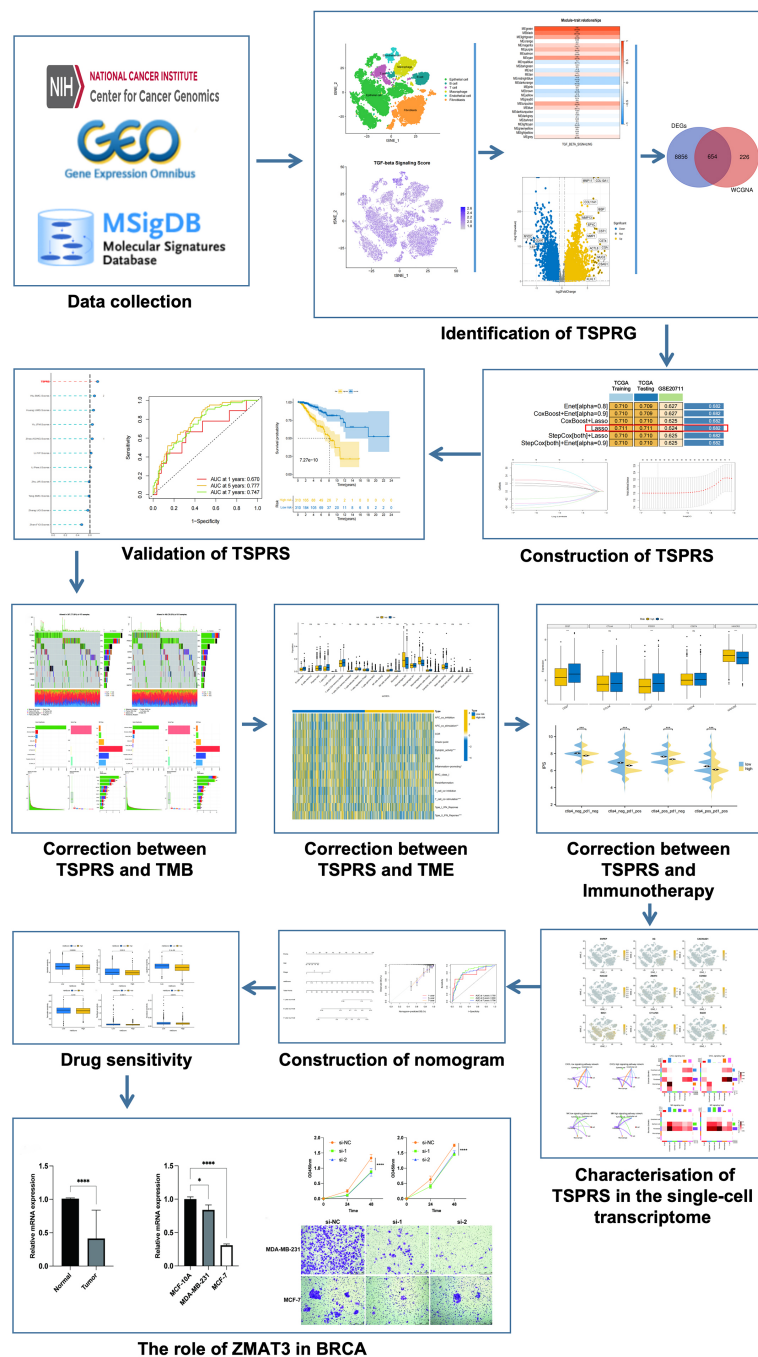


FIGURE 1

Flowchart for analysis of prognostic signatures associated with the TGF- $\beta$  signalling pathway. TSPRG, TGF- $\beta$  signaling pathway-related genes; TSPRS, TGF- $\beta$  signaling pathway-related signature; TMB, tumor mutation burden.

Furthermore, the Tumor Immune Dysfunction and Exclusion (TIDE) computational framework (accessible at <http://tide.dfci.harvard.edu/>) was applied to appraise the potential for immune escape mechanisms in patient groups stratified by high and low prognostic risk.

Immune phenotype scores (IPS), retrieved from The Cancer Immunome Atlas (TCIA) database (available at <https://tcia.at/home>), were used to anticipate the efficacy of immunotherapeutic

interventions in TCGA-BRCA patient subsets, delineated by the aforementioned risk stratification.

## TSPRS and drug sensitivity analysis

To facilitate the customization of therapeutic regimens, we employed the “oncoPredict” package (13). This predictive model



was applied to estimate the chemosensitivity of patients with BRCA, stratified by varying TSPRS. OncoPredict executes a comparative analysis between the gene expression profiles of patient-derived tissue samples and established cancer cell line repositories, thereby calculating the half-maximal inhibitory concentration (IC50) as a metric of chemotherapeutic potency.

### Cell culture and small-interfering RNA transfection

The human breast cancer cell lines MCF-10A, MDA-MB-231 and MCF-7 were procured from the cell bank of the Chinese Academy of Science. MDA-MB-231 and MCF-7 cells were cultured in high glucose DMEM medium, supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin Solution (P/S), in an incubator set at 37°C with a 5% CO2 atmosphere. The sequences of small-interfering RNA (siRNA) targeting ZMAT3 were cloned into MDA-MB-231 and MCF-7 cells. Using GP-transfect-Mate (GenePharma, China), the siRNA transfection process was conducted as instructed by the manufacturer. The sequences of the siRNAs are shown in [Table 1](#).

### Cell transwell migration testing

After cell transfection was completed, the serum-free cell suspension was spread evenly in the upper chamber of Transwell (Corning, USA), and DMEM medium containing 20% FBS was added to the lower chamber. After 8 hours of incubation, the upper chamber was fixed with 4% paraformaldehyde, and the cells that did not cross the polycarbonate membrane were gently scraped off with a cotton swab. After gentian violet staining and washing with PBS, cells at the bottom of the chambers were photographed in different fields of view using a microscope, and cells were counted using imageJ.

### CCK-8 proliferation assay

The transfected cells were inoculated into 96-well plates at a concentration of 200  $\mu$ L containing 3000 cells per well, and 3-6 replicate wells were set up in each group. The surrounding circle of wells should not be used as sample wells in principle, and 100  $\mu$ L of PBS was added to each well. 20  $\mu$ L of CCK-8 solution was added to each well at 0h, 24h, and 48h, respectively, and the absorbance at 450 nm was measured by enzyme labeling instrument after incubation for 1-4 hours in the incubator.

TABLE 1 The sequences of the siRNA targeting ZMAT3.

| Gene      | Sequence            |
|-----------|---------------------|
| si1-ZMAT3 | GGGAATGAGTTTAAGATGA |
| si2-ZMAT3 | GGCTCAGGCTCACTATCAA |

### mRNA expression analysis

We collected 32 pairs of breast cancer tissue and matched adjacent normal tissue from the Department of Breast Care Surgery, The First Affiliated Hospital, Guangdong Pharmaceutical University.

Total RNA was extracted from clinical samples and cells using TRIzol, after which it was reverse-transcribed into cDNA with a reverse transcription kit (Tsingke,China). The reaction conditions were set at 25°C for 10 minutes, 50°C for 15 minutes, and 85°C for 5 minutes, followed by Real-time Quantitative PCR. The PCR amplification conditions were as follows: pre-denaturation at 95°C for 1 minute, denaturation at 95°C for 30 seconds, and annealing at 60°C for 20 seconds, repeated for 40 cycles. The relative expression of the target gene was calculated using the  $2^{-(\Delta\Delta Ct)}$  method. The primer sequences are shown in [Table 2](#).

### Statistical analysis

Data manipulation and statistical analyses were performed utilizing the R statistical computing environment (version 4.1.2). Comparative analyses between distinct groups were executed employing the non-parametric Wilcoxon rank-sum test. To quantify the strength and direction of the associations, Spearman’s rank correlation coefficient was calculated for three sets of relationships: between TMB and risk scores, between immune cell infiltration scores and risk scores, and between immune cell infiltration scores and gene expression profiles, respectively. Statistical significance was established at a p-value threshold of less than 0.05.

## Results

### Differential expression and genetic variation mapping of TGF- $\beta$ signaling pathway genes in breast cancer

We first performed differential expression analysis of 54 TGF- $\beta$  signaling pathway genes (TSPG) in breast cancer tissues and adjacent normal tissues. We found that 44 TSPG were differentially expressed ([Supplementary Figures S1A, B](#)). Protein-protein interaction network analysis constructed on the basis of the String database showed that there was a close association between all TSPG except BCAR3, RRAB31 and SLC20A1 ([Supplementary Figure S1C](#)). In the subsequent phase of our investigation, we assessed the frequency of somatic mutations within a cohort of 54 TSPG in breast cancer cohorts. A waterfall plot was constructed to illustrate the mutational landscape, which identified CDH1 as the

TABLE 2 The primer sequences for ZMAT3.

| Gene  | Primer sequence           |
|-------|---------------------------|
| ZMAT3 | F-ATGCAGCAAATAGCTGTCTCTC  |
|       | R-GGGACTGGAACAACCTGGAGTAG |



gene with the highest mutation frequency, followed in descending order by ARID4B, TJP1, and APC (Supplementary Figure S1D). Furthermore, we scrutinized the prevalence of copy number variations (CNV) within the TSPGs. Our findings indicated a significant amplification in copy numbers predominantly for SMURF2, PPP1CA, and KLF10, while SKI, ID3, and RHOA were the genes most notably subjected to copy number losses (Supplementary Figure S1E).

## Characterization of the TSP activity in the single-cell transcriptome

From five primary breast cancer patients, we acquired single-cell RNA-seq data encompassing 25,550 cells. The Harmony algorithm was applied to mitigate batch effects, which facilitated the integration of the quintet of samples. Subsequently, we employed PCA and t-SNE on the most variable 2,000 genes to achieve dimensionality reduction. This stratified the cells into 23 distinct clusters at a resolution parameter of 0.8 (Figure 2A). Utilizing marker genes, six clusters were characterized, representative of diverse cellular identities: Epithelial cells, T cells, B cells, macrophages, Endothelial cells, and Fibroblasts (Figure 2B). A heatmap was generated to display the three most prominent marker genes within each cellular subset (Figure 2C).

In three out of the six cellular phenotypes—namely, Endothelial cells, Fibroblasts, and Epithelial cells—a notably elevated activity of TSP was detected (Figures 2D, E). TGF- $\beta$  signaling plays a crucial role in endothelial cells by regulating angiogenesis, vascular homeostasis, and endothelial function. Through its receptors, TGF- $\beta$  activates the SMAD pathway, affecting endothelial cell proliferation, migration, and differentiation. In fibroblasts, a primary target of TGF- $\beta$ , it promotes ECM protein synthesis and fibrosis by inducing collagen and other ECM components, aiding tissue repair and fibrosis. In the TME, fibroblasts, especially cancer-associated fibroblasts (CAFs), use TGF- $\beta$  signaling to promote tumor progression and metastasis. Additionally, TGF- $\beta$  regulates epithelial-mesenchymal transition (EMT) in epithelial cells, a process vital for embryonic development, tissue repair, and cancer metastasis, by inducing epithelial cells to acquire mesenchymal traits, enhancing their migratory and invasive capabilities (14–16).

We then identified 322 marker genes in the high TSP activity group and 45 marker genes in the low TSP activity group. Subsequent KEGG enrichment analysis revealed that the marker genes in the high TSP activity group were mainly enriched in focal adhesion, proteoglycans in cancer, and tight junction pathways (Figure 2F). The marker genes in the low TSP activity group were mainly enriched in the Antigen processing and presentation pathway (Figure 2G).

## Identification of TSP activity-related modular genes

The ssGSEA method is frequently employed to evaluate alterations in biological processes and the activity of pathways

within individual specimens. We utilized this algorithm in the present investigation to derive a quantification of TSP activity for each sample within the TCGA-BRCA cohort. Notably, the tumor specimens exhibited a markedly reduced TSP activity in comparison to the adjacent non-tumor tissues (as depicted in Figure 3A). This derived score was then incorporated as a phenotypic variable for downstream WGCNA. The WGCNA approach was subsequently applied to the TCGA-BRCA data to pinpoint gene modules that show significant correlations with TSP activity. After removing outlier samples, we constructed co-expression networks (Figure 3B). The optimal soft threshold of power=4 was selected to ensure a scale-free topological network (Figure 3C). A total of 27 modules were obtained by setting the minimum module gene count to 50 and MEDissThres to 0.15 (Figure 3D). Our findings indicated that the MEgreen module was strongly correlated with the TSP activity score in bulk RNA-seq (Figure 3E). Moreover, the scatter plot of gene significance (GS) versus module membership (MM) for the blue module displayed a significant correlation (Figure 3F), suggesting that genes within the green module may have a functional significance associated with the TSP.

The volcano plot (Figure 3G) illustrates the DEGs between breast tumor and adjacent normal tissues in the TCGA-BRCA bulk RNA-seq ( $|\log FC| > 0.5$  and  $p_{\text{adjust}} < 0.05$ ). We intersected the 880 genes in the green module with the DEGs from the bulk RNA-seq, finally identifying a total of 654 genes (Figure 3H), named TSPRG. GO enrichment analysis of TSPRG showed a significant enrichment in biological processes (BP), including extracellular matrix organization and extracellular structure organization, as well as in cellular components (CC) such as collagen-containing extracellular matrix, and in molecular functions (MF) including extracellular matrix structural constituent (Supplementary Figure S2A). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of TSPRG showed a significant enrichment in Focal adhesion, Protein digestion and absorption, Proteoglycans in cancer, and ECM–receptor interaction (Supplementary Figure S2B).

## Development of a prognosis signature through comprehensive machine learning integration

To develop a unified signature associated with the TSP, we employed an ensemble of 101 machine learning algorithms to evaluate the nine genes identified as prognostic by univariate Cox regression analysis (Figure 4A). Within the TCGA training cohort, we constructed 101 predictive models using a ten-fold cross-validation scheme and calculated the C-index for each combination of training and validation groups (Figure 4B).

Out of the 101 models, we calculated the C-index of each machine learning algorithm combination in the three datasets and ranked each algorithm combination according to its average C-index. The results show that the six algorithm combinations, Enet [ $\alpha=0.8$ ], CoxBoost+Enet [ $\alpha=0.9$ ], CoxBoost+Lasso, Lasso, StepCox[both]+Lasso, and StepCo[both]+Enet [ $\alpha=0.9$ ], exhibit good prediction ability. However, among these six algorithm

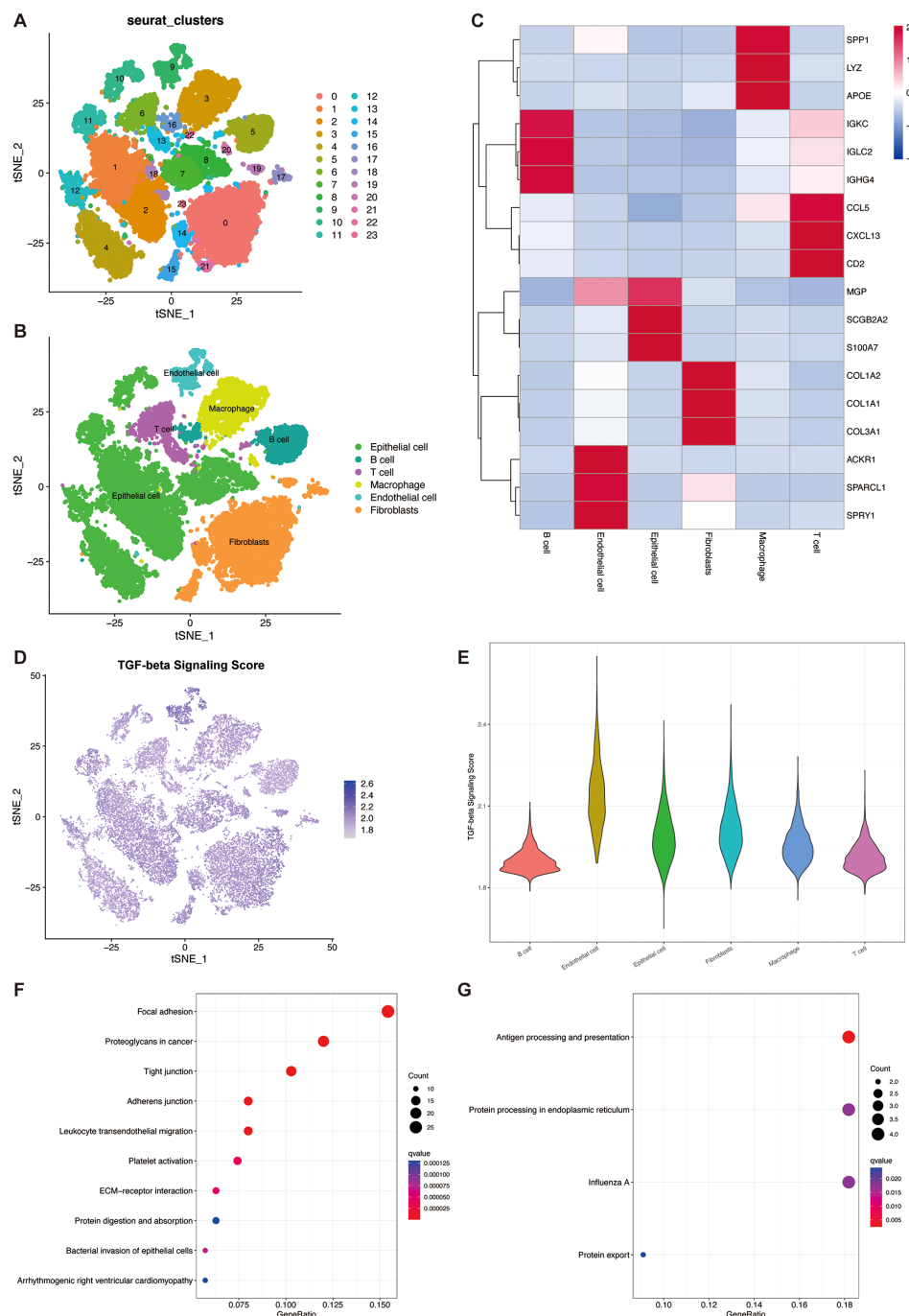


FIGURE 2

TGF- $\beta$  pathway activity in the single cell transcriptome. **(A)** t-SNE plot showing the cell clusters. **(B)** t-SNE plot showing the cell types. **(C)** Heatmap showing the top 3 marker genes in each cell types. **(D)** t-SNE plot showing the distribution of TSP scores. **(E)** Violin plot of TGF- $\beta$  pathway activity scores in each cell type. **(F)** Bubble plots for KEGG enrichment analysis of high TSP score groups. **(G)** Bubble plots for KEGG enrichment analysis of low TSP score groups.

combinations, only the Lasso algorithm showed the best prediction ability in the training set and the internal validation set. The Lasso algorithm achieved an area under curve (AUC) of 0.711 on both the TCGA training and test sets, and 0.624 on the GSE20711 validation set. These results show that Lasso's performance is on par with other models like Enet and CoxBoost, and it remains consistent across datasets. By reducing some coefficients to zero for feature

selection, the Lasso model simplifies and enhances interpretability, focusing on identifying key genes in the TSP. These benefits led us to select the Lasso model for constructing TSPRS.

Using a tenfold cross-validation framework, we identified the optimal  $\lambda$  value of 0.001900647 in the LASSO analysis by minimising the partial likelihood deviation (Figures 4C, D). A prognostic signature was developed employing the nine genes

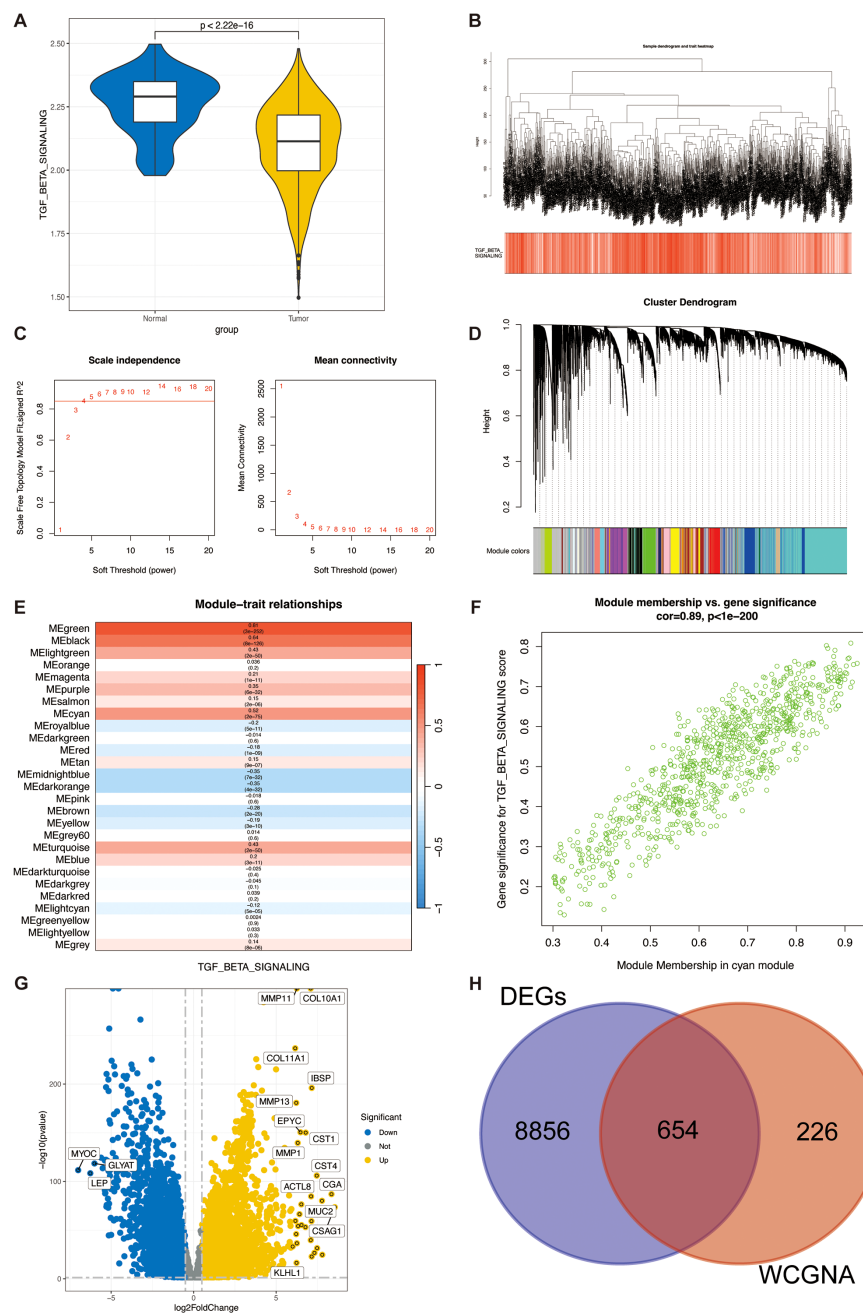


FIGURE 3

Identification of TSPRG. (A) Violin plots of TGF- $\beta$  pathway activity scores in tumour tissue and adjacent paracancerous normal tissue. (B) Dendrogram showing the hierarchical clustering of TCGA-BRCA samples. The bottom heatmap represents the TSPR score of each sample calculated by the ssGSEA algorithm. (C) The determination of the optimal soft threshold in WGCNA analysis. (D) Cluster dendrogram from the WGCNA analysis. (E) Module-trait heatmap showing that the MEgreen module was closely related to the TSPR score feature. (F) Scatter plot showing the relationship between gene significance (GS) and module membership (MM) in the green module. (G) Volcano plot showing the results of the differential analysis of TCGA-BRCA tumor samples and normal samples. (H) Venn plot showing the overlapping genes between the MEgreen module and DEGs in bulk RNA-seq. DEGs, differentially expressed genes; WGCNA, weighted gene co-expression network analysis.

that retained non-zero coefficients subsequent to the LASSO regression analysis (Table 3). For each patient, a risk score was computed, facilitating the stratification of patients into high-risk or low-risk categories predicated on the median value of the calculated risk scores. Kaplan-Meier curves show that SDC1, NACAD, ZMAT3, CCND2, XG and SGCE are associated with prognosis in breast cancer patients (Supplementary Figures S3A–I). It was

observed that there was a proportional escalation in mortality concomitant with an increase in the risk score (Supplementary Figures S4A–L). Furthermore, comparative analyses of OS rates within the training cohort, the internal validation cohort, and the external GSE20711 dataset revealed that patients categorized within the high-risk bracket had significantly inferior OS outcomes compared to their low-risk group (Figures 4E, G, I). In a similar

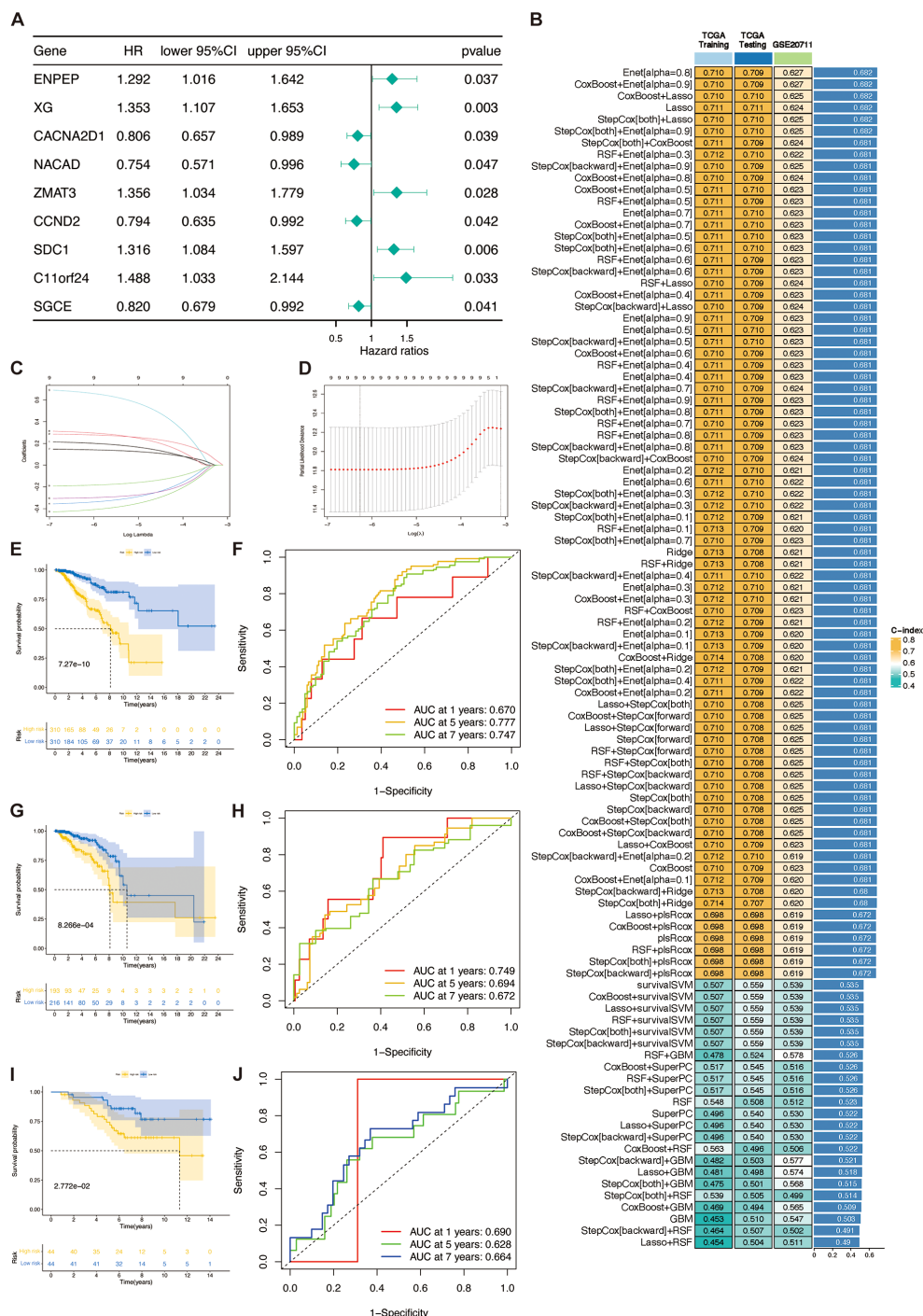


TABLE 3 The nine genes that constitute TSPRS.

| Gene Symbol | Full Name                                                     | Function                                                                                                                                 | Known Biological Role in Breast Cancer                                                                                                    | References |
|-------------|---------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------|------------|
| ENPEP       | Glutamyl Aminopeptidase                                       | Involved in blood pressure regulation.                                                                                                   | May be related to tumor microenvironment and angiogenesis.                                                                                | (41)       |
| XG          | Xg Glycoprotein                                               | Blood group antigen                                                                                                                      | Specific role in breast cancer is unclear.                                                                                                |            |
| CACNA2D1    | Calcium Voltage-Gated Channel Auxiliary Subunit Alpha2delta 1 | Regulates calcium channel activity.                                                                                                      | Increasing the infiltration of immune cells and up-regulating the expression of immune checkpoints.                                       | (42)       |
| NACAD       | NAC Alpha Domain Containing                                   | Involved in protein transport                                                                                                            | Specific role in breast cancer is unclear.                                                                                                | (43)       |
| ZMAT3       | Zinc Finger Matrin-Type 3                                     | Involved in p53 signaling pathway                                                                                                        | May play a role in tumor suppression and cell cycle regulation.                                                                           | (40)       |
| CCND2       | Cyclin D2                                                     | Regulates cell cycle                                                                                                                     | Promotes cell proliferation, potentially related to breast cancer progression.                                                            | (44)       |
| SDC1        | Syndecan 1                                                    | Participates in cell proliferation, cell migration, and cell-matrix interactions through its receptor for extracellular matrix proteins. | Interacts with various ligands and receptors involved in tumor progression, affecting cancer stem cell function, cell proliferation, etc. | (45, 46)   |
| C11orf24    | Chromosome 11 Open Reading Frame 24                           | Involved in cell cycle progression.                                                                                                      | Specific role in breast cancer is unclear.                                                                                                | (47)       |
| SGCE        | Sarcoglycan Epsilon                                           | A single pass transmembrane protein forming part of the dystrophin-associated glycoprotein complex.                                      | Specific role in breast cancer is unclear.                                                                                                | (48)       |

vein, assessments of PFS and DFS demonstrated a markedly improved prognosis for patients in the low-risk group relative to those in the high-risk group (Supplementary Figures S4O, P).

Validation of TSPRS

The analysis of the ROC curve indicated that for the training cohort, the AUC for the TSPRS was 0.670, 0.777, and 0.747 at 1, 5, and 7 years, respectively. For the internal validation cohort, these values were 0.749, 0.694, and 0.672, and for the GSE20711 dataset, the AUCs were 0.690, 0.628, and 0.664 (Figures 4F, H, J). These metrics underscore the superior prognostic efficacy of the TSPRS.

The comparative analysis of prognostic performance was conducted between TSPRS and ten established prognostic signatures, as devised by Wu et al., Zhan et al., Huang et al., Li et al. (first instance), Yu et al., Tang et al., Li et al. (second instance), Zhang et al., Zhu et al., and Zhao et al. (17–26), with respect to patient outcomes in BRCA. Our analysis revealed that TSPRS surpasses the aforementioned prognostic models, evidenced by a significantly elevated C-index (Figure 5A). These findings reinforce the remarkable prognostic precision of TSPRS in the context of anticipating clinical outcomes for BRCA patients.

Then we observed the expression of the angiogenic marker VEGF and the cell proliferation indicator KI67 in the high- and low-risk groups, and the results showed that there were higher levels of VEGF and KI67 expression in the high-risk group, suggesting that the tumors of the patients in the high-risk group had higher levels of angiogenic and proliferative capacity than those in the low-risk group (Figures 5B, C).

Given the routine utilization of clinical characteristics to prognosticate outcomes for patients with BRCA, we conducted an assessment of the association between the TSPRS and a spectrum of clinical parameters. In the TCGA-BRCA dataset, we observed significantly higher risk scores in patients with age greater than or equal to 60 than in patients with age less than 60 (Figure 5D), but did not observe differences in risk scores in stage, T, N, or M (Supplementary Figures S5A–E). We also found that TSPRS showed strong prognostic power in ER+/PR+ patients, TNBC patients, and subgroups with different clinicopathologic features (Figures 5E–O). However, this good predictive ability was not observed in patients with M1-staged and HER2+ breast cancer, probably due to the small sample size (Supplementary Figures S5F, G).

Association of TSPRS with mutation landscapes

TMB is generally defined as the number of non-synonymous mutations per megabase pair (Mb) of somatic cells in a given genomic region. Tumor mutational load is a quantitative biomarker that reflects the total number of mutations carried by tumor cells, indirectly reflecting the ability and degree of neoantigen production by tumors, and has been shown to predict the efficacy of immunotherapy for a variety of tumors (27, 28). Tumor cells with a high TMB have a higher level of neoantigens, and studies have shown that patients with a high TMB are more likely to benefit from ICIs therapy (29).

The ten genes with the highest mutation rates differed significantly between the two groups (Figures 6A–D). TMB was



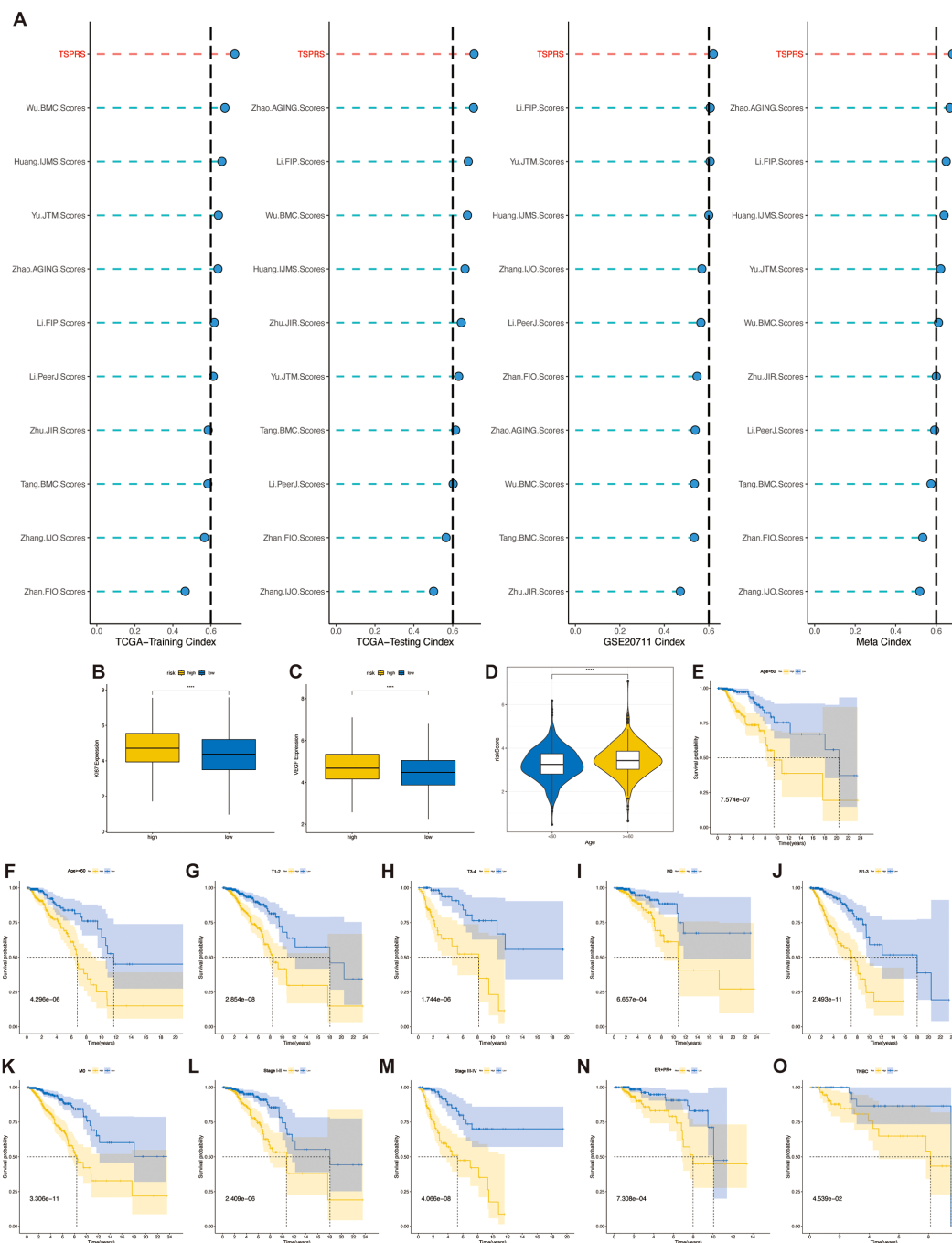


FIGURE 5

Clinical relevance of TSPRS. (A) C-index comparison of TSPRS with ten published prognostic signatures for breast cancer patients. (B) Differences in KI-67 expression levels between high and low risk groups. (C) Differences in VEGF expression levels between high and low risk groups. (D) Differences in risk scores between patients younger than 60 years and those older than or equal to 60 years. (E–O) Kaplan-Meier curves showing the stable performance of TSPRS in the subgroups of BRCA patients, including age, T, N, M, stage, ER+/PR+ and TNBC. \*\*\*\*,  $P < 0.0001$ .

significantly higher in the high-risk group than in patients in the low-risk group (Figure 6E), and there was a significant positive correlation between the risk score and TMB (Supplementary Figure S4S). Suggesting that patients in the high-risk group may benefit from ICIs treatment. To understand whether the risk profile or TMB was a better predictor of survival, we divided the samples into high and low mutation subgroups based on median

TMB. KM survival curves showed no statistically significant difference between the survival of patients in these two groups (Figure 6F). However, in the combined analysis of TMB and TSPRS, both low TMB in the high-risk group and high TMB in the high-risk group possessed shorter survival times, suggesting a worse prognosis (Figure 6G). This suggests the stronger predictive ability of TSPRS.

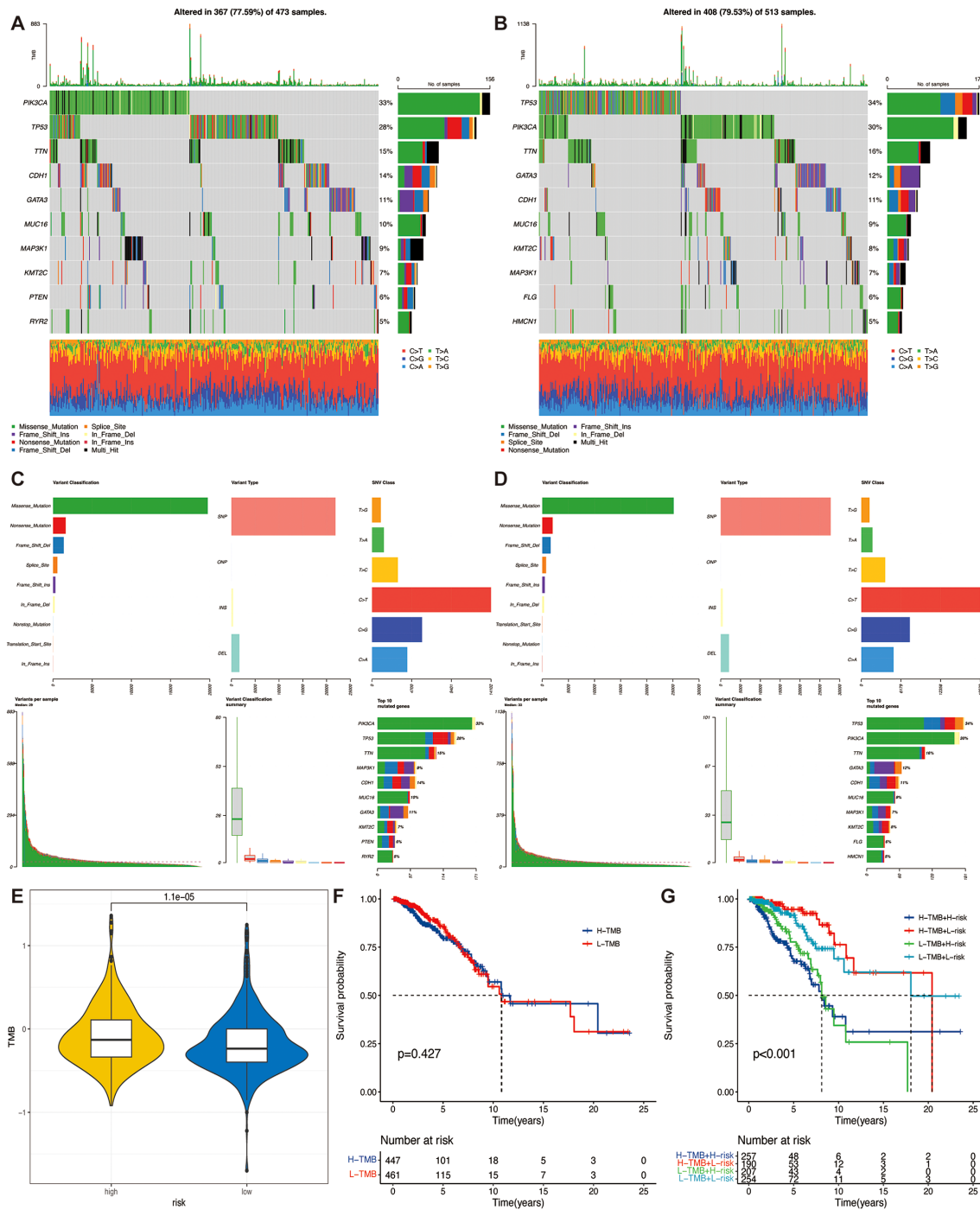


FIGURE 6 Correlation of TSPRS with TMB. (A) Waterfall plot of the top ten genes' TMB status in the low-risk groups, and (B) the high-risk group. (C) Summary of the maf files of the low-risk groups, and (D) the high-risk group. (E) Differences in TMB between high and low risk groups. (F) Kaplan-Meier curve of different TMB levels. (G) Kaplan-Meier curves of different TMB and risk levels. TMB, tumor mutation burden.

Association of TSPRS with the breast cancer microenvironment

To evaluate the status of immune cell infiltration within breast cancer specimen, we utilized the Estimation of Stromal and Immune cells in Malignant Tumor tissues using Expression data (ESTIMATE) algorithm. This computational approach facilitated the quantification

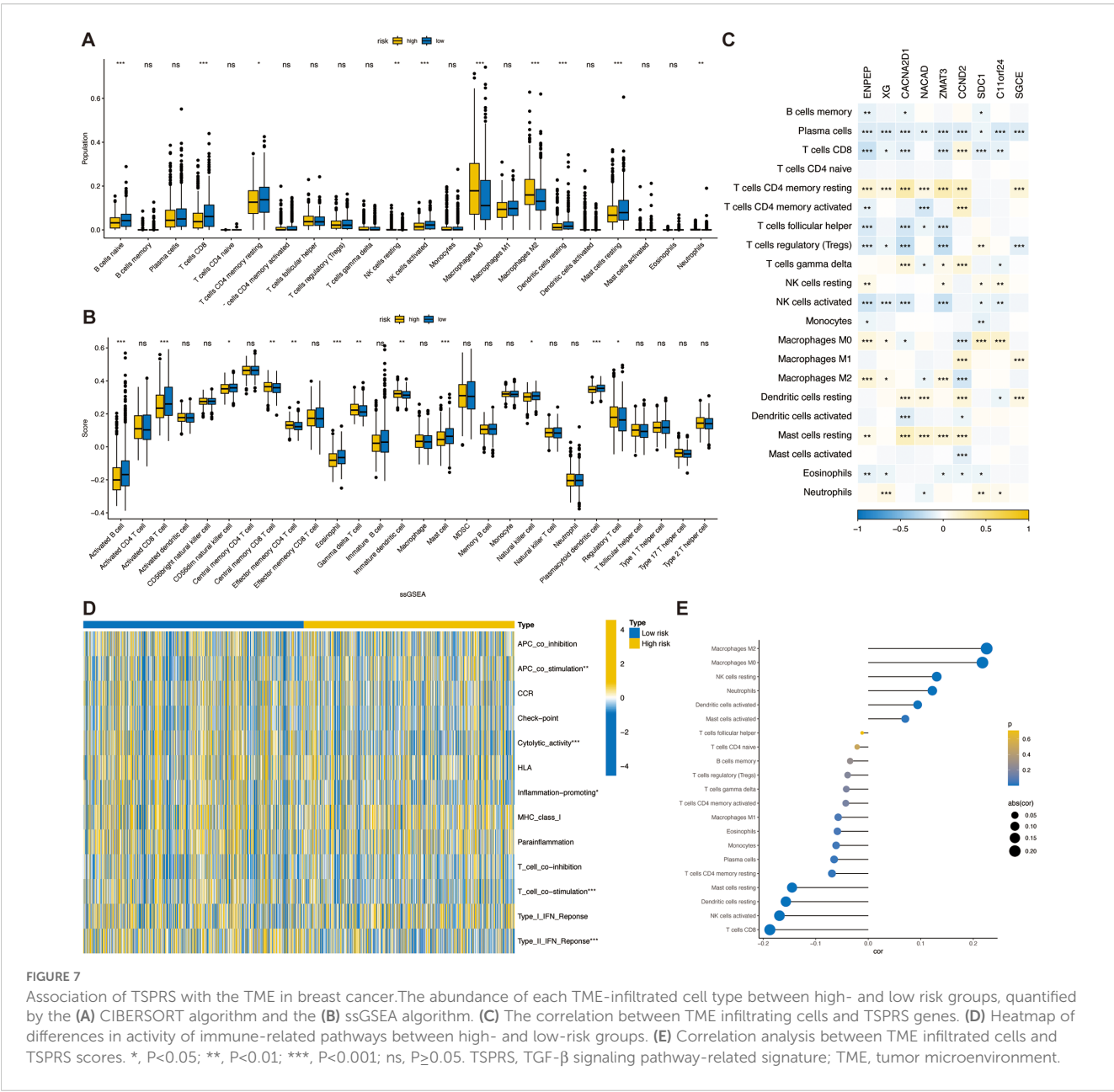
of the immune score, stromal score, ESTIMATE score, and an estimation of tumor purity across different TSPRS risk stratifications. Contrary to expectations, a comparative analysis between high-risk and low-risk cohorts did not yield statistically significant differences for these four scores (Supplementary Figures S5I-L).

To delve into the disparities in the infiltration of specific immune cell types between the high- and low-risk groups, we

quantitatively assessed the prevalence of various infiltrating immune cells within each sample by employing the CIBERSORT algorithm. This analytical tool allows for a refined characterization of the immune cell composition by deconvolving gene expression profiles from bulk tumor transcriptomic data. We found lower levels of CD8+ T cells, NK cells activated and higher levels of M2 macrophages in the high-risk group compared to the low-risk group. This suggests a higher level of immunosuppression in the high-risk group (Figure 7A). Concordant outcomes were procured through the application of the ssGSEA algorithm, serving as a validation method (Figure 7B). Moreover, within the TSPRS, we identified nine genes that exhibited a strong correlation with the presence of tumor-infiltrating immune cells. Notably, *ENPEP*, *XG*, *CACNA2D1*, *ZMAT3*, and *SDC1* demonstrated a significant inverse correlation with activated CD8+ T cells and NK cells. Additionally,

*ENPEP*, *XG*, and *ZMAT3* showed a significant positive correlation with M2 macrophages (Figure 7C).

CD8+ cytotoxic T lymphocytes (CTLs) are the preferred immune cells for targeting cancer. However, during cancer progression, immune tolerance and suppression within the TME lead to CTL dysfunction and exhaustion, promoting adaptive immune resistance. M2 macrophages and regulatory T cells (Tregs) form immune barriers against CD8+ T cell-mediated antitumor immune responses. In the breast cancer microenvironment, TGF- $\beta$  can induce macrophages to transform into M2 macrophages, which possess immunosuppressive and tumor-promoting characteristics. These cells secrete various immunosuppressive factors, including TGF- $\beta$ . TGF- $\beta$  inhibits CXCR3 expression in CD8+ T cells (30), thereby limiting their infiltration into tumors. This finding is consistent with our research results. Given the role of TGF- $\beta$  signaling in the immunosuppressive



**FIGURE 7** Association of TSPRS with the TME in breast cancer. The abundance of each TME-infiltrated cell type between high- and low-risk groups, quantified by the (A) CIBERSORT algorithm and the (B) ssGSEA algorithm. (C) The correlation between TME infiltrating cells and TSPRS genes. (D) Heatmap of differences in activity of immune-related pathways between high- and low-risk groups. (E) Correlation analysis between TME infiltrated cells and TSPRS scores. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns,  $P \geq 0.05$ . TSPRS, TGF- $\beta$  signaling pathway-related signature; TME, tumor microenvironment.

TME, combining TGF- $\beta$  signaling inhibitors with ICIs can enhance antitumor immune responses.

Additionally, using the ssGSEA algorithm, we obtained immune-related pathway scores. The high-risk group demonstrated significantly stronger activity in APC co-stimulation. The low-risk group demonstrated significantly stronger activity in cytolytic activity, inflammation promoting, T cell co-stimulation, and type II IFN response pathways (Figure 7D).

Next, we investigated the correlation between TSPRS and immune cell infiltration by Spearman's correlation analysis, and in agreement with previous results, the risk score was positively correlated with M2 macrophages, and negatively correlated with NK cells activated and CD8+ T cells, suggesting that the risk score predicts the immune status in breast cancer tissues (Figure 7E).

## The relationship between the TSPRS and immunotherapy response

Prior studies have linked higher immune checkpoint levels to better responses to ICIs (25). We compared immune checkpoint expression in TSPRS risk subgroups. The results indicated that in the high-risk group, CD27 and PDCD1 expression levels were lower, while HAVCR2 expression levels were higher, compared to those in the low-risk group (Figure 8A). Further analysis of IPS scores from the TCIA database showed higher scores correlate with improved ICI response, including treatments with PD-1 and CTLA4 inhibitors across four categories: (1) ips\_ctla4\_pos\_pd1\_pos, (2) ips\_ctla4\_pos\_pd1\_neg, (3) ips\_ctla4\_neg\_pd1\_pos, and (4) ips\_ctla4\_neg\_pd1\_neg. Our findings reveal that the low-risk group had significantly higher IPS scores and

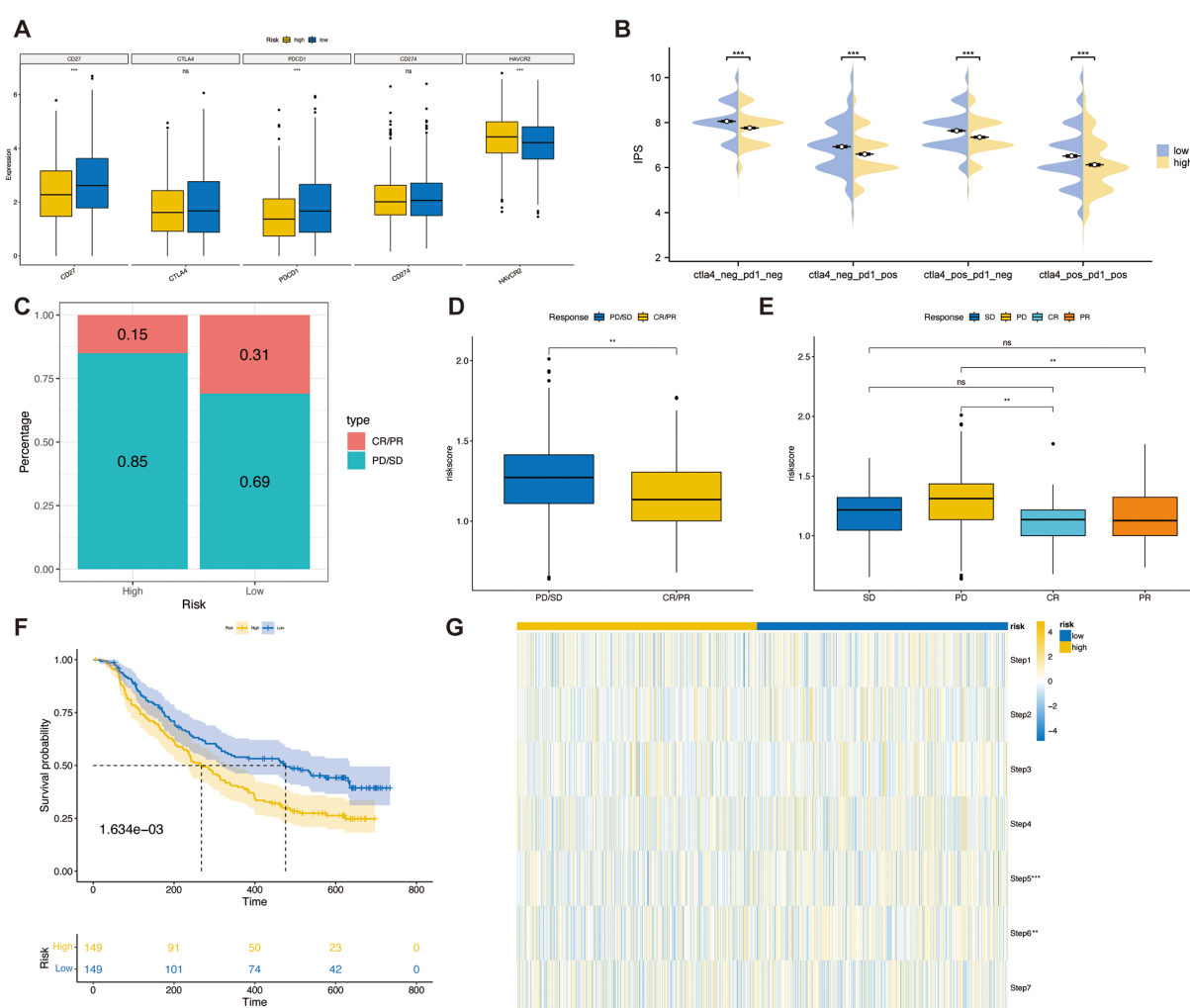


FIGURE 8

The relationship between TSPRS and immunotherapy response. (A) The expression of immune checkpoints in high-risk and low-risk groups. (B) The IPS score between high-risk and low-risk groups. (C) The proportion of CR/PR or SD/PD patients receiving immunotherapy in the high- and low-risk groups of the IMvigor210 cohort. (D) A boxplot showing the difference in risk score between patients with CR/PR and those with SD/PD in the IMvigor210 cohort. (E) A boxplot showing the variation in risk score between patients with CR, PR, SD and PD in the IMvigor210 cohort. (F) KM survival curves for high and low risk groups in the IMvigor210 cohort. (G) Heatmap showing the difference in the seven-step anti-cancer immunity cycle activity between high- and low-risk groups. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns,  $P \geq 0.05$ . CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.

better responses to all four ICIs treatment regimens compared to the high-risk group (Figure 8B).

To validate TSPRS's prognostic value in predicting immune therapy outcomes, we analyzed the IMvigor210 cohort treated with atezolizumab. Using TSPRS, we assigned risk scores and categorized patients into high-risk and low-risk groups. The low-risk group showed a higher rate of complete or partial responses (CR/PR), whereas the high-risk group mainly had progressive or stable disease (PD/SD, Figure 8C). Risk scores were significantly lower in patients with PD/SD compared to those with CR/PR (Figure 8D). Specifically, patients with progressive disease (PD) had notably higher risk scores than those achieving responses (Figure 8E). In the IMvigor210 dataset, shorter survival times were associated with the high-risk group (Figure 8F). These results support TSPRS's ability to forecast outcomes of immune-based treatments, suggesting better therapeutic outcomes for low-risk patients.

Finally, we evaluated the activity of the anticancer immune cycle in high and low risk groups by the Tracking Tumor Immunophenotype (TIP) database in order to gain a comprehensive understanding of the anticancer role of immune cells and to guide immunotherapy (31). The seven steps are release of cancer cell antigens (Step 1), cancer antigen presentation (Step 2), priming and activation (Step 3), trafficking of immune cells to tumors (Step 4), infiltration of immune cells into tumors (Step 5), recognition of cancer cells by T cells (Step 6) and killing of cancer cells (Step 7). The results showed that the activities of Steps 5 and 6 of the anti-cancer immune cycle were significantly higher in the low-risk group than in the high-risk group (Figure 8G).

## The correlation of the TSPRS with single-cell characteristics

To elucidate the influence of TSPRS within the TME at the level of single-cell transcriptomics, we conducted an examination of the expression profiles of a cohort of genes, namely *ENPEP*, *XG*, *CACNA2D1*, *NACAD*, *ZMAT3*, *CCND2*, *SDC1*, *C11orf24*, and *SGCE*, across diverse cellular phenotypes. Our findings delineated that the aforementioned genes predominantly manifested expression within fibroblasts, with *CCND2* additionally exhibiting pronounced expression in T cells (Figure 9A).

KEGG enrichment analysis showed that the differential genes in the high and low risk groups were mainly enriched in the pathways of Focal adhesion, Proteoglycans in cancer, and Leukocyte transendothelial migration (Figure 9B). Gene Set Enrichment Analysis (GSEA) enrichment analysis showed that the high-risk group was mainly involved in pathways such as EPITHELIAL\_MESENCHYMAL\_TRANSITION (Figure 9C).

In addition, we found that cells within the high- and low-risk groups had different communication patterns (Figure 9D). In the CXCL pathway, the high-risk group was dominated by macrophage-endothelial cell communication, whereas the low-risk group was dominated by fibroblast-T cell communication (Figures 9E, F). In the MK pathway, the high-risk group was dominated by fibroblast-fibroblast communication, whereas the

low-risk group was dominated by fibroblast-endothelial cell communication (Figures 9G, H).

## Establishment and validation of a nomogram

To evaluate the independence of TSPRS as a prognostic indicator for BRCA, a series of Cox proportional hazards regression analyses—both univariate and multivariate—were performed on overall survival (OS) metrics within the TCGA-BRCA (Figures 10A–D). The results demonstrated that TSPRS constituted a considerable hazard element for OS based on the single-variable analysis. Further, TSPRS preserved its status as an autonomous prognostic indicator in the multiple-variable analysis, suggesting its strong predictive capacity for BRCA patient outcomes.

To enhance the clinical utility of the TSPRS, stepwise regression was employed to examine the interplay between clinical attributes and TSPRS. This analysis facilitated the integration of age, disease stage, and TSPRS into a predictive nomogram (Figure 10E). The C-index affirmed the nomogram's consistent and formidable predictive performance, surpassing that of other clinical parameters (Figure 10F). Calibration plots corroborated the congruence between the nomogram's forecasts and the actual clinical outcomes (Figure 10G). Within the training cohort, the AUC values for the nomogram were impressive, registering at 0.780, 0.830, and 0.796 for the 1-, 5-, and 7-year marks, respectively (Figure 10H). The internal validation set yielded AUC values of 0.854, 0.786, and 0.806 at the corresponding 1-, 5-, and 7-year intervals, underscoring the nomogram's high predictive precision (Figure 10I). Collectively, these results endorse the TSPRS-informed nomogram as a robust and precise instrument for the tailored prognostication of BRCA patients.

## Analysis of the correlation between the TSPRS and drug sensitivity

We analyzed the correlation between TSPRS and drug sensitivity using the R package 'oncoPredict', which showed that the IC50 of the high-risk group for the PIK3CA kinase inhibitors Alpelisib and Pictilisib (Figures 11A, B), the tyrosine kinase inhibitor Lapatinib (Figure 11C), the estrogen receptor modulator Tamoxifen (Figure 11D), and the conventional chemotherapeutic agents Paclitaxel and Docetaxel were lower than those of the low-risk group (Figures 11E, F).

## ZMAT3 is associated with breast cancer growth and invasion

Multivariate Cox regression analyses in the TCGA dataset showed that *XG* and *ZMAT3* were independent prognostic risk factors for breast cancer, and *ZMAT3* had higher HR values, suggesting that it may be a key gene influencing the prognosis of



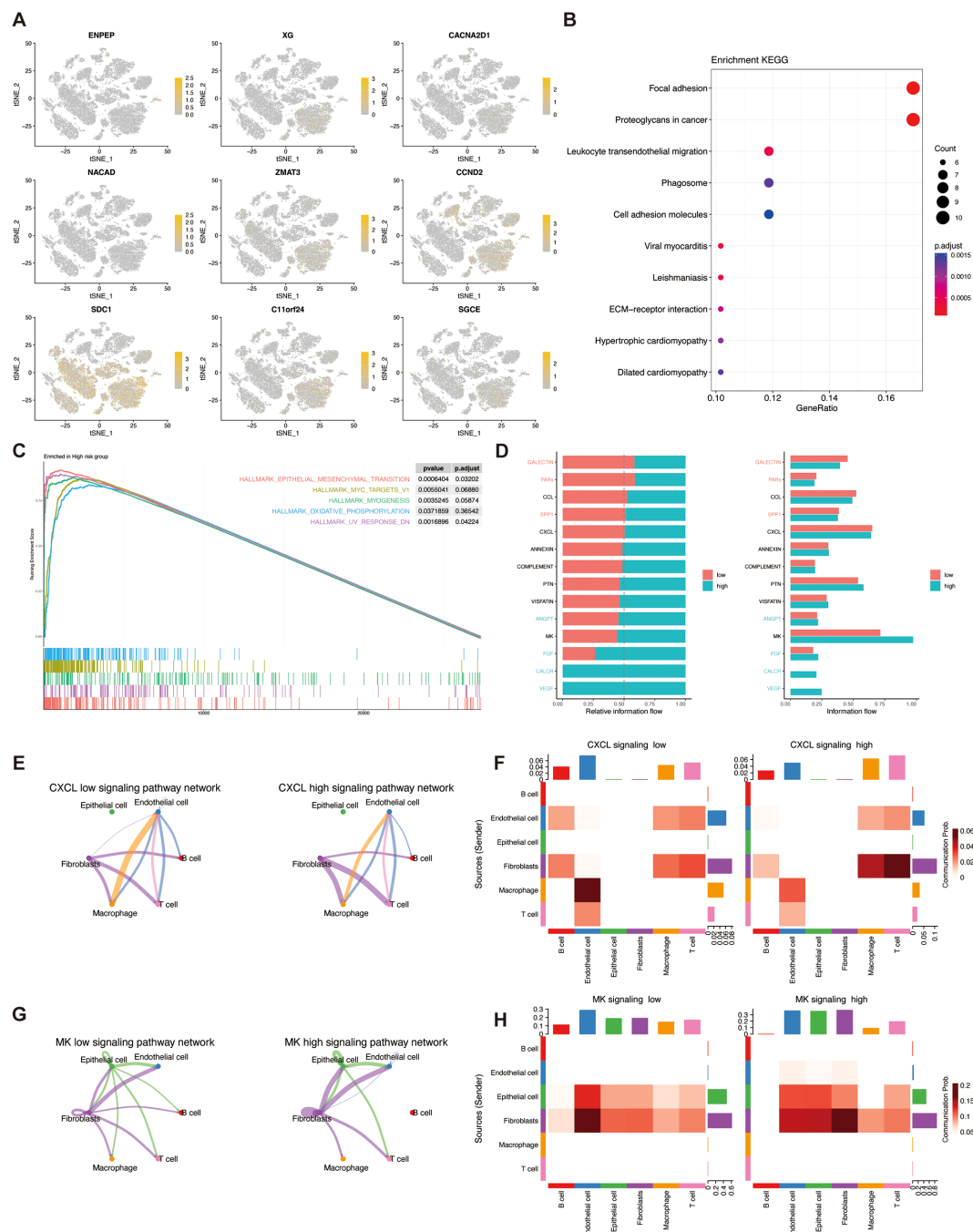
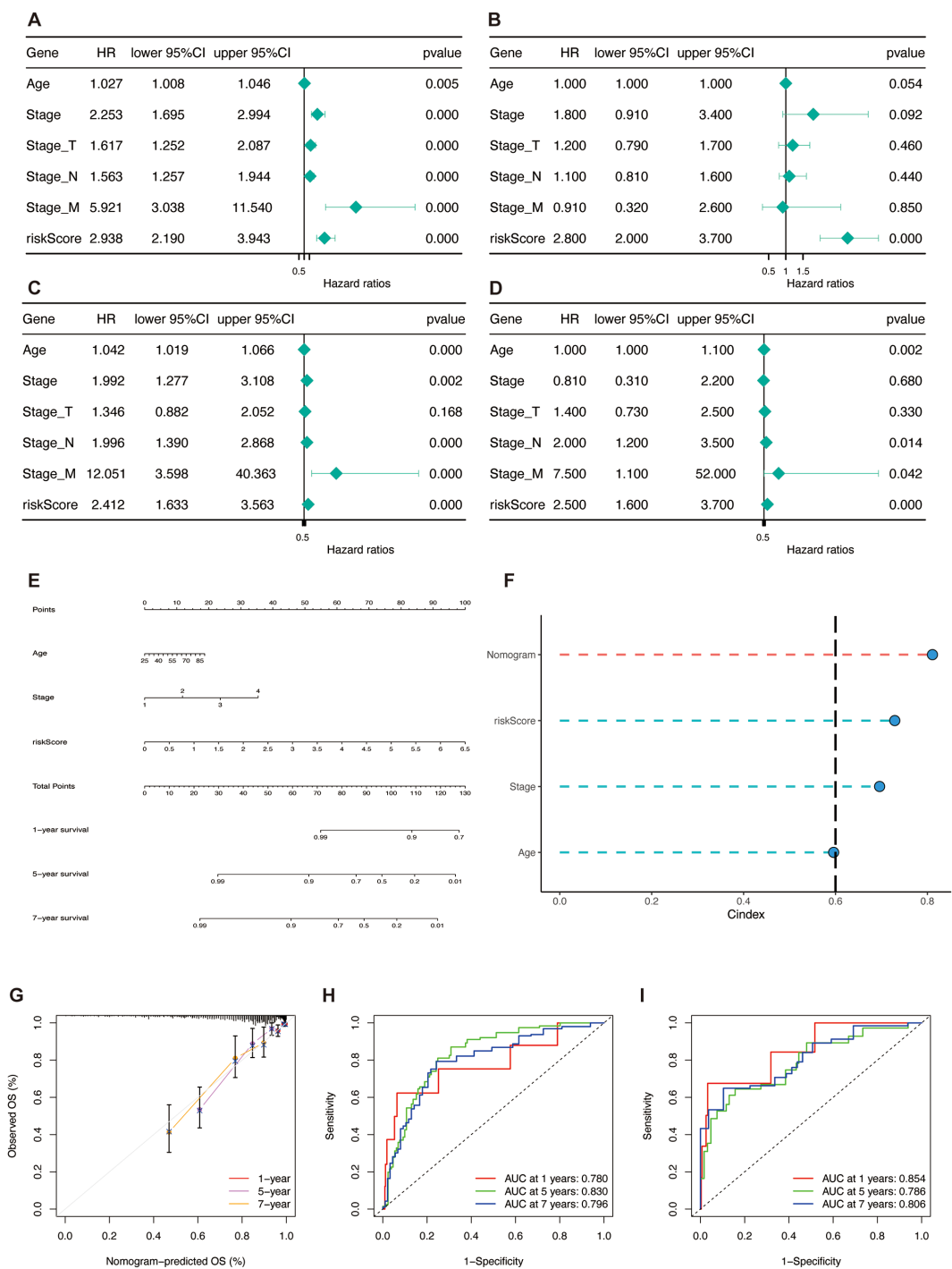


FIGURE 9

The correlation of TSPRS with single-cell characteristics. **(A)** The expression of ENPEP, XG, NACAD, CACNA2D1, ZMAT3, CCND2, SDC1, C11orf24 and SGCE in different cell types by single-cell RNA-seq analysis. **(B)** KEGG analysis of DEGs between the high-risk and low-risk cells. **(C)** GSEA enrichment analysis of the high-risk groups. **(D)** Comparative analysis of the intensity of cellular signaling pathways in high- and low-risk groups. **(E)** CXCL pathway network loops in high and low risk groups. **(F)** Heatmap of the role of different cell types in the CXCL pathway network in high and low risk groups. **(G)** MK pathway network loops in high and low risk groups. **(H)** Heatmap of the role of different cell types in the MK pathway network in high and low risk groups. KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, Gene Set Enrichment Analysis.

breast cancer (Supplementary Figure S5M). The results of the GSEA enrichment analysis showed that the high-expression ZMAT3 group was predominantly in the Cellular senescence (Supplementary Figure S5N). In the TCGA dataset, the expression level of ZMAT3 in tumors was significantly lower than that in adjacent normal tissues (Supplementary Figure S5O). In the

32 matched pairs of clinical samples we collected from, the qPCR results were consistent with TCGA (Figure 12A). The mRNA expression level of ZMAT3 in human breast cancer cell lines MDA-MB-231 and MCF-7 was significantly lower than that in human breast epithelial cell line MCF-10A (Figure 12B). Transfection of siRNAs targeting ZMAT3 was performed in



**FIGURE 10** Construction and validation of the nomogram. (A, B) Univariate and multivariate Cox regression analyses of clinical pathology variables and risk scores with OS in the TCGA training set and (C, D) the TCGA test set. (E) Construction of the nomogram based on TSPRS and clinical characteristics, including age and stage. (F) C-index comparison of the nomogram, TSPRS, stage and age. (G) Calibration curve of the nomogram for 1, 5 and 7-year OS. (H) ROC curves showing the predictive performance of the nomogram in 1, 5, and 7-year OS in the TCGA training set and (I) the TCGA testing set.

MDA-MB-231 and MCF-7 cells to investigate the regulatory role of ZMAT3 expression in breast cancer cell progression (Figures 12C, D). Western blot confirmed that ZMAT3 was successfully knocked down (Figure 12E). The proliferation of MDA-MB-231 and MCF-7 cells was significantly inhibited when ZMAT3 was knocked down in

CCK-8 assays (Figures 12F, G). Additionally, knockdown of ZMAT3 significantly inhibited the invasion and migration ability of breast cancer (Figures 12H, 13A, B). Overall, our findings indicated that ZMAT3 may promote the proliferation and invasion of breast cancer.

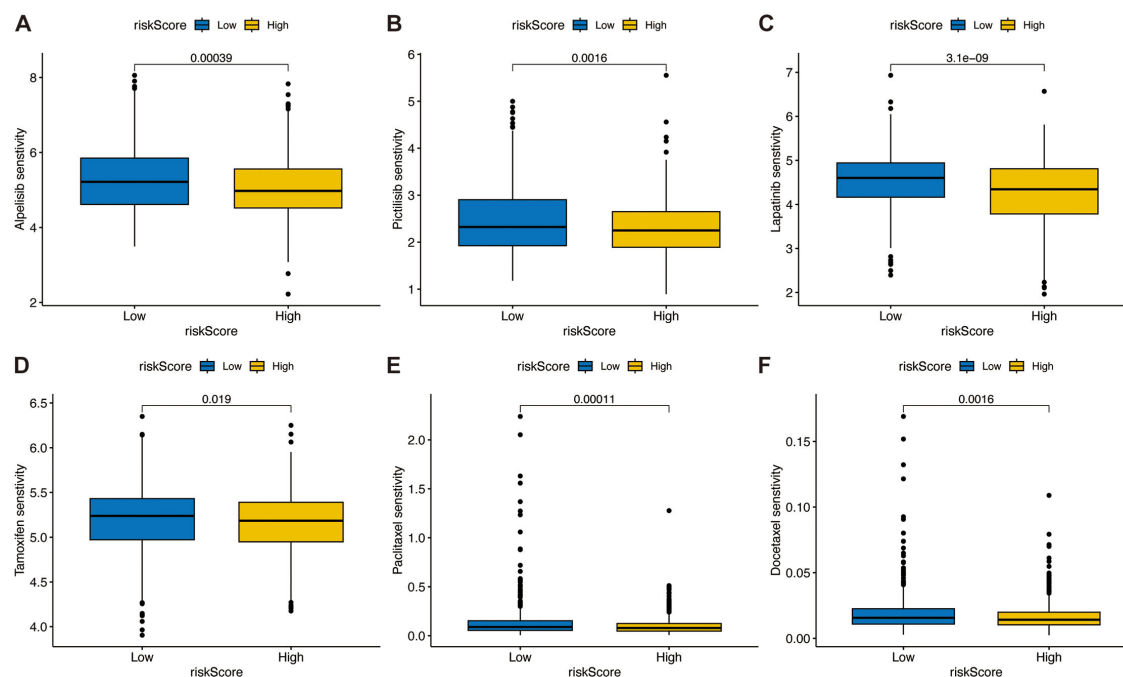


FIGURE 11

Association between the TSPRS and drug sensitivity. The box plots show the half-maximal inhibitory concentration of PIK3CA kinase inhibitors (A) Alpelisib and (B) Pictilisib, the tyrosine kinase inhibitor (C) Lapatinib, the estrogen receptor modulator (D) Tamoxifen, and conventional chemotherapy agents (E) Paclitaxel and (F) Docetaxel in high and low risk groups.

Given that the attenuation of ZMAT3 expression diminished the proliferative and invasive propensities of breast cancer cells, we evaluated the sensitivity of ZMAT3 to a spectrum of pharmacological compounds. Our findings reveal a pronounced inverse correlation between ZMAT3 expression and the IC<sub>50</sub> values of Cediranib, JQ1, NU7441, and RO-3306, with correlation coefficients all below -0.5 (Figures 13C–F). Subsequent molecular docking analyses of ZMAT3 with these agents yielded binding energies of -6.6 kcal/mol for Cediranib, -5.9 kcal/mol for JQ1, -7.9 kcal/mol for NU7441, and -6.7 kcal/mol for RO-3306, respectively, underscoring the potential for efficacious interaction with ZMAT3 (Figures 13G–J).

## Discussion

The TGF- $\beta$  signaling pathway plays a crucial dual role in tumor progression and the shaping of the tumor immune microenvironment. Previous studies have reported that anti-TGF- $\beta$ /PD-L1 bispecific antibodies, such as YM101 and BiTP, exhibit strong antitumor effects in mouse tumor models (32, 33). However, despite extensive research on individual or several genes within this pathway, our understanding of its overall activity and infiltration characteristics in the TME is still limited. Unraveling the functions of TGF- $\beta$  signaling pathway activities within the TME could advance our knowledge of the tumor immune microenvironment and guide more precise personalized immunotherapy approaches.

In this study, we first performed a single-cell level analysis to assess the TGF- $\beta$  signaling pathway activity across various cell types

within the breast cancer microenvironment. The highest activity was noted in endothelial cells, fibroblasts, and epithelial cells. At the bulk transcriptome level, we found that the pathway's activity was significantly higher in normal tissue compared to breast cancer tissue. Through WCGNA analysis, we identified 880 module genes closely associated with TGF- $\beta$  signaling pathway activity. Crossing these with differentially expressed genes in breast cancer yielded 654 module differential genes.

To establish a prognostic feature linked to the TSP with enhanced predictive accuracy, we compared the C-index of various models using ten machine learning algorithms and 101 algorithm combinations. We ultimately utilized the Lasso method to construct a prognostic feature comprising nine genes: *ENPEP*, *XG*, *NACAD*, *CACNA2D1*, *ZMAT3*, *CCND2*, *SDC1*, *C11orf24*, and *SGCE*. This feature's predictive capability was validated in training, internal, and external validation sets. Compared to ten previously published prognostic signatures, our TSPRS demonstrated the highest C-index, indicating superior predictive power. We used the TSPRS to assign risk scores to breast cancer patients, dividing them into high and low-risk groups based on the median risk score. Patients in the low-risk group had a longer OS and higher immune cell infiltration, while the high-risk group exhibited a more potent immune-suppressive TME, leading to reduced patient survival times (34). Both univariate and multivariate COX regression analyses confirmed that TSPRS is an independent prognostic indicator for breast cancer. Additionally, a nomogram integrating TSPRS with clinical pathological characteristics showed excellent performance in predicting 1-year, 5-year, and 7-year OS rates for breast cancer patients.

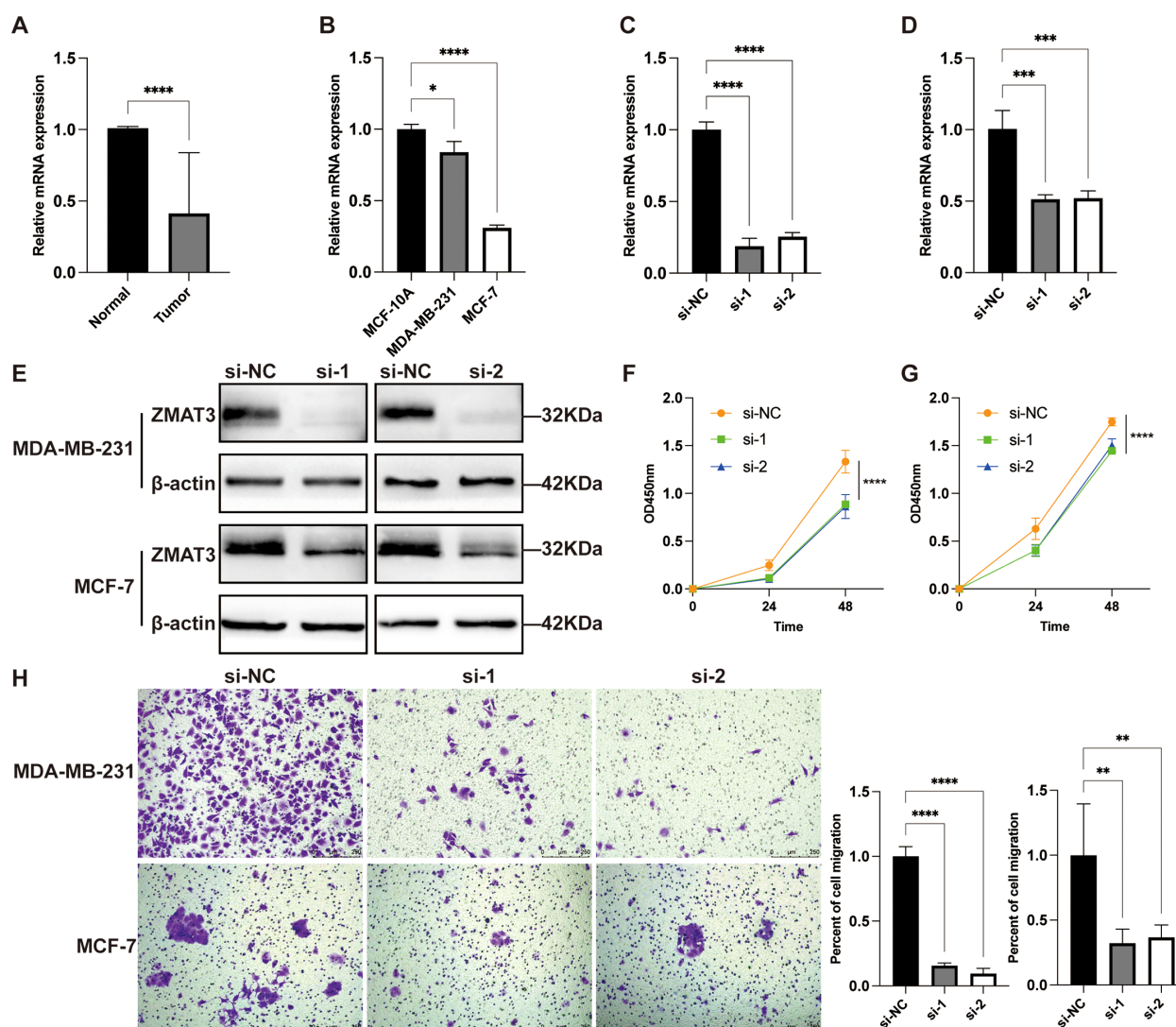


FIGURE 12

The role of ZMAT3 in breast cancer. (A) Expression levels of ZMAT3 in tumor tissues and normal tissues in 32 matched pairs of clinical samples. (B) Expression levels of ZMAT3 in MCF-10A, MDA-MB-231 and MCF-7 cells. (C) Validation of ZMAT3 knockdown at the mRNA level in MDA-MB-231 cells. (D) ZMAT3 knockdown was verified at the mRNA level in MCF-7 cells. (E) ZMAT3 knockdown was verified at the protein level in MDA-MB-231 and MCF-7 cells. (F) CCK-8 assay to detect changes in proliferative capacity of MDA-MB-231 cells after ZMAT3 knockdown. (G) CCK-8 assay to detect changes in proliferative capacity of MCF-7 cells after knockdown of ZMAT3. (H) Migration assay to detect changes in invasion ability of MDA-MB-231 and MCF-7 cells after knockdown of ZMAT3. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ .

The molecule PD-L1 on the surface of tumor cells can interact with the PD-1 molecule on T cells' surface, thereby evading T-cell mediated immune surveillance. By using specific antibodies to block the interaction between PD-1 and PD-L1, the proliferation and cytotoxic functions of T cells can be enhanced, thus playing an anti-tumor role. The levels of immune cell infiltration and PD-L1 expression in TNBC are significantly higher than in other subtypes of breast cancer, indicating that PD-1/PD-L1 inhibitors may have potential therapeutic value in TNBC. Therefore, several clinical studies on ICIs treatment for breast cancer are targeted at TNBC, but other subtypes of breast cancer also possess potential for ICIs treatment. In terms of immunotherapy, our study found that the low-risk group with breast cancer showed higher PD-1 expression levels, greater infiltration of CD8+ T cells and NK

cells, and higher IPS, suggesting that these patients might benefit more from ICIs treatment. TSPRS could thus potentially predict immunotherapy response in breast cancer patients. Although data on immunotherapy in breast cancer are scarce, the application of IMV210 indirectly supports TSPRS's predictive capacity for immunotherapy.

To effectively use TSPRS in managing breast cancer, we suggest applying this model for risk assessment post-diagnosis. By analyzing TSPRS scores, patients can be quickly categorized as high or low risk, guiding treatment decisions. For instance, our study shows that low-risk patients, with higher immune checkpoint expression, may benefit more from single immune checkpoint inhibitor treatments. Conversely, high-risk patients, more responsive to PI3CA kinase inhibitors, tyrosine kinase



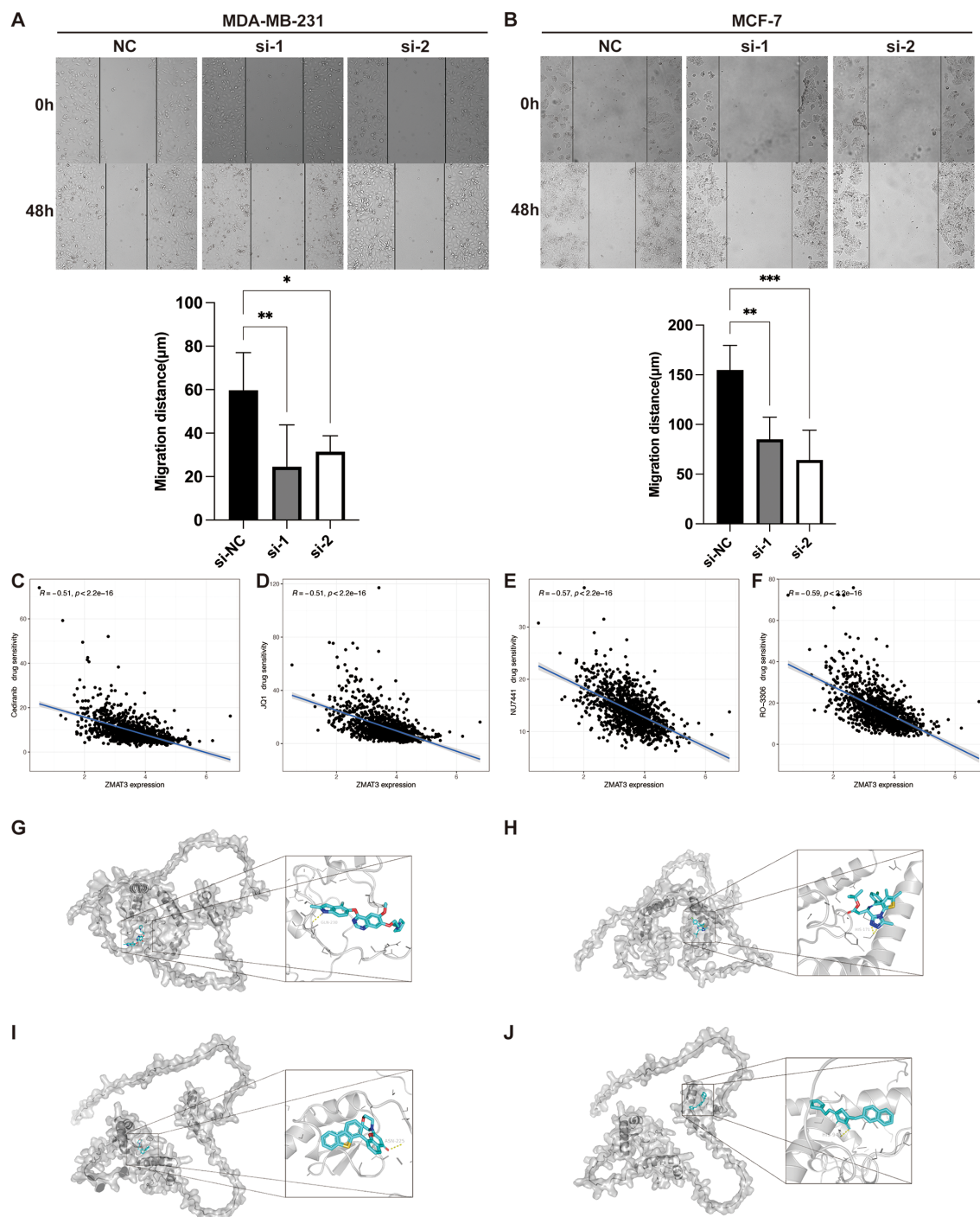


FIGURE 13

The role of ZMAT3 in breast cancer. (A, B) Wound-Healing assay to detect changes in migration ability of MDA-MB-231 and MCF-7 cells after knockdown of ZMAT3. (C) Scatterplot of correlation between Cediranib, (D) JQ1, (E) NU7441, and (F) RO-3306 sensitivity and ZMAT3 expression levels. Molecular docking representations of ZMAT3 with (G) Cediranib, (H) JQ1, (I) NU7441, and (J) RO-3306. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

inhibitors, estrogen receptor modulators, and standard chemotherapies, and with elevated HAVCR2 levels, might require a combined approach of HAVCR2-targeted immunotherapy and chemotherapy to enhance treatment efficacy.

Focusing on individual genes, our multivariate COX regression analysis identified ZMAT3 and XG as independent prognostic risk factors for breast cancer, with ZMAT3 having the highest HR value.

ZMAT3 (Zinc Finger Matrin-Type 3) codes for an RNA-binding protein that contains a zinc finger structure. Despite research indicating that ZMAT3, as a target gene of p53, plays a crucial role in p53-mediated tumor suppression (35), it is recognized as a tumor suppressor factor (36). However, the molecular mechanism through which it operates within cells still needs to be elucidated. For instance, in colorectal cancer, silencing ZMAT3 can promote



the proliferation of cancer cells by increasing the inclusion of CD44 variant exons (37). In LUAD, ZMAT3 suppresses tumor growth by inhibiting cell proliferation without inducing cell apoptosis (38). However, research also shows that unlike p53, the absence of ZMAT3 does not affect the development of lymphomas driven by c-Myc or LUAD driven by KRAS mutations (39).

To date, no studies have reported on the role of ZMAT3 in breast cancer. Contrary to previous reports, we found that ZMAT3 is an independent risk factor for poor prognosis in breast cancer, with patients exhibiting high levels of ZMAT3 expression having significantly lower OS compared to those with low expression levels. It has been reported that ZMAT3 is directly transcriptionally controlled by p53 in various cell types (38). In tumors lacking p53, such as breast cancer, ZMAT3 expression is reduced, which is consistent with our experimental results. In the 31 pairs of clinical samples we collected, ZMAT3 expression levels were significantly lower in breast cancer tissues compared to adjacent normal tissues.

p53 primarily induces cell cycle arrest through p21 and 14-3-3 $\sigma$  and apoptosis through target genes such as FAS, PUMA, and NOXA. However, the molecular mechanisms by which cells decide to enter growth arrest or apoptosis following p53 activation are not fully understood. By identifying transcripts affected by ZMAT3 knockdown, researchers found (40) that FAS and 14-3-3 $\sigma$  mRNA are regulated by ZMAT3 in a p53-independent manner. Additionally, ZMAT3 deficiency is associated with increased cell death and reduced cell cycle arrest in response to DNA damage. When we used siRNA transfection to reduce ZMAT3 expression in breast cancer cells, the proliferation and invasion capabilities of these cells were significantly inhibited. GSEA analysis showed that high ZMAT3 expression is associated with enrichment of the cellular senescence pathway. Cellular senescence is a stress response that typically leads to cell cycle arrest. However, in cancer, the senescence mechanism may be exploited by cancer cells to evade apoptosis, which could be one of the mechanisms by which high ZMAT3 expression promotes breast cancer progression.

Inhibiting ZMAT3 may enhance the sensitivity of tumor cells to chemotherapy and radiotherapy by inducing DNA damage. Furthermore, ZMAT3 could serve as a potential target for targeted therapy in breast cancer. Developing inhibitors or small molecule drugs against ZMAT3 could provide new treatment options for breast cancer patients, especially those with high ZMAT3 expression and poor prognosis.

Despite these insights, our study is not without limitations. It is a retrospective study based on public data and requires further validation through large-scale, multi-center prospective studies. To enhance the reliability of TSPRS, we plan to validate this prognostic marker in independent prospective cohorts or clinical trials. Additionally, a more comprehensive integration of clinicopathological features is necessary to fully analyze the clinical value of the risk signature. We recommend designing and implementing prospective studies in future research to evaluate the prognostic predictive ability of TSPRS in different patient populations and to explore its potential application in clinical practice. The role of ZMAT3 in the development and progression of breast cancer also warrants further experimental investigation.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Author contributions

YZ: Conceptualization, Formal analysis, Methodology, Resources, Writing – original draft, Writing – review & editing. LL: Methodology, Writing – review & editing, Data curation. WC: Conceptualization, Writing – original draft, Writing – review & editing. LinL: Writing – review & editing, Methodology. RZ: Writing – review & editing, Supervision. WH: Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. YC: Project administration, Resources, Writing – review & editing.

## Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the National Natural Science Foundation of China (No.22074024), Natural Science Foundation of Guangdong Province (No. 2023A1515012573), National Undergraduate Training Program for Innovation and Entrepreneurship & Student Research Training Program (No. 202310573005).

## Acknowledgments

We sincerely acknowledge TCGA and GEO database for providing their platforms and contributors for uploading their meaningful datasets. In the preparation of this manuscript, we employed OpenAI's GPT-4 model to assist with grammatical correction. The specific details of the model used are as follows: Name: GPT-4, Version: 4.0, Source: OpenAI. Thanks to Ms. Linna Dai from Johns Hopkins University, Baltimore, USA, for manuscript correcting and polishing.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2024.1488137/full#supplementary-material>

### SUPPLEMENTARY FIGURE 1

(A) Differential expression analysis of TSPG in breast cancer and adjacent normal tissues. (B) Protein-protein interaction network analysis of TSPG in the STRING database. (C) Mutation frequency analysis of TSPG in breast cancer. (D) Frequencies of CNV gain, loss, and non-CNV among TSPG.

### SUPPLEMENTARY FIGURE 2

Enrichment analysis of TSPRG. (A) GO enrichment of TSPRG. (B) KEGG enrichment of TSPRG.

### SUPPLEMENTARY FIGURE 3

Kaplan-Meier curves for (A) SDC1, (B) NACAD, (C) ZMAT3, (D) CCND2, (E) CACNA2D1, (F) XG, (G) SGCE, (H) ENPEP, and (I) C11orf24 in the TCGA dataset.

### SUPPLEMENTARY FIGURE 4

The distribution of the risk score, overall survival status of patients and signature gene expression heatmap in (A–C) the TCGA training set, (D–F) TCGA internal validation set, (G–I) TCGA entire set, and (J–L) GSE20711. (M) Kaplan-Meier curves of OS according to TSPRS in the TCGA entire set. (N) ROC curves showing the specificity and sensitivity of TSPRS in predicting 1, 5, and 7-year OS in the TCGA entire set. (O) Kaplan-Meier curves of DFS according to TSPRS in the TCGA entire set. (P) Kaplan-Meier curves of PFS according to TSPRS in the TCGA entire set. (R) Risk coefficients for the nine genes that make up the TSPRS. (S) Scatterplot of correlation between TMB and riskScore.

### SUPPLEMENTARY FIGURE 5

(A) Heatmap of correlations with clinical features based on TSPRS. Differences in risk scores for (B) T, (C) N, (D) M, and (E) stage in high- and low-risk groups. Kaplan-Meier curves for (F) M1 and (G) HER2+ patients. Differences in (I) stromal score, (J) immune score, (K) estimate score, (L) tumor purity in high and low risk groups. (M) Forest plot of multivariate Cox regression analysis. (N) GSEA analysis of high and low ZMAT3 expression groups. (O) Difference in expression of ZMAT3 in TCGA in breast cancer and adjacent normal tissue.

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RECEIVED 14 August 2024

ACCEPTED 06 January 2025

PUBLISHED 24 January 2025

## CITATION

Fang Q, Chen S, Chen X, Zou W, Chen D, Huang Y and Wu C (2025) Mature tertiary lymphoid structure associated CD103+ CD8+ Trm cells determined improved anti-tumor immune in breast cancer.  
*Front. Oncol.* 15:1480461.  
doi: 10.3389/fonc.2025.1480461

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# Mature tertiary lymphoid structure associated CD103+ CD8+ Trm cells determined improved anti-tumor immune in breast cancer

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**Background:** Although tertiary lymphoid structures (TLS) play crucial roles in the anti-tumor immune response and are associated with favorable prognoses in many solid tumors, the precise mechanisms by which TLSs enhance anti-tumor immunity remain poorly understood. The current study aimed to explore the relationship between the maturity of tertiary lymphoid structures and their key immune cells in combating breast cancer.

**Patients and methods:** In this study, we utilized immunofluorescence and H&E staining to detect tumor-resident memory T cells (Trm) and assess the maturity of TLS, analyzing their distribution and proportion in an annotated cohort of 95 breast cancer patients.

**Results:** The presence of tumor-associated TLSs was correlated with an improved prognosis in patients with breast cancer. The proportion of CD8+CD103+ resident memory T cells and natural killer (NK) cells within the TLSs was significantly higher than that in areas outside of these structures. Additionally, the proportions of CD103+ CD8+ Trm cells and NK cells were significantly increased with the gradual maturation of TLS. Furthermore, the secretion function of effector molecules by CD8+ CD103+ Trm cells and NK cells within TLSs was significantly enhanced, indicating a strong correlation between the effector function of CD103+ CD8+ Trm and NK cells and the maturity of TLSs.

**Conclusion:** Our study identifies potential additional prognostic information for the clinical prognosis of breast cancer patients, underscoring the prognostic significance of immune cells within TLS, with a particular focus on CD103+ CD8+ Trm cells and NK cells.

## KEYWORDS

breast cancer, tertiary lymphoid structures, CD103+ CD8+ Trm cells, prognosis factors, tumor immunology

## Background

Breast cancer is the most frequently diagnosed cancer in women and affects a large percentage of women (1). Although significant advancements have been made in the early detection and treatment of breast cancer, it continues to be the second leading cause of cancer-related deaths in women, following lung cancer (2). While breast cancer is generally considered less immunogenic than cancers like melanoma or lung cancer, it remains among the top ten most immune-infiltrated tumors according to Mandal et al (3). This highlights that, despite its comparatively lower immunogenicity, immune infiltration plays a significant role in the breast cancer microenvironment. Nonetheless, recent studies have consistently demonstrated that the tumor microenvironment of breast cancer encompasses a varied array of cell populations from both the innate and adaptive immune systems. These populations have proven to be of biological and clinical significance to different extents (4). To identify effective therapeutic strategies against BC progression, it is necessary to provide a more profound elucidation of the histological features.

Over the past few decades, cancer immunotherapy has transformed from a promising treatment approach into a substantial clinical reality (37828275). Traditional treatments like surgery, chemotherapy, and radiotherapy primarily target the tumor cells directly, often overlooking the critical roles played by the TME and the immune system in cancer progression and response to treatment. The TME consists of various components, including immune cells, fibroblasts, endothelial cells, extracellular matrix, and signaling molecules, all of which can influence tumor growth, metastasis, and resistance to therapy (5, 6). Recent studies and clinical trials, especially those involving immunotherapy, have underscored the importance of considering the immune contexture in cancer treatment. Immunotherapy, such as immune checkpoint inhibitors (ICIs), has demonstrated significant success by targeting the interactions between tumor cells and immune cells, thereby reactivating the immune system to recognize and attack cancer cells. This success highlights the potential of therapies that modulate the immune environment to enhance cancer treatment efficacy (7, 8).

Ectopic accumulations of immune cells including B and T cell is initially identified as tertiary lymphoid structures (TLSs) that develops in the context of chronic inflammation or cancer (9). TLSs often exhibit features of conventional secondary lymphoid organs, including high endothelial-like vessels that express peripheral node addressin (PNAd), a T-cell zone with mature DCs, and adjacent B-cell zone with follicular DCs and B cells (9, 10). The presence of TLSs is documented in association with control of cancer growth and better prognosis in a wide variety of primary and metastatic solid tumors of human (10, 11). To date, how BC-associated TLSs augment anti-tumor immunity remains incompletely understood.

In this study, we reported the finding that tissue-resident memory CD8<sup>+</sup> Trm cells within tumor-associated TLS correlate with improved prognosis in patients with breast cancer. We identified that CD103<sup>+</sup> CD8<sup>+</sup> Trm cells within tumor-associated TLS contributed to anti-tumor immune response in breast cancer. To sum up, this study further revealed relationship between CD103<sup>+</sup> CD8<sup>+</sup> Trm cells, NK cells and TLS, shedding light on the role of CD103<sup>+</sup> CD8<sup>+</sup> Trm cells and NK cells within TLS in promoting cancer immunotherapy.

## Materials and method

This study is a retrospective study (approval number: GASTO-22-01-007, November 2022). An annotated cohort of 95 untreated primary invasive breast carcinoma and ductal breast carcinoma, collected from female patients diagnosed and treated at Huizhou Centers Hospital between 2017 and 2018, was analyzed. Oral informed consent was obtained from the patients. A pathologist specializing in breast cancer reviewed the clinical and pathological features of the patients involved in this study. The experiments were conducted in accordance with government policies and the Helsinki Declaration. The Ethics Committee of Huizhou Central People's Hospital approved the study. [Supplementary Table 1](#) presents detailed clinical and pathological information on the patients. The methods used in this study are described in the section of Materials and Methods.

### Tertiary lymphoid structures evaluation

Tertiary lymphoid structures (TLS) were both qualified and quantified using Hematoxylin and Eosin (H&E) staining or immunofluorescence, as detailed in prior studies (12, 13), with the number of TLS per square millimeter area being the specific measure utilized. For H&E-stained evaluation, A TLS was considered as germinal centers positive (mature TLS) if the TLS showed the characteristic morphology of proliferating centroblasts. For Immunofluorescence evaluation, TLS were identified as lymphocyte aggregates with histological features that included B cells (CD20), T cells (CD3), follicular dendritic cells (CD21 and CD23) in the intratumor, peritumor, and para-tumor areas. TLS was evaluated for its level of maturation in GC tissue. E-TLS, PFL-TLS, and SFL-TLS. These types differed in the presence of follicular dendritic cells (FDC) and their markers. Three types of TLS were identified: E-TLS, which are early TLS, consist of aggregates of CD20<sup>+</sup> B cells without FDC. PFL-TLS, which are primary follicle-like TLS, have differentiated CD21<sup>+</sup> FDC. SFL-TLS, which are secondary follicle-like TLS, have a germinal center that is notably visible through the presence of CD21<sup>+</sup> CD23<sup>+</sup> FDC.

### Calculation of tertiary lymphoid structure density

The determination of TLS density at intratumoral, peritumoral, and para-tumoral sites in breast cancer involved using the microscope with an eyepiece field of view of 22, counting TLS per 10X field. The calculation involved a diameter (d) of 0.22 mm, leading to an S of  $\pi/4 \cdot d^2$  (3.8 mm<sup>2</sup>). Following this, TLS density was calculated throughout the whole slide, as has been described in previously studies (14, 15).

### Immunofluorescence evaluation

Sections of 4-μm thickness were prepared from paraffin-embedded human breast cancer tissues for immunofluorescence staining, employing antibodies against human CD20 (1:500, Abcam, Cat#



ab78237), CD3 (1:100, Abcam, ab5690, Cat# ab5690), CD21 (1:200, Abcam, Cat# ab75985), CD23 (1:400, Abcam, Cat# ab135386), CD8 (1:150, Abcam, Cat# ab237710), CD103 (1:500, Abcam, Cat# ab129202), Granzyme B (1:200, Abcam, Cat# ab255598), IFN- $\gamma$  (1:50, proteintech, Cat# 15365-1-AP), CD56 (1:600, Abcam, Cat# ab220360), IgG (1:200, Abcam, Cat# ab181236), cleaved-caspase 3 (1:100, Abcam, Cat# Ab32042), CK-7 (1:1000, Abcam, Cat# ab181598), Ki-67 (1:600, Abcam, Cat# ab16667) followed by HRP-conjugated anti-rabbit IgG (ThermoFisher). For immunofluorescence staining, primary and secondary antibodies were incubated at 37°C for 2 hours. the sections staining was performed on an Alexa Fluor<sup>TM</sup> 488/555/647 Tyramide SuperBoost<sup>TM</sup> Kit (ThermoFisher). Positive cells were detected by confocal microscopy (Carl Zeiss LSM800). For cell counting in fluorescence-stained samples, we used QuPath, an open-source software designed for digital pathology image analysis. QuPath enabled us to perform automated cell counts across designated regions of interest, ensuring accuracy and reproducibility in the results. Specific fluorescent markers were optimized to minimize signal overlap, and cells were counted based on the presence of specific markers, ensuring precise identification and quantification across the designated regions of interest.

## The TCGA TCGA search protocol

Go to the TCGA homepage (tap me to enter) — Lunch Data Portal — Download Data — Data Matrix — Filter Settings: select Disease (BRCA - Breast Cancer), Data Type (RNA Seq), and Platform: genome-wide mRNA level (Illumina mRNA-seq).

## Statistical analysis

All data are presented as mean  $\pm$  SEM. Non-compliance with parameter test conditions necessitated the use of the Mann-Whitney U test for statistical analyses, which were performed using two-tailed tests. The parameter test conditions used Welch's ANOVA test. Kaplan-Meier overall survival and disease-free survival analyses of breast patients was performed using log rank. Correlation analyses were performed a Pearson, the two variables analyzed in these correlation analyses were normally distributed. Statistical analyses in the table I used a  $\chi^2$  test. P values < 0.05 are statistically significant. The Cox proportional hazards regression model was utilized to compute HR and 95% CI, with statistical significance set at P < 0.05. Two or three independent experiments was performed in the data. Statistical analyses were executed with R (version 4.0.1), Caseviewer 2.2, QUPATH 0.4.3, and GraphPad Prism 8.0.

## Result

### Clinicopathological characteristics of patients with breast cancer

A total of 105 postoperative primary breast cancer patients were retrospectively recruited for the study, with their initial characteristics

outlined in [Table 1](#); [Supplementary Table 1](#). All participants in the study were female, with a median diagnosis age of 50 years, ranging between 29 and 95 years. The number of tumors per patient ranged from 1 to 4, with the median being 1 tumor. Sizes of the tumors varied from 0.6 cm to 8 cm, with a median measurement of 2.5 cm. There were 76 tumors at stages I-II and 29 at stages IIIA-IIIC. In terms of menopausal status, there were 53 patients who were premenopausal and 52 who were postmenopausal.

## Identification of tertiary lymphoid structure

The initial evaluation of the presence and localization of TLS involved the examination of H&E-stained sections obtained from 105 patients with breast cancer at Huizhou Central Hospital. TLSs presented in many solid tumors and aggregated lymphocytes were considered as the typical structure of TLSs. From our H&E-staining of breast cancer, we observed the presence of multi-location in which TLS was found within tumor tissue (intratumor TLS, iTLS) or peritumoral region (peritumoral, pTLS) or distant normal tissue (para-tumoral TLS, para-TLS) ([Figure 1A](#)). To evaluate the TLS density, we quantified the numbers of TLSs in 5 random microscope bright light fields, and TLS density was calculated as the number of TLS per mm<sup>2</sup> in different regions of tumor tissue. The distribution of TLS density was presented in [Figure 1B](#). Our data showed that most TLSs located in the peritumor compared with those in intra-tumor and para-tumor in most patients with breast cancer ([Figure 1C](#)). We also examined the presence of iTLS relative to pTLS density and found a higher pTLS density in cases presence of iTLS ([Figure 1D](#)). Notably, the tumor tissue of relapsed or premenopausal breast cancer patients exhibited a low pTLS density. Still, we did not find any difference in pTLS density between different histological type ([Figure 1E](#)). Additionally, we also performed immunofluorescence staining (IF), found that TLS with a characteristic morphology of germinal centers was a hallmark of secondary follicle-like TLSs (SFL-TLS), containing mature CD21+ CD23+ follicular dendritic cells (FDC). Primary follicle-like TLSs (PFL-TLS) contained mature CD21 + FDC and lacking germinal centers (CD23-). Early TLSs (E-TLS) showed early lymphocytic aggregate without any apparent FDC networks or germinal centers ([Figure 1F](#)). In summary, we evaluated the TLS densities in breast cancer patients, and found that pTLS was the highest density among iTLS, pTLS and para-TLS.

## TLS density predicts survival of breast cancer

To investigate the prognostic effect of pTLS density on HCC patients, a threshold for separating patients with low and high pTLS densities was examined by using median of pTLS densities ([Figure 2A](#)). Kaplan-Meier survival analyses revealed that high pTLS density (> 0.0526 pTLS/mm<sup>2</sup>, n=54) significantly correlated with improved OS in 105 HCC patients from Huizhou Central Hospital ([Figure 2A](#)). Univariate analyses demonstrated a significant correlation between high pTLS density and longer OS (hazard ratio, 0.214; 95% CI, 0.047-0.981). When we grouped stage I

separately from stages II-III, we observed a significant relationship with OS (Figure 2B). The findings were confirmed by the validation cohort of BRCA TCGA data set. In tumors with high pTLS densities, the presence of TLS still correlated with better Over Survival and Disease-Free Survival of breast cancer patients, suggesting its association with the best prognostic outcomes (Figure 2C). Taken together, these data indicate that pTLSs is a favorable prognostic factor for breast cancer patients.

Peritumoral tertiary lymphoid structure was associated with CD103+CD8+ tissue-resident memory T cell infiltration

Tumor-infiltrating lymphocytes (TILs) represent a more robust antigen-experienced, antitumor immune response, we hypothesized that TLS may support the activation of TIL attack against tumor

cells. Immunohistochemistry using anti-CD103 antibody showed that CD103+ T cells were scattered around areas of lymphocyte aggregation as seen with anti-CD20 staining; these aggregates were considered TLSs (Figure 3A). Most CD8+ T cells had infiltrated not only into the intra-tumor, but also into the TLS of peritumor regions. Conversely, most CD103+ T cells were located within the border of TLS in peritumor regions and were considered tissue-resident memory T cells (Figure 3A). Immunofluorescence double staining for CD8 and CD103 showed that many CD103+ T cells express CD8 (Figure 3A). Interestingly, a significant increase in the percentage of CD103+ CD8+ T cluster was observed in the set of TLS-high group compared with TLS-low one (Figure 3B). These results indicated that CD103+ CD8+ Trm cells, one subtype of CD8+ T cells, infiltrated within TLS. It came to the same conclusion using the TCGA data set regarding breast cancer, tissue-resident memory T signature was related to TLS signature (Figure 3C). To examine the function of CD103+ CD8+ Trm cells, we analyzed the

TABLE 1 Associations between clinical factors in patients with breast cancer.

| Characteristics         | Low-mTLS (N=51) | High-mTLS (N=54) | Total (N=105) | P-value | FDR  |
|-------------------------|-----------------|------------------|---------------|---------|------|
| TNM stages              |                 |                  |               | 1       | 1    |
| I-II                    | 37 (35.24%)     | 39 (37.14%)      | 76 (72.38%)   |         |      |
| IIIA-IIIC               | 14 (13.33%)     | 15 (14.29%)      | 29 (27.62%)   |         |      |
| Age (median, 50)        |                 |                  |               | 0.02    | 0.24 |
| <50                     | 31 (29.52%)     | 19 (18.10%)      | 50 (47.62%)   |         |      |
| ≥50                     | 20 (19.05%)     | 35 (33.33%)      | 55 (52.38%)   |         |      |
| menopause               |                 |                  |               | 0.02    | 0.37 |
| Before                  | 32 (30.48%)     | 21 (20.00%)      | 53 (50.48%)   |         |      |
| After                   | 19 (18.10%)     | 33 (31.43%)      | 52 (49.52%)   |         |      |
| pathological type       |                 |                  |               | 1       | 1    |
| breast ductal carcinoma | 20 (19.05%)     | 21 (20.00%)      | 41 (39.05%)   |         |      |
| invasive breast cancer  | 31 (29.52%)     | 33 (31.43%)      | 64 (60.95%)   |         |      |
| Tumor number            |                 |                  |               | 0.2     | 1    |
| 1≤<1                    | 21 (20.00%)     | 30 (28.57%)      | 51 (48.57%)   |         |      |
| >1                      | 30 (28.57%)     | 24 (22.86%)      | 54 (51.43%)   |         |      |
| Size (cm)               |                 |                  |               | 0.38    | 1    |
| <2.5                    | 23 (21.90%)     | 30 (28.57%)      | 53 (50.48%)   |         |      |
| ≥2.5                    | 28 (26.67%)     | 24 (22.86%)      | 52 (49.52%)   |         |      |
| ER                      |                 |                  |               | 0.06    | 0.8  |
| positive                | 40 (38.10%)     | 32 (30.48%)      | 72 (68.57%)   |         |      |
| negative                | 11 (10.48%)     | 22 (20.95%)      | 33 (31.43%)   |         |      |
| PR                      |                 |                  |               | 0.1     | 1    |
| positive                | 38 (36.19%)     | 31 (29.52%)      | 69 (65.71%)   |         |      |
| negative                | 13 (12.38%)     | 23 (21.90%)      | 36 (34.29%)   |         |      |

(Continued)

TABLE 1 Continued

| Characteristics      | Low-mTLS<br>(N=51) | High-mTLS<br>(N=54) | Total (N=105) | <i>P</i> -value | FDR |
|----------------------|--------------------|---------------------|---------------|-----------------|-----|
| CerB2                |                    |                     |               | 0.53            | 1   |
| positive             | 37 (35.24%)        | 43 (40.95%)         | 80 (76.19%)   |                 |     |
| negative             | 14 (13.33%)        | 11 (10.48%)         | 25 (23.81%)   |                 |     |
| Her2                 |                    |                     |               | 0.44            | 1   |
| positive             | 5 (20.00%)         | 2 (8.00%)           | 7 (28.00%)    |                 |     |
| negative             | 8 (32.00%)         | 10 (40.00%)         | 18 (72.00%)   |                 |     |
| Ki67 (Median=40%)    |                    |                     |               | 0.15            | 1   |
| <40                  | 28 (26.67%)        | 21 (20.00%)         | 49 (46.67%)   |                 |     |
| >=40                 | 23 (21.90%)        | 33 (31.43%)         | 56 (53.33%)   |                 |     |
| sentinel node        |                    |                     |               | 1               | 1   |
| negative             | 33 (31.43%)        | 35 (33.33%)         | 68 (64.76%)   |                 |     |
| positive             | 18 (17.14%)        | 19 (18.10%)         | 37 (35.24%)   |                 |     |
| axillary lymph nodes |                    |                     |               | 0.67            | 1   |
| negative             | 36 (34.29%)        | 35 (33.33%)         | 71 (67.62%)   |                 |     |
| positive             | 15 (14.29%)        | 19 (18.10%)         | 34 (32.38%)   |                 |     |

Data were expressed as n (%) and median. TLS, Mature tertiary lymphoid structures. Statistical analyses the table used a  $\chi^2$  test. *P* values in bold are statistically significant.

difference in cytokine production by CD103+ CD8+ Trm cells from 8 tumors. And we found that most Granzyme B+ CD103+ CD8+ Trm cells infiltrating within TLS rather than non-TLS one in breast cancer (Figure 3D). Additionally, we conducted a comparison of the number of GZMB+ CD8+ Trm cells within TLS versus TLS outside areas. This analysis confirmed the presence of significantly more effector (GZMB+) CD8+ Trm cells within mature TLS compared to TLS outside regions (Figure 3E), indicating a strong correlation between the effector function of CD103+ CD8+ Trm and TLS. Kaplan-Meier survival analyses revealed that high The CD103+ CD8+ Trm cells signature significantly correlated with improved prognosis in patients (Figure 4A). Furthermore, The Kaplan–Meier curves indicated that patients with high TLS signature and CD103+ CD8+ Trm cells signature in breast cancer conferred significantly best prognosis than those with low TLS signature and CD103+ CD8+ Trm cells signature (Figure 4B).

Peritumoral tertiary lymphoid structure with multiple effector immune cells infiltration was associated enhancement of anti-tumor immunity in breast cancer

To unveil potential functions of TLS of breast cancer, we searched for signs of proliferation and apoptosis occurring in tumor cells, identified by their typical large nucleus and CK7+ phenotype, using an antibody that detects Ki-67 and cleaved caspase 3. Figure 5A illustrates labeling of cleaved caspase 3 in a TLS+. The density of cleaved caspase 3+ tumor cells was significantly higher in tumors around TLS.

Recent study has previously revealed that presence of intratumoral TLS correlated with multiple tumor-infiltrating immune cells. We also examined the tumor-infiltrating immune cells in breast cancer tissues by immunofluorescent staining (Figure 5B). In addition, we determined the association between pTLS and tumor-infiltrating immune cells. pTLS-high tumors were significantly associated with increased CD56+ NK cells which secreted effector molecule Granzyme B and IFN- $\gamma$  (Figure 5B). As Of note, a typical large nucleus tumor by conjugated anti-human IgG was observed as illustrated in a TLS+ tumor (Figure 5C). We addressed the question of the mechanisms that may be at work in inducing apoptosis of tumor cells. Since NK cells are the main effectors of antibody-dependent cellular cytotoxicity (ADCC), we evaluated the overall density of CD56+ NK cells on breast cancer tissues and found that tumors with both high numbers of apoptotic cells and a high percentages of IgG-stained tumor cells were more infiltrated by CD56+ NK than tumors with high numbers of apoptotic cells but low percentages of IgG-stained tumor cells (Figure 5C). Additionally, using the TCGA data set regarding breast cancer, we also found that TH1, effector T cell, effector memory T cell and central memory T cell signature were positively related to TLS signature (Figure 5D). Taken together, TLS with multiple effector immune cells infiltration was associated enhancement of anti-tumor immunity in breast cancer.

Discussion

The current study delves into the relationship between tertiary lymphoid structures (TLS) and their associated immune cells in

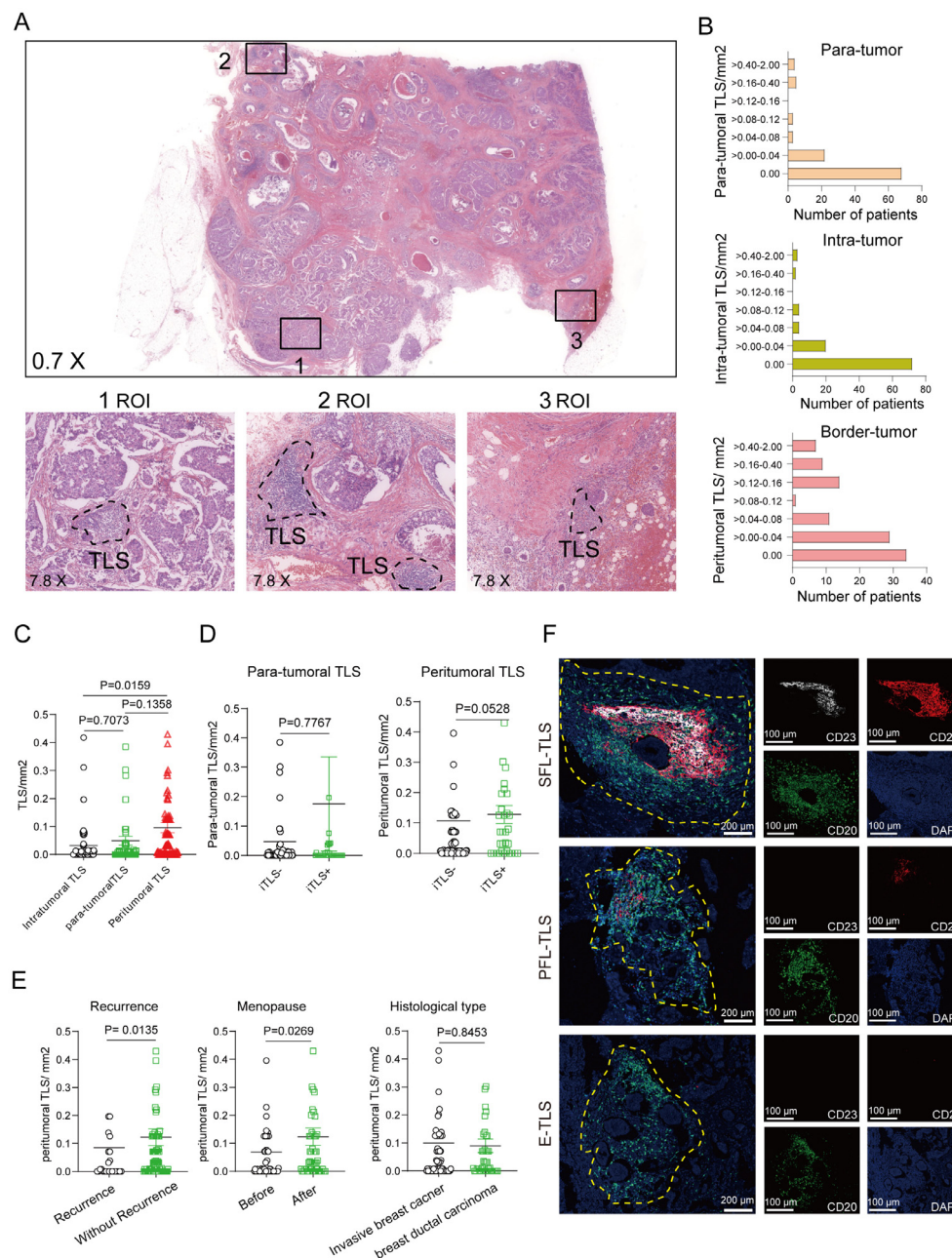


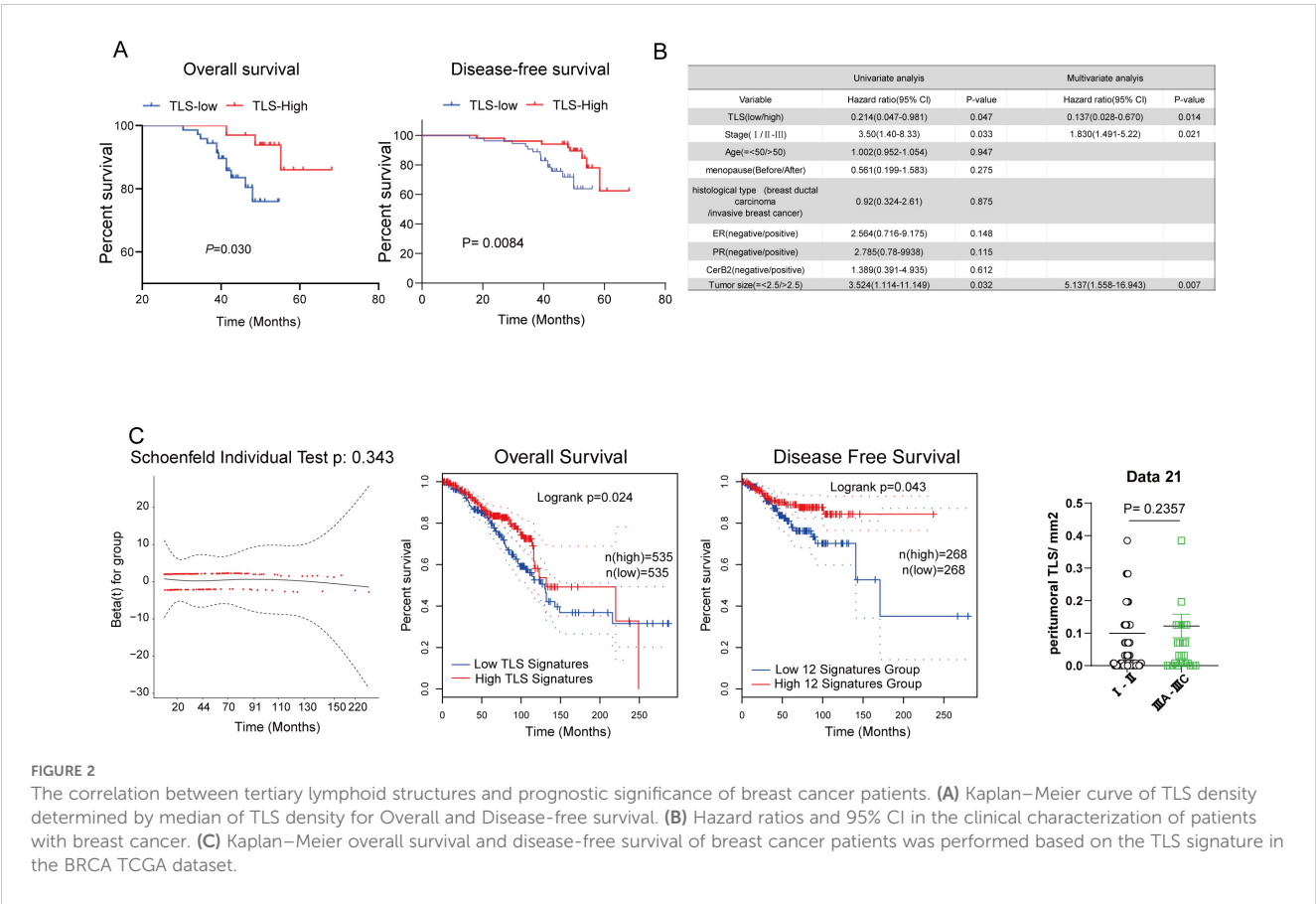
FIGURE 1

Detection and identification of tertiary lymphoid structures. Characterization of breast cancer-associated TLS. **(A)** Representative images of H&E-stained breast cancer tissue showing 3 regions of interest (ROI) in tumors. **(B, C)** The distribution of TLS density in intratumor, para-tumor and peritumor of breast cancer tissues was evaluated throughout the whole slide. **(D)** The density of pTLS was compared between breast cancer patients present or absent with iTLS. **(E)** The relationship between recurrence, Menopause, and Histological type. **(F)** the co-stained immunofluorescence of CD20+, CD21+, and CD23+. Data are presented as means  $\pm$  SEM. Significance was determined by Welch's ANOVA **(C)** two-tailed Mann-Whitney U **(D, E)**.

breast cancer, aiming to shed light on the mechanisms underlying their role in anti-tumor immunity. While prior research has acknowledged the importance of TLS in various solid tumors and their correlation with better prognoses, the precise mechanisms remained elusive (9, 16). Firstly, unlike previous studies that primarily focused on the presence or absence of TLS, our research delves deeper by assessing the maturity of TLS and its

correlation with key immune cells, particularly CD103+ CD8+ Trm and NK cells. Secondly, our study not only confirms the favorable prognosis associated with the presence of TLS in breast cancer patients but also introduces potential additional prognostic markers—CD103+ CD8+ Trm cells and NK cells within TLS. Finally, by demonstrating the enhanced effector function of CD103+ CD8+ Trm cells and NK cells within mature TLS, we establish a strong





**FIGURE 2** The correlation between tertiary lymphoid structures and prognostic significance of breast cancer patients. **(A)** Kaplan–Meier curve of TLS density determined by median of TLS density for Overall and Disease-free survival. **(B)** Hazard ratios and 95% CI in the clinical characterization of patients with breast cancer. **(C)** Kaplan–Meier overall survival and disease-free survival of breast cancer patients was performed based on the TLS signature in the BRCA TCGA dataset.

correlation between immune cell functionality and TLS, offering new insights into their collective role in anti-tumor immunity.

To the best of our knowledge, this study is the first large comprehensive report on the importance of CD103+ CD8+ Trm cell and TLS in breast cancer. We found that TLSs are mainly present in peritumoral carcinoma *in situ* in breast cancer. Although there is no standardized classification of TLS maturity, we used the classification method to classify the TLSs into three mature stages, including E-TLSs, PFL-TLSs, and SFL-TLSs. We found that TLS with a characteristic morphology of germinal centers was a hallmark of secondary follicle-like TLSs (SFL-TLS), containing mature CD21+ CD23+ follicular dendritic cells (FDC). Primary follicle-like TLS (PFL-TLS) contained mature CD21+ FDC and lacking germinal centers (CD23-). Early TLS (E-TLS) showed early lymphocytic aggregate without any apparent FDC networks or germinal centers. For the first time, we divided patients into two levels based on the mature state of TLS; 1) mature TLS: only E-TLS or no TLS with no PFL-TLS and SFL-TLS; 2) immature TLS: PFL-TLS and SFL-TLS in the tumor, and without E-TLSs. The results showed that patients in mature TLS had the best over survival than that in immature TLS. This was consistent with the findings in primary pancreatic adenocarcinoma, colorectal cancer and lung squamous cell carcinoma that found that patients with GC reaction had a better progression-free survival, and overall survival (15–17).

This indicates that mature TLS play pivotal roles in the anti-tumor immune response.

The recent successful treatment of immunotherapy in various solid and hematological tumors suggests that immunotherapy has a better antitumor effect, and cytotoxic CD8+ lymphocytes infiltrated in tumors have been widely studied and associated with improved survival in most patients (18, 19). Many studies have now found that there are also lymphocyte aggregates in the tumor microenvironment, which resemble secondary lymphoid structures called tertiary lymphoid structures (TLS); the presence of tertiary lymphoid structures has been closely associated with a good prognosis for patients in a variety of tumors (9, 10, 20). Our study also found that TLS in breast cancer can be associated with good prognosis. Prolonged exposure to inflammatory infiltration and modulation by cytokines and chemokines can promote the formation of tertiary lymphoid structures in peripheral tissues, and a large number of studies have found tertiary lymph node formation in tissues with autoimmune diseases, transplant rejection and chronic diseases (21). The vast majority of tumor-associated TLS have antitumor functions and are indicators of a good tumor prognosis. Several studies have pointed out the infiltration of TLS defined by HEV, CD4 or TFH cells, B-cells and mature DCs TH1 and cytotoxic T-cells are associated and promote antitumor effects (9). Numerous studies have confirmed the better antitumor effect of



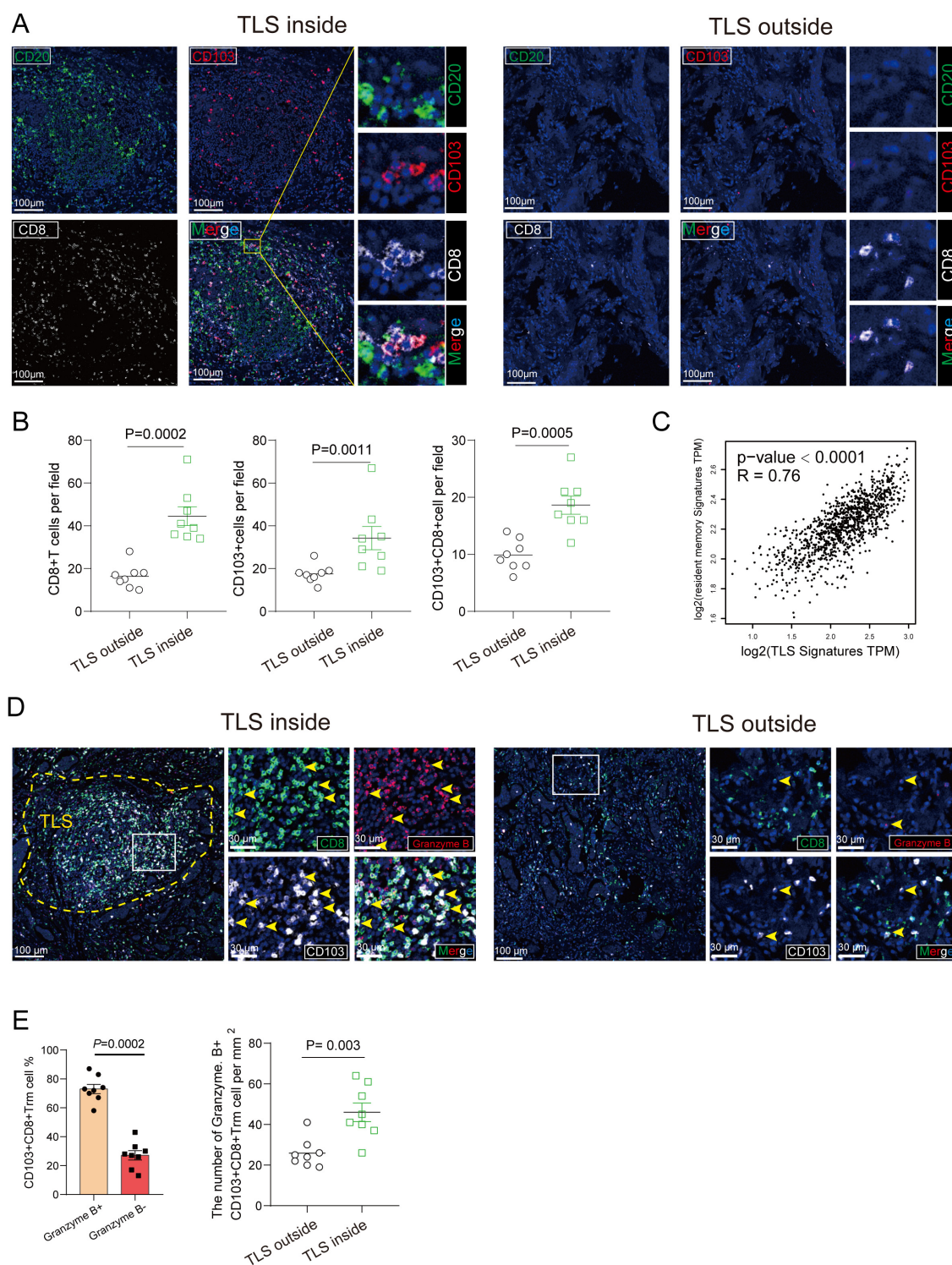


FIGURE 3

CD103+ CD8+ Trm cells distribution and its association with TLS. **(A)** The representative image of tumor section within mature TLS or TLS outside tissue from Breast cancer patients was stained by multiplex Immunofluorescence showing colocalization of CD103+ CD8+ Trm cells and CD20+ B cells. Scale bars, 100  $\mu\text{m}$ . **(B)** The number of CD103+ cells, CD8+ T cell and CD103+ CD8+ Trm cells per  $\text{mm}^2$  was showed inside and outside the TLS area. **(C)** The correlation between resident memory T cell signature and TLS signature. P values and R values were determined based on the analysis of Spearman's correlation. **(D)** Multiplex Immunofluorescence of Granzyme **(B)** CD103+ CD8+ Trm cells were shown. The representative multi-immunofluorescence of breast cancer tissue was stained with CD8 (green), CD103 (gray) and Granzyme B (red). Scale bar, 100  $\mu\text{m}$ . **(E)** The quantitation was shown. Data are presented as means  $\pm$  SEM. Significance was determined by two-tailed Mann-Whitney U **(B)**.

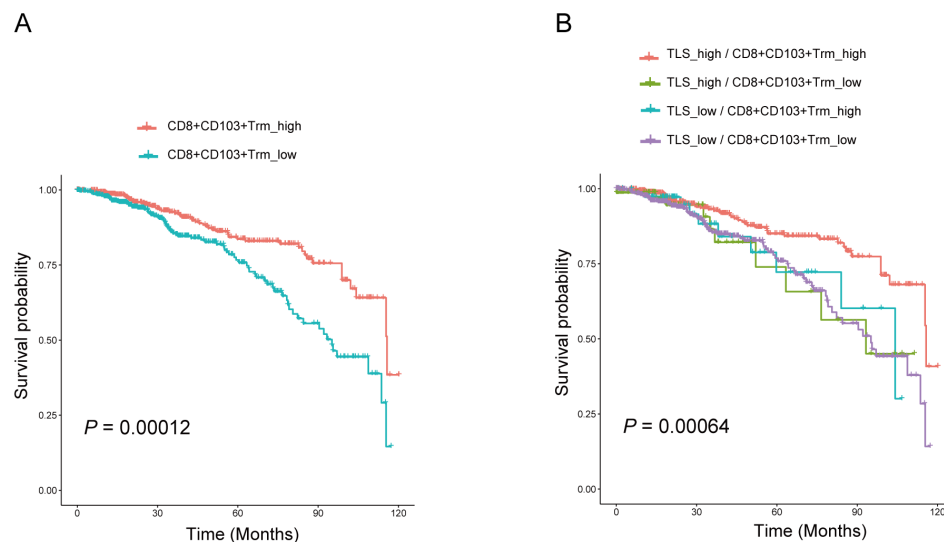


FIGURE 4

CD103+CD8+ Trm cells and TLS correlated with better prognosis in patients with breast cancer. (A) Kaplan–Meier overall survival and disease-free survival of breast cancer patients was performed based on the CD103+CD8+ Trm cells signature in the BRCA TCGA dataset. (B) Overall survival of patients with breast cancer based on CD103+CD8+ Trm cells signature and TLS signature according to the BRCA TCGA dataset. Analysis was performed using Kaplan–Meier.

tumor infiltrating CD8+ T cells, for this reason, researchers found an independent antitumor function of TLS by excluding the role of CD8+ T cells through multifactorial analysis (10). In TLS with low DC infiltration, it has a poor prognostic effect even though it contains a large number of CD8+ T cells (22, 23). Interestingly, we found that TLS in breast cancer was able to correlate with a good prognosis, which was closely associated with the density of CD103+CD8+ Trm cells and NK cell infiltration in TLS, and also found that CD103+CD8+ Trm cells and NK cells secreted a large number of effector molecules such as IFN- $\gamma$  and Granzyme B. Suggesting that the CD103+CD8+Trm cells and NK cells within TLS favor anti-tumor immune responses.

Although the exact mechanism by which tissue-resident memory T cells are preferentially localized within TLS remains to be fully elucidated, it has been reported that CXCL13 plays a pivotal role as the primary molecular driver behind the formation of TLS in the tumor microenvironment (TME) (15, 24, 25). Activated CD103+ cytotoxic T lymphocytes (CTLs) play a crucial role in the recruitment of B cells to the tumor microenvironment through the secretion of CXCL13. Tumors characterized by a high mutation load and rich in CD8+ T cells exhibit elevated levels of CXCL13 and CD103, correlating with significantly increased B cell presence across various tumor types. This enhanced B cell infiltration is particularly pronounced in tumors with abundant TLS, suggesting a vital role for activated CD103+ CTLs in mediating this process (25). A prior study examining the distribution of CD8+CD103+ Trm cells in gastric carcinoma revealed similar outcomes. CD103+ T cells were predominantly located around TLSs, and patients exhibiting a high expression of CD103 (CD103 High) displayed

an increased number of TLSs (33735485). Furthermore, tumor infiltrating CD103+Trm cell was prognostic factor for gastric cancer patients (26). However, this study mainly focused on the relationship between TLS maturity and CD103+CD8+ Trm in breast cancer. Importantly, CD103+CD8+ Trm cells in TLSs served as an independent prognosticator of breast cancer. Additionally, TLS and CD103+CD8+ Trm cells located within TLS were linked to enhanced overall survival rates in breast cancer patients. Immune cells are essential components of tumor environment, reflecting the immunogenicity of tumor, and they are important biomarkers for immunotherapy for patients with breast cancer (27, 28). It has been reported that in invasive breast cancer there is no difference in the distribution of immune cells between the intratumor and peritumor, 32 but in our study, peritumoral immune cells were more abundant than intratumor in breast cancer. Specifically, we found that CD103+CD8+ Trm cells and NK cells were more in the peritumor than in the peritumor. Previously studies have shown a decreasing trend of TILs in metastatic breast cancer compared to primary breast cancer (29, 30). Interestingly, we found that TLS in breast cancer was able to correlate with good prognosis, and the density of CD103+CD8+ Trm cells and NK cell infiltration in TLS were closely correlated. The combination of TLS with CD103+CD8+ Trm cells predicted a better good prognosis. This study investigated the relationship between infiltration of different immune subpopulations of TLS at different areas in breast cancer and patient prognosis. Future studies are still needed to determine the role of immune cells in breast cancer.

In conclusion, our data highlight that the proportion of CD103+CD8+ Trm cells within pTLSs was significantly increased with the



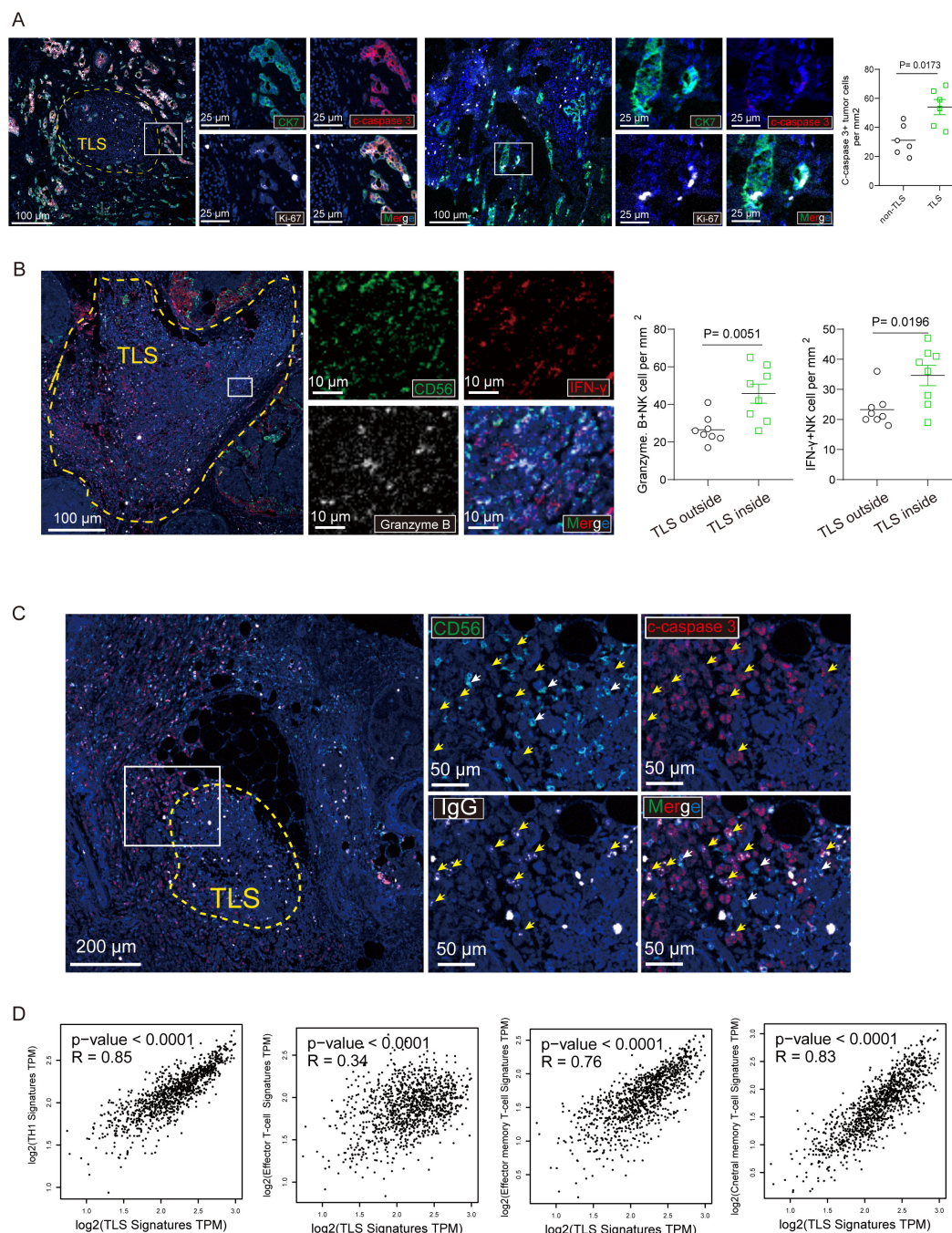


FIGURE 5

CD103+ CD8+ Trm cells and NK cells within mature TLS secreted effector molecules. **(A)** The representative immunostaining of breast cancer tissue with mature TLS was stained with CK7, cleaved caspase-3 (c- caspase-3) and Ki-67. Scale bar, 100  $\mu$ m. the number of c- caspase-3 tumor cells per mm<sup>2</sup> was shown. **(B)** The representative multi-immunofluorescence of breast cancer tissue with mature TLS was stained with CD56 (green), Granzyme B (gray) and IFN- $\gamma$  (red). Scale bar, 100  $\mu$ m. **(C)** The representative immunostaining of breast cancer tissue with TLS was stained with CD56, cleaved caspase-3 (c- caspase-3) and immunoglobulin G (IgG). Scale bar, 100  $\mu$ m. **(D)** The correlation between TLS signature and TH1 signature, Effector T-cell signature, Effector memory T-cell signature, central memory T-cell signature, respectively. P values and R values were determined based on the analysis of Spearman's correlation.

maturation of TLSs. TLS with multiple effector immune cell infiltration was associated with enhanced anti-tumor immunity in breast cancer. These results indicate a close relationship between CD103+ CD8+ Trm cell and pTLS maturity, suggesting a strong correlation between the effector function of CD103+ CD8+ Trm

and NK cells and the maturity of pTLSs. Furthermore, patients with a combination feature of TLS and CD103+ CD8+ Trm cells showed a good prognosis. The combination of pTLS maturity and CD103+ CD8+ Trm cells proportion could be used as a biomarker to predict the prognosis of breast cancer patients.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The studies involving humans were approved by Huizhou Central People's Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from gifted from another research group. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

## Author contributions

CW: Funding acquisition, Project administration, Resources, Writing – review & editing. SC: Data curation, Investigation, Visualization, Writing – original draft, Writing – review & editing. QF: Conceptualization, Methodology, Software, Writing – review & editing. XC: Formal Analysis, Methodology, Software, Supervision, Writing – review & editing. WZ: Formal Analysis, Validation, Writing – review & editing. DC: Validation, Visualization, Writing – review & editing. YH: Resources, Visualization, Writing – review & editing.

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## Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by project grants from Guangdong Association Study of Thoracic Oncology (GASTO-22-01-007).

## Conflict of interest

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2025.1480461/full#supplementary-material>

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RECEIVED 16 September 2024

ACCEPTED 23 December 2024

PUBLISHED 20 February 2025

## CITATION

Bai C, Xin X, Yang Y, Qu F and Fan Z (2025)  
Breast acinic cell carcinoma with weak  
progesterone receptor expression: a case  
report and literature review.  
*Front. Oncol.* 14:1497272.  
doi: 10.3389/fonc.2024.1497272

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# Breast acinic cell carcinoma with weak progesterone receptor expression: a case report and literature review

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**Rationale:** Acinic cell carcinoma (AcCC) of the breast is an extremely rare malignant epithelial tumor characterized by acini cell differentiation, clinical low-grade malignancy, and a molecular triple-negative subtype.

**Patient concern:** A 47-year-old female presented with a 1-month history of a painless mass in her right breast.

**Diagnosis:** Ultrasound imaging, mammography and magnetic resonance imaging revealed a lesion, approximately 3.0cmx1.5cm in size, in the right breast, which was considered to be a malignancy. After the surgery, the AcCC of the breast was confirmed histologically.

**Interventions:** Right breast mastectomy and sentinel lymph node biopsy were performed. Adjuvant chemotherapy included 4 cycles of doxorubicin hydrochloride (Adriamycin) and cyclophosphamide followed by 4 cycles of docetaxel (Taxotere).

**Outcomes:** The patient was discharged from the hospital after surgery. There was no sign of recurrence during a 9-month follow-up period.

**Lessons:** Acinic cell carcinoma (AcCC) of the breast is an extremely rare malignant epithelial tumor that can be accurately diagnosed based on histopathologic morphology and immunohistochemistry. The weak positive progesterone receptor (PR) expressed in this case is extremely rare, which may provide a new research direction for the endocrine therapy of AcCC. Both AcCC and microglandular adenosis(MGA) exhibit microglandular growth, and the relationship between them remains unclear. Differentiation between them not only relies on histomorphology and pathological immunohistochemistry but also depends on clinical manifestations and other presentations. Optimal treatment of AcCC is the same as that for invasive breast cancer. The prognosis is generally good, with adjuvant therapy after surgery.

## KEYWORDS

breast cancer, acinic cell carcinoma, progesterone receptor, adjuvant chemotherapy, microglandular adenosis

## Introduction

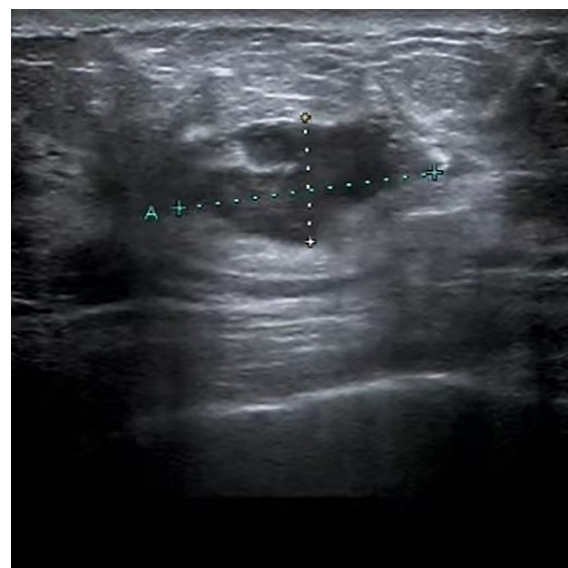
Breast cancer, is a heterogeneous disease, originating from the breast epithelial cells, and it falls under the category of adenocarcinomas. In the latest WHO classification, breast cancer is divided into 18 types, including invasive ductal carcinomas of no special type and 17 special types. Pathologists have identified specific structures and cytological patterns that align with certain clinical presentations and/or outcomes, termed “histological special types,” which account for 25% of all breast cancers, include micropapillary carcinoma, apocrine carcinoma, metaplastic carcinoma, medullary carcinoma, adenoid cystic carcinoma, secretory carcinoma, acinic cell carcinoma, and so on. Special types of breast cancer hold significant clinical importance due to their unique biological characteristics. However, there is a lack of standardized diagnostic criteria for these special types of breast cancer. Acinic Cell Carcinoma (AcCC) is an extremely rare special type of breast cancer characterized by acinic cell differentiation and low malignancy. Its molecular subtype is triple-negative (estrogen receptor-negative, progesterone receptor-negative, and Her-2 negative) predominantly, with immunohistochemical features often positive for S-100 and EMA, high expression of lysozyme, and stain positive for periodic acid-Schiff (PAS) with diastase (PAS-D). Here, we report the clinical case of a 47-year-old female patient with breast AcCC with weak progesterone receptor (PR) expression and review the relevant literature.

## Case information

A 47-year-old female presented with an accidental discovery of a broad bean-sized lump in her right breast in September 2021. The lump was painless, without nipple discharge or nipple retraction. She visited a local hospital and underwent a core needle biopsy, which suggested invasive carcinoma. She was then referred to our hospital on Nov 13, 2021, for further treatment. Physical examination: A round, hard mass approximately 3.0 cm × 1.5 cm in size was palpable in the upper inner quadrant of the right breast, with poorly defined borders, no orange peel-like changes, and no enlarged lymph nodes in the bilateral axilla and supraclavicular region. Imaging examination: Ultrasound of breast and axilla: A hypoechoic mass with a size of 25.4 mm × 12.5 mm was located near three o'clock position in the upper quadrant of the right breast, with unclear boundaries and irregular shape, with blood flow signals observed, indicative of a prompt right breast BI-RADS IVb tumor (Figure 1). Some lymph nodes in the right axilla had lost normal structure, with the largest 9.7 mm × 5.9 mm (Figure 2).

**Abbreviations:** AcCC, Acinic cell carcinoma; MRI, magnetic resonance imaging; DWI, Diffusion-weighted MRI; ADC, Antibody-drug conjugate; CT, computed tomography; ER, estrogen receptor; PR, progesterone receptor; Her-2, human epidermal growth factor receptor 2; CK, cytokeratin; GCDFF-15, gross cystic disease fluid protein 15; EMA, epithelial membrane antigen; Syn, synaptophysin; AR, androgen receptor; CgA, chromogranin A; TNBC, triple-negative breast cancer; MGA, microglandular adenosis; PAS, Periodic Acid-Schiff; PAS-D, periodic acid-Schiff with diastase; OFS, ovarian function suppression.

**Mammography:** The structure of the local glands in the upper quadrant of the right breast was slightly disordered with increased density BI-RADS 0; lymph node shadows were seen in the right axilla with increased density (Figure 3). Breast magnetic resonance imaging (MRI) plain scan + enhanced + diffusion: Irregular-shaped abnormal signal shadow in the upper quadrant of the right breast, approximately 2.7 × 2.2 cm, T1 fat-suppressed image showed iso-intensity, T2 fat-suppressed image showed slight hyper-intensity. Diffusion-weighted MRI (DWI) showed hyper-intensity. Antibody-drug conjugate (ADC) showed a low signal, lobulation, and burr at the edge of the lesion, with an uneven enhancement and a time-signal intensity curve exhibiting a rapid-rising type. The right breast tumor was classified as BI-RADS-MR V. With enhancement, a small lymph node shadow was seen in the right axilla of approximately 0.7 cm. Results did not rule out the possibility of axillary lymph node metastasis, and a right axillary lymph node biopsy was performed, and no cancer invasion was seen. There were no clear signs of metastasis on chest and abdomen computed tomography (CT) and whole-body bone imaging, with no surgical contraindications. On Nov 23, 2021, a right breast mastectomy and sentinel lymph node biopsy were performed. Two sentinel lymph nodes were evaluated during the operation. No cancer metastasis was found. Postoperative pathology: breast acinic cell carcinoma (Figure 4), volume 2.3 cm × 2.0 cm × 1.5 cm, with no cancer invasion in vessels, nerves, superficial fascia, or breast tissue in the remaining quadrant. Immunohistochemistry (Supplementary Figures 1, 2): PR (weak + 20%) (Supplementary Figure 3), ER (-) (Supplementary Figure 4), Her-2 (0) (Supplementary Figure 5), CK7 (+), GATA3 (+), GCDFF-15 (+), EMA (+), S-100 (+), CK5/6 (partly +), p63 (-), Ki-67 (+70%) (Supplementary Figure 6), Syn (-), CgA (-), AR (-), ECadherin (+) (Supplementary Figure 7), lysozyme



**FIGURE 1**  
Ultrasound image of the breast: A mass, measuring 25.4 mm by 12.5 mm, was identified in the upper quadrant of the right breast, near the three o'clock position. This mass has indistinct margins and an irregular contour.

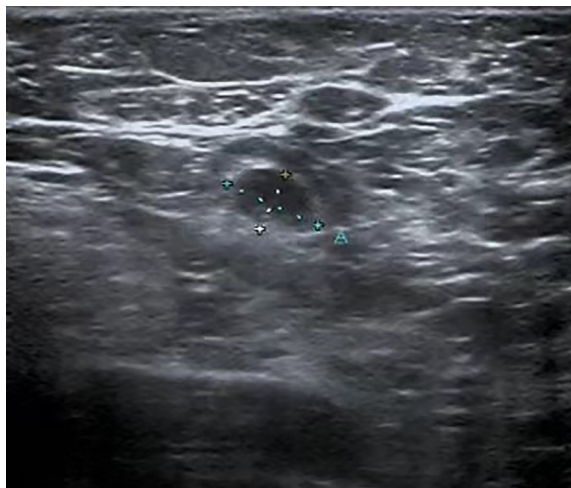


FIGURE 2

Ultrasound image of the axilla: The largest lymph node in the right axilla, measuring 9.7mm by 5.9mm, has lost its normal architecture.

(+) (Supplementary Figure 8), and special stain: D-PAS (+). Currently, the patient has successfully undergone eight cycles of AC-T adjuvant chemotherapy, 4 cycles of doxorubicin hydrochloride (Adriamycin) and cyclophosphamide followed by 4 cycles of docetaxel (Taxotere). Her condition remains stable, and there have been no indications of tumor recurrence observed during subsequent follow-ups.

## Discussion

Breast cancer is a heterogeneous disease, originating from malignant tumors of breast epithelial cells, and it belongs to adenocarcinomas. In the latest WHO classification, breast cancer is divided into 18 types, including invasive ductal carcinomas of no special type (IDC-NST) and 17 special types. Special types of breast cancer account for 25% of all breast cancers (1), include micropapillary carcinoma, apocrine carcinoma, metaplastic carcinoma, medullary carcinoma, adenoid cystic carcinoma, secretory carcinoma, acinic cell carcinoma, and so on (1, 2). Pathologists have identified that over 90% of tumor entities with recognized special type morphological characteristics are pure special types. Special types of breast cancer are of significant clinical importance due to their unique biological features. However, there is a lack of standardized diagnostic criteria for special types of breast cancer due to the scarcity of reports and low interobserver reproducibility (3).

AcCC is a rare malignant epithelial tumor that belongs to salivary gland-like carcinoma, which is a special type of invasive cancer (4, 5). AcCC is a low-grade malignant salivary gland epithelial tumor with serous acinar cell differentiation, accounting for 17% of salivary gland tumors (6). The parotid gland is the primary site of origin, with approximately 2%-3% of parotid tumors being primary adenoid cystic carcinomas (7). It was first breast AcCC reported by Roncaroli in 1996 (8), and most of the literature since then has been case reports. We found 52 case reports in

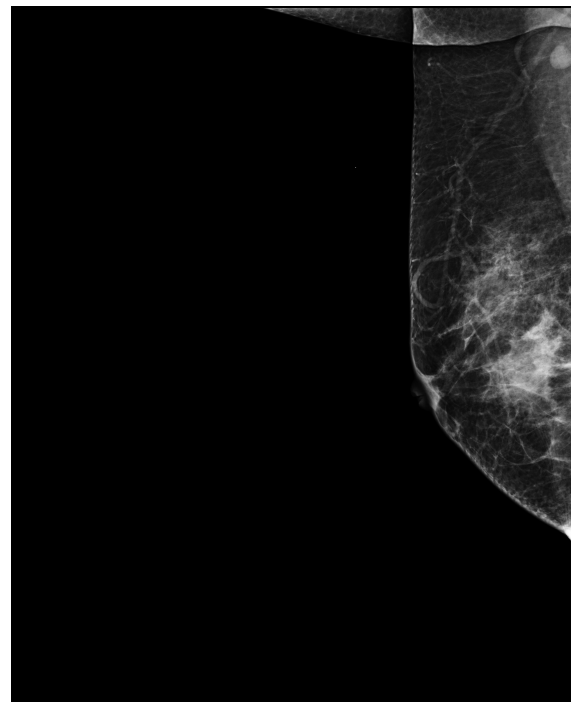


FIGURE 3

X-ray image of right breast mass and axillary lymph nodes: The structure of the local glands in the upper quadrant of the right breast was slightly disordered with increased density; lymph node shadows were seen in the right axilla with increased density.

English on PubMed, including the only male breast AcCC reported by Shimao et al. (9). According to a recent report, The mean patient age at diagnosis was 51 years (median age, 49; range, 23–80 years), with a higher incidence on the right breast, though no specific quadrant preference has been observed, as evidenced in this case (4). Physical examination and imaging showed Nodules with unclear borders, irregular morphology, and slightly hard texture (10, 11). In addition, axillary metastasis is rare in AcCC cases, and the pathological results of our patient did not show axillary

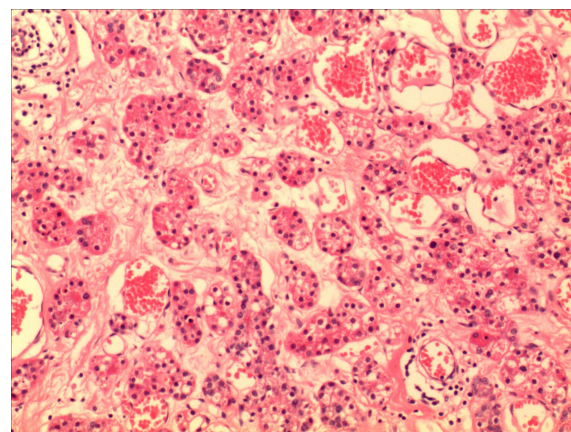


FIGURE 4

The pathological image shows acinar hyperplasia, with the acini being irregular and composed of multilayered cell arrangements.



metastasis (4, 12). There is no characteristic description of AcCC in the existing literature resembling those of breast cancer, with irregular morphology, poorly defined borders, visible blood flow signals, and nonhomogeneous enhancement in enhanced MR, so the accurate diagnosis relies on pathological examination.

Breast tissue shares histomorphology and functional similarities with most secretory glands, e.g., the salivary glands and the mammary glands are composed of tubular alveoli with the same histological structure (4). It is, therefore, not surprising that breast tumors present as salivary gland-like carcinomas, including acinic cell carcinoma, polymorphous adenocarcinoma, mucoepidermoid carcinoma, secretory carcinoma and adenoid cystic carcinoma (5, 13). Genomic abnormalities in some types of primary salivary gland tumors can also be detected in salivary gland-like primary tumors of the breast (e.g., polymorphous adenocarcinoma with HMGA2 or PLAG1 rearrangement; Secretory carcinoma with T (12; 15) fusion with ETV6-NTRK3; or adenoid cystic carcinoma with t(6; 9), resulting in MYB-NFIB fusion) (14). AcCC commonly occurs in salivary glands and resembles salivary gland acinic cell carcinoma morphologically when found in the breast. AcCC of the salivary glands exhibits a distinct morphological presentation, characterized by well-defined tumor margins, a prominent capsule, and a variety of morphological growth patterns (15). Typical breast AcCC shows adenoalveolar cell differentiation with cytoplasm enriched with enzyme-producing granules (4). The cytoplasm is mostly eosinophilic but can also be basophilic or a mixture of eosinophilic and basophilic (16). However, breast AcCC is still somewhat different from salivary gland AcCC, with smaller borders and less invasion compared to salivary gland AcCC (13, 17). Compared with salivary gland AcCC, breast AcCC is more similar to triple-negative breast cancer (TNBC) with no special types. For example, mutation of TP53, which is the most common mutation pattern in breast cancer, was found in 80% of AcCC cases, but this mutation was not detected in salivary gland AcCC (18); however, AcCC aggressiveness is far less than triple-negative breast cancer (4); in terms of clinical manifestations and morbidity are also quite different (5).

AcCC exhibits various growth patterns, among which the cells arranged in solid with or without microglandular growth pattern are predominant, and which is one of the characteristics of microglandular adenosis (MGA) of the breast (4). AcCC of the breast can show local morphological features similar to those of MGA; the presence of rare, rough, bright eosinophilic cytoplasmic granules in the breast epithelium is a characteristic feature of the tumor, mainly found in MGA lesions and AcCC (16). Therefore, the cytological and histological features are particularly important in the diagnosis of AcCC. MGA is a benign breast lesion, and 4 cases of breast AcCC have been reported to occur on the basis of MGA (10–13), while the real relationship between these two diseases remains unclear (16). Scholars hold diverse opinions on the relationship between them. With Rosen suggesting that breast AcCC is invasive carcinoma with acinic cell differentiation arising in microglandular adenosis (19), Geyer et al. pointed out that MGA and AcCC represent the low-grade spectrum of relatively inert TNBC lesions and share some common molecular features but that they are two distinct lesions (20); and Conlon N advocated that AcCC occurs in the

stroma of MGA, and the resection margin should be re-examined if MGA lesions are present when breast-conserving surgery is performed for AcCC (16). However, this phenomenon is not absolute in rare case reports, so the differentiation between the two depends not only on histomorphology and pathological immunohistochemistry but also on clinical and other presentations.

Detailed descriptions of immunohistochemical characterization of breast AcCC have been reported in some of the breast AcCC literature, and the classical immunohistochemical features of breast AcCC: high expression of S-100, EMA, lysozyme and positive for PAS (21). The basic manifestations of AcCC reported in the existing literature are triple negative. AcCC belongs to the triple-negative breast cancer (TNBC) subtype; the number of AcCC that do not show triple negative is less than about 10% (4, 16). S-100 and EMA positivity are the main markers for distinguishing AcCC from serous carcinoma; typical breast AcCC shows acinar cell differentiation and lysozyme-positive cytoplasm rich in enzyme-producing granules, which are positive to the special staining PAS and PAS-D (4). The testing for the myoepithelial marker p63 was negative, confirming the invasive nature of this tumor type (21). As with other epithelial tumors, AcCC was positive for broad-spectrum cytokeratins and low molecular weight cytokeratins (e.g., CK7 and CK18). As with no specific type of triple-negative breast cancer, high molecular weight cytokeratins (e.g., CK5/6) can also be detected. Proliferation rates, as measured by Ki-67, show a wide positive range (5–71%); however, the majority of studies have reported that AcCC exhibits low to moderate Ki67 expression (range 5–30%) (4). In addition, GATA3 and GCDFP-15, which are apocrine differentiation markers, are expressed in about 1/2 of the AcCC cases. Furthermore, there are cases showing mutations in CTNBN1 (encoding  $\beta$ -adhesin), but no mutations in CDH1 (encoding E-Cadherin) have been identified (22, 23). The case we report in this article shows a general agreement with the above description. The special point is that the progesterone receptor (PR) is weakly positive and shows positive for E-cadherin, though no genetic testing has been performed, and our case also showed 70% of ki-67 high expression. Due to the rarity of AcCC and the limited number of reported cases, there is a lack of clear diagnostic criteria for AcCC, both in salivary gland cancers and breast cancers. A meta-analysis has indicated that among 333 salivary gland AcCC patients, a DOG1 expression rate of 55% was observed, suggesting a strong correlation between the expression of this marker and the final diagnosis of AcCC. So DOG1 can serve as an immunohistochemical marker for salivary gland AcCC (24), and pathological evidence along with immunohistochemistry can provide a diagnostic basis for breast AcCC.

With advancements in molecular biology and immunohistochemistry, there is increasing evidence to support the presence of individuals with the ER-negative/PR-positive subtype, which represents a very small minority and is more commonly found in younger patients (<49 years of age). The prognosis of ER-negative/PR-positive is worse than that of ER-positive/PR-positive or ER-positive/PR-negative breast cancers and is superior to that of ER-negative/PR-negative breast cancers (16). 31% of ER-negative/PR-positive patients are ERBB2-positive, which is not significantly different from the ERBB2 status between ER-negative/PR-negative subtypes (25.3% vs 29.5%). Some researchers have also suggested that

ER-negative/PR positivity is due to inadequate tissue fixation or failure of immunohistochemical detection technology in ER-positive/PR-positive breast cancers (25–27). However, with the optimization of immunohistochemical technology, there has been a decrease in ER-negative/PR-positive breast cancers. Conlon also reported a case of ER-negative/PR-positive AcCC (16).

The PR-RANK-RANKL pathway is responsible for progesterone-mediated abnormal proliferation of breast epithelial cells (28). It has been shown that inhibition of RANKL directly leads to a reduction in early hormone-induced breast epithelial tumorigenesis (29). PR is involved in molecular subtypes and plays a decisive factor in therapeutic decisions. However, its complex role in different contexts needs to be specifically elucidated. The growth of breast cancer cells that express positive for ER and/or PR depends on estrogen and/or progesterone. The expression of ER and/or PR is a key determinant of whether patients can benefit from endocrine therapy targeting the effects of estrogen, such as selective ER modulators (SERMs, e.g., tamoxifen), aromatase inhibitors (letrozole, anastrozole, exemestane), and estrogen receptor downregulation (fulvestrant). The WHO defines luminal A breast cancer as hormone receptor (HR) positive, HER2 negative, with Ki-67 less than 14% and PR greater than 20%, while luminal B is defined as estrogen receptor (ER) positive, HER2 negative, with Ki-67 greater than 14% or PR less than 20%. PR is an independent prognostic factor for breast cancer, and even though clinically, PR-positive breast cancer consistently responds better to endocrine therapy than PR-negative breast cancer (30), the poor prognosis of PR-negative breast cancer may be related to endocrine resistance (28). However, PR expression is not yet predictive of the efficacy of endocrine drugs in clinical trials and may be related to the fact that the ER+/PR- genome has the same gene expression pattern as the ER+/PR+ and ER-/PR- genomes (28). Currently, the combined use of ovarian function suppression (OFS) with hormone receptor inhibitors has also led to breakthroughs in endocrine therapy. In the subgroup with PR expression of 20–49%, exemestane plus OFS showed a more favorable disease-free survival (DFS) compared to tamoxifen plus OFS, with HR = 0.43. Similar trends were observed in the PR < 20% and > 50% subgroups, with HRs of 0.59 and 0.75, respectively. Therefore, upgrading endocrine therapy or switching to alternative endocrine drugs may overcome endocrine resistance.

Generally speaking, TNBC is more aggressive and has a poorer prognosis compared to Her2-overexpressing and hormone receptor-positive breast cancers. Due to the diversity in tumor histological histology, TNBC encompasses a range of malignant tumors with varying clinical behaviors. Similarly, the lack of HER-2 expression is categorized into HER-2 low expression and ultra-low expression (31). Histologically distinct subtypes of TNBC, such as adenoid cystic carcinoma, medullary carcinoma, and apocrine carcinoma, tend to have a better prognosis than other types of invasive ductal carcinomas (32, 33). However, there are fewer reports on AcCC of the breast (2, 34). Recently, there have been reports of AcCC with poor prognosis, including cases with distant metastasis to the lungs, bones, liver, and even the peritoneum (1, 10). Among the 68 cases reported in the literature, four had local metastasis, six developed distant metastasis after adjuvant therapy, and three died due to tumor progression (4, 30). Given the

morphological variations within and between tumors (pure and mixed types) in the published studies, it is challenging to draw definitive conclusions about the prognosis of breast AcCC from the existing literature. Some experts believe that low-grade pure AcCC is a more indolent tumor type, similar to MGA esions, and is associated with an indolent biological behavior, thus less likely to benefit from aggressive adjuvant chemotherapy and has a better prognosis. In contrast, high-grade AcCC or AcCC mixed with other types of breast cancer may exhibit more aggressive behavior (35). However, due to the limited number of AcCC cases reported and the lack of observer reproducibility, further research is needed to identify specific high-risk factors for poor prognosis.

Treatment for AcCC is similar to that for invasive breast cancer, and surgery can be breast-conserving or mastectomy, based on tumor characteristics and patient preference. Most patients receive chemotherapy, and hormone receptor-positive cases undergo endocrine therapy. Some patients also received neoadjuvant therapy for breast cancer, but neoadjuvant therapy is not the first choice for AcCC treatment due to the relative inertia nature of AcCC and the relatively low ki-67, which makes neoadjuvant therapy ineffective (4). Curigliano's study showed that TNBC subtypes are highly sensitive to platinum salts and taxanes (36). This patient received AC-T adjuvant chemotherapy after surgery and completed all cycles with no signs of local recurrence or distant metastasis. Due to the rarity of AcCC, evidence for its molecular genomics is scarce, and targeted therapies against specific signaling pathway targets could be used in the future when sufficient data are available. The use of immunosuppressive agents could also serve as a reference for individualized treatment.

## Conclusion

AcCC of the breast is a rare malignant tumor of the breast; due to its rarity, there is still limited evidence on its molecular genomics, making pathological evidence and the immunohistochemical analysis extremely valuable in this disease. Accurate diagnosis of AcCC can be made on the basis of pathohistological morphology and especially immunohistochemical expression. In most cases, high expression of S-100, EMA, lysozyme, and positive for PAS is observed, and most of the cases showed triple negative. The weakly positive expression of (PR) in this case is extremely rare and is accompanied by positive E-cadherin expression. The cases in this article provide new evidence for the field of endocrine therapy of AcCC and are beneficial to its development. E-Cadherin, encoded by CDH1, suggests that it may serve as a novel signal in the research field of AcCC, providing a new target for targeted therapy. Unfortunately, no genetic testing was performed in this case. Both AcCC and MGA exhibit microglandular growth patterns, but the relationship between them remains unclear. The expression of cohesin and type IV collagen was different, with MGA positive, while the AcCC was the opposite. However, in rare cases, this phenomenon is not absolute, so the differentiation depends not only on histomorphology and pathological immunohistochemistry but also on clinical presentation and other manifestations. The treatment is the same as invasive breast cancer, and the prognosis is generally good, with further adjuvant treatment after surgery. With the gradual development of the



breast cancer endocrine field, endocrine drugs can also be added according to the expression of PR.

## Data availability statement

All data generated or analyzed during this study are included in this published article.

## Ethics statement

Ethical approval was obtained from the First Hospital of Jilin University Ethics Committee. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

## Author contributions

CB: Validation, Writing – original draft, Writing – review & editing. XX: Visualization, Writing – review & editing. YY: Formal analysis, Investigation, Writing – review & editing. FQ: Resources, Supervision, Validation, Writing – review & editing. ZF: Supervision, Validation, Visualization, Writing – review & editing.

## Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The study was funded by the Doctor of Excellence Program (DEP) and the First Hospital of Jilin University (JDYY-DEP-2023019).

## Acknowledgments

The authors are grateful to the surgical department of the First Hospital of Jilin University for providing the patient's data.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2024.1497272/full#supplementary-material>

### SUPPLEMENTARY FIGURE 1

Breast acinic cell carcinoma pathology (HE \* 400).

### SUPPLEMENTARY FIGURE 2

Breast acinic cell carcinoma pathology (HE \* 400).

### SUPPLEMENTARY FIGURE 3

Immunohistochemistry: Weak PR receptor expression.

### SUPPLEMENTARY FIGURE 4

Immunohistochemistry: ER is negative.

### SUPPLEMENTARY FIGURE 5

Immunohistochemistry: Her-2 is negative.

### SUPPLEMENTARY FIGURE 6

Immunohistochemistry: Ki-67 expressed 70%.

### SUPPLEMENTARY FIGURE 7

Immunohistochemistry: E-Cadherin is positive.

### SUPPLEMENTARY FIGURE 8

Lysozyme is positive, which is a marker of serous differentiation.

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RECEIVED 13 September 2024

ACCEPTED 19 May 2025

PUBLISHED 11 June 2025

## CITATION

Qi X, Zhang M, Zhu J and Wang H (2025)  
Case report of concurrent ulcerative colitis  
and bilateral breast cancer: a literature review  
of the bidirectional association between  
autoimmune diseases and breast cancer.  
*Front. Oncol.* 15:1495731.  
doi: 10.3389/fonc.2025.1495731

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# Case report of concurrent ulcerative colitis and bilateral breast cancer: a literature review of the bidirectional association between autoimmune diseases and breast cancer

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A 58-year-old female patient with bilateral breast cancer developed unexpected hematochezia at a frequency of approximately 10 episodes per day following adjuvant chemotherapy, with the emergency endoscopy reporting superficial ulcers throughout the entire colon, suggesting a diagnosis of ulcerative colitis (UC). Given the absence of preexisting autoimmune history or detectable autoantibodies, we supposed that the onset of UC was closely related to the chemotherapy. The complex bidirectional relationship between autoimmune rheumatic diseases and cancer continues to be elucidated. Variations in autoimmune disease type, duration, and specific clinical/laboratory features may modulate cancer risk, either increasing or decreasing susceptibility to certain malignancies. These associations could potentially inform type-specific cancer screening strategies. Furthermore, the widespread use of immune checkpoint inhibitors across multiple tumor types, along with their associated inflammatory syndromes, has significant implications for the development and management of autoimmune rheumatic diseases. Herein, we report this case, which could be one of the few bilateral breast cancer cases to be reported with ulcerative colitis, and conducted a literature review of the bidirectional association of breast cancer and autoimmune diseases.

## KEYWORDS

ulcerative colitis, bilateral breast cancer, adjuvant chemotherapy, target therapy, autoimmune disease

Introduction

Ulcerative colitis (UC), one of the two primary forms of inflammatory bowel disease (IBD), is characterized by mucosal inflammation extending continuously from the rectum to the proximal colon (1). While the exact etiology of IBD remains incompletely understood, current evidence suggests a multifactorial pathogenesis involving molecular and cellular mechanisms, microbial factors, microbiome interactions, genetic predisposition, and immune dysregulation (2). Similar to other chronic inflammatory conditions, UC is associated with an elevated risk of malignancy. Notably, colitis-associated cancer (CAC)—colorectal cancer being the most prevalent—demonstrates risk stratification based on factors including age at diagnosis, disease duration, and disease severity (3). Given the elevated cancer risk associated with autoimmune diseases (AIDs), there has been growing attention on the long-term management of patients with pre-existing AIDs (4). Recent studies have yielded divergent findings regarding the cancer risks of different AIDs, with some conditions, such as rheumatoid arthritis (RA), potentially conferring a reduced risk rather than an increased one (5). Using breast cancer as an example, recent research has revealed that the impact of autoimmune disorders on breast cancer development extends far beyond the initially recognized association with thyroid disease, particularly the involvement of both anti-thyroid peroxidase (anti-TPO) and anti-thyroglobulin (anti-TG) antibodies (6, 7). Furthermore, mounting evidence confirms a bidirectional interplay between AIDs and cancer, wherein certain malignancies may significantly alter susceptibility to subsequent autoimmune conditions. This relationship is exemplified by a nationwide population-based cohort study (Chen et al.), which revealed that breast cancer patients showed markedly reduced incidence rates of systemic lupus erythematosus (SLE), RA, and Sjögren’s syndrome (SS) compared to matched controls (8, 9).

Case presentation

A 58-year-old female patient underwent sequential radical mastectomies, first on the right breast (20 September 2023) and then followed by the left breast (8 November 2023). Histopathological analysis revealed the following: Right breast: invasive ductal carcinoma (Estrogen receptor (ER) >90%,

Progesterone receptor (PR) 60%, Human epidermal growth factor receptor 2 (Her2) 1+, Ki-67 10-20%), pathological stage pT2N0Mx; Left breast: predominantly ductal carcinoma *in situ* with focal invasive ductal carcinoma (ER <1%, PR <1%, HER2 3+, Ki-67 40%), pathological stage pT1bN0Mx (Table 1). The patient initiated the AC-THP adjuvant chemotherapy regimen, completing four cycles (cyclophosphamide 0.8g d1 + doxorubicin liposome 50mg d1, Q3W). During the fifth cycle (trastuzumab 560mg d0 + pertuzumab 840mg d0 + albumin-bound paclitaxel 400mg d1), she developed acute hematochezia (approximately 10 episodes per day). Emergency endoscopy demonstrated diffuse superficial colonic ulcers (Figure 1), though a biopsy was refused by the patient due to bleeding concerns. Given the result of negative stool cultures and *C. difficile* toxin assays, the absence of serum autoimmune antibodies (Table 2), and the clinical improvement following glucocorticoid and mesalazine therapy, combined with the endoscopic findings, the patient was diagnosed with UC. Follow-up endoscopy (January 2, 2025) showed complete ulcer resolution (Figure 2). The complete treatment timeline summarizing the main events is shown in Figure 3.

Discussion

Bilateral breast cancer represents a distinct and relatively uncommon form of malignant breast neoplasia, characterized by

TABLE 1 Details in postoperative histo-pathological information.

| Pathological description and immunohistochemistry | Right breast              | Left breast                                                            |
|---------------------------------------------------|---------------------------|------------------------------------------------------------------------|
| Pathological type                                 | Invasive ductal carcinoma | ductal carcinoma in situ, with some areas of invasive ductal carcinoma |
| Histological grade                                | grade II                  | grade II-III                                                           |
| Lymphocyte infiltration                           | <5%                       | 30%                                                                    |
| Vascular tumor emboli                             | +                         | –                                                                      |
| ER                                                | >90%                      | <1%                                                                    |
| PR                                                | 60%                       | <1%                                                                    |
| Her-2                                             | +                         | +++                                                                    |
| Ki-67                                             | 10%-20%                   | 40%                                                                    |
| P53                                               | 5%                        | 80%                                                                    |
| EGFR                                              | <1%                       | 10%                                                                    |
| CK5/6                                             | <1%                       | <1%                                                                    |
| AR                                                | >90%                      | 70%                                                                    |
| Lymph nodes                                       | 0/19                      | 0/11                                                                   |
| Surgical margin                                   | Negative                  | Negative                                                               |
| Pathological staging                              | pT2N0Mx                   | pT1bN0Mx                                                               |
| Remarks                                           |                           | Paget's disease of the nipple                                          |

ER, Estrogen receptor; PR, Progesterone receptor; Her2, Human epidermal growth factor receptor 2; EGFR, Epidermal growth factor receptor; AR, Androgen receptor.

**Abbreviations:** UC, Ulcerative colitis; IBD, Inflammatory bowel disease; CAC, Colitis-associated cancer; AIDs, Autoimmune diseases; AI, Aromatase inhibitors; TPO, Thyroid peroxidase; TG, Thyroglobulin; SBBC, Synchronous bilateral breast cancer; ICIs, Immune checkpoint inhibitors; MMP-1, Matrix metalloproteinase-1; TNF- $\alpha$ , Tumor necrosis factor- $\alpha$ ; TGF- $\beta$ , Tumor growth factor - $\beta$ ; NIS, Sodium/iodine symphathetic apparatus; TR $\alpha$ 2, Thyroid hormone receptor  $\alpha$ 2; BRCA1, Breast cancer 1; RA, Rheumatoid arthritis; DMARDs, Disease-modifying antirheumatic drugs; CXCR-4, Chemokine receptor -4; PAR-1, Protease-activated receptor-1; SDF-1, Stromal cell-derived factor-1; SLE, Systemic lupus erythematosus; SS, Systemic sclerosis; pSS, Primary Sjögren’s syndrome; T1DM, Type-1 diabetes mellitus; PsO, Psoriasis; MS, Multiple sclerosis.



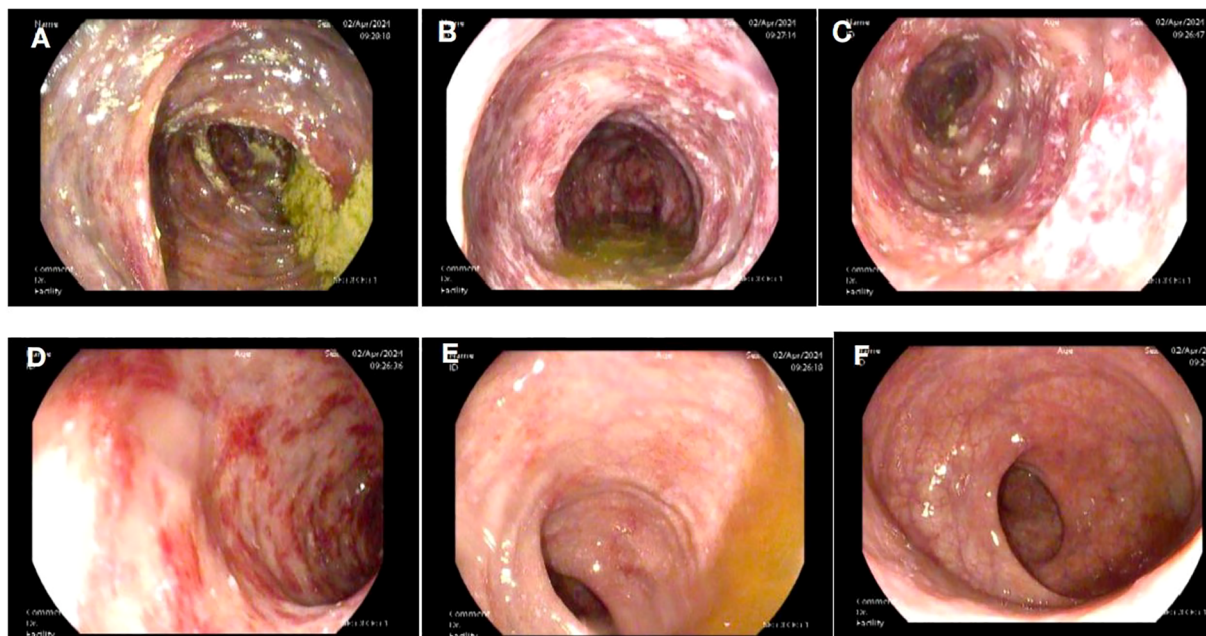


FIGURE 1

Superficial ulcers involving the whole colon. Superficial ulcers in the ascending colon (A), transverse colon (B), descending colon (C), and sigmoid colon (D). No obvious ulcers in the rectosigmoid junction (E) and rectum (F).

independent primary tumors originating from the ductal and/or lobular epithelium in both breasts, either synchronously or metachronously. Since Kilgore's initial description in 1921 (10), reported cases have progressively increased, with synchronous bilateral breast cancer (SBBC) constituting  $\leq 5\%$  of all breast malignancies (11). UC, an idiopathic chronic inflammatory disorder of the colonic mucosa (with potential pancolonic involvement), typically manifests with relapsing-remitting hematochezia (12). We herein present a rare case of synchronous bilateral breast cancer complicated by UC. The mechanisms underlying CAC involve multiple molecular pathways that may include genetic mutations, such as TP53 gene alterations; inflammatory signaling, such as TLR4-MyD88-dependent NF- $\kappa$ B activation, which perpetuates a pro-inflammatory microenvironment; the prostaglandin pathway, such as COX-2-mediated PGE2 production, promoting angiogenesis and immune modulation via PI3K signaling; metabolic reprogramming, such as PI3K/AKT activation by cytokines that facilitates GLUT4 translocation, enhancing TH2 and TH17 lymphocyte metabolism; genetic regulation, such as modifications in key regulatory genes (IL23R and rs10889677 [c.\*309C>A]) of the PI3K/AKT signaling pathway. These molecular alterations interact with immune components (macrophages, neutrophils, and T-regulatory cells) and cellular processes (autophagy), ultimately driving CAC development (13).

Similarities in the pathologies of autoimmune diseases and cancers can be traced back at least 30 years. Autoimmune diseases result from the breakdown of peripheral immune tolerance, specifically, the disruption of the delicate balance between immune tolerance and immune activation. This

equilibrium is normally maintained by specialized cell subsets, such as regulatory T cells (Tregs) and B cells (Bregs), macrophages, fibroblasts, and myeloid-derived suppressor cells (MDSCs) (14). When this balance is disturbed, it leads to pathological immune overactivation. While the recruitment of tolerogenic immune cell subsets and evasion of immune surveillance are recognized as the hallmarks of cancer, often termed "cancer tolerance", malignant cells achieve this through multiple mechanisms. These include the expression of immune checkpoint proteins, impaired antigen presentation, epithelial-to-mesenchymal transition (EMT), and dysregulated RNA editing. A very important part of this complex network is the microbiome, as both the microbiome and its metabolites can exert pro-tumor function, and a gut microbiota imbalance can cause gastrointestinal cancers or IBD (15). Inflammatory cytokines and growth factors mediate cell proliferation, and proteinases, especially the collagenase, matrix metalloproteinase-1 (MMP-1), contribute to disease progression by remodeling the extracellular matrix and modulating the microenvironment (16). Chronic inflammation caused by autoimmune diseases or the anti-rheumatic therapies and oncogenic virus infections, such as human papillomavirus, hepatitis B and C viruses, and Epstein-Barr virus, which are difficult to treat, may contribute to the development and progression of cancer (8). Autoimmunity-induced chronic inflammation and tissue damage may produce inflammatory mediators, such as TNF- $\alpha$ , interleukin (IL)-6, tumor growth factor (TGF)- $\beta$ , and IL-10, leading to the failure of Treg-mediated immune tolerance and stimulating the development of malignant tumors through various mechanisms such as DNA damage, inactivation of tumor-suppressor genes, triggering of angiogenesis, and enhancing invasiveness (17).



TABLE 2 Indicators of autoimmune antibodies.

| Antibodies                                            | Abbreviation | Index | Normal range |
|-------------------------------------------------------|--------------|-------|--------------|
| Thyroid antibodies                                    |              |       |              |
| Thyroid globulin antibodies                           | TG           | (-)   | (-)          |
| Thyroid peroxidase antibodies                         | TPO          | (-)   | (-)          |
| ANCA                                                  |              |       |              |
| Anti-neutrophil cytoplasmic antibodies                | ANCA         | (-)   | (-)          |
| Anti-nuclear antibodies (ANAs)                        |              |       |              |
| Anti-nuclear ribonucleoprotein/Smith antibodies       | nRNP/Sm      | (-)   | (-)          |
| Anti-Smith antibodies                                 | Sm           | (-)   | (-)          |
| Anti-Sjögren's syndrome-A antibodies                  | SS-A         | (-)   | (-)          |
| Anti-Sjögren's syndrome-B antibodies                  | SS-B         | (-)   | (-)          |
| Anti-Ro-52 antibodies                                 | KRO          | (-)   | (-)          |
| Anti-Jo-1 antibodies                                  | Jo-1         | (-)   | (-)          |
| Anti-Scleroderma-70 antibodies                        | scl-70       | (-)   | (-)          |
| Anti-PM-Scl antibody                                  | PM-scl       | (-)   | (-)          |
| Anti-centromere antibodies                            | CENPB        | (-)   | (-)          |
| Anti-proliferating cell nuclear antigen antibodies    | PCNA         | (-)   | (-)          |
| Anti-double-stranded deoxyribonucleic acid antibodies | dsDNA        | (-)   | (-)          |
| Anti-nucleosome antibodies                            | Nucleosome   | (-)   | (-)          |
| Anti-histone antibodies                               | Histones     | (-)   | (-)          |
| Anti-ribosomal P protein antibodies                   | ARPA         | (-)   | (-)          |

Conversely, anti-tumor immune responses may cross-react with self-tissues, leading to the development of autoimmunity (6). Previous studies indicate that patients with pre-existing autoimmune diseases often exhibit shortened survival, primarily attributable to the direct or indirect immune-related adverse events (irAEs) induced by anti-tumor therapies such as chemotherapy, radiation therapy, and, notably, the accumulated application of immune checkpoint inhibitor in the management of advanced tumors (18), leading to the flare-up or onset of autoimmune diseases (8, 19, 20). Numerous chemotherapeutic agents have been implicated in the development of autoimmune-like manifestations, especially bleomycin, gemcitabine, carboplatin, and paclitaxel have been associated with scleroderma-like syndromes and Raynaud's phenomenon (21). Aromatase inhibitors (AIs), a mainstay of hormone receptor-positive breast cancer treatment, frequently induce arthralgias (22). Radiotherapy targeting the head and neck regions can induce xerostomia that

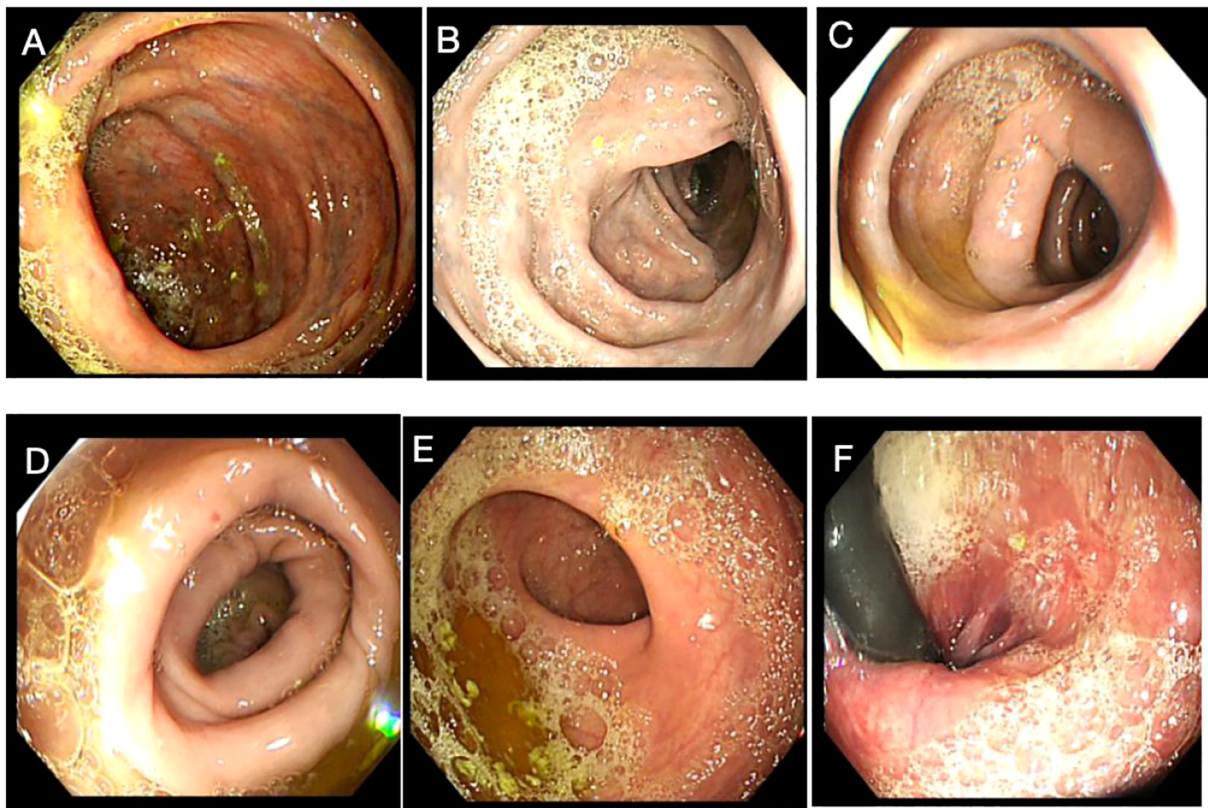
clinically resembles primary Sjögren's syndrome. Emerging evidence suggests that approximately 50% of breast cancer patients may develop radiation-induced cutaneous fibrosis at treatment sites, though current studies remain limited by small cohort sizes and methodological constraints (23). In patients with SLE, low levels of transforming growth factor-beta 1 (TGF-β1) correlate with increased disease severity. Therefore, radiotherapy-induced TGF-β release may have therapeutic potential in SLE by compensating for this deficiency (24). ICIs induce uncontrolled T-cell activation, resulting in systemic inflammatory syndromes that may involve multiple organ systems. These irAEs can manifest as autoimmune-like pathologies affecting the skin, endocrine, gastrointestinal, cardiovascular, pulmonary, and neurological systems (25, 26). The patient in this case report received multiple chemotherapy, targeted, and endocrine therapies. While treatment-induced colitis has not been previously reported with these specific regimens, the potential for immune-related adverse events, including autoimmune-like manifestations such as colitis, cannot be ruled out.

### Autoimmune thyroid diseases

The association between breast cancer and thyroid autoimmunity has attracted sustained research interest owing to the shared biological characteristics of thyroid and mammary gland tissues. A previous meta-analysis (27) demonstrated an elevated breast cancer risk among patients with autoimmune thyroiditis, particularly in those with anti-thyroid peroxidase (anti-TPO) and anti-thyroglobulin (anti-TG) antibodies. This association may stem from shared iodine-concentrating capacity via the sodium/iodide symporter (NIS) in both epithelial tissues or the abundance of TSH receptor-rich adipose tissue in breast parenchyma (28). While NIS protein expression is too limited to serve as a primary autoantigen, current evidence highlights the following two key antigenic links: thyroid peroxidase and its mammary homolog lactoperoxidase (both expressed in breast tissue) (29) and thyroid hormone receptor α 2 (TRα2), which modulates breast cancer signaling pathways. Notably, TRα2 overexpression correlates with improved survival outcomes in patients with breast cancer and is particularly upregulated in BRCA1-associated breast cancers, serving as a positive prognostic marker for both 5-year and overall survival. Additional mechanistic insights include the presence of estrogen receptors in abnormal thyroid tissue, potentially explaining thyroid dysfunction in breast cancer patients and shared endocrine stimuli affecting both organs (30, 31). These findings suggest diagnostic and therapeutic opportunities through targeting thyroid-breast antigenic connections (6).

### Rheumatoid arthritis

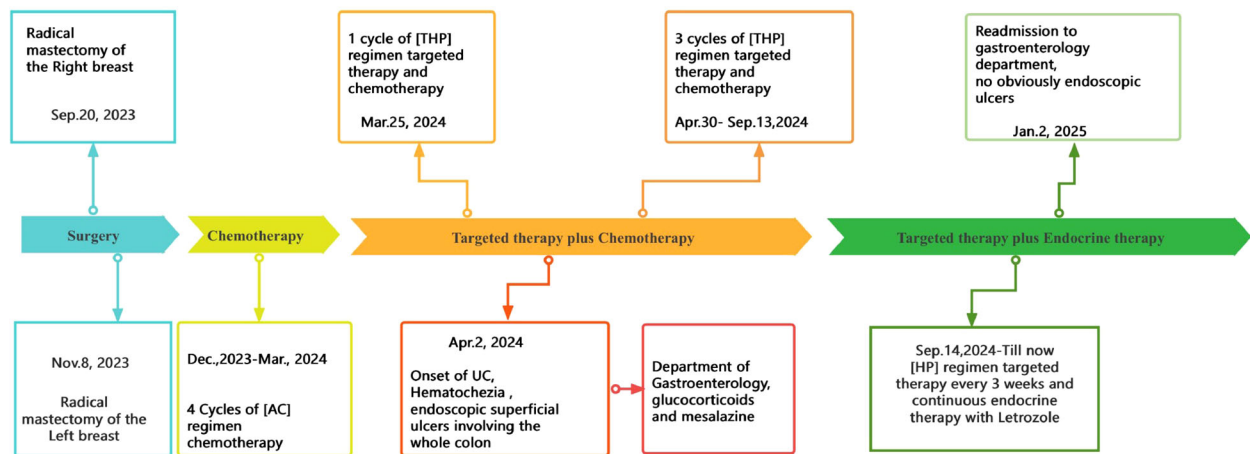
RA has been associated with an increased risk of hematological and solid malignancies for many years, even more common during



**FIGURE 2**  
Endoscopic findings upon remission of the colitis. No obvious ulcers were detected in the ascending colon (A), transverse colon (B), descending colon (C), sigmoid colon (D), rectosigmoid junction (E), and rectum (F).

treatment with disease-modifying anti-rheumatic drugs (DMARDs) and biotherapy (32). However, in recent years, multiple studies have demonstrated that RA does not uniformly elevate cancer risk, but rather exhibits tumor-specific and sex-dependent variations in malignancy incidence. Patients with RA have an increased risk of non-Hodgkin lymphoma, but a lower risk

of gastric cancer (33). Men have a reduced risk of rectal and renal malignancies, while women have a reduced risk of gastric and rectal cancers, and a reduced risk of hepatic malignancies is found in both men and women (34). Regarding hematological tumors, the risk of lymphoma was significantly higher in RA patients of both sexes, but the incidence of leukemia was significantly lower in women (35).



**FIGURE 3**  
Complete timeline summarizing the main events of the patient's treatment.

Epidemiological studies demonstrate a bidirectional reduction in disease risk between RA and breast cancer. Women with RA show decreased breast cancer incidence, while breast cancer survivors exhibit similarly reduced RA risk (5, 36). Notably, adjuvant antihormonal therapies (tamoxifen or aromatase inhibitors) do not increase RA risk compared to other breast cancer treatments, a finding replicated in Chinese populations (7). The MMP-1 system reveals important pathophysiological connections between these conditions. Initially characterized for its collagenolytic activity, MMP-1 now demonstrates pleiotropic functions through interactions with Chemokine receptor CXCR-4 and protease-activated receptor-1 (PAR-1). These interactions establish critical signaling networks. The MMP-1/CXCR4 axis modulates activated fibroblast behavior in both RA and cancer. The MMP-1/PAR-1 autocrine/paracrine loop amplifies extracellular matrix remodeling and cellular responses. Emerging therapies targeting MMP-1 and associated G protein-coupled receptors highlight shared pathogenic mechanisms between autoimmunity and oncogenesis (10). While more specific than previous agents, these therapies underscore the fundamental biological parallels between RA and certain malignancies.

## Systemic lupus erythematosus

The association between SLE and breast cancer remains controversial. While early studies suggested a protective effect of SLE against breast cancer, more recent investigations have yielded conflicting results. A Brazilian cohort study (n=100) reported elevated breast and cervical cancer incidence in patients with SLE vs. the general population (37). Large international studies demonstrated reduced breast cancer risk in patients with SLE (38, 39). Age-adjusted analyses show comparable or slightly increased breast cancer risk in patients with SLE vs. controls (40). A multicenter international study (n=16,409) found no SLE-specific factors explaining cancer risk variations (41). Longitudinal data indicate that age is the primary breast cancer risk factor in patients with SLE (41). A Mendelian randomization analysis revealed population-specific effects, with a significant risk reduction in East Asian cohorts (OR 0.85, 95% CI 0.78-0.93) but no association in European populations (42). A five-gene SLE prognostic signature (RACGAP1, HMMR, TTK, TOP2A, and KIF15) effectively stratified breast cancer survival risk (43). These contradictory findings highlight the need for more large prospective cohort studies, population-specific risk assessments, and mechanistic investigations into SLE-related immunomodulatory effects.

## Other autoimmune diseases

A study based on a Spanish population showed an increased overall cancer risk in patients with systemic sclerosis (SSc) compared with the general population. A high risk in lung, breast cancer, and hematological malignancies was also reported, with the

presence of anticentromere antibodies an independent favorable factor for decreasing cancer risk (44). A similar outcome was reported in a large-scale cohort study conducted in China (4). For primary Sjögren's syndrome (pSS), there could be geographical differences in its association with breast cancer risk; patients with pSS in European countries exhibited a lower risk of breast cancer, different from Asia and Argentina (44). Dedousis et al. conducted a survival analysis that found that there was a higher prevalence of rheumatoid arthritis, Crohn's disease, ulcerative colitis, and systemic lupus erythematosus in patients with breast cancer compared to age-matched cohorts in the general population. The presence of an autoimmune diagnosis was associated with a lower overall survival (OS) in stages I–III breast cancer and improved OS in patients with stage IV disease, suggesting that anti-tumor immunity plays an important role in advanced breast cancer and could potentially be exploited to improve the effectiveness of immunotherapy (45). High breast cancer risk was also found in type 1 diabetes mellitus (T1DM) and psoriasis (Pso) (5, 46), whereas breast cancer patients with multiple sclerosis suffered a modest progression during breast cancer treatment (47). It is generally accepted that during the 3 to 5 years before and after the diagnosis, patients with inflammatory myopathies have the highest risk of developing certain types of cancers, with adenocarcinoma being the most common histological type, among which the breasts could be a susceptible site (48). In autoimmune retinopathy, anti-TULP1 AABs disrupt protein translocation to the outer segments and are involved in photoreceptor degeneration in a manner similar to the degeneration induced by mutations in the TULP1 gene. The detection of anti-TULP1 AABs in patients with breast cancer suggests that anti-TULP1 AABs have the potential to be a biomarker for cancer-associated autoimmune retinopathy (49).

## Conclusion

Breast cancer and autoimmune diseases represent two prevalent conditions that threaten women's health, with growing evidence suggesting their complex bidirectional relationship. This case highlights the co-occurrence of breast cancer and UC, offering several important insights. While colorectal cancer remains the predominant malignancy associated with UC, this case suggests the potential oncogenic effects of UC beyond the colon. The patient's diverse therapeutic regimens (chemotherapy, targeted agents, and endocrine therapy) may have contributed to UC development, consistent with established evidence linking anticancer treatments and autoimmune-like manifestations. However, there is also a limitation as the exact causal relationship between breast cancer and UC in this case remains undetermined. This also calls for clinical vigilance regarding autoimmune manifestations in cancer patients and the requirement for large-cohort studies to elucidate the disease-disease interactions



and treatment-related autoimmune effects to provide guidelines for oncologists, immunologists, and gastroenterologists to optimize care for patients with overlapping cancer and autoimmune diseases.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## Author contributions

QX: Writing – original draft. MZ: Writing – original draft. JZ: Writing – original draft. HW: Conceptualization, Writing – review & editing.

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## Funding

The author(s) declare that financial support was received for the research and/or publication of this article. The work was funded by the National Natural Science Foundation of China (Grant No. 82070206); Tianjin Key Medical Discipline (Specialty) Construction Project (Grant No. TJYXZDXK-053B); Tianjin Health Research Project (Grant No. TJWJ2023MS013).

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