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Epithelial-to-Mesenchymal Transition in Breast Cancer: A Multi-Level Study Integrating Patient Tissues, Sera, and Cellular Systems

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ABSTRACT

Background: Epithelial-to-mesenchymal transition (EMT) is a key process in breast cancer (BC) progression, contributing to tumor plasticity, invasion, and therapeutic resistance. Increasing evidence suggests that EMT does not occur as a binary switch but rather as a spectrum of intermediate or hybrid states influenced by tumor-microenvironment interactions.

Methods: EMT-associated epithelial and mesenchymal markers were analyzed using western blot in breast cancer tissues and paired adjacent normal samples to assess expression patterns and heterogeneity. Associations with tumor microenvironment (TME) components were investigated using the TIMER database. The functional effects of patient-derived sera from primary and metastatic breast cancer patients, as well as from healthy donors, were evaluated in malignant (MDA-MB-231) and non-transformed (MCF10A) mammary epithelial cells using proliferation and migration assays. EMT-markers and EMT regulation were further examined and three-dimensional tumorsphere cultures using quantitative PCR (qPCR). To capture EMT complexity at a broader scale, bioinformatic analyses integrating several databases were performed. Finally, metalloprotease activity was assessed in BC tissues and sera by gelatin zymography.

Results: Breast cancer tissues displayed marked high heterogeneity, with unexpected positive correlations between epithelial and mesenchymal markers, supporting the existence of hybrid EMT states. EMT-related markers were frequently expressed at comparable or higher levels in adjacent normal tissues, highlighting the non-exclusive association of EMT features with malignant compartments. Breast cancer patient-derived sera promoted proliferation and migration in cellular models, whereas healthy sera exerted suppressive effects. In 3D tumorspheres, EMT regulation reflected a highly plastic hybrid state characterized by reduced proliferation, partial epithelial features, activation of EMT transcription factors, increased stemness, and extracellular matrix remodeling. Moreover, the 3D system showed greater sensitivity to serum-induced effects compared with 2D cultures. Bioinformatic integration identified a stringent breast cancer-specific EMT subset of 37 genes with both diagnostic and prognostic relevance, significantly associated with aggressive clinicopathological features. Finally, zymography analyses revealed widespread gelatinolytic activity, with circulating pro-MMP-2 emerging as a potential indicator of disease progression.

Conclusions: Collectively, these findings demonstrate that EMT in breast cancer is a dynamic, context-dependent, and highly plastic process shaped by microenvironmental interactions, and systemic circulating factors. EMT-related features cannot be fully captured by individual markers alone, highlighting the need for integrative experimental and bioinformatic approaches to better reflect EMT complexity and its clinical relevance.

INTRODUCTION

Cancer and carcinogenesis

Despite decades of research, cancer remains a leading cause of morbidity and mortality worldwide, causing approximately one in every six deaths globally, according to the WHO (World Health Organization, 2025), which defines cancer as “a large group of diseases that can start in almost any organ or tissue of the body when abnormal cells grow uncontrollably, go beyond their usual boundaries to invade adjoining parts of the body and/or spread to other organs”.

It's well known that tumorigenesis is driven by the progressive accumulation of genetic and epigenetic anomalies that deregulate cellular homeostasis, including proliferation, apoptosis, differentiation, and DNA repair.

The biological and clinical behavior of cancer cells depends on multiple factors, including the tissue of origin, tumor microenvironment, and interactions with the immune system, so that cancer is a highly heterogeneous disease. Recent advances in -omics, immunotherapy, and precision medicine have significantly improved the understanding of tumor biology and led to the development of novel therapeutic strategies. Nevertheless, many challenges remain, particularly in treatment resistance and disease recurrence.

Classical multistep model of carcinogenesis

Despite the cancer heterogeneity, carcinogenesis is usually divided into three main and sequential phases: initiation, promotion, and progression (**Figure 1**).

Initiation is a rapid and irreversible process involving the accumulation of mutations in key genes, termed proto-oncogenes and tumor suppressors. Proto-oncogenes normally encode for growth factors (e.g., Platelet-Derived Growth Factor: PDGF) (Witsch et al., 2010), growth factor receptors (e.g., Human Epidermal Growth Factor Receptor 2: HER2) (Slamon et al., 1987), transcription factors (e.g., C-myc) (Miller et al., 2012), or enzymes that promote cell division and survival (BRAF) (Davies et al., 2002). Mutations or overexpressions of proto-oncogenes induce aberrant cell proliferation. On the other hand, tumor suppressor genes act as brakes, encoding proteins that slow down cell division (e.g., Tumor protein p53) (Harris, 1996) and DNA damage repair (e.g., Breast Cancer gene 1 and Breast Cancer gene 2: BRCA1 and BRCA2) (Khalizieva et al., 2024). Under specific conditions, they can also trigger cell death (e.g., Phosphatase and tensin homolog: PTEN) (Goberdhan & Wilson, 2003). When tumor suppressor genes are lost or inactivated, cells can escape normal control and grow aberrantly.

Gene mutations can occur from inherited genetic conditions or be triggered by chemical, physical, or viral carcinogens (e.g., DNA viruses such as HPV and HBV, or RNA viruses like EBV). If not repaired efficiently, these alterations are fixed after cell replication. Consequently, the cell becomes “initiated”, even if it is still morphologically indistinguishable from a normal cell.

Promotion represents a slow, reversible process during which initiated cells receive a proliferative stimulus, generating different clones with a high proliferative rate and susceptibility to further mutations. These cells may lead to preneoplastic changes, such as hyperplasia, dysplasia, or metaplasia, which may regress, remain unchanged, or, if additional mutations drive malignant transformation, progress to cancerous lesions.

Progression, the third stage of carcinogenesis, involves the acquisition by tumor cells of a motile, aggressive, and invasive phenotype, allowing them to degrade the basement membrane, migrate, and disseminate in distant organs (Scott et al., 1984).

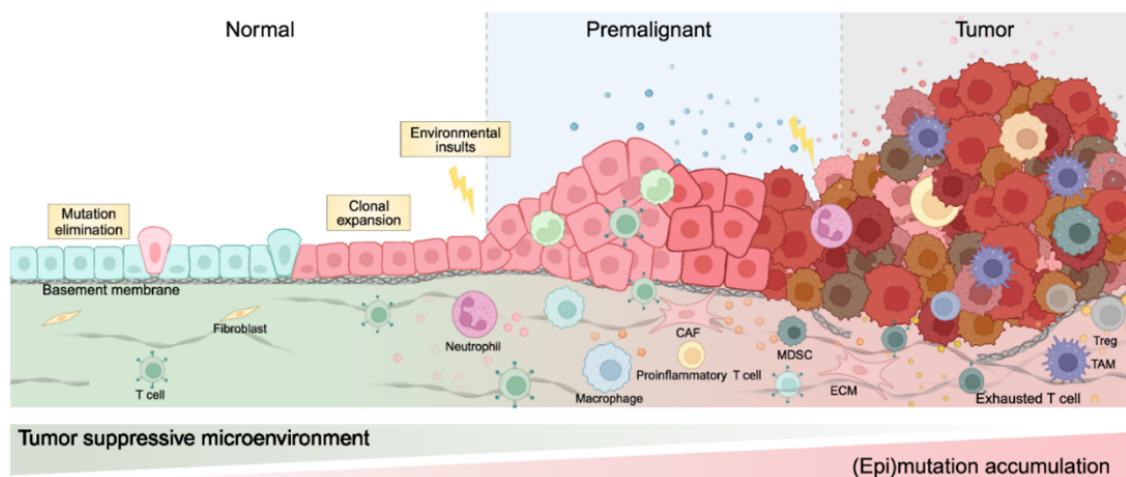


Figure 1. Multistage tumorigenesis model - (S. Zhang et al., 2024)

Cancer stem cell niche model of carcinogenesis

An additional mechanism of carcinogenesis has been proposed by Barcellos-Hoff et al., who introduced the concept of a so-called “cancer niche”, an early and necessary step required for initiated cells to evolve into cancer. According to this model, cancer development depends not only on genetic alterations within epithelial cells but also on the progressive construction of a permissive microenvironment (**Figure 2**). Indeed, the cell’s microenvironment can promote cancer growth and malignancy. The main differences observed in the tumor microenvironment (TME) embrace the lack of efficient immunosurveillance, which enhances a pro-inflammatory environment. Moreover, a

gradual stiffening of the extracellular matrix (ECM) and the transformation of fibroblasts into Cancer-Associated Fibroblasts (CAFs), support cancer progression. In fact, CAFs play a key role in cancer malignancy by continuously synthesizing and remodeling the ECM, leading to its deposition around the tumor. This phenomenon, called desmoplasia, creates a physical barrier around the cancer cells, contributing to their drug resistance (D. Yang et al., 2023).

Based on Barcellos-Hoff model, the cancer niche is initially established (precancer niche) when biological or chemical promoters recruit or induce host stromal cells to interact with genetically initiated cells. This hypothesis is supported by the observation that stromal tissue from women with dense breast tissue shows a loss of CD36 expression and a gene expression profile closely resembling that observed in breast cancer patients (DeFilippis et al., 2012).

Following the initial interaction between stromal, normal, and initiated cells, the second step consists of the niche expansion, which corresponds to the progressive remodeling of the microenvironment through the production of secreted factors, such as cytokines, chemokines, and exosomes, that support the clonal expansion of initiated cells. Concurrently, the activation of oncogenic signaling pathways further amplifies niche expansion, promoting inflammatory cells' recruitment and stromal reprogramming.

The third and final step of this model is niche maturation, which is required for cancer progression. This step involves systemic changes occurring in bone marrow, cellular activation and mobilization in response to tumor growth. Although the molecular mechanisms are not yet understood, several candidates have been proposed, including Tumor Growth Factor- β (TGF- β), stromal cell-derived factor 1 (SDF-1), and tumor-derived exosomes. In particular, TGF- β appears to play a central role by driving the conversion of mesenchymal stem cells (MSCs) into cancer-associated fibroblasts (CAFs), which, in turn, promote tumor progression.

Moreover, since cancer development increases significantly with age, Barcellos-Hoff et al. propose that aging itself may promote niche construction and expansion, due to the decline in immune and inflammatory regulatory systems, but also because senescent cells are characterized by a pro-inflammatory secretory profile. According to this model, randomly initiated cells may remain dormant until age-related systemic inflammation reaches a threshold sufficient to support niche establishment needed for cancer progression (Barcellos-Hoff et al., 2013).

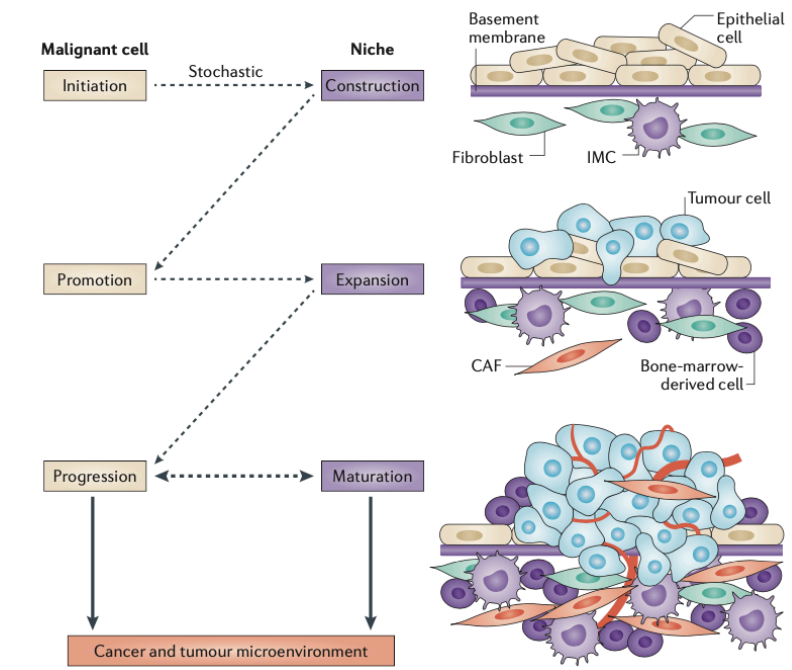


Figure 2. Classic and alternative multistep carcinogenesis model - (Barcellos-Hoff et al., 2013)

The hallmarks of cancer

To simplify the process of understanding cancer, in its nature as a heterogeneous and complex disease, the first six characteristics common to all tumors were identified and described as the “Hallmarks of Cancer” in 2000 (**Figure 3**) and are listed below.

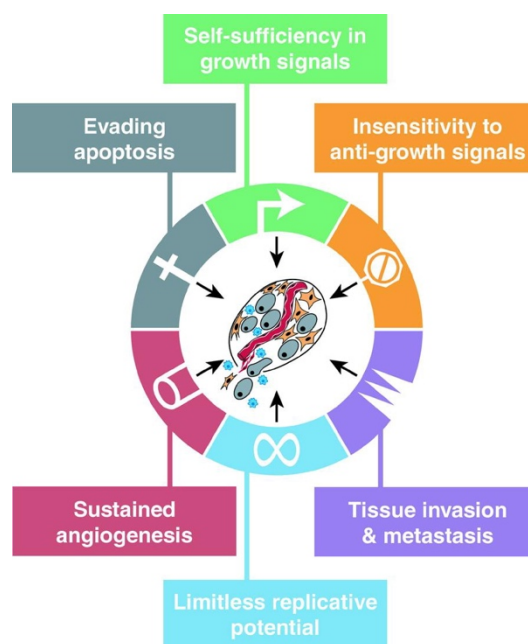


Figure 3. 2000s six Hallmarks of Cancer - (Hanahan & Weinberg, 2000)

This set of biological capabilities collectively sustains tumor initiation, progression, and therapeutic resistance. One of the earliest described hallmarks is self-sufficiency in growth signals, whereby cancer cells proliferate independently of normal regulatory cues. This is achieved through oncogenic mutations that activate proliferative signaling pathways and through the autocrine or paracrine production of growth factors, such as Platelet-Derived Growth Factor (PDGF) in glioblastomas and TGF- α in sarcomas (Anzano et al., 1983; Lokker et al., 2002). In addition, tumor cells frequently overexpress growth factor receptors, including EGFR or HER2, further amplifying mitogenic signaling, particularly in breast and gastric cancers (Slamon et al., 1987; Uribe et al., 2021).

Another critical hallmark is insensitivity to anti-growth signals, which typically constrain cell proliferation and preserve tissue homeostasis. Tumor cells evade these controls primarily by interacting with normal tumor suppressor pathways, as in the case of the retinoblastoma (pRb) pathway. Loss of pRb function results in uncontrolled E2Fs' activity, a family of transcription factors essential for regulating the genes required for progression from G1 to S phase (Weinberg, 1995). Moreover, the antiproliferative effects of TGF- β , which normally inhibit cyclin-CDK activity (Datto et al., 1997; Hannon & Beach, 1994) and suppress C-MYC expression, are frequently abolished in cancer due to downregulation or mutation of TGF- β receptors, leading to unchecked proliferation (Kim et al., 2023; Massagué & Sheppard, 2023).

Tumor progression also requires the acquisition of limitless replicative potential. While normal somatic cells undergo replicative senescence due to progressive telomere shortening, cancer cells bypass this limitation by reactivating telomerase. Telomerase, particularly its catalytic subunit Telomerase Reverse Transcriptase (TERT), maintains telomere length and enables indefinite cell division. Although TERT expression is primarily restricted to germ, stem, and immune cells (Cong et al., 2002), it becomes aberrantly activated in approximately 85% of human cancers, representing a key mechanism underlying cellular immortality (Akincilar et al., 2016).

Evasion of apoptosis enables tumor cells to survive despite genomic instability, oncogenic stress, and therapeutic insults. Apoptotic pathways are commonly disrupted through alterations in both intrinsic and extrinsic mechanisms, including mutations in caspases, overexpression of anti-apoptotic BCL-2 family members, loss of pro-apoptotic proteins such as BAX and BAK, and impairment of death receptor signaling (Birkinshaw & Czabotar, 2017; Guicciardi & Gores, 2009; Lopez & Tait, 2015). Tumor cells often overexpress various inhibitors that block both intrinsic and extrinsic apoptotic pathways, contributing to resistance to cancer therapies. This resistance is generally driven by increased expression of anti-apoptotic BCL-2 family proteins and reduced levels of pro-apoptotic proteins, which together enable cancer cells to evade the effects of chemotherapy and radiation (Tian

et al., 2024). Moreover, resistance to apoptosis can depend on mutations in the TP53 tumor suppressor gene, which is able to affect both intrinsic and extrinsic apoptosis pathways. p53 is inactivated in approximately 50% of human cancers and in nearly all cultured tumor cell lines (Tuval et al., 2024).

Sustained angiogenesis represents another essential hallmark, because tumors require a functional vascular network to supply oxygen and nutrients and to support continued growth and dissemination. Tumor-associated hypoxia, through hypoxia-inducible factors (HIFs), induces the activation of angiogenic genes, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), PDGF, TGF- β , and angiopoietins, thereby promoting the formation and remodeling of tumor vasculature (Semenza, 2003; Vimalraj, 2022). Beyond classical angiogenesis, tumors can exploit alternative vascularization strategies, that collectively enhance vascular plasticity and are frequently associated with invasive phenotypes and EMT-related programs (R. Zhang et al., 2024).

Focus on tissue invasion and metastasis

Importantly, when tumor mass remains localized to its tissue of origin, the disease can be considered relatively non-aggressive. The transition to full malignancy is marked by the acquisition of invasive properties, allowing cancer cells to penetrate surrounding tissues and ultimately initiate the metastatic cascade. (Dillekås et al., 2019). During tissues invasion, cells manage to detach from the basal lamina, to which they are typically anchored, and embark on an intricate journey, becoming circulating tumor cells (CTCs). CTCs first enter the bloodstream via a process known as intravasation, and this mechanically stressful process involves mechanical changes in cancer cells as nuclear squeezing, which can physically determine genomic rearrangements and increase metastatic potential (Denais et al., 2016).

CTCs are highly heterogeneous, and only a subset of cells likely possessing the specific characteristics necessary to succeed in various steps of the metastatic process can form metastases in specific target organs. For example, single CTCs are less efficient at initiating metastases compared to multicellular clusters of CTCs, that contain also stromal and immune cells, contributing to enhanced survival in circulation (Aceto et al., 2014).

When CTCs arrive at small capillaries, they become physically trapped. This event can cause microvascular rupture or induce cell extravasation. Organs such as the liver and bone, characterized by highly permeable vasculature, are particularly prone to metastatic colonization because CTCs can extravasate into these tissues with relative ease. In contrast, other organs present more restrictive environments, where tight endothelial junctions and basement membranes pose significant barriers to transmigration, requiring genetic and molecular adaptations by cancer cells.

Extravasation is a multifaceted process that involves dynamic interactions between ligands and receptors, chemokine gradients, and various circulating non-tumor cells. Among the key mediators, integrins play a critical role in guiding CTCs to specific extravasation and colonization sites. By promoting anchorage-independent survival, integrins support the successful establishment of metastatic lesions in distant tissues (Hamidi & Ivaska, 2018).

Moreover, it is well known that the majority of CTCs primarily originate from epithelial tissues. A specific process allows these cells to acquire a distinct phenotype typical of mesenchymal cells when necessary and then revert to a more epithelial phenotype once they reach their target organ. This remarkable ability is known as “epithelial-to-mesenchymal plasticity”. The initial transition towards a more mesenchymal phenotype equips these cells with a set of characteristics associated with metastatic behavior, such as the ability to migrate, invade, and survive when not adhered to other cells or surfaces. This phase is commonly referred to as epithelial-to-mesenchymal transition (EMT) (Nieto et al., 2016).

Tumor cells exhibiting an incomplete EMT phenotype are particularly well-suited to survive in the bloodstream and during colonization of the secondary site, thereby increasing the likelihood of metastasis. Furthermore, the different stages of EMT occur within distinct microenvironments and interact with varying populations of stromal cells. For example, metastatic cells with a strongly mesenchymal phenotype tend to proliferate in proximity to endothelial and inflammatory cells. In particular, these tumor cells secrete high levels of chemokines and signaling proteins that attract immune cells and promote angiogenesis, thereby creating a specialized, highly vascularized, and inflammatory niche (Pastushenko et al., 2018). Also, hypoxia (Rankin & Giaccia, 2016), metabolic stress, and increased matrix stiffness (Lamouille et al., 2014) serve as key stimuli that activate the EMT program in cancer cells.

Despite Stephen Paget’s groundbreaking theory in 1889, which likened metastatic cells to ‘seeds’ that spread throughout the body, but only ‘take root’ in certain organs, the mechanisms dictating this organ specificity remain largely unclear even today. Recent research often refers to the concept of the “pre-metastatic niche” (PMN), underscoring the idea that primary tumors can precondition distant sites for the arrival and implantation of cancer cells (Peinado et al., 2017).

Moreover, it’s well known that cancer cells can release exosomes that deliver pro-metastatic factors, such as proteins, RNA and microRNAs (miRNAs), which promote vascular permeability, extracellular matrix (ECM) remodeling, and immune suppression, thereby facilitating metastatic dissemination. For instance, exosome-associated miR-10b, has been shown to enhance metastatic potential in breast cancer cells (R. Singh et al., 2014). Additionally, exosome-mediated signaling can

activate oncogenic pathways, such as EGFR-signaling, further supporting metastatic progression (Higginbotham et al., 2011). Exosomes also carry EMT-inducing factors or enzymes involved in extracellular matrix (ECM) remodeling, such as matrix metalloproteinases (MMPs). Indeed, in cancer metastasis, MMPs play a fundamental role in ECM disassembly. MMPs are also involved in cell spreading and survival, immune response, resistance to apoptosis, and angiogenesis (Kessenbrock et al., 2010; Shuman Moss et al., 2012). Moreover, MMPs are overexpressed in most cancer types and are associated with poor prognosis (Hadler-Olsen et al., 2013). Together with the action of chemokines and growth factors, these changes contribute to the development of the PMN (Fares et al., 2020).

Emerging hallmarks of cancer

However, over the years the definition of cancer hallmarks has evolved, leading to a more detailed and dynamic vision of cancer, in which genomic instability and altered cellular metabolism, as well as the cells of the tumor microenvironment, strongly influence and positively or negatively modulate tumor growth. In this context, the seminal update by Hanahan and Weinberg in 2011 introduced several emerging hallmarks, including the ability of tumor cells to evade immune destruction, the deregulation of cellular metabolism, often resulting in the Warburg effect, persistent tumor-promoting inflammation, and genomic instability (**Figure 4A**) (Hanahan & Weinberg, 2011). More recently, the latest version of the “Hallmarks of Cancer” included four additional aspects: epigenetic reprogramming, the plasticity of the tumor phenotype, the effect of senescence on tumor progression, and the influence of the microbiome on tumor progression. (**Figure 4B**) (Hanahan, 2022).

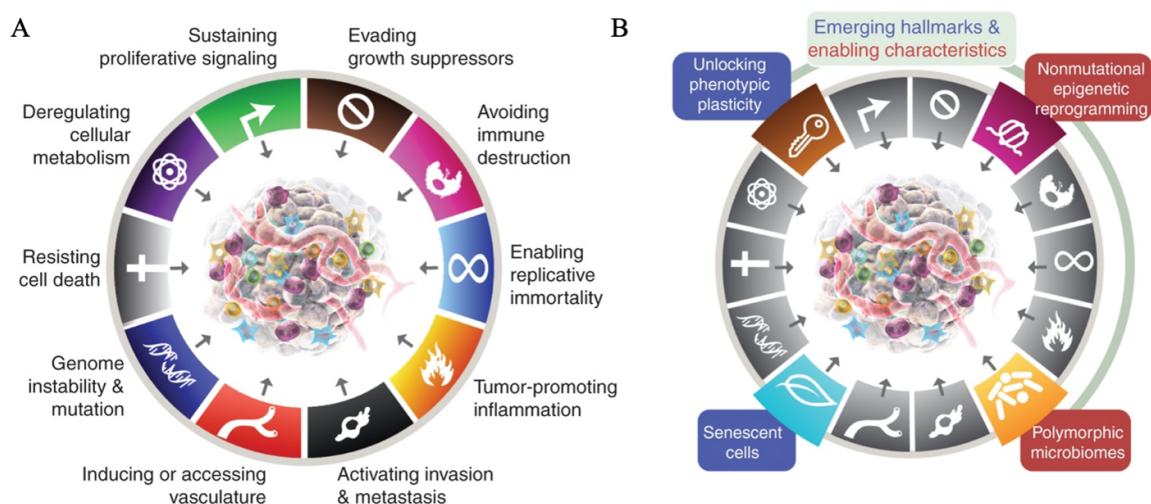


Figure 4. Schematic representation of the “Hallmark of Cancer” updated to 2011 (A) and to 2022 (B) - (Hanahan, 2022)

Among the most recent hallmarks, the remarkable plasticity of cancer cells is fundamental to tumor progression and responsible for inter- and intra-tumor heterogeneity.

Focus on phenotypic plasticity

Cellular plasticity refers to the capacity of cells to reversibly modify their phenotype in response to environmental stimuli, without requiring genetic alterations.

Indeed, unlike normal cells, tumor cells can escape differentiated states, either by reverting to a progenitor-like state or by avoiding terminal differentiation, thereby maintaining a partially differentiated state. During this event, cells can also become dormant, hiding in protective niches and retaining the potential to resume proliferative expansion when needed (Phan & Croucher, 2020). However, in many tumor types, dedifferentiation and blocked differentiation often overlap, rendering them indistinguishable. An additional mechanism by which tumor cells can exhibit plasticity is transdifferentiation. Through this event, cells that were destined to assume a specific phenotype are able to completely change their differentiation program to alternative cell lineages (Hanahan, 2022).

In this context, it's essential to highlight the crucial role that cancer stem cells (CSCs) can play in cancer progression. Indeed, recent research has underlined the role of CSCs in promoting angiogenesis through the release of angiogenic factors and exosomes. For example, CSCs in breast cancer have been found to secrete stromal cell-derived factor-1 and VEGF to facilitate angiogenesis. Additionally, emerging evidence suggests that tumor cells possess the ability to differentiate into phenotypes of endothelial cells (ECs) that are engaged in tumor angiogenesis (Butti et al., 2019).

One of the most emblematic manifestations of this phenotypic plasticity is the epithelial-to-mesenchymal transition, a developmental program aberrantly reactivated in cancer that perfectly exemplifies how cancer cells exploit phenotypic plasticity to enhance invasion, survival, and metastatic competence.

The epithelial-to-mesenchymal transition

The epithelial-to-mesenchymal transition (EMT) is a biological process in which epithelial cells lose their polarized phenotype and acquire mesenchymal properties.

In fact, cells can usually be described as having an epithelial or mesenchymal phenotype, based on their shape, polarization, gene expression, and other characteristics. In particular, the epithelial phenotype is characterized by a polygonal, flat morphology, which is achieved and maintained by intermediate filaments and the actin cytoskeleton (Axelrod, 2006). On the other hand, the mesenchymal phenotype is characterized by an irregular, more elongated shape (Voulgari & Pintzas, 2009) and anterior-posterior polarity, rather than the basal-apical polarity that characterizes the epithelial phenotype. In this case, plasma membranes are oriented toward the lumen in the apical portion and away from it in the basal portion (M. Singh et al., 2018), and each membrane is characterized by distinct proteins that support specific processes (J. M. Lee et al., 2006). For example, epithelial cells are characterized by increased Keratin expression, while mesenchymal cells exhibit higher Vimentin expression, which helps them migrate and invade nearby tissues (Williams et al., 2008). Moreover, this polarization difference is essential for biological processes such as vesicle transport, exo- and endocytosis (Voulgari & Pintzas, 2009).

Furthermore, epithelial cells can contact each other via tight junctions (TJs), adherens junctions, gap junctions, or desmosomes (Huang et al., 2012). In the same way, they use tight junctions to merge into a single layer, forming the epithelial tissue (Fletcher & Mullins, 2010). Still, when these junctions are interrupted, epithelial cells lose their apical-basal polarity. In particular, tight junction instability leads to a decrease in the expression of epithelial proteins, such as Claudin, Occludin, E-cadherin, and Zone of occlusion 1 (ZO-1), and an increase in the expression of proteins like Vimentin, Fibronectin, and N-cadherin, which are markers of the mesenchymal phenotype (Huang et al., 2012).

EMT is a fundamental process for proper cell differentiation and can be divided into three types based on its function (Kalluri & Weinberg, 2009). Indeed, the type I EMT is essential in embryonic differentiation, promoting neural crest stratification, mesodermal and pro-intestinal formation, endocardial morphogenesis, and the growth of various cell types and tissues (Nieto et al., 2016). The type II EMT is involved in wound healing and the inflammatory process, helping restore endothelial and epithelial integrity. The last one, the type III EMT, plays key roles in cancer progression, enabling cancer cells to acquire invasive and metastatic behavior and enhancing cell survival (Mittal, 2018).

Indeed, particularly during cancer development, epithelial cells acquire distinct mesenchymal traits within the primary tumor, allowing them to invade nearby tissues and then to spread to distant sites.

Once mesenchymal cancer cells have reached distant sites, they may undergo the reverse process, called mesenchymal-to-epithelial transition (MET). In this opposite process, mesenchymal cells re-acquire their epithelial phenotype and are free to grow at the secondary tumor site. This mechanism is fundamental to the onset and progression of metastases (Brabletz et al., 2021).

EMT activation further promotes this invasive phenotype and reduces tumor cell sensitivity to chemotherapy by modifying the tumor microenvironment. Notably, cells in a near-mesenchymal state exhibit greater resistance to treatments such as chemotherapy and immunotherapy (Cai et al., 2021; Dongre & Weinberg, 2019), and higher baseline EMT status is associated with poorer prognosis following oxaliplatin treatment (Malagoli Tagliazucchi et al., 2023).

EMT-related signaling pathways

Among the signaling pathways involved in EMT activation, the most important are the TGF- β , WNT, NOTCH, and HIPPO pathways (**Figure 5**).

Canonical TGF- β signaling induces the phosphorylation of SMAD2 and SMAD3, which subsequently form heteromeric complexes with SMAD4 and translocate into the nucleus, where they activate transcriptional programs that include master regulators of EMT, including Snail, Zeb, and Twist families. TGF- β signaling displays a context-dependent dual function in oncogenesis: it functions as a potent tumor suppressor during early stages of tumor development, while switching to a pro-tumorigenic driver once malignant cells accumulate oncogenic alterations. In advanced cancers, sustained TGF- β activity promotes EMT, invasiveness, stem-like properties, angiogenesis, and immune evasion (Deng et al., 2024; Peng et al., 2022).

The WNT/ β -catenin pathway is critically involved in maintaining mesenchymal characteristics and stabilizing post-EMT cellular states. Upon activation, β -catenin accumulates in the nucleus, where it promotes the expression of mesenchymal genes while repressing epithelial markers (Ghafoor et al., 2025). Functional crosstalk between WNT and TGF- β signaling pathways sustains hybrid EMT states, thereby enhancing intratumoral heterogeneity and resistance to therapy (Xue et al., 2025).

NOTCH signaling exerts highly context-dependent effects in cancer, acting predominantly as an oncogenic pathway in breast, pancreatic, and lung malignancies, while displaying tumor-suppressive functions in specific tissues or genetic backgrounds.

The HIPPO pathway, primarily through its downstream effectors YAP (yes-associated protein) and TAZ (Transcriptional coactivator with PDZ-binding motif), plays a complex and context-specific role in EMT regulation. Depending on tissue type and signaling crosstalk, HIPPO signaling can either restrain or facilitate EMT (Johnson & Halder, 2014). When HIPPO pathway activity is suppressed,

YAP and TAZ translocate to the nucleus and cooperate with EMT-associated transcription factors. In cancer, aberrant YAP/TAZ activation contributes to increased tumor aggressiveness, metabolic rewiring, angiogenesis, and metastatic spread, frequently in coordination with TGF- β and WNT signaling pathways (Fu et al., 2022).

These EMT-associated signaling pathways are commonly activated by extrinsic cues such as chronic inflammation, oxidative stress, and metabolic alterations within the tumor microenvironment.

Collectively, these signaling pathways converge on a limited set of transcriptional regulators that act as the final executors of EMT programs.

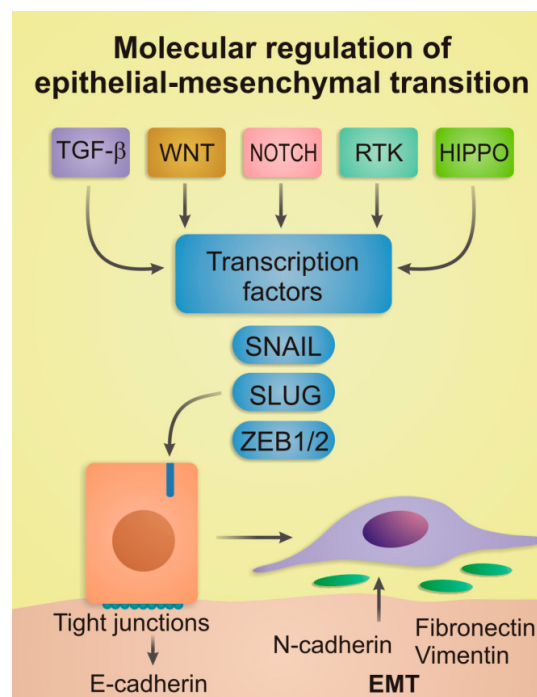


Figure 5. EMT-related signaling pathway and their downstream targets - (Bastos et al., 2025)

EMT-related transcriptional factors

The progression of EMT is regulated by transcriptional factors (TFs) known as EMT-TFs, which primarily control cell polarity, motility, and intercellular adhesion (Lu & Kang, 2019). As shown in **Figure 6**, several pathways are involved in the activation of the EMT-TFs, such as those mediated by integrins and tyrosine kinase receptors (RTKs). The latter can also trigger TGF- β R signaling, which in turn promotes EMT-TFs expression. Moreover, integrin–RTK cooperation results in the coordinated activation of shared signaling networks, including Src family kinases (SFKs), focal adhesion kinase (FAK), and RTK phosphorylation. Through this crosstalk, downstream pathways involving YAP, c-Jun/Fos, mammalian target of rapamycin (mTOR), and Forkhead box O (FOXO) are engaged, ultimately enhancing EMT, invasion, proliferation, and survival (Cooper & Giancotti,

2019). One of the principal outcomes is profound actin cytoskeletal remodeling, which enables cell migration and the formation of invasive structures termed invadopodia.

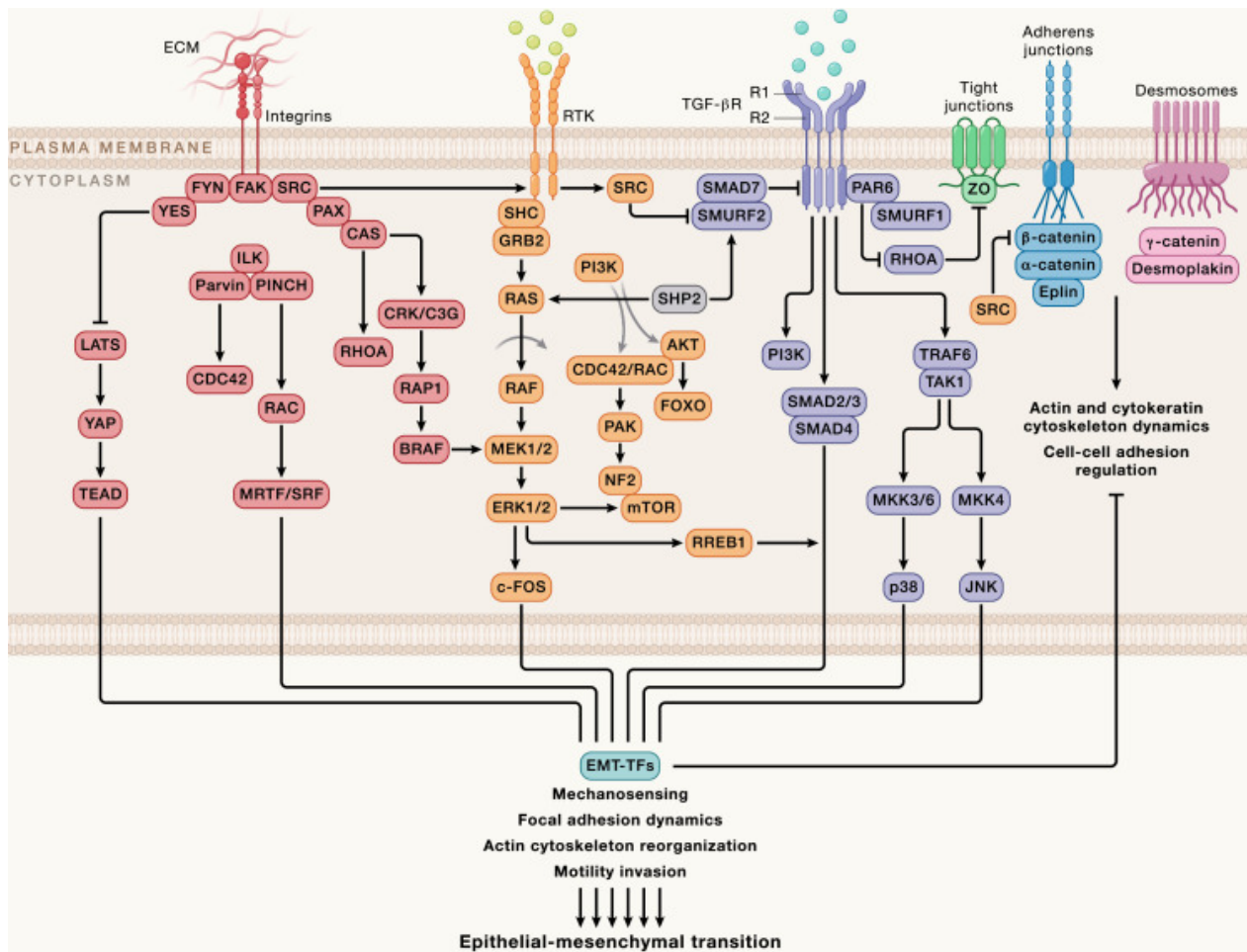


Figure 6. Schematic representation of the major signaling pathways converging on the epithelial-to-mesenchymal transition - (C. X. Zhang et al., 2025)

EMT-TFs include TFs such as the Snail family with Snail1 and Snail2 and Twist1 and Twist2, members of the basic helix-loop-helix (BHLH) family, and Zeb1 and Zeb2, zinc-finger E-box-binding homeobox transcription factors. All these TFs can upregulate the expression of mesenchymal genes and, on the other hand, downregulate the expression of epithelial ones.

Snail1 and Snail2 play pivotal roles in EMT, both during embryogenesis and in cancer progression (Barrallo-Gimeno & Nieto, 2005), binding to the E-box sequence within the CDH1 gene, which encodes for E-cadherin, marker of the epithelial phenotype. In this way, Snail family represses E-cadherin by recruiting the polycomb repressor complex (PRC). The presence of the PRC mediates epigenetic silencing of E-cadherin by promoting histone hypermethylation and deacetylation, thereby reducing gene expression (Herranz et al., 2008). Furthermore, Snail1 expression levels serve as a potential prognostic marker. Indeed, Snail1 can upregulate markers of the mesenchymal phenotype,

such as Vimentin and Fibronectin, downregulate E-cadherin, and inhibit the formation of tight junctions between the cells (Kajita et al., 2004). Similarly, Snail2 contributes to the loss of cell adhesion and polarity, thereby enhancing migratory and invasive capabilities (W. Zhou et al., 2019). Additionally, sustained expression of Snail2 has been shown to reduce E-cadherin levels, thereby promoting metastasis in breast and ovarian cancers (Li et al., 2020).

The expression of Snail proteins in specific physiological contexts can be triggered by multiple signaling pathways, including TGF- β , Wnt, Notch, and growth factors acting through receptor tyrosine kinases (RTKs) (Peinado et al., 2007), highlighting the complexity and multifaceted regulation of the Snail-driven EMT program. Moreover, both factors also collaborate with other transcriptional regulators to modulate gene expression; for instance, Snail1 works alongside ETS1 to activate matrix metalloproteinases expression (Jordà et al., 2005).

Zeb family contributes to EMT by downregulating the expression of TJs genes and components of the cell polarity complex (Aigner et al., 2007); in particular, Zeb1 contributes to the repression of CDH1 by recruiting DNA methyltransferase 1 (DNMT1) to its promoter region, thereby sustaining its methylation and silencing E-cadherin expression (Fukagawa et al., 2015). Simultaneously, the Zeb family can upregulate mesenchymal markers such as N-cadherin and Vimentin (Bindels et al., 2006). Even in this case, the expression of Zeb proteins is induced by several signaling pathways, including TGF- β , Wnt, and various growth factors that activate the Ras-MAPK pathway (Lamouille et al., 2014). Additionally, Twist1 can work together with Snail1 to drive Zeb1 expression, often initiating a feedback loop that further activates Snail (Dave et al., 2011). Zeb1 and Zeb2 promote EMT and a mesenchymal phenotype in tumor-initiating cells, facilitating invasion, metastatic spread, and cellular dedifferentiation (Chaffer et al., 2013). Clinically, high levels of Zeb1 expression are associated with poor prognosis and survival across multiple solid tumor types, including breast, colorectal, and pancreatic cancers (B. Chen et al., 2019) and with effective tumor invasion and metastasis (Krebs et al., 2017).

During the EMT, several signaling pathways can activate the Twist family, including HIF-1 α under hypoxic conditions (M.-H. Yang et al., 2008). This way, Twist1, acting as Snail1, can have an adverse effect on cell adhesion and a positive impact on cell motility, acting as a suppressor of E-cadherin expression and increasing N-cadherin levels (Meng et al., 2018). As the other factors already cited, Twist overexpression is correlated with worse prognosis and metastasis (T. K. Lee et al., 2006). Moreover, Twist1 can lead to the direct or indirect inactivation of p53, promoting proliferation despite a possible DNA damage. Furthermore, Twist1, by blocking p53, disrupts the negative feedback loop in which miR-200 and miR-34 regulate Zeb1 and Snail1 expression. The culmination of this circuit

is the induction of a CSC phenotype, due to the activation of stemness genes such as SOX2 and KLF4 (Puisieux et al., 2014).

Beyond Snail, Zeb, and Twist families, a variety of other transcription factors also play essential roles in EMT during tissue development and cancer progression. For instance, forkhead box (FOX) family members, such as Foxc1, Foxc2, and Foxq1, modulate negatively proteins involved in cell-cell junctions and cell polarity complexes, thereby promoting mesenchymal differentiation (Myatt & Lam, 2007; Ou-Yang et al., 2015).

Additionally, SRY-related HMG-box genes (SOX) together with Snail promote EMT (Sakai et al., 2006). Recent studies have identified paired-related homeobox 1 (PRRX1) as a novel and significant regulator of EMT (Guo et al., 2015).

EMT-related proteins

In cancer progression, EMT is also characterized by several different proteins related to this process, such as:

- E-cadherin and N-cadherin: during complete EMT, there is usually a downregulation of E-cadherin, which will be expressed again during the reverse process of EMT: the mesenchymal-to-epithelial transition (Chao et al., 2012). This way, metastatic cells can take root within the metastatic niche. N-cadherin, on the other hand, behaves in precisely the opposite way (Gravdal et al., 2007). Furthermore, when normal E-cadherin levels decrease, β -catenin migrates to the nucleus, activating the Wnt/ β -catenin pathway, which induces the expression of Snail, Zeb, and Twist families, and inhibits the transcription of CDH1. Despite this, downregulation of E-cadherin levels is not strictly necessary for EMT, and restoring its regular expression is not sufficient to halt or reverse the transition (Loh et al., 2019);
- CD44: several studies have shown that during EMT, a “standard” isoform of CD44 is expressed, which, unlike its “variable” isoform (CD44v), is more highly expressed in BC and correlates directly with N-cadherin expression levels (Brown et al., 2011);
- Discoidin Domain Receptor type 2 (DDR2): a collagen-specific tyrosine kinase receptor, which can reflect the changes in the extracellular matrix during EMT. Indeed, in adults, DDR2 is typically expressed in fibroblasts and in smooth muscle cells of blood vessels; in BC, DDR2 overexpression is involved with increased invasiveness (Evtimova et al., 2003). In particular, DDR2 is involved in ERK2 activation, which, in turn, by reducing the ubiquitination of Snail1, increases its half-life and promotes the invasion and metastasis processes (K. Zhang et al., 2013);

- β -catenin: generally located in the tight junctions of epithelial cells, it contributes to the stabilization of E-cadherin and N-cadherin at the membrane level. The loss of cell polarity and adhesion during EMT promotes the migration of β -catenin to the nucleus, where it promotes transcription of genes involved in EMT, such as CD44 and metalloproteinases (Z.-Q. Cao et al., 2019);
- Vimentin: cytoskeletal protein expressed in various organisms, belongs to the type III intermediate filament family. Vimentin is often linked to the onset of various diseases and, in addition, with multiple types of cancer (lung, breast, prostate, colorectal, and even melanoma). Indeed, Vimentin is generally associated with mesenchymal phenotype and, for this reason, it is used as an EMT marker. In BC, Vimentin is regulated by SIP-1 (Smad Interacting Protein 1) (Bindels et al., 2006) and is more expressed in patients with stage IV disease (Patel et al., 2015);
- α -Smooth Muscle Actin (α -SMA): it is involved in embryogenesis and wound healing; in adults, it is expressed in smooth muscle cells of blood vessels and in myoepithelial cells. The involvement of α -SMA in tumor progression has been demonstrated in BC, especially in basal-like subtypes (Damonte et al., 2007). Indeed α -SMA expression is used as a marker of CAFs (Barb et al., 2023) and α -SMA-positive breast cancer cell populations correlate with tumor progression, metastatic potential and worse prognosis (Findakly et al., 2020).
- Cytokeratins-18 (CK-18): is a main epithelial intermediate filament protein involved in maintaining cellular structure. Its downregulation is commonly associated with EMT and chemoresistance. Furthermore, CK-18 downregulation promotes migratory and invasive properties in epithelial cancer cells (Fortier et al., 2013). In breast epithelial cells, loss of CK-18 is required for TGF- β -induced EMT (Jung et al., 2016). Moreover, reduced CK-18 expression has been associated with the acquisition of CSC-like traits in cancer cells (Mallini et al., 2014).

EMT-related epigenetic regulation

Beyond the canonical regulating pathways, EMT induction is frequently controlled by epigenetic modifications. For example, as mentioned above, one of the most considerably characterized events associated with EMT induction is the hypermethylation of the CDH1 promoter, mediated by EMT-TFs recruiting DNA methyltransferases (DNMTs), leading to stable repression of E-cadherin expression. Conversely, active DNA demethylation driven by Ten-Eleven Translocation (TET)

enzymes is required for MET during somatic cell reprogramming, highlighting the dynamic and reversible nature of DNA methylation (X. Hu et al., 2014).

In parallel, histone acetylation represents a key epigenetic modification associated with chromatin relaxation and transcriptional activation. Transcriptional coactivators such as p300 and CREB-binding protein (CBP) facilitate the expression of EMT-associated genes by establishing a permissive chromatin environment, thereby promoting tumor progression and metastatic dissemination (Xin et al., 2019). Conversely, histone deacetylases (HDACs) contribute to EMT by being recruited by EMT-TFs to epithelial gene promoters, resulting in chromatin condensation and transcriptional silencing, particularly of epithelial markers such as E-cadherin (Peinado et al., 2004).

In addition to acetylation-based mechanisms, histone lysine methylation, catalyzed by lysine methyltransferases (KMTs), provides an additional layer of epigenetic control over EMT. Depending on the specific lysine residue and the degree of methylation, this modification can be associated with either transcriptional activation or repression by selectively recruiting chromatin-associated regulatory proteins. In this context, EZH2, the catalytic subunit of the Polycomb Repressive Complex 2 (PRC2), mediates transcriptional repression via trimethylation of histone H3 at lysine 27 (H3K27me3) and has been implicated in the silencing of epithelial genes such as CDH1, thus promoting EMT (Q. Cao et al., 2008). Other KMTs, including DOT1L and G9a, have also been linked to EMT regulation. DOT1L-mediated H3K79 methylation has been associated with transcriptional activation of EMT-related transcription factors and the acquisition of mesenchymal and stem-like features in cancer cells (Cho et al., 2015), whereas G9a catalyzes repressive H3K9 methylation and cooperates with EMT-inducing transcription factors to silence epithelial genes such as EPCAM (M.-W. Chen et al., 2010) and E-cadherin (Dong et al., 2012), thereby promoting EMT and metastatic progression.

Histone demethylation further contributes to EMT-associated chromatin plasticity. Lysine-specific demethylase 1 (LSD1) removes methyl groups from histone H3 at lysine 4 or lysine 9, thereby functioning either as a transcriptional repressor or activator depending on the chromatin context. LSD1 can be recruited by EMT-TFs, such as Snail, to epithelial gene promoters, leading to the repression of epithelial markers and EMT induction (T. Lin et al., 2010). Conversely, when associated with chromatin-repressive complexes such as Mi-2/NuRD (Y. Wang et al., 2009), or the co-repressor Sin3A (Y. Yang et al., 2018), LSD1 has been reported to contribute to the maintenance of epithelial identity, underscoring its dual role in EMT regulation.

In addition to chromatin-modifying enzymes, microRNAs (miRNAs) can also regulate the EMT process by targeting its transcription factors. For example, miR-34 (Siemens et al., 2011), miR-203

(Moes et al., 2012), miR-29b (Ru et al., 2012), and miR-30 (J. Zhang et al., 2012) can suppress Snail1 expression in breast, colorectal, prostate, and hepatocyte tumor cells. Furthermore, the miR-200 family and miR-205 play key roles in downregulating Zeb1 and Zeb2 expression. Although miR-200 and miR-205 bind to different target sites of Zeb sequences, they act cooperatively to more effectively suppress Zeb expression (Gregory et al., 2008). Notably, this regulatory system is part of a double-negative feedback loop: while the miR-200 family inhibits Zeb expression and activity, Zeb proteins repress the transcription of miR-200 family members (Bracken et al., 2008). Another well-known miRNA that regulates Zeb1 is miR-1199-5p, which binds Zeb1 directly, leading to its downregulation (Diepenbruck et al., 2017).

New vision of EMP: Hybrid EMT

However, as noted before, EMT is not a binary event; instead, cells may occupy an intermediate or “hybrid” state (Youssef et al., 2024). In this case, tumor cells undergoing partial EMT can migrate as clusters of circulating cells both in vivo and in vitro, whereas complete EMT cell lines spread as single cells (Aiello et al., 2018). Usually, early hybrid EMT is regulated by pathways that promote EMT without the loss of E-cadherin. These hybrid phenotypes are stabilized by inhibitory feedback loops between EMT-TFs and their regulatory miRNAs (He et al., 2018). Moreover, other agents, such as nuclear factor erythroid 2 related factor 2 (NRF2), which was recently identified as a phenotypic stability factor (PSF), have been shown to promote and maintain hybrid EMT states by regulating CDH1, Zeb1, Snail1, and miRNAs (Bocci et al., 2019).

Furthermore, it's known that epithelial carcinoma cells progressively acquire EMT characteristics in continuity rather than a defined transition. Interestingly, metastatic ability has been primarily associated with cells exhibiting a late hybrid EMT, as observed in triple-negative breast cancer, rather than with cells with early hybrid states, as found in skin squamous cell carcinoma (Huang et al., 2013). Taken together, these findings indicate that a universal, sequential EMT program doesn't exist.

Additionally, it's important to note that these hybrid states are associated with increased stem cell properties, phenotypic plasticity, tumorigenicity, drug resistance, and metastasis (Vipparthi et al., 2022). Indeed, intermediate EMT reflects epithelial-mesenchymal plasticity (EMP), allowing tumor cells to dynamically adapt to environmental cues, including signals from the metastatic niche or therapeutic pressure (J. Yang et al., 2020).

A recent study conducted by Grosse-Wild et al. showed that cells exhibiting intermediate EMT also demonstrated enhanced migratory and invasive ability in vitro and a higher propensity to form metastases in vivo compared with cells with a complete epithelial or mesenchymal phenotype. Moreover, the same study introduced an immunostaining method that can quantitatively assess EMT

heterogeneity (EMH) and EMT scores in tumor specimens. Notably, hybrid EMT states combined with elevated EMH were associated with poorer survival in stage III breast cancer patients, especially within hormone receptor-negative subtypes. Taken together, these findings underscore the prognostic relevance of EMH driven by intermediate EMT plasticity, which outperforms single-marker-based evaluation (Grosse-Wilde et al., 2015).

Moreover, bioinformatics-based EMT timing analysis revealed that epithelial cells are more able to progress toward a hybrid state rather than undergoing a direct transition into a fully mesenchymal phenotype and that the hybrid state is the most stable along the EMT spectrum.

Along this continuum, the TME also plays a key role in the regulation of the EMT hybrid state. Indeed, during this spectrum, immune infiltration progressively increases. Specifically, hybrid EMT is usually dominated by mesenchymal stem cells (MSCs), inflammatory CAFs (iCAFs), and myoblastic cancer-associated fibroblast-like (myCAF-like) population. Instead, the mesenchymal state reveals an enrichment of CD4⁺ and CD8⁺ T cells, monocytes, and macrophages (Malagoli Tagliazucchi et al., 2023). Furthermore, within the TME, besides CAFs, also interferon signaling, hypoxic regions, and endothelial cells frequently localize together in tumor cells displaying a hybrid EMT signature (Tyler & Tirosh, 2021).

Moreover, a tight association links the EMT program with the acquisition of stemness, through which tumor cells gain properties typically attributed to cancer stem cells. These traits include tumor-initiating ability, resilience to cellular stress, immune escape, and resistance to multiple therapeutic agents. Across patient cohorts and experimental systems, elevated EMT scores consistently correlate with the expression of CSC markers and with functional indicators of stemness (Lei et al., 2024). Notably, these phenotypic advantages are most evident in intermediate EMT states, where epithelial cell–cell adhesion is retained while mesenchymal programs associated with survival and extracellular matrix remodeling are activated. Such hybrid configurations support self-renewal, dormancy, and tolerance to anticancer therapies (den Hollander et al., 2024). Consistent with this model, CSC populations have been identified across a wide range of human cancers, and experimental induction of EMT in carcinoma cells has been shown to enhance their tumorigenic potential (Batlle & Clevers, 2017). In breast cancer, clones occupying intermediate EMT states simultaneously express both epithelial and mesenchymal gene modules and exhibit increased tumorigenicity, metastatic capacity, and *in vivo* recurrence, providing direct functional evidence that partial EMT triggers stemness-driven disease progression (L. Zhang et al., 2023).

Several studies of skin and breast tumors found that these two tumor types contain multiple epithelial/mesenchymal (E/M) subpopulations, each characterized by unique chromatin landscapes

and gene expression profiles, which are spatially distributed across specific tumor regions (Pastushenko et al., 2018). A more recent and extensive analysis of 67 biopsies from breast cancer patients identified three spatial phenotypes: EMT-high, EMT-low, and EMT-patched. These three phenotypes displayed distinct cell-cycle gene expression profiles, and EMT-high regions were markedly enriched in fibroblasts, further supporting the key role of stromal interactions in promoting EMT. Moreover, the EMT-patched phenotype is characterized by areas of elevated EMT within EMT-low tumors. This pattern highlights the strong intra-tumoral heterogeneity, in which only a few subregions undergoing EMT are sufficient to influence metastasis and resistance to therapy (Klughammer et al., 2024).

Furthermore, application of the clustering classification and sorting tree (CCAST) algorithm, performed by Karacosta et al., resolved eight distinct cellular clusters based on the expression of CDH1 (E-cadherin), VIM (Vimentin), CD44 and CD24 (Cluster of Differentiation 44 and 24), MUC1 (Mucin-1, a glycoprotein), and TWIST. Three clusters corresponded to epithelial phenotypes, defined by the expression of CDH1, CD24, and MUC1. The other three clusters represented intermediate EMT, characterized by the concomitant expression of CDH1 and VIM, with TWIST detected only in some cases of the more advanced intermediate population. Only a single fully mesenchymal cluster was identified, marked by high VIM and CD44 expression, whereas CD24 levels were down-regulated, suggesting cancer stem-like features.

Moreover, concerning mesenchymal-to-epithelial transition (MET), the MET-associated cluster showed re-expression of MUC1, TROP2 (Trophoblast Cell Surface Antigen 2, a glycoprotein), and Cytokeratin-8 (CK-8) while remaining CDH1-negative. The same study also revealed that the mesenchymal state most frequently arises from intermediate EMT instead of directly from epithelial states. In contrast, the transition from the mesenchymal state back to the intermediate one is relatively rare (Karacosta et al., 2019).

Breast cancer

Among cancers, breast cancer (BC) is the leading cause of cancer mortality in women. According to the World Health Organization, in 2022, there were an estimated 2.3 million women diagnosed with breast cancer and 670,000 deaths globally (World Health Organization, 2025). In particular, BC accounts for roughly 80% of cases in high-income regions such as Asia, Europe, and North America (International Agency for Research on Cancer, 2022). Over the past three decades, global breast cancer incidence has risen by 128% (Xu et al., 2023), and projections suggest an additional 40% increase by 2040 (Arnold et al., 2022).

The elevated incidence of breast cancer observed in countries with a high Human Development Index (HDI) appears to be linked to hormonal and lifestyle-related risk factors. Furthermore, it also correlates with better outcomes, as evidenced by a lower mortality rate, thanks to easy access to healthcare and screening programs. The different healthcare situation also helps explain why nations with lower HDI, despite lower incidence, experience higher mortality rates, as shown in **Figures 7A** and **7B** (World Health Organization, 2025).

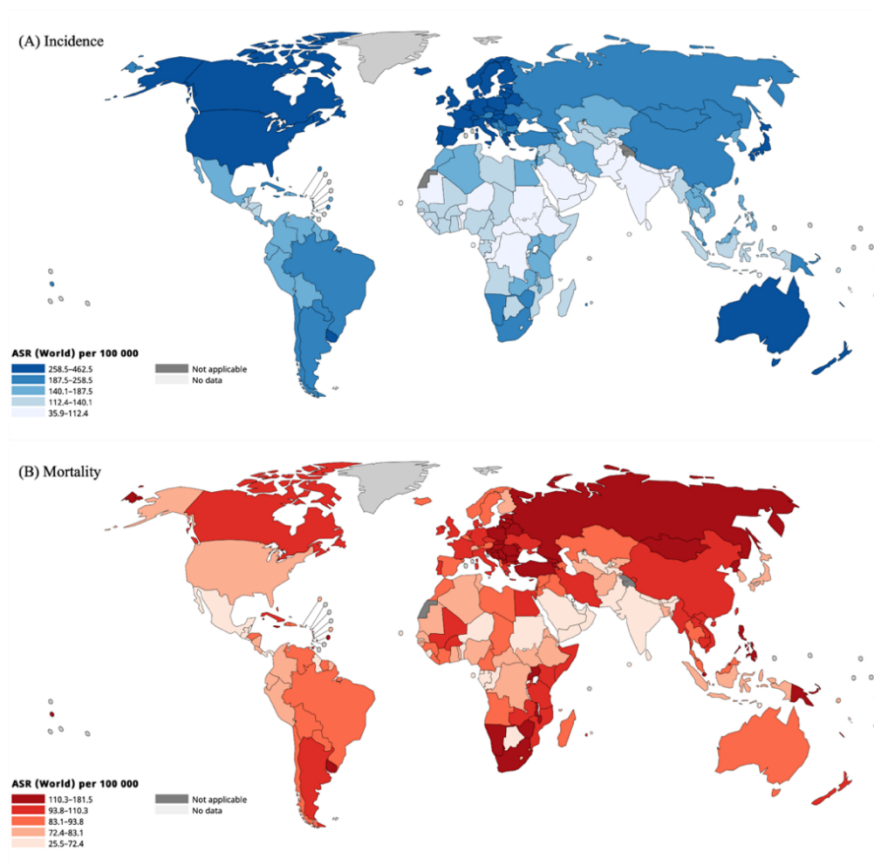


Figure 7. Global incidence (A) and mortality (B) of breast cancer - (*International Agency for Research on Cancer, 2022*)

It's well known that the onset of breast cancer is associated with a wide variety of risk factors of different kinds.

These undoubtedly include factors related to gender (women are more likely to develop the disease than men) and age, as the likelihood of developing this cancer increases with age, peaking at menopause (around 50-55 years of age), then slowing down and resuming its increase after the age of 60. This trend could also be due to the continuous hormonal stimulation that the epithelium undergoes over the years.

Nutrition and lifestyle factors are also significant risk factors for breast cancer, first and foremost, obesity, especially after menopause, a diet rich in saturated fats, high alcohol consumption, and stress. On the other hand, a healthy diet and physical activity seem to reduce the risk of cancer.

Reproductive and endocrine factors play a crucial role, including early menarche (before age 12), late menopause (after age 55) or hormone therapy used during menopause, absence of pregnancies, and prolonged use of oral contraceptives (Key et al., 2001).

In addition to the risk factors already mentioned, family history is one of the most critical factors, as it is present in between 5% and 10% of breast cancer cases. In particular, the BRCA-1 and BRCA-2 (BRCAst CAncer gene) tumor suppressors, which are physiologically involved in cell cycle regulation and DNA repair mechanisms and are located on chromosomes 13 and 17, respectively, are implicated in the onset of hereditary breast cancer.

Women with a mutation in one of these two genes have a 60-80% chance of developing hereditary breast cancer during their lifetime. However, these same genes are not associated with the onset of sporadic cancer (K. Yoshida & Miki, 2004).

Breast cancer classification

Breast cancer can be classified based on the anatomical location of onset (**Figure 8**). In this case, it's possible to recognize the ductal carcinoma (DC), which accounts for about 80% of breast cancer cases and affects ductal cells, epithelial cells lining the mammary ducts, responsible for milk transport and secretion. On the other hand, the lobular carcinoma (LC) only accounts for about 10-15% of all breast cancer cases and affects lobular cells, epithelial cells forming the lobules of the mammary gland.

Based on its invasive capacity, breast cancer is classified as carcinoma in situ (or non-invasive) and invasive. Invasive carcinoma is characterized by the uncontrolled proliferation of cancer cells, which, after degrading and crossing the basement membrane, migrate through the bloodstream and

metastasize to other regions (IDC and ILC). IDC, in particular, is the most common type of BC, accounting for 80% of invasive breast cancer.

Non-invasive carcinoma, on the other hand, although also characterized by uncontrolled proliferation, does not cross the basement membrane and therefore does not migrate, and thus does not manifest an invasive phenotype (DCIS and LCIS).

Other, less common forms of breast cancer are mucinous, papillary, cribriform, tubular, and intraductal carcinoma in situ, all of which have a favorable prognosis (American Cancer Society, 2025).

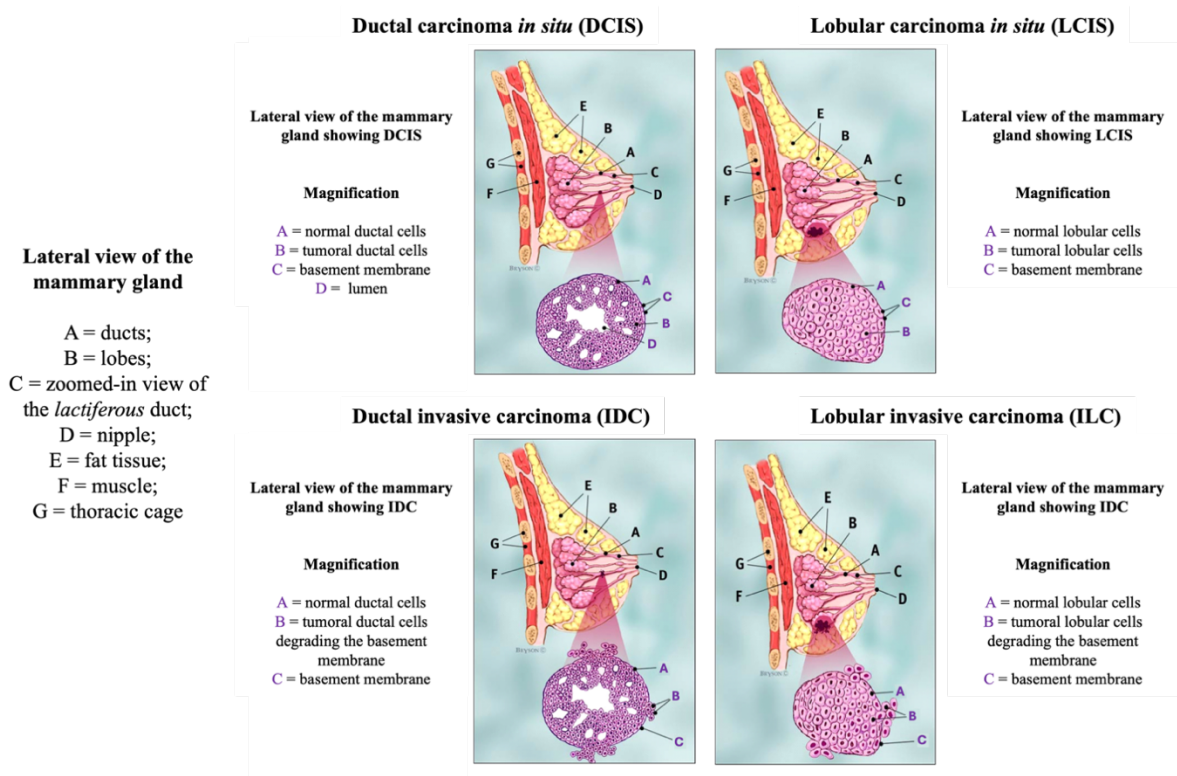


Figure 8. Schematic representation of breast cancer subtypes – (Adapted from Bryson Biomedical Illustration)

Staging and prognosis

The TNM system is a classification system that allows tumors to be cataloged based exclusively on their anatomical characteristics. This system classifies the extent of the primary tumor (T = tumor), the presence or absence of metastasis to regional lymph nodes (N = node), and the presence or absence of distant metastasis (M = metastasis).

Specifically, breast cancer can be classified into five stages:

- Stage 0: the cancer is in situ;
- Stage I: early-stage tumor, less than 2 cm in diameter and not involving the lymph nodes;

- Stage II: may be a tumor larger than 2 cm in diameter but without lymph node involvement, or a tumor still in the early stages and measuring less than 2 cm in diameter but with involvement of the axillary lymph nodes;
- Stage III: locally advanced tumor of varying size involving the axillary lymph nodes or those located along the sternum and/or tissues adjacent to the breast;
- Stage IV: tumor already in the metastatic stage and has spread to other organs, usually bones, lungs, brain, or liver.

In addition to TNM classification and histological subtype, the following factors certainly play an essential role in breast cancer prognosis:

- The expression of hormone receptors: estrogen receptor (ER) and progesterone receptor (PR);
- The overexpression of HER2/neu (Human Epidermal Growth Factor Receptor 2), an oncogene that codes for a tyrosine kinase receptor, whose activation induces cell proliferation and survival; HER2, in particular, is overexpressed and/or amplified in approximately 20-30% of breast cancer cases, which also present as more aggressive forms with a worse prognosis;
- The cell proliferation index, assessed by Ki-67 expression, i.e. the percentage of cells in the S phase of the cell cycle. High Ki-67 values are associated with a worse prognosis and early recurrence (Kondov et al., 2018).

Moreover, to better understand and address the BC, several classifications were used over the years based on molecular characterization, as shown in **Figure 9**.

In early 2000, Perou and Sørli created an intrinsic classification based on a 50-gene expression signature called PAM50. This classification distinguishes four subtypes of breast cancer based solely on ER status (Perou et al., 2000).

Regarding ER⁺ types of tumors, it's possible to distinguish:

- Luminal A: shows the activation of ESR1 that encodes for ER α , GATA3 (that maintains epithelial differentiation in breast tissues), and FOXA1 (that helps maintain the luminal phenotype and supports estrogen-driven transcription). The histology of this type of tumor is classical and lobular;
- Luminal B: is characterized by PIKCA (a kinase involved in cell growth), ESR1, ERBB2, and ERBB3 (which encodes mutually for HER2 and HER3) mutations. This type of tumor has a micropapillary and atypical lobular histology.

On the other hand, ER- subtypes were:

- Basal-like: characterized by BRCA and TP53 mutations, genetic instability, and a medullary-like histology with poorly differentiated cells;
- Human Epidermal growth factor Receptor 2 (HER2)-enriched: enriched for HER2, it shows GRB7 (Growth factor Receptor Bound protein 7) amplification and PIK3CA mutation.

As shown in **Figure 9**, this classification included normal-like and claudin-low types; however, studies indicate that the normal-like type is often an artefact due to the high proportion of non-neoplastic cells in the sample. The Claudin-low type, although shown in the figure, is not part of the classical PAM50 classification because these tumors require additional gene-expression signatures beyond PAM50 for classification and often overlap with metaplastic tumors (Hennessy et al., 2009).

Nowadays, clinical practice uses a classification based on the expression levels of the Estrogen Receptors (ER), the Progesterone Receptors (PR) and the Human Epidermal Growth Factor Receptor 2 (HER2).

- Luminal A-like: strong expressions of estrogen and progesterone receptors and negative expression of HER2. It's a low-grade tumor with a good prognosis;
- Luminal B-like HER2+: estrogen and progesterone receptors are present but at a lower rate than the luminal A-like type. The tumor has a good response to targeted therapies, and the prognosis is intermediate;
- Luminal B-like HER2-: as the luminal B-like type, the expression of estrogen receptors and progesterone receptors is present but lower than in the Luminal A-like type with a negative HER2. This tumor shows a lobular pleiomorphic histology with an intermediate prognosis;
- HER2-enriched (non-luminal): just like the triple negative type, the HER2-enriched type doesn't express the estrogen and progesterone receptors but has a positivity for HER2 receptors. The disease is aggressive but responds well to targeted therapies, with an intermediate prognosis;
- Triple negative: estrogen receptors, progesterone receptors, and HER2 are all negative. It is usually of a specific histologic type and the prognosis is poor except in some cases (Harbeck et al., 2019).

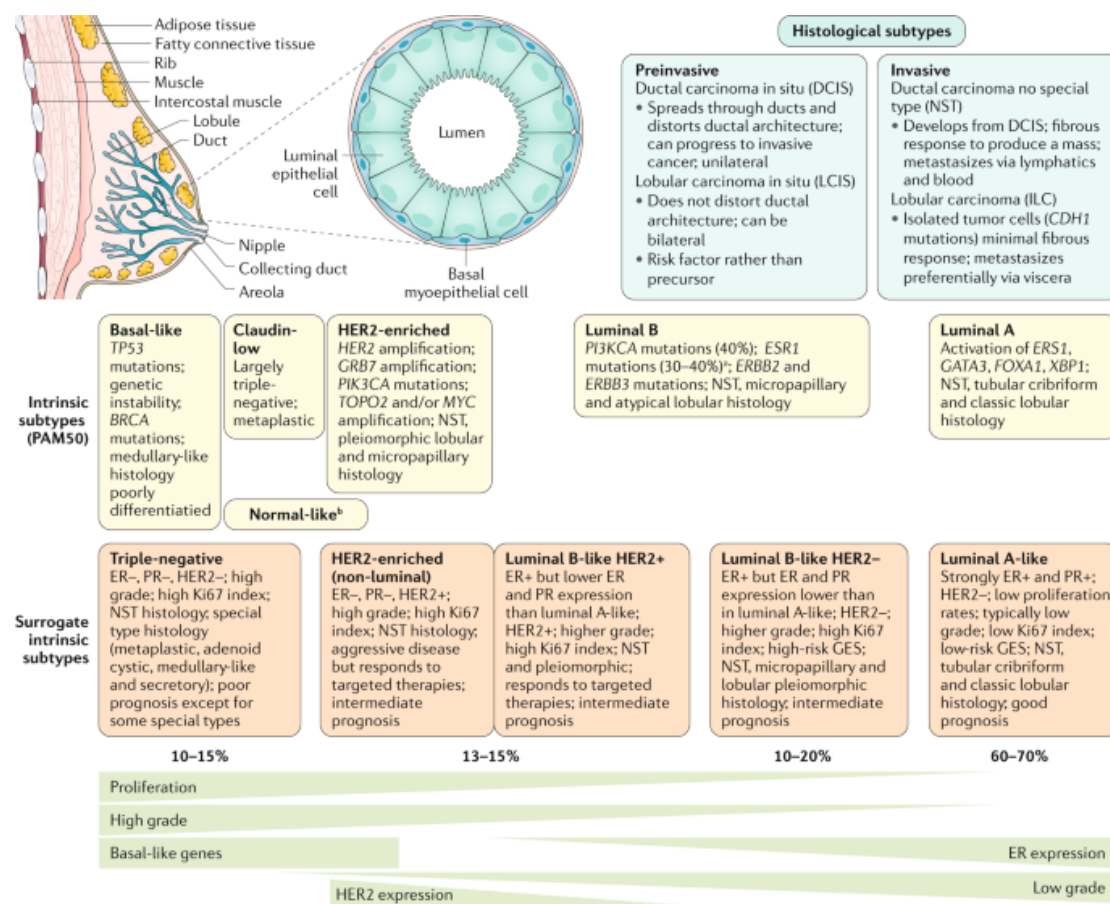


Figure 9. Breast cancer types and their histological and intrinsic classification - (Harbeck et al., 2019)

Despite these classifications, nowadays there is no unanimous agreement on the alignment between immunohistochemical classification and gene expression-based subtyping.

For example, in a study by Nielsen et al., all HER2+ tumors, including both hormone receptor positive (ER+/PR+) and negative (ER-/PR-) cases, were classified within a single HER2+ subclass (Nielsen et al., 2004). This grouping was supported by evidence showing that HER2 amplified breast cancers display comparable genomic alterations (“Comprehensive Molecular Portraits of Human Breast Tumours,” 2012) and clinical outcomes (Carey et al., 2006), largely independent of their hormonal receptor status.

Moreover, beyond the traditional classification of breast cancer based on histopathological features and receptor status, increasing evidence highlights the contribution of additional molecular and functional markers. These emerging biomarkers provide a more nuanced view of tumor heterogeneity, capturing dynamic biological programs that are not fully reflected by conventional classification schemes.

As an example, Cheang et al. proposed an extended immunohistochemical classification that incorporates Ki-67 alongside ER, PR, and HER2 to refine breast cancer subtyping. In hormone receptor-positive tumors (ER+/PR+), cases were stratified into prognostically distinct subgroups based on Ki-67 and HER2 expression. Specifically, ER+/PR+/HER2- tumors were further subdivided into Ki-67-low and Ki-67-high categories, with elevated Ki-67 levels being associated with poorer clinical outcomes irrespective of systemic treatment (Cheang et al., 2009). This observation is consistent with the cancer hallmark of increased tumor proliferation. Moreover, this approach allows intrinsic luminal B tumors to be further resolved into two subsets: ER+/PR+/HER2-Ki-67+ and ER+/PR+/HER2+Ki-67+. This underscores the importance of combining Ki-67 with ER, PR, and HER2 to better discriminate between biologically and clinically distinct hormone receptor-positive breast cancers.

In this context, claudin-low breast cancers have been characterized as a subset of triple-negative tumors (ER-/PR-/HER2-) distinguished by reduced expression of tight junction components, including claudins 3, 4, and 7, as well as the calcium-dependent adhesion molecule E-cadherin. This subtype accounts for approximately 7–14% of breast cancer cases. At the molecular level, claudin-low tumors are characterized by the enrichment of gene expression programs associated with EMT, stem-like properties, and tumor-initiating cells, alongside a marked depletion of epithelial traits and low expression of proliferation-related genes. Although some degree of responsiveness to chemotherapy has been reported, claudin-low breast cancers are generally associated with unfavorable clinical outcomes and reduced overall survival (Prat et al., 2010).

In addition to the conventional markers, interferon signaling has also been introduced as a defining feature of a recently identified subtype within triple-negative breast cancers. The overexpression of interferon-regulated genes characterizes this interferon-rich subgroup and accounts for approximately 10% of breast tumor cases (Z. Hu et al., 2006).

Also, the prognostic significance of BCL2 expression has been extensively explored in breast cancer. Tumors exhibiting moderate to high BCL2 positivity (BCL2+) are strongly associated with favorable pathological features, including low mitotic index, high histological differentiation, and absence of p53 expression or tumor necrosis. Clinically, patients with BCL2+ tumors tend to experience more favorable short-term outcomes, although long-term breast cancer-specific survival appears comparable to that of patients with BCL2- tumors (Joensuu et al., 1994).

Lastly, TP53 has also been included as an essential marker in BC prognosis and therapy responsiveness. Indeed, it's known that among all breast cancer types, PR- also negative for TP53 expression are the ones associated with the worst prognosis (Olivier et al., 2006). Accumulating

evidence indicates that TP53 dysfunction contributes to the development of anti-estrogen resistance in ER+ tumors (Rahko et al., 2006), while ER- tumors with reduced or absent TP53 expression may exhibit poor response to chemotherapy (Lønning et al., 2007).

In summary, despite the identification of numerous novel biomarkers and the refinement of classification strategies, the intrinsic heterogeneity of breast cancer remains evident. The molecular and phenotypic subtypes described, ranging from ER/PR/HER2 to the aforementioned other markers, often overlap and are not strictly mutually exclusive. This complexity underscores that variations in clinical outcomes are primarily driven by the activation of fundamental cancer hallmarks, rather than by individual biomarkers alone, highlighting the challenges in achieving an entirely unambiguous breast cancer classification.

Breast cancer therapy

Over the past decades, advances in the molecular classification of BC have profoundly transformed its therapeutic landscape, leading to a progressive expansion of available treatment options beyond conventional approaches. The identification of distinct biological subtypes has enabled the development of increasingly effective therapies, significantly improving patient survival and quality of life. With new therapies, treatment strategies are no longer based solely on tumor stage, but on molecular features that guide therapeutic decision-making.

Indeed, local treatment strategies have evolved towards minimally invasive approaches, with breast-conserving surgery, and the excision of lymph nodes is now necessary only when the Sentinel Lymph Node Biopsy (SLNB) is positive. Moreover, when patients present positive lymph nodes, radiation therapy is conventionally used as a post-surgical treatment. The benefits of radiotherapy are well recognized, but several related risks have been observed, including lymphedema and increased heart and lung toxicity (Poortmans et al., 2015; Thorsen et al., 2016).

The integration of neoadjuvant therapies has further reduced surgical morbidity by enabling tumor downstaging and allowing more conservative interventions, without compromising oncological outcomes (Xiong et al., 2025; Wang & Wu, 2023).

Despite the new therapies' achievements, systemic therapies remain a fundamental step in BC management, administered as chemotherapy in neoadjuvant, adjuvant, or metastatic settings depending on disease characteristics. Standard regimens based on taxanes and anthracyclines, with the addition of platinum compounds in selected aggressive subtypes such as triple-negative BC, have demonstrated improved response rates and optimal disease control, although overall survival benefits remain variable (von Minckwitz et al., 2014; Yu et al., 2020). Notably, the use of molecular profiling

and multigene assays has become essential for identifying patients most likely to benefit from chemotherapy, particularly in ER+, HER2+, and triple-negative tumors (Wang & Wu, 2023).

Endocrine therapy is a clear example of personalized treatment and must be directly linked to the study of hormone receptor expression (Du et al., 2021). In fact, those with high expression of both receptors are highly responsive to hormonal therapies, whereas tumors with low expression of one or both receptors show only partial endocrine sensitivity (Goldhirsch et al., 2007). Overall, assessing ER and PR together provides a more accurate differentiation of breast cancer subtypes than evaluating either receptor in isolation. Therefore, in patients with hormone receptor positivity (ER or PR staining $\geq 1\%$), both aromatase inhibitors and tamoxifen are optimal treatment strategies. The selection between aromatase inhibitors and tamoxifen is guided by receptor status, menopausal state, and patient-specific risk factors (Dowsett et al., 2010).

HER2 positivity is associated with relative resistance to endocrine therapies, reflecting the inverse correlation between HER2 and ER/PR expression levels (Qu  nel et al., 1995). In ER+ or PR+ tumors, HER2 overexpression can limit the effectiveness of hormone therapy, likely due to reduced receptor expression or increased proliferative activity rather than HER2 itself (Konecny et al., 2003). Among the main subtypes, ER+/PR+/HER2- tumors show the best prognosis and hormone responsiveness, whereas ER-/PR-/HER2+ and ER-/PR-/HER2- tumors are generally aggressive and poorly responsive to endocrine treatments. This clinical limitation has been successfully addressed by the development of HER2-targeted therapies. Indeed, the introduction of HER2-targeted agents, including Trastuzumab, Pertuzumab, and Lapatinib, used combined to each other or to other drugs, has dramatically improved outcomes in HER2-positive disease, both in early and metastatic settings, establishing targeted therapy as a fundamental component of BC treatment (Fernandez-Martinez et al., 2020; Verma et al., 2012). In particular, recent data have shown a significant improvement in pathological complete response (pCR) when Pertuzumab is added to the combination of Trastuzumab and Docetaxel (a taxane) (Gianni et al., 2012). Indeed, Docetaxel, Trastuzumab, and Pertuzumab are considered the standard anti-HER2 therapy in a positive and metastatic HER2 breast cancer.

More recently, immunotherapy has emerged as a promising therapeutic option, particularly in triple-negative BC also characterized by Programmed Death-Ligand 1 (PD-L1) positivity. Immune checkpoint inhibitors such as Pembrolizumab and Atezolizumab, especially when combined with chemotherapy or HER2-targeted agents, have shown encouraging improvements in progression-free and overall survival, although long-term follow-up is still required to define their long-term efficacy (Schmid et al., 2020; Cortes et al., 2020; Loi et al., 2019; Emens et al., 2020; Nanda et al., 2020).

In parallel, the use of gene therapy has gained importance in recent years, including strategies such as microRNA, DNA or RNA vaccination, gene editing, and targeting transcription factors (Dastjerdi et al., 2022).

In particular, a phase I clinical trial tested the safety and efficacy of targeting the human HER2 gene promoter, demonstrating the safest approach and HER2 expression in up to 90% of patients. Promising results were also obtained by inhibiting cell development and proliferation by using microRNA in anti-cancer therapy, and it's believed it could play an essential role in the treatment of breast cancer in the future (X. Lin et al., 2017).

EMT in breast cancer: biological features and therapeutic consequences

Given the marked heterogeneity and aggressive potential of breast cancer, together with the challenges associated with its targeted treatment, understanding the cellular and molecular mechanisms underlying tumor progression is crucial. Among these, the EMT has emerged as a key process contributing to invasion, metastasis, and therapy resistance in breast cancer.

Beyond its well-established role in tumor progression, EMT also reflects the intrinsic heterogeneity of breast cancer, characterized by high plasticity that significantly influences patient prognosis and therapeutic response. Indeed, as observed in other cancer types, breast tumor cells frequently adopt a hybrid EMT state. Experimental evidence shows that cells undergoing hybrid EMT, when transplanted into mice, are capable of initiating tumors and display stem-like properties that are not always found in cells with fully epithelial or fully mesenchymal phenotypes. In the same study it was demonstrated that driving BC cells toward a fully mesenchymal state through *Zeb1* expression and activation of noncanonical Wnt signaling produced a cell population with limited tumor-initiating capacity. In contrast, cells exhibiting high levels of *Snail1*, together with activation of the canonical Wnt pathway, adopted a hybrid EMT phenotype and showed enhanced tumorigenic potential. These findings suggest that tumor-initiating ability in breast cancer is closely associated with hybrid EMT, driven by *Snail1*, whereas the acquisition of a fully mesenchymal phenotype via *Zeb1* correlates with reduced stem-like properties (Kröger et al., 2019). In fact, the increased plasticity of hybrid EMT cells enables dynamic transitions along the epithelial–mesenchymal spectrum useful to adapt to diverse microenvironmental condition at secondary sites (Pastushenko & Blanpain, 2019). For instance, as reported by Harper et al., in Her2 positive BC, a hybrid cell population with high levels of *Twist1* and cytokeratins 8 and 18, but low E-cadherin, was able to disseminate at early stages of tumor progression (Harper et al., 2016).

Moreover, Yamashita et al. also demonstrated a paradox in the expression of epithelial and mesenchymal markers in breast cancer. Indeed, their study showed the correlation between high levels of E-cadherin and Vimentin with a poorer prognosis. As shown in **Figure 10**, patients co-expressing E-cadherin and Vimentin had a 10-year RFS (**Figure 10A**) and OS (**Figure 10B**) of 64%, whereas patients with other expression patterns showed 78% and 83%, respectively. Further investigations comparing E-cadherin and Vimentin expression in primary tumor and metastatic lymph nodes revealed that patients with high levels of both markers in metastatic lymph nodes had 5-year RFS and OS of 50%. Conversely, patients with different expression profiles for the two proteins showed a 5-year RFS of 69% and a 5-year OS of 82% (Yamashita et al., 2018).

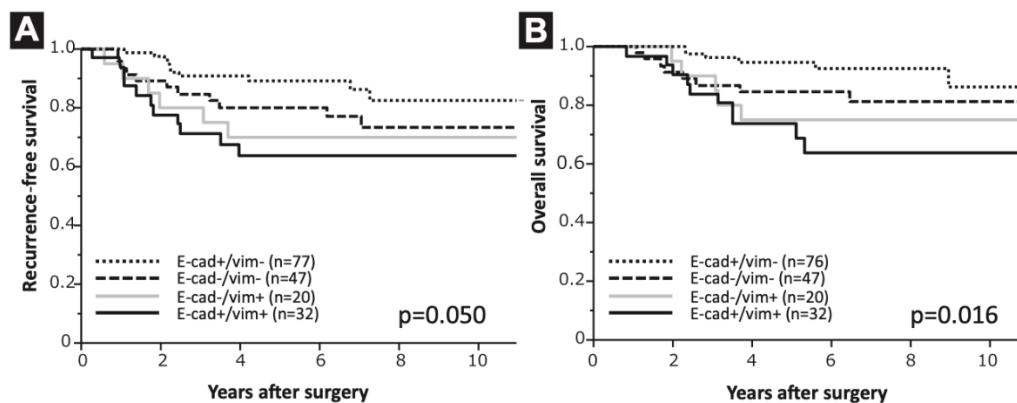


Figure 10. Kaplan-Meier curves for RFS (A) and OS (B) referring to the expression of E-cadherin and Vimentin in the primary tumor. Patients were divided into four groups based on the expression of the two proteins - (Yamashita et al., 2018)

Since EMT is a key process in BC progression together with its pronounced heterogeneity and plasticity, recent therapeutic strategies have increasingly focused on targeting EMT and its reverse program. These approaches aim to reprogram cancer cells from a more invasive, therapy-resistant mesenchymal-like state toward a more epithelial, therapy-sensitive phenotype, thereby improving treatment efficacy and potentially limiting metastatic progression.

Among the most well-characterized of this therapeutic concept is eribulin mesylate, a microtubule inhibitor indicated in metastatic breast cancer. Beyond its antimetabolic effects, eribulin has been shown to induce MET, increasing epithelial markers such as E-cadherin and reducing mesenchymal markers like Vimentin and Zeb1. Preclinical and patient-derived models confirm that MET induction by eribulin is associated with reduced metastatic potential and resensitize tumors to other therapies. (T. Yoshida et al., 2014; LIM et al., 2020).

Additional strategies under investigation include histone deacetylase inhibitors (HDACis), which can restore epithelial-associated microRNAs such as miR-203 and reverse EMT-driven drug resistance (Meidhof et al., 2015).

Moreover, direct inhibition of EMT transcription factors such as Snail, Zeb1, or Slug using small molecules or siRNA-based approaches (S.-H. Lee et al., 2010; Vistain et al., 2015; Takemura et al., 2025) and repurposed agents like metformin and salinomycin, which modulate metabolism, inhibit EMT, and reduce stem-like resistance (K. Cheng & Hao, 2016; Y. Zhou et al., 2015).

Also targeted therapies that inhibit oncogenic pathways driving EMT can also promote MET and restore chemosensitivity. An example are c-MET inhibitors, such as Crizotinib (Cañadas et al., 2014).

Furthermore, miRNA-based approach, particularly related to the miR-200 family, function in a feedback loop with Zeb1/Zeb2 to restore the epithelial phenotype and decrease cell motility (Hill et al., 2013).

Finally, multi-targeted epigenetic therapy (MTET) combines polyphenols, hormone modulators, kinase inhibitors, and other agents to revert partial EMT toward a fully epithelial state, demonstrating promising tumor responses without compromising quality of life (Nezami et al., 2015).

The role of Vimentin in BC treatment is also crucial: recent studies have shown that patients with high Vimentin levels are more responsive to chemotherapy than those with lower levels (Dine et al., 2016). Conversely, in patients with HER2+ BC treated with Trastuzumab, high levels of Vimentin are associated with resistance to therapy. In particular, high levels of Vimentin and N-cadherin are observed in Trastuzumab-resistant cells, suggesting that this resistance may be associated with EMT (Burnett et al., 2015).

Taken together, the increasing complexity and diversity of therapeutic options in breast cancer underscores the critical importance of patient stratification and precision medicine. In this context, understanding both molecular and phenotypic tumor heterogeneity is essential to guide treatment selection, maximize therapeutic efficacy, and minimize unnecessary toxicity. Among the factors contributing to this heterogeneity, the epithelial-to-mesenchymal transition plays a pivotal role. As said before, EMT is often associated with the modulation of epithelial and mesenchymal markers. These changes not only correlate with enhanced invasion, metastasis, and therapy resistance, but also exemplify how phenotypic plasticity can influence patient response to treatment, further highlighting the need for precise biomarker-driven therapeutic strategies.

AIM

The epithelial-to-mesenchymal transition (EMT) is a dynamic cellular program involved in embryonic development, wound healing, and tumorigenesis. In breast cancer (BC), EMT plays a central role in tumor invasion and metastasis, which account for the majority of BC-related deaths. Through a cascade of biological events, EMT enables cancer cells to lose epithelial characteristics and acquire migratory and invasive properties, ultimately facilitating the dissemination and colonization of secondary sites. Consequently, understanding the complexity of EMT and identifying EMT-associated gene signatures and biomarkers could provide useful prognostic and predictive tools in breast cancer and other tumor types, allowing for the distinction between easily treatable tumors and more aggressive ones, with a focus on personalized therapies.

Despite extensive research, no clinically approved gene signatures specifically focused on EMT-related genes are currently available. This limitation is particularly relevant in early disease stages, such as ductal carcinoma in situ (DCIS), where the identification of tumors with a high risk of recurrence or progression to invasive or metastatic disease would significantly influence therapeutic decision-making, as shown by Knudsen et al., who reported that EMT-associated genes are consistently upregulated in invasive breast cancer compared to pure DCIS (Knudsen et al., 2012). Similarly, a meta-analysis by Gröger et al. identified 365 EMT-related genes across multiple datasets, of which 130 were differentially expressed in breast cancer cells. These genes were mainly associated with pathways involved in adhesion, migration, differentiation, proliferation, angiogenesis, wound healing, and metabolism (Gröger et al., 2012).

Taken together, these findings highlight the intrinsic heterogeneity of both EMT and breast cancer and support the need for a personalized medicine approach, in which EMT-related features are evaluated at the individual patient level.

For these reasons, this thesis aimed to investigate the contribution of EMT to breast cancer heterogeneity by analyzing key EMT markers in patient-derived breast cancer tissues and serum samples and by assessing the effects of breast cancer patient sera on EMT-related phenotypes, using both tumor and normal cell lines in monolayer and in three-dimensional systems. Furthermore, this study aimed to extend the analysis beyond individual markers by applying bioinformatic approaches to define EMT-related gene signatures. By integrating experimental and *in silico* analyses, this work seeks to identify EMT-associated features that may support patient-specific prognostic stratification and contribute to the development of personalized therapeutic strategies in breast cancer.

MATERIALS AND METHODS

Experimental procedures

Tissue and sera samples from breast cancer patients and healthy patients

This study was conducted on 95 breast tissue samples and 48 serum samples taken from patients who suffered breast cancer, hospitalized at “La Maddalena Hospital” of Palermo. Healthy sera were collected from non-pathological donors.

Tumor specimens, from patients with BC who underwent surgery for tumor resection, containing at least 80% tumor cells, were stored at -80°C until protein extraction was performed. The frozen tissues were homogenized in an ice bath with 50 mM Tris-HCl pH 7.5, 0.003% penicillin, 0.005% streptomycin, and then incubated overnight at 4°C with rotation. Tissue lysates were centrifuged several times at 10.000 rpm for 20 min to remove cellular debris, as previously reported. The protein content in the tissue extracts was quantified using the Bradford assay (Bradford, 1976).

The study was conducted in accordance with all required ethical standards, with the informed consent of patients and with the approval of the Institutional Review Board (N° 515/2008, May 13, 2008) from “La Maddalena Hospital”.

Electrophoresis SDS-PAGE and Western Blotting

Aliquots containing 20 μg of cell lysates from BC tissues were separated by electrophoresis on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels, under reducing conditions. After electrophoresis, performed at 150 V, Western Blot was performed using a nitrocellulose membrane for 1 h at 100V and stained with Ponceau S (Sigma-Aldrich, St. Louis, MO, USA). Membranes were blocked with 5% milk in T-TBS solution 0,05% Tween-20 for 1 h at room temperature and then incubated overnight at 4°C with a mouse monoclonal antibody for Vimentin (Santa Cruz Biotechnology, Dallas, TX, USA - sc-6260), E-cadherin (Cell Signaling Technology, Danvers, MA, USA - #14472), Cytokeratin-8 (Santa Cruz Biotechnology, Dallas, TX, USA - sc-8020), Cytokeratin-18 (Santa Cruz Bio-technology, Dallas, TX, USA - sc-51582), alfa-Smooth Muscle Actin (Sigma-Aldrich, St. Louis, MO, USA- #A5228) and β -Actin (Santa Cruz Biotechnology, Dallas, TX, USA - sc-47778). Following incubation with the mouse peroxidase-linked antibody (Promega, Fitchburg, WI, USA - W402B or Invitrogen, Waltham, MA, USA - #31430), the reaction was detected using the ECL detection system on a ChemiDoc™ MP System (Bio-Rad, Milano, Italy). Antibody specificity was verified by using only the secondary antibody as a negative control and by confirming the presence of the expected band as reported in the datasheet. Proper protein loading was ascertained through Ponceau Red staining and β -Actin immunoblotting. Band intensities were quantified using *ImageJ*

software. Western blot quantification was normalized against the β -Actin signal. Correlations between epithelial and mesenchymal markers and clinicopathological features were evaluated using Pearson's test. To reduce technical variability, all patient samples were processed and analyzed in parallel blots.

Zymography

Zymography, based on the application of SDS-PAGE, enables the detection of enzymatic activities within a sample, such as those of matrix metalloproteinases MMP-2 and MMP-9. The method is based on the incorporation of a specific substrate into the polyacrylamide gel matrix, allowing enzymes to exert their activity after electrophoretic separation (Troeberg & Nagase, 2003). In this study, gels were prepared with a 7.5% acrylamide concentration and supplemented with 0.1% final concentration of gelatin, as the substrate. 18 μ g of total protein for BC tissues, and 10 μ l of each serum sample, previously diluted 1:25, were loaded on the gels, allowing optimal separation. After electrophoresis, performed at 150 V for approximately 1 hour, the gels were washed for 1 hour in a renaturation buffer containing 50 mM Tris-HCl pH 7.5 and 2.5% Triton X-100 and then incubated with an activation buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 5 mM CaCl₂ for 18 h at 37°C to allow activation of the gelatinases. Finally, the gels were stained with 0.2% Coomassie Brilliant Blue G-250 in 40% methanol and 10% acetic acid and destained in 7% methanol and 5% acetic acid. Areas of enzymatic activity were detected as clear bands of lysis on the blue background of the stained gelatin. Band intensity was quantified using *ImageJ* software.

Cell cultures

Two different cell lines were used for this study: triple-negative MDA-MB-231 breast cancer cells and MCF-10A, an immortalized mammary epithelial cell line. MDA-MB-231 cells were maintained in culture with DMEM (Dulbecco's Modified Eagle Medium, Corning, New York, USA or Gibco, Waltham, MA, USA) enriched with 10% Fetal Bovine Serum (FBS) (Sial, Rome, Italy), previously heat-inactivated, and 1% antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin) (Corning, New York, USA). MCF-10A were maintained in culture with DMEM-F12 (Gibco, Waltham, MA, USA) enriched with 5% Horse Serum (HS) (Linus), 20 ng/ml of human Epithelial Growth Factor (hEGF, Sigma Aldrich, St. Louis, MO, USA), 0.05 mg/ml of Insulin (Sigma Aldrich, St. Louis, MO, USA), 500 ng/ml Hydrocortisone (Merck KGaA, Darmstadt, Germany) and 1% antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin) (Corning, New York, USA). Both cell lines were incubated at 37 °C in a humidified atmosphere with 5% CO₂.

Wound healing assay

To evaluate cell migration, MDA-MB-231 cells were plated in a 48-well plate, grown until confluence, and starved for 15h to synchronize the cell population. A sterile 200 μ L pipette tip was used to create a scratch in the cell layer. After wound generation, cells were treated with 1% of Fetal Bovine Serum, Horse Serum, or breast cancer sera. Imaging was performed under a phase-contrast microscope at 40x magnification (Olympus IX70; Olympus Corporation, Tokyo, Japan) at 0h, 6h, and 24 h after wound generation to evaluate wound closure (**Figure 11**). The percentage of wound closure was calculated as the wound area at a given time compared to the initial wound surface. For each treatment, experiments were performed in triplicate.

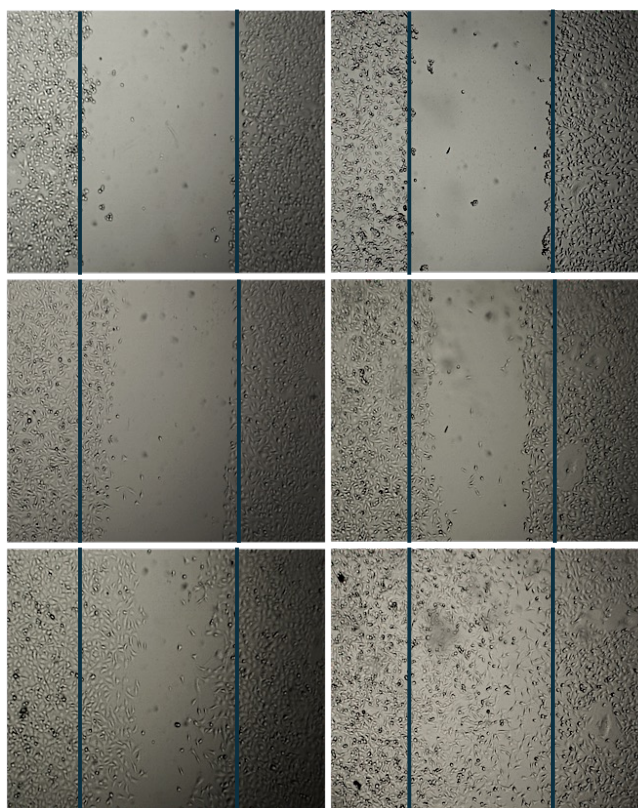


Figure 11. Representative wound-healing assay of MDA-MB-231 treated with FBS (left), BC serum (right), and observed at three different time stages (0h, 6h, and 24h).

MTT Viability Assay

The proliferative activity of cells treated with the analyzed sera was determined using the MTT assay. To perform the MTT assay, both cell lines were seeded in 96-well multiwell plates (Sarstedt, Nümbrecht, Germany, or Corning Costar®, NY, USA) at a concentration of 10,000 cells/well in 200 μ L of complete DMEM medium containing 1% treatment. For each treatment, experiments were performed in triplicate to obtain statistically significant results. After 48 hours of treatment, the

medium was removed from the wells. After three washes with PBS, 100 μ L of complete DMEM medium and 20 μ L of Thiazolyl Blue Tetrazolium Bromide (MTT) (Merck, Darmstadt, Germany) (5 mg mL⁻¹, dissolved in PBS: phosphate-buffered saline) were added. The plate was then incubated in the dark for 2 hours at 37 °C in a humidified atmosphere with 5% CO₂.

After removing the medium containing the MTT, each well was washed three times using 100 μ L of PBS. Following washing, 100 μ L of DMSO were added to each well to promote cell lysis and solubilize the formazan crystals produced, resulting in a purple color. Plates were shaken for 10 minutes at room temperature in the dark. Then, the color intensity, evaluated as absorbance, was measured at a wavelength of 570 nm using a 96-well plate reader (Spark® 20M Tecan Trading AG, Switzerland). After subtracting the absorbance values recorded in the blank, the percentage of cell viability of the treated cells was calculated relative to the untreated control cells, which were assumed to have 100% cell viability.

MDA-MB-231 and MCF-10A derived tumorspheres

MDA-MB-231 and MCF-10A tumorspheres were cultured using Ultra-Low Attachment 24-well plates (Corning Costar®, NY, USA) in Gibco Dulbecco's Modified Eagle Medium: DMEM/F-12 (Gibco, Waltham, MA, USA) supplemented with 2% B27 Supplement (Life Technologies, Waltham, MA, USA), 1% GlutaMAX™-100× (Gibco, Waltham, MA, USA), 20 ng/ml of human Epidermal Growth Factor (hEGF, Sigma Aldrich, St. Louis, MO, USA) and 10 ng/ml of human Fibroblast Growth Factor-Basic (hBFGF, Sigma Aldrich, St. Louis, MO, USA). In both cases, 50,000 cells were seeded per well. Images were obtained using a confocal microscope Eclipse TS100 (Nikon, New York, NY, USA).

Quantitative Real-Time PCR (RT-qPCR)

RNA was extracted using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. RT-qPCR was performed using an NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal). The qPCR was completed using LightCycler® 480 SYBR® Green I Master (Roche Diagnostics GmbH, Basel, Switzerland) and LightCycler® 480 Multiwell Plate 96, white (Roche Diagnostics GmbH, Basel, Switzerland). All the assays were performed in duplicate. As a housekeeping control, the HPRT gene was used. Data analysis was performed by using Microsoft Excel.

Bioinformatic Analysis

The Tumor IMmune Estimation Resource database analysis

As a comprehensive database for the analysis and visualization of relationship between immune infiltrate levels and several variables, the Tumor IMmune Estimation Resource (TIMER) database (<https://cistrome.shinyapps.io/timer/>) was applied to determine the relationship between EMT markers expression levels and immune infiltration based on the CIBERSORT algorithm. In particular, the focus was on tumor-infiltrating immune cells, including B cells, CD4⁺ T cells, CD8⁺ T cells, neutrophils, macrophages, dendritic cells and cancer associated fibroblasts.

EMTome

EMTome (<http://www.emtome.org>, accessed June 20, 2024) is a resource that provides gene lists associated with both EMT and MET across different cancer types. EMTome is organized into four main modules: EMT core signature, EMT-related gene retrieval, EMT-related gene interactome, and EMT-signature score. In this study, the EMT core signature module was employed to identify a gene set specifically involved in the EMT of BC. This module enables the exploration of cancer type-specific EMT signatures and their associated genes or gene products. EMT signatures are available in tabular format, with genes hyperlinked to external databases (e.g., NCBI GeneCards) for detailed annotation. Additionally, relevant gene sets can be downloaded directly from the platform in text or GMT file formats (Vasaikar et al., 2021).

UALCAN

Differential expression of EMT-related genes between breast cancer (BC) and normal tissues was evaluated using UALCAN, a freely accessible online platform for in silico analyses (<https://ualcan.path.uab.edu/>, accessed June 20, 2024). For the present study, data from the The Cancer Genome Atlas (TCGA) database were analyzed, including a total of 1211 patients, comprising 1,097 BC specimens and 114 normal breast tissues. The expression levels of queried genes are normalized as transcripts per million (TPM). For each gene of interest, UALCAN provides graphical representations of expression levels in normal and cancer tissues as box-and-whisker plots, displaying minimum, first quartile, median, third quartile, and maximum values. Welch's T-test estimated the significance of differences in expression levels between normal and primary tumors, and a p value ≤ 0.05 was considered as significant (Chandrashekar et al., 2017).

Kaplan-Meier plotter

The prognostic significance of mRNA expression of selected EMT-related genes was evaluated using the Kaplan-Meier plotter online tool (<https://kmplot.com/analysis/>, accessed June 20, 2024), which correlates gene expression levels with patient survival outcomes, including distant metastasis-free survival (DMFS), overall survival (OS), and relapse-free survival (RFS). Patients were stratified into high- and low-expression groups based on gene expression levels, using the optimal cutoff option and the JetSet probe selection. When the optimal cut-off is selected, all possible cutoff values between the lower and upper quartiles are computed, and the best performing threshold is used as cut-off. For each analysis, the platform reports the corresponding p-value and false discovery rate (FDR). Moreover, Jetset selects the optimal probe set for each gene using a scoring method established to assess each probe set for specificity, coverage and degradation resistance (Lánczky & Györffy, 2021).

STRING

Biological connectivity between the EMT-selected genes was explored by using STRING (<https://string-db.org/>, accessed June 20, 2024). This online database allows the study of direct (physical), and indirect (functional) protein-protein interactions derived from different sources (Genomic Context Predictions, High-throughput Lab Experiments, (Conserved) Co-Expression, Automated Textmining, Previous Knowledge in Databases, Coverage) (Szklarczyk et al., 2015).

bcGenExMiner and GOBO

The correlation between the mRNA expression of selected EMT-related genes and different clinical-pathological parameters (such as ER/PR/HER receptor status and lymph node involvement) was assessed using the Breast cancer Gene-Expression Miner v4.5 database (bcGenExMiner v4.5, <http://bcgenex.ico.unicancer.fr>, accessed June 20, 2024), an on-line statistical mining tool for annotated breast cancer transcriptomic data, including DNA microarrays ($n = 11552$) and RNA-seq ($n = 5023$). bcGenExMiner enables the exploration of gene expression, prognostic, and correlation analyses, providing various types of plots (Jézéquel et al., 2012). The association between selected EMT genes and tumor grading (G1/G2/G3) was evaluated using the Gene expression-based Outcome for Breast Cancer Online (GOBO) database (accessed June 20, 2024). GOBO is based on a dataset of 1881 breast cancer samples generated on Affymetrix U133A microarrays (Ringnér et al., 2011).

RESULTS AND DISCUSSION

Proteomic analysis of Vimentin, α -SMA, Cytokeratin-18, and E-cadherin in BC tissues

As previously stated, the EMT contributes to nearly all the hallmarks of cancer, and its role in tumorigenesis has been demonstrated in various cancers, including breast cancer (BC). Despite its complexity, due to context-dependent EMT mechanisms, a comprehensive understanding of this process remains challenging, especially in patient tissues. To investigate EMT-related features in breast cancer tissues, the expression levels of canonical EMT markers, including epithelial markers (Cytokeratin-18 and E-cadherin) and mesenchymal markers (Vimentin and α -SMA), were analyzed by western blotting in a cohort of 95 BC samples (**Figures 12-13-14-15**). Clinical and pathological characteristics of the enrolled patients, including tumor grade and histological subtype, are summarized in **Supplementary Table 1**.

The Cytokeratin-18 antibody (**Figure 12**) revealed a predominant immunoreactive band at the expected molecular weight (MW) of approximately 42 kDa, along with additional bands ranging from 35 to 55 kDa, consistent with the presence of different isoforms and/or post-translationally modified forms of the protein.

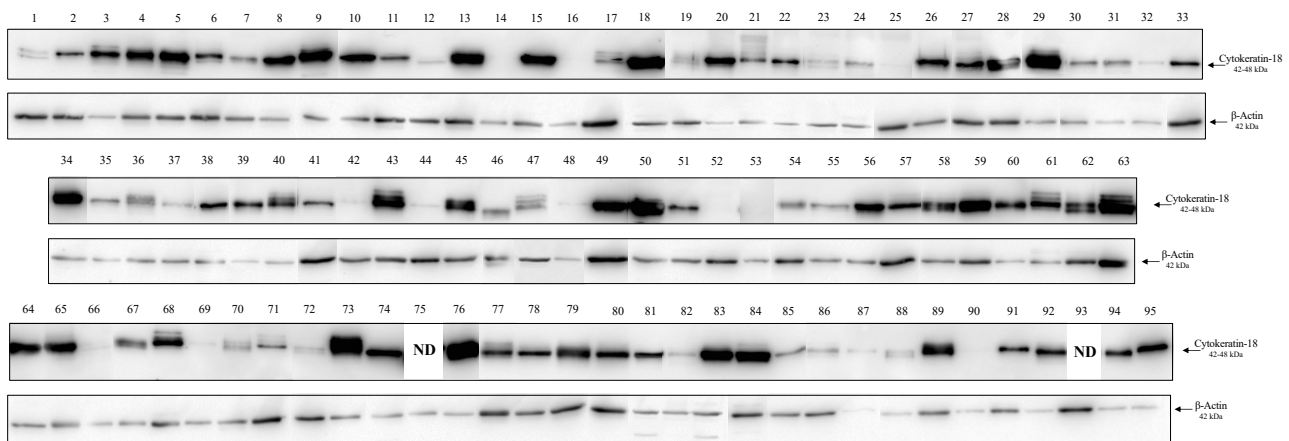


Figure 12. Western blot analysis performed on 95 BC tissue protein extracts to evaluate Cytokeratin-18 (42-48 kDa) expression levels. β -actin (42 kDa) was used as an internal control. ND = not detected

Analysis of E-cadherin expression (**Figure 13**) revealed two main immunoreactive bands: a higher MW band of approximately 110 kDa, corresponding to the precursor form of E-cadherin, and a lower MW band of approximately 90 kDa, corresponding to the mature protein form.

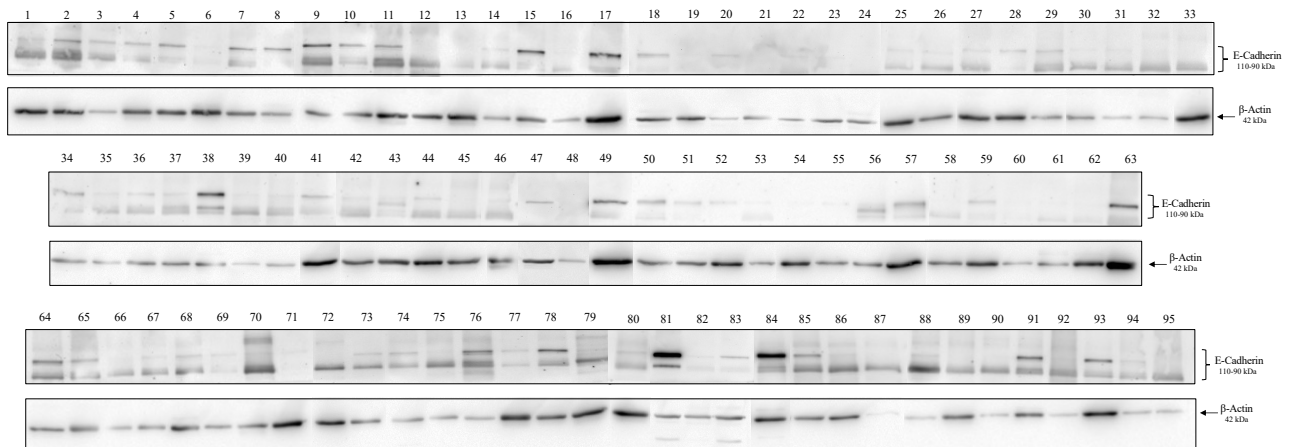


Figure 13. Western blot analysis performed on 95 BC tissue protein extracts to evaluate E-cadherin (110-90 kDa) expression levels. β -actin (42 kDa) was used as an internal control

The α -SMA antibody (**Figure 14**) detected a single band at the expected MW of approximately 42 kDa.

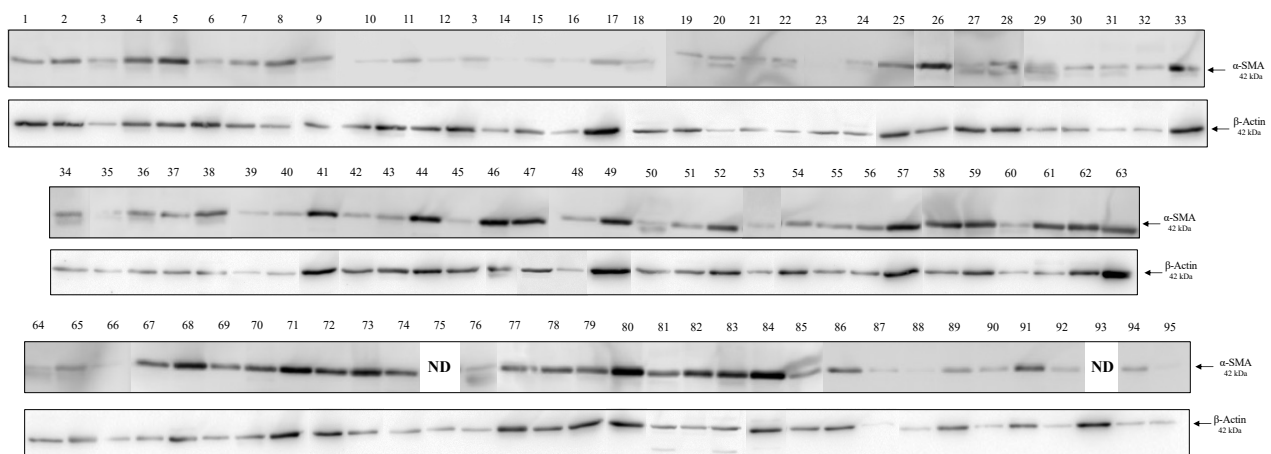


Figure 14. Western blot analysis performed on 95 BC tissue protein extracts to evaluate α -SMA (42 kDa) expression levels. β -actin (42 kDa) was used as an internal control. ND = not detected

Vimentin expression (**Figure 15**) was characterized by two major bands at approximately 50 and 40 kDa, together with several minor bands ranging from 38 to 55 kDa, likely representing different isoforms and/or proteolytic products. Proteolytic processing of Vimentin and Cytokeratin-18 has been reported during apoptosis (Schutte et al., 2004; Davis et al., 2019; Byun et al., 2001), while Vimentin isoforms have been linked to disease specificity in other cancers, such as renal carcinoma (Satelli & Li, 2011).

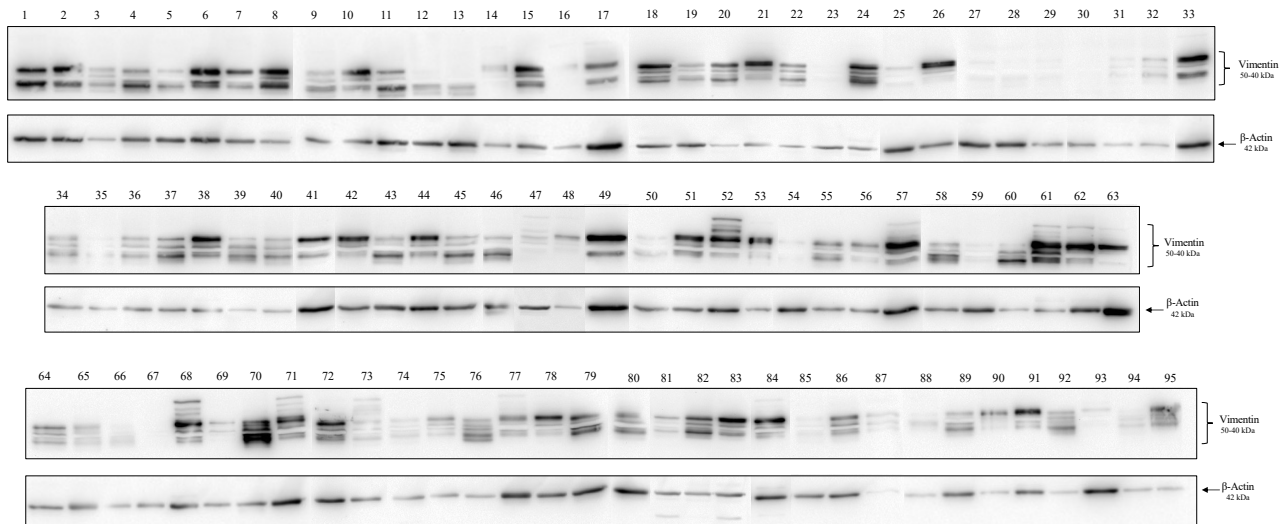


Figure 15. Western blot analysis performed on 95 BC tissue protein extracts to evaluate Vimentin (50-40 kDa) expression levels. β -actin (42 kDa) was used as an internal control

Interestingly, a marked inter-sample heterogeneity in the expression of all analyzed markers was observed, both qualitatively, in terms of isoform patterns, and quantitatively, in terms of band intensities (**Figure 16**). Given the intrinsic heterogeneity of BC tissues and the variable proportions of neoplastic and stromal components, protein expression levels, quantified using ImageJ software, were normalized to β -Actin (ACTB) expression in each tissue extract to allow a more objective comparison among samples.

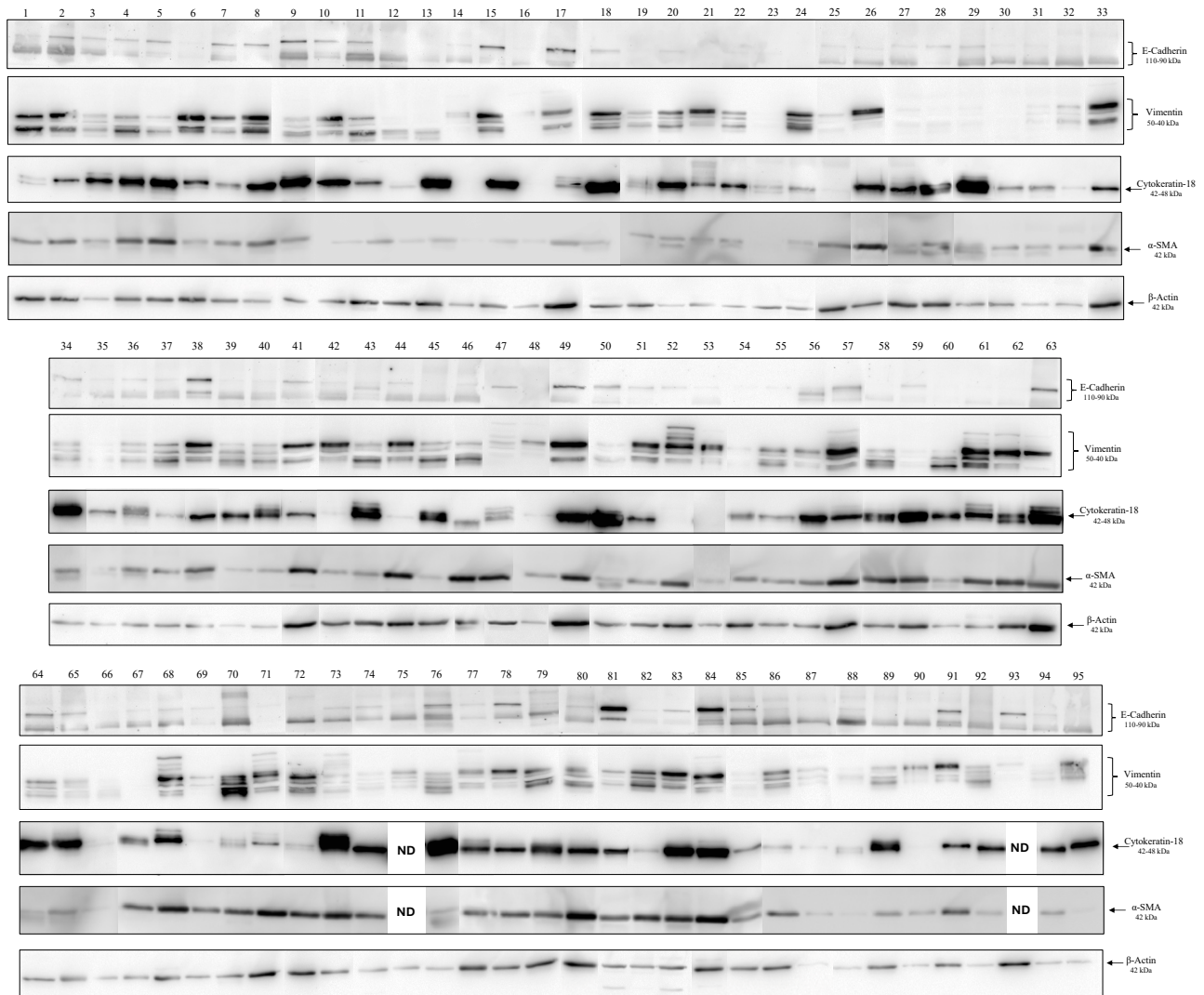


Figure 16. Western blot analysis performed on 95 BC tissue protein extracts to evaluate E-cadherin (110-90 kDa), Vimentin (50-40 kDa), Cytokeratin-18 (42-48 kDa), and α -SMA (42 kDa) expression levels. β -actin (42 kDa) was used as an internal control. ND = not detected

Overall, quantitative analysis of the immunoreactive bands revealed interesting positive correlations between Vimentin and E-cadherin expression levels (**Figure 17A**) and between Vimentin and Cytokeratin-18 (**Figure 17B**). A positive correlation was also found between α -SMA and E-cadherin (**Figure 17C**). However, no correlation was observed between the epithelial markers themselves or between the mesenchymal markers.

These results, which are unexpected when interpreted according to the classical EMT model, suggest that in BC cells, both epithelial and mesenchymal phenotypes coexist within the tumor mass and reflect the activation of multiple, context-dependent mechanisms.

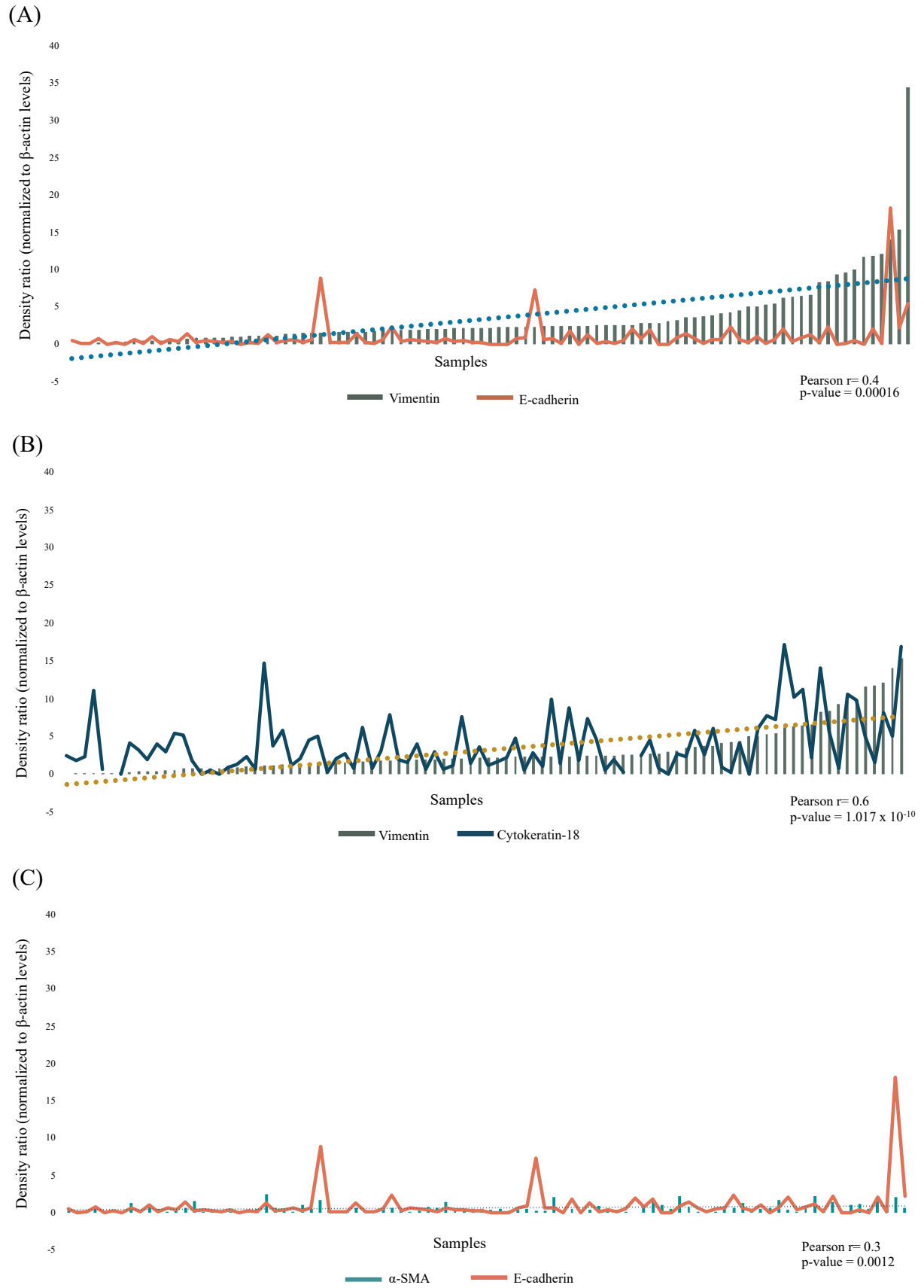


Figure 17. Histogram of E-cadherin & Vimentin (A) and Vimentin & Cytokeratin-18 (B), and α -SMA & E-cadherin (C) expression levels, normalized for β -actin levels, in the cohort of 95 BC tissues analyzed (each lane corresponds to a BC patient). Patients were sorted by increased levels of Vimentin expression. The dashed line shows the trend of the positive correlation between the selected markers

To evaluate the potential prevalence of epithelial or mesenchymal phenotypes among BC samples, the relationship between Vimentin and E-cadherin expression levels was investigated. A Vimentin/E-cadherin ratio <1 would indicate a predominantly epithelial phenotype, while a higher ratio would suggest a predominantly mesenchymal phenotype. Interestingly, only a small fraction of samples (13/95, corresponding to 14% of patients) exhibited a Vimentin/E-cadherin ratio <1 . In contrast, 22 samples (23% of patients) displayed a Vimentin/E-cadherin ratio >10 . The majority of samples (60/95, corresponding to 63% of patients) showed an intermediate phenotype, with Vimentin/E-cadherin ratios ranging from 1 to 10 (**Figure 18**).

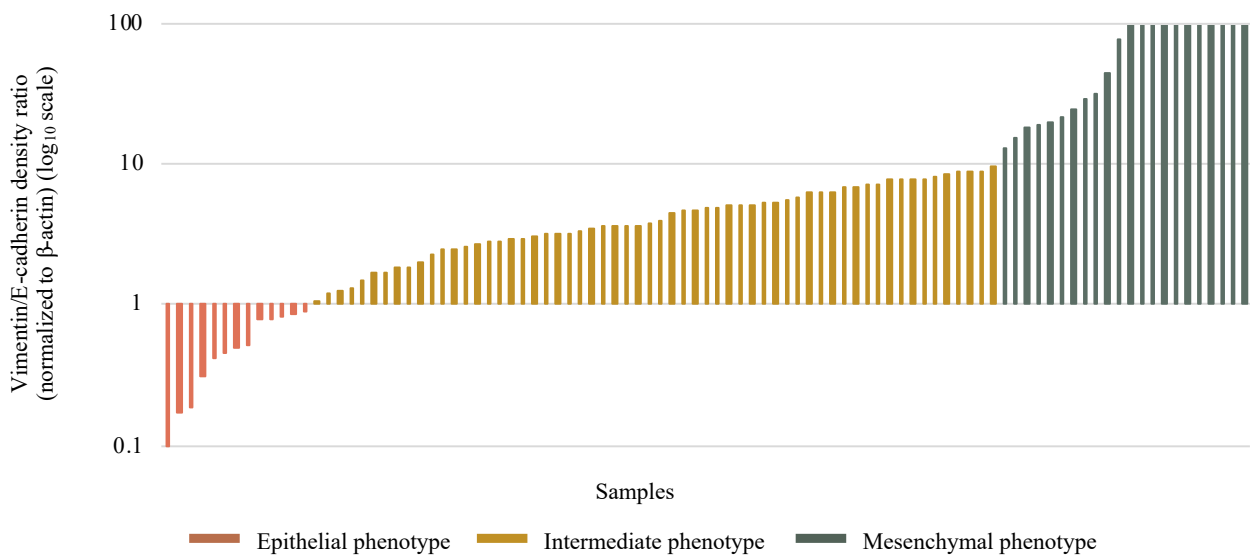


Figure 18. Vimentin/E-cadherin ratio on a $\log_{10}$ scale. Vimentin/E-cadherin ratio <1 (orange), Vimentin/E-cadherin ratio between 1 and 10 (yellow), Vimentin/E-cadherin ratio >10 (green). Each lane corresponds to a breast cancer patient

A similar distribution pattern was observed when evaluating the expression ratios of other epithelial and mesenchymal markers, such as the α -SMA/E-cadherin ratio (**Figure 19**) and the Vimentin/Cytokeratin-18 ratio (**Figure 20**). Although the expression patterns were not perfectly overlapping, the prevalence of intermediate phenotypes remained consistent across the comparisons.

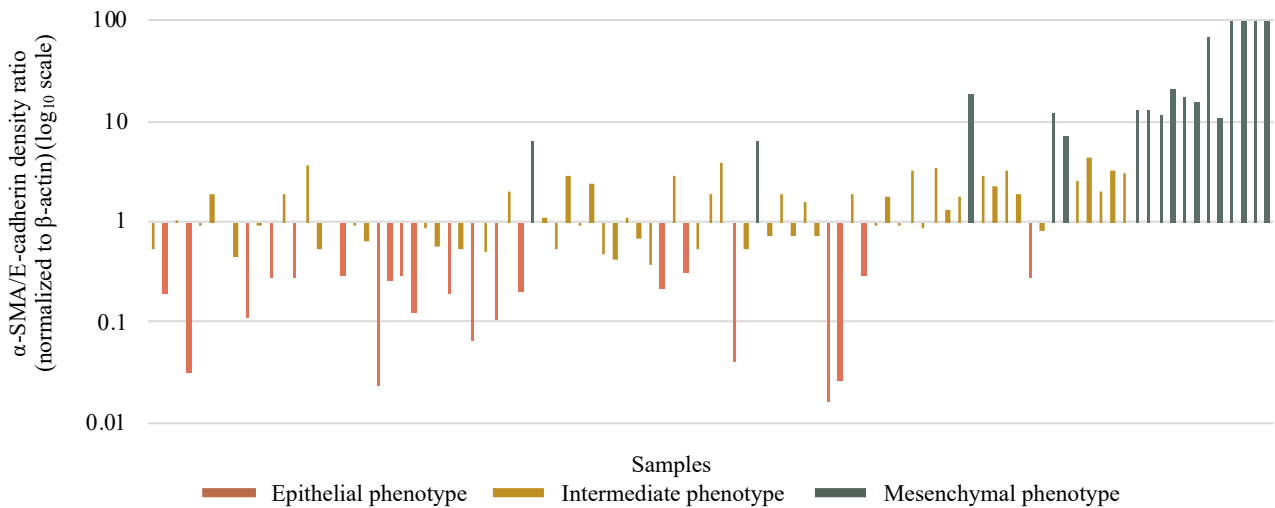


Figure 19. α -SMA/E-cadherin ratio on a log₁₀ scale. α -SMA/E-cadherin ratio <1 (orange), α -SMA/E-cadherin ratio between 1 and 10 (yellow), α -SMA/E-cadherin ratio >10 (green). Each lane corresponds to a breast cancer patient

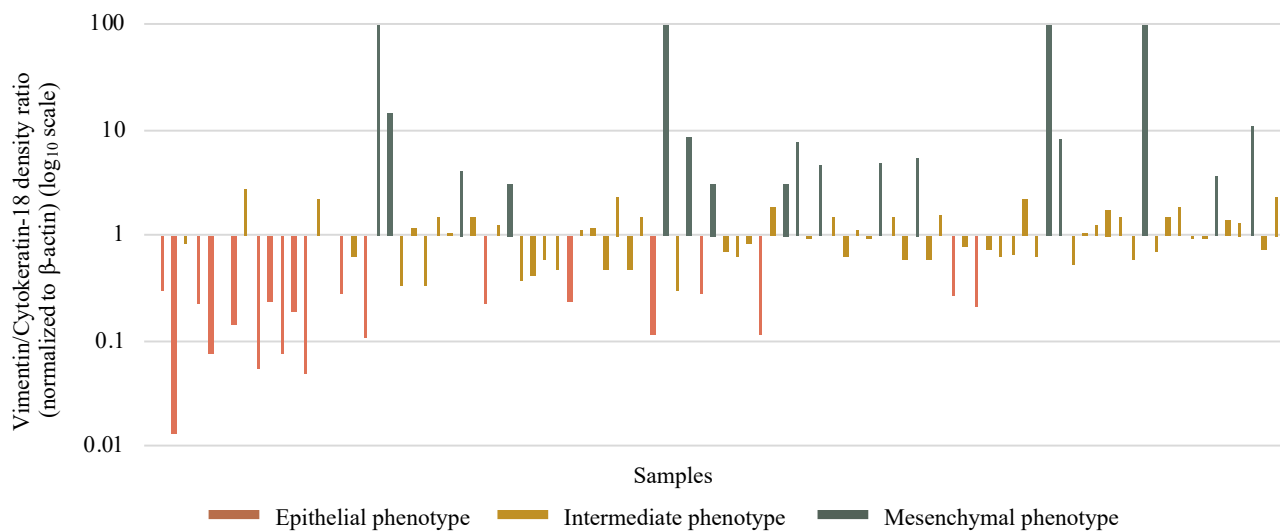


Figure 20. Vimentin/Cytokeratin-18 ratio on a log₁₀ scale. Vimentin/Cytokeratin-18 ratio <1 (orange), Vimentin/Cytokeratin-18 ratio between 1 and 10 (yellow), Vimentin/Cytokeratin-18 ratio >10 (green). Each lane corresponds to a breast cancer patient

These findings indicate that the expression of epithelial and mesenchymal markers is not strictly mutually exclusive, and in the majority of BC tissues analyzed the EMT is partial, characterized by a high degree of cellular plasticity, which is crucial for enabling cancer cells to adapt to changes in the

tumor microenvironment. Moreover, epithelial plasticity can contribute to tumor heterogeneity, which refers to variations observed between tumors of the same type in different patients and among cancer cells within a single tumor. The high heterogeneity of EMT markers expression patterns was also confirmed by limited correlation with clinicopathological characteristics, with no significant association, except for Ki-67, which significantly correlated with both Vimentin and Cytokeratin-18 (Table 1).

Pearson correlation	Age	E-Cadherin	Vimentin	Cytokeratin-18	α -SMA	Grading	HER2	ER%	PGR%	Ki67%
Age	-	-	-	-	-	-	-	-	-	-
E-Cadherin	-	-	$r = 0,378$ $p = 0,00016$	$r = 0,208$ $p = 0,045$	$r = 0,332$ $p = 0,0011$	-	-	-	-	-
Vimentin	-	$r = 0,378$ $p = 0,00016$	-	$r = 0,609$ $p = 1,017E-10$	-	-	-	-	-	$r = -0,326$ $p = 0,0043$
Cytokeratin-18	-	$r = 0,208$ $p = 0,045$	$r = 0,609$ $p = 1,017E-10$	-	-	-	-	-	-	$r = -0,246$ $p = 0,035$
α -SMA	-	$r = 0,332$ $p = 0,0011$	-	-	-	-	-	-	-	-
Grading	-	-	-	-	-	-	-	$r = -0,328$ $p = 0,002$	$r = -0,391$ $p = 0,00020$	$r = 0,503$ $p = 5,011E-10$
HER2	-	-	-	-	-	-	-	-	-	-
ER%	-	-	-	-	-	$r = -0,328$ $p = 0,002$	-	-	$r = 0,6887408$ $p = 1,66E-13$	$r = -0,308$ $p = 0,0071$
PGR%	-	-	-	-	-	$r = -0,391$ $p = 0,00020$	-	$r = 0,6887408$ $p = 1,66E-13$	-	$r = -0,242$ $p = 0,037$
Ki67%	-	-	$r = -0,326$ $p = 0,0043$	$r = -0,246$ $p = 0,035$	-	$r = 0,503$ $p = 5,011E-10$	-	$r = -0,308$ $p = 0,0071$	$r = -0,242$ $p = 0,037$	-

Table 1. Pearson correlation of EMT markers and clinicopathological characteristics.
r indicates the correlation coefficient, p the p-value

Proteomic analysis of Vimentin, α -SMA, Cytokeratin-8, and E-cadherin in BC tissues and matched normal tissues, and immune cell infiltration analysis

The proteomic analysis was also extended to a cohort of 24 surgical BC specimens and their matched adjacent normal tissues, collected from patients diagnosed with infiltrating ductal carcinoma of histological grade G2/G3. As shown in **Figure 21**, a marked inter-sample heterogeneity was observed between tumoral tissues and their corresponding normal counterparts. Notably, for some samples, no detectable signal was observed for specific markers. In several cases, EMT-related markers were expressed at comparable levels in both tumoral and normal tissues or were even more abundant in the adjacent normal samples. Overall, epithelial markers such as E-cadherin and Cytokeratin-8, as well as the loading control β -actin, displayed relatively low expression levels in the normal tissues. In contrast, mesenchymal markers, including Vimentin and α -SMA, exhibited a more homogeneous distribution across both tumoral and normal samples.

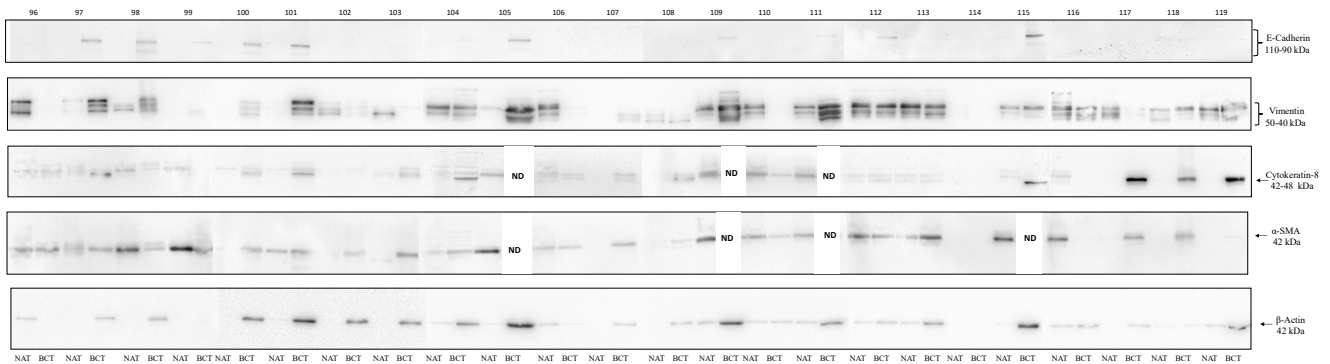


Figure 21. Western blot analysis performed on 24 surgical breast cancer (BC) specimens and their matched adjacent normal tissues to evaluate E-cadherin (110-90 kDa), Vimentin (50-40 kDa), Cytokeratin-8 (42-48 kDa), α -SMA (42 kDa), and β -actin (42 kDa) expression. ND = not detected

These findings suggest additional layers of complexity in the regulation and dynamics of EMT during breast cancer progression. A critical factor to consider is the cellular origin of each marker detected in bulk tissue lysates. Indeed, infiltration of stromal cells from the tumor microenvironment may contribute to the observed expression patterns and could partially explain the heterogeneity and overlap between tumoral and normal samples.

Alternatively, these results may reflect early molecular alterations occurring in histologically normal adjacent tissues, potentially induced by initial oncogenic events or paracrine signalling from the tumor microenvironment. Although the BC tissues analyzed in this study were enriched in tumor cells, the experimental approach does not allow discrimination between protein expression derived from distinct cellular compartments. This limitation is particularly relevant for mesenchymal markers. For instance, a caspase-3–cleaved Vimentin variant secreted by tumor-associated macrophages has been shown to correlate with tumor malignancy and lymph node metastasis (Miao

et al., 2025). Similarly, α -SMA expression has been extensively characterized in cancer-associated fibroblasts (CAFs), where it correlates with tumor progression, metastatic potential, prognosis, and overall survival (Barb et al., 2023; Cui et al., 2024).

To better resolve the cellular origin and spatial distribution of these EMT-related markers, further analyses using immunohistochemistry (IHC) on tissue sections would be instrumental, allowing precise localization within epithelial, stromal, and immune compartments.

However, complementary *in silico* analyses can provide valuable insights into the potential cellular contributors to the expression patterns detected in tissue samples. In this context, the relationship between EMT marker expression and immune cell infiltration, including cancer-associated fibroblasts, macrophages, endothelial cells, CD8⁺ T cells, and CD4⁺ T cells, was investigated using the TIMER database. This approach allows inference of cell-type-specific contributions based on correlation analyses across large transcriptomic datasets. The results revealed significant positive correlations between *VIM* (Vimentin) expression and multiple components of the immune microenvironment (**Figure 22**), supporting the notion that Vimentin signals detected in bulk tissues may, at least in part, derive from stromal and immune cell populations. As expected, *ACTA2* (α -SMA) expression showed a strong positive correlation with cancer-associated fibroblast infiltration, further reinforcing its established role as a CAFs-associated marker. In contrast, *CDH1* (E-cadherin) and *KRT18* (Cytokeratin-18) exhibited only weak correlations with immune cell infiltration, consistent with their predominantly epithelial expression profiles.

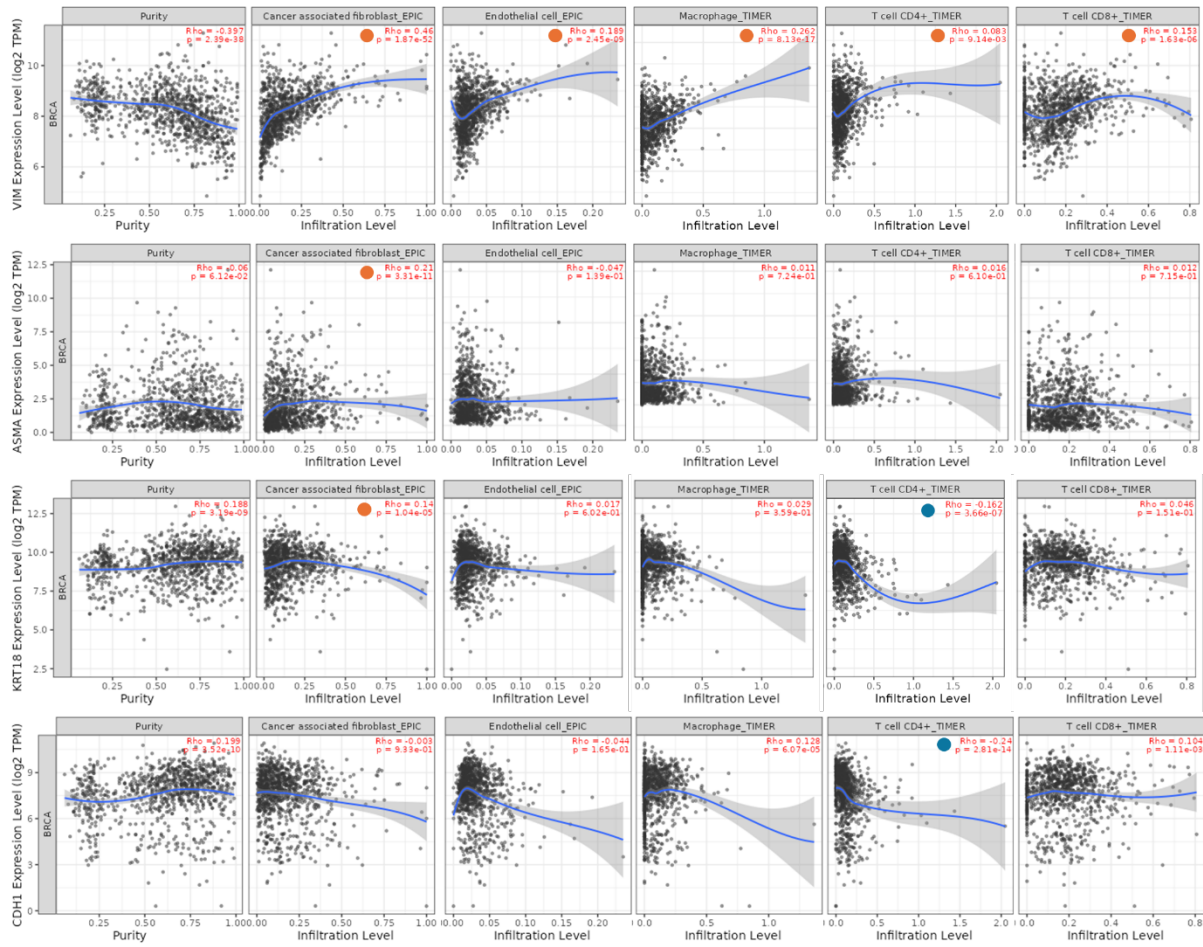


Figure 22. Correlation analysis between *VIM* (Vimentin), *ACTA2* (α -SMA), *KRT18* (Cytokeratin-18), and *CDH1* (E-cadherin) expression and immune cell infiltration, including cancer-associated fibroblasts, macrophages, endothelial cells, CD8⁺ T cells, and CD4⁺ T cells, using the TIMER database. Orange circles indicate significant positive correlations, whereas blue circles indicate significant negative correlations. Each panel represents a scatterplot depicting the correlation between gene expression levels and the estimated abundance of the indicated immune or stromal cell population

Contribution of breast cancer sera in cell proliferation, migration, and EMT markers expression

Analyses performed on breast cancer tissue samples revealed heterogeneous expression of EMT-related markers, indicating that tumor aggressiveness and biological behavior may be highly patient-specific. Although EMT represents a key biological process underlying tumor invasion and progression, the direct correlation of EMT markers expression and tumor aggressiveness remains challenging due to its dynamic, context-dependent, and heterogeneous nature within tissues.

To overcome these limitations, the investigation was extended to patient-derived sera as a complementary and functionally oriented approach. Serum-based analyses represent a minimally invasive and clinically applicable alternative to tissue biopsies and may capture circulating factors associated with tumor biology. In this context, rather than attempting to suppose tumor aggressiveness solely from EMT markers expression, the ability of breast cancer sera to functionally modulate cancer

cell proliferation and migration was explored using an in vitro breast cancer cell model. The MDA-MB-231 cells were used as a prototype of a highly proliferative and metastatic tumor model. This strategy could provide an indirect yet biologically relevant readout of aggressive tumor behavior and may serve as a practical tool for patient stratification and the development of personalized therapeutic approaches.

Cell proliferation was assessed in MDA-MB-231 cells by MTT assay after 48 h of treatment with 1% sera from primary breast cancer patients ($n = 22$), previously analyzed by western blot, or sera from healthy donors ($n = 30$), and compared to cells treated with 1% Fetal Bovine Serum (FBS), used as a control. All experimental conditions were performed in triplicate to ensure reproducibility and statistical robustness.

Interestingly, treatment with breast cancer sera induced a significant increase in cell proliferation in 6 out of 22 samples (32%) (**Figure 23A**). In contrast, treatment with sera from healthy donors resulted in a significant reduction in cell proliferation in 26 out of 30 samples (87%). Collectively, although a high degree of inter-sample heterogeneity was observed, these findings indicate that proliferative responses in cancer cells are selectively driven by tumor-associated soluble factors present in sera from breast cancer patients.

To evaluate whether BC sera could also induce a proliferative response in normal-like epithelial cells, potentially reflecting tumor-host crosstalk mediated by circulating factors, MTT assays were performed using MCF10A, an immortalized but non-transformed mammary epithelial cell line. Consistent with the results obtained in MDA-MB-231 cells, treatment of MCF10A cells with breast cancer sera increased cell proliferation in the majority of samples (**Figure 23B**).

Conversely, exposure to sera from healthy donors resulted in reduced cell proliferation, mirroring the response observed in the cancer cell model. Overall, sera from patients with primary breast cancer induced a significant increase in cell proliferation in both MDA-MB-231 (**Figure 23C**) and MCF10A cells (**Figure 23D**), whereas sera from healthy donors consistently suppressed cell proliferation across both cellular models.

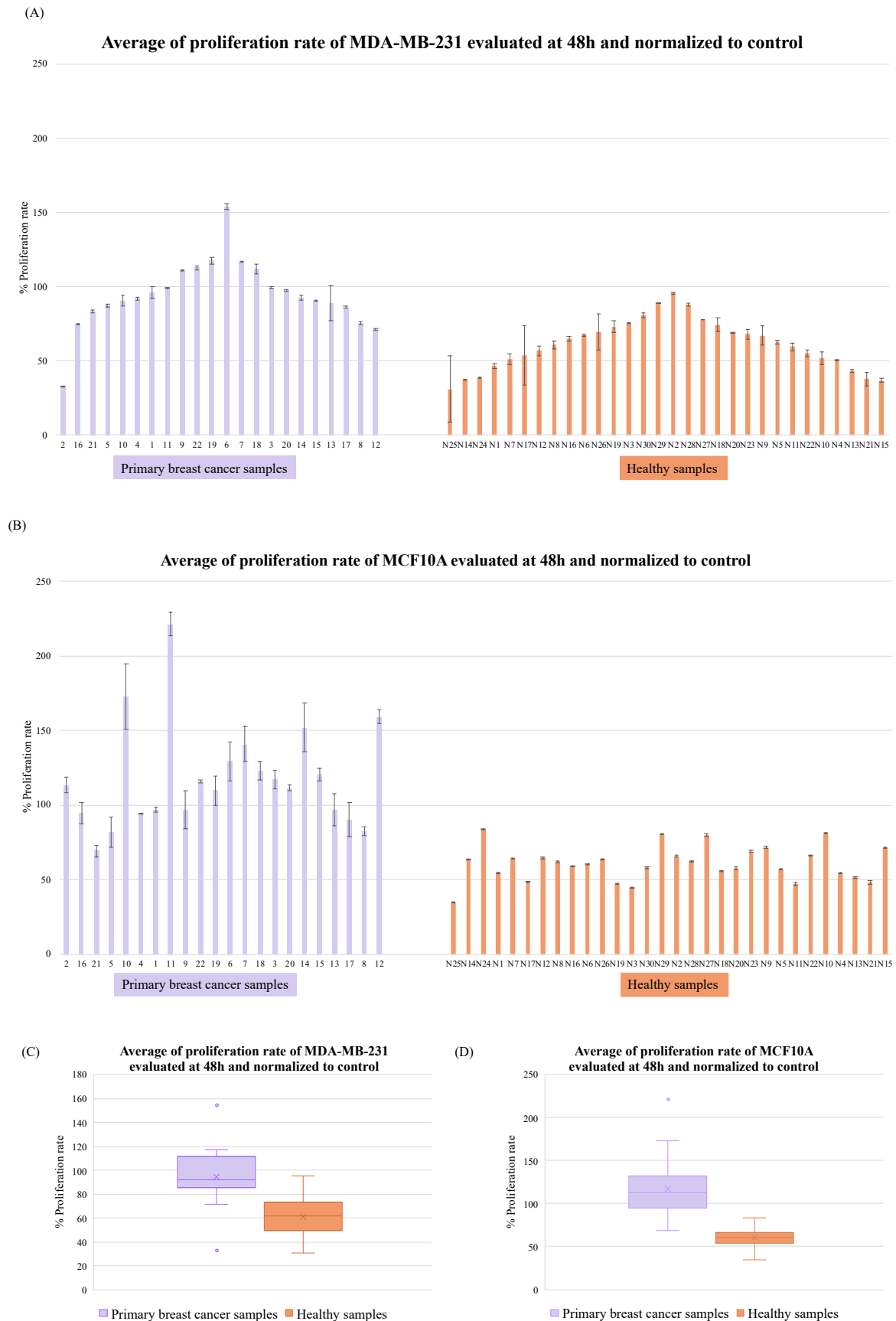


Figure 23. MTT assay results showing the proliferation rate of MDA-MB-231 (A–C) and MCF10A (B–D) cells after 48 hours of treatment, normalized to control. Each bar represents treatment with primary breast cancer sera (violet) or healthy sera (orange) (A, B). Samples were ordered to highlight the distribution of biological responses. Box plots summarize the proliferation rate 48 hours after treatment with primary breast cancer sera (violet) or healthy sera (orange) (C, D)

To further support the hypothesis that breast cancer sera may contain malignancy-associated circulating factors capable of stimulating cell proliferation, a subset of sera from patients diagnosed with metastatic breast cancer (n=26) was analyzed (**Figure 24A-D**). Notably, sera derived from metastatic patients induced a higher proliferative response compared to sera from patients with primary tumors when tested on MDA-MB-231 cells (**Figure 24C**). In contrast, this enhanced stimulatory effect was not observed in MCF10A cells, where metastatic sera elicited a proliferative response comparable to that induced by sera from patients with primary breast cancer (**Figure 24D**). These findings suggest that stage-dependent circulating factors may preferentially influence tumor cell behaviour, with a limited effect on normal-like epithelial cells.

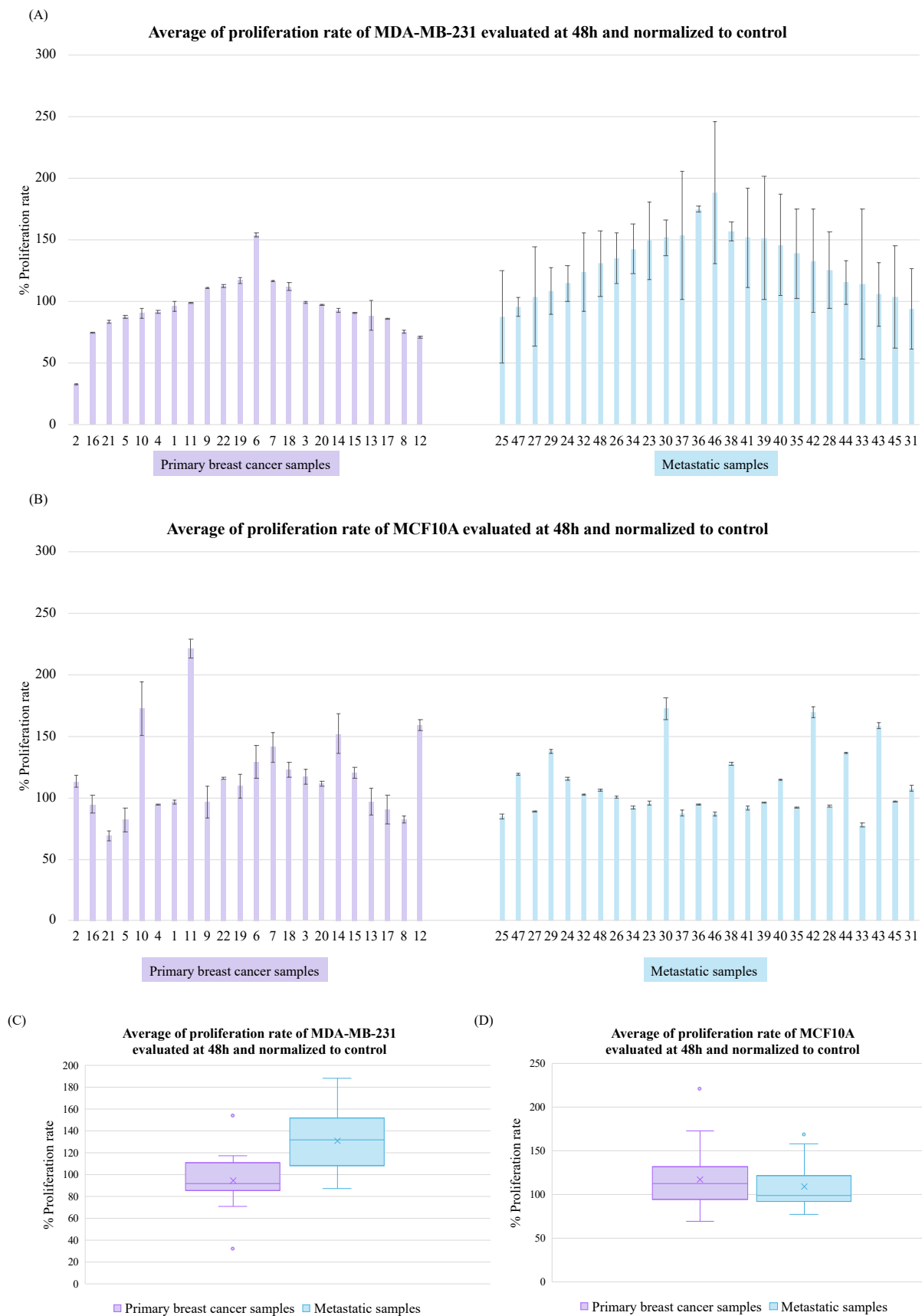


Figure 24. MTT assay results showing the proliferation rate of MDA-MB-231 (A–C) and MCF10A (B–D) cells after 48 hours of treatment, normalized to control. Each bar represents treatment with primary breast cancer sera (violet) or metastatic breast cancer sera (blue) (A, B). Samples were ordered to highlight the distribution of biological responses. Box plots summarize the proliferation rate 48 hours after treatment with primary breast cancer sera (violet) or metastatic sera (blue) (C, D)

The next step was to evaluate whether patient-derived sera could also modulate cancer cell migration, a functional hallmark of tumor aggressiveness, often associated with EMT. Cell migration was assessed in MDA-MB-231 cells using a wound-healing assay after 6 and 24 hours of treatment with 1% sera from primary breast cancer patients ($n = 22$), previously analyzed by western blot, or sera from healthy donors ($n = 12$), and compared to cells treated with 1% Fetal Bovine Serum (FBS) as a control. A negative control, consisting of cells treated with serum-free medium, was also included. All experimental conditions were performed in triplicate to ensure reproducibility and statistical robustness.

The most pronounced effects were observed after 24 hours of treatment and are summarized in **Figure 25A**. Data were normalized to the 1% FBS treatment and presented on a logarithmic scale, with values above 1 indicating increased migration compared to FBS-treated cells and values below 1 indicating reduced migratory activity. The majority of primary breast cancer sera induced enhanced wound closure after 24 hours compared to both control and untreated cells. Consistent with the proliferation assays, treatment with healthy sera generally reduced wound closure compared to FBS-treated cells, with all values below 1 (**Figure 25B**). However, migration in healthy serum-treated cells was consistently higher than in cells treated with serum-free medium (NT), indicating that while human serum exerts a general stimulatory effect, the enhanced migration observed with breast cancer sera is not a nonspecific response but is attributable to factors specifically associated with malignancy.

Overall, as shown in **Figure 25C**, sera from primary breast cancer patients promoted cancer cell migration compared to sera from non-pathological donors. Therefore, the observed modulation on proliferation and migration likely reflects the differential enrichment of bioactive factors in each serum sample.

An interesting perspective for future investigation would be to determine whether the observed effects on proliferation and migration are, at least in part, mediated by extracellular vesicles, such as exosomes, which are known to carry bioactive molecules capable of modulating tumor and stromal cell behaviour.

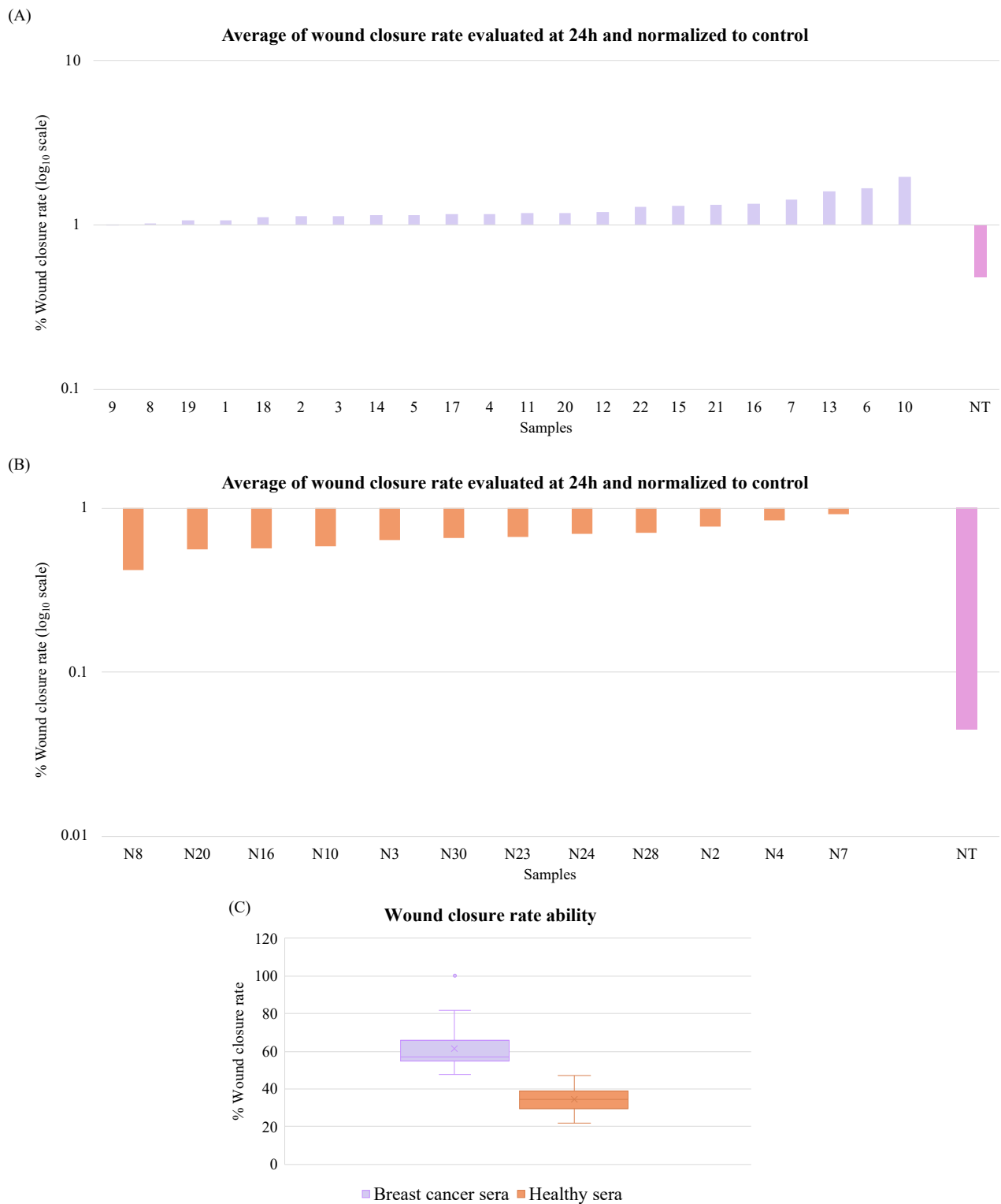


Figure 25. Comparison between the effect of BC-related sera (violet) and healthy sera (orange) in the wound-healing assay on MDA-MB-231. Average wound closure rate at 24h, normalized to the control on a log₁₀ scale. The last lane is the untreated well. Each lane relates to the migratory ability of a human serum-treated well, corresponding to primary breast cancer serum samples (A) and healthy serum samples (B); Box plots showing the average migratory ability (%) of BC sera (violet) and healthy sera (orange) on MDA-MB-231 at 24h after wound induction (C)

The functional effects of patient-derived sera on both proliferation and migration suggest that circulating factors in breast cancer sera can actively modulate aggressive cellular phenotypes. To further explore whether these functional responses were accompanied by changes in EMT markers, including Vimentin and E-cadherin expression, western blot analyses were performed on MDA-MB-231 cells following the scratch and the concomitant treatment with the breast serum samples (n = 22). As shown in **Figure 26A**, the expression of Vimentin and E-cadherin was modulated across the different breast serum treatments, indicating that distinct sera exerted differential effects on EMT-related markers. Interestingly, a weak positive correlation between Vimentin and E-cadherin levels was observed (Pearson's $r = 0.32$), similar to what was seen in breast cancer tissue samples. However, despite the enhanced migratory response induced by BC sera, no direct association with Vimentin expression was detected; in fact, both Vimentin and E-cadherin levels were often reduced compared to control cells (**Figure 26B**).

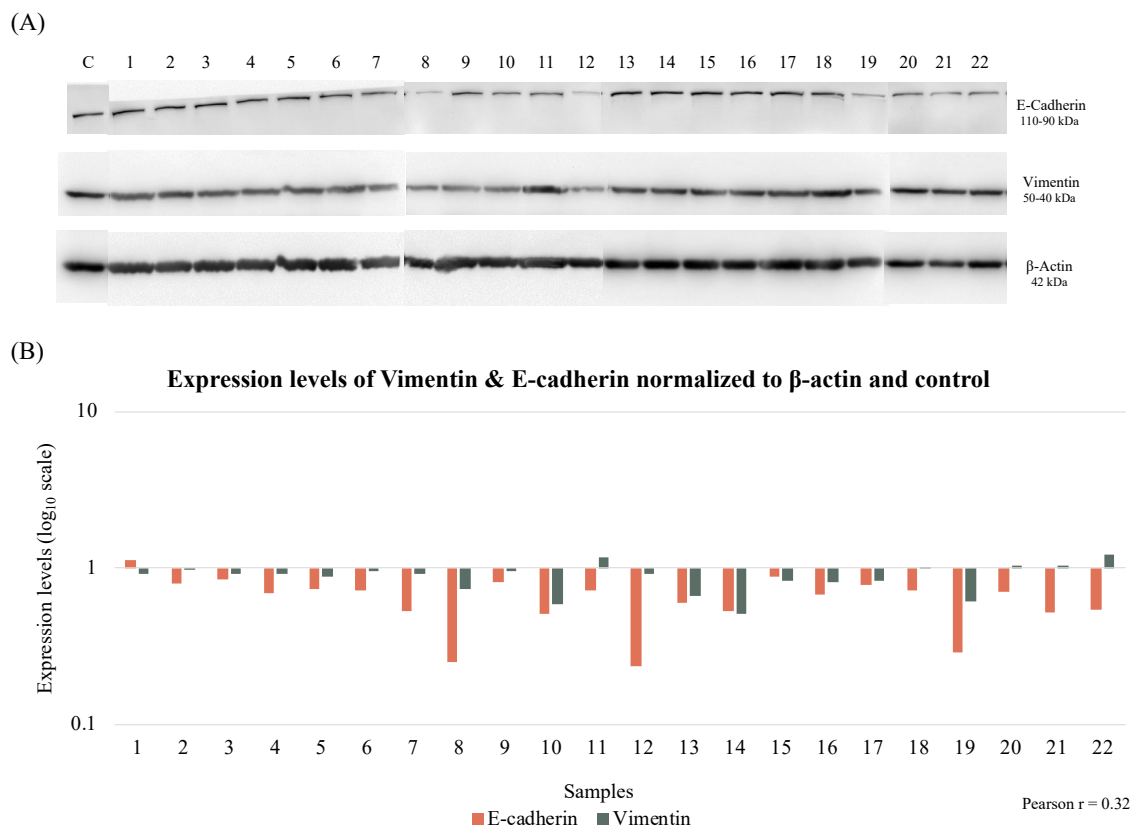


Figure 26. Modulation of EMT markers expression after BC sera treatment. Western blot analysis performed on MDA-MB-231 cells after treatment with breast cancer serum samples to evaluate E-cadherin (110-90 kDa) and Vimentin (50-40 kDa) expression levels. β -actin (42 kDa) was used as an internal control. C represents the control. Each lane corresponds to a different breast serum-treated well (A); E-cadherin and Vimentin expression levels were normalized to β -actin and to the control condition and displayed on a $\log_{10}$ scale. Each lane corresponds to wells treated with a different breast cancer serum (B)

These findings underscore the complexity of EMT in breast cancer and suggest that the expression of a few canonical EMT markers alone is insufficient to fully capture the functional migratory and proliferative phenotypes induced by tumor-associated sera.

3D Culture of Mammary Cells: Spheres Formation and Assessment

While experiments conducted on monolayer (2D) cells provided valuable insights into the effects of BC sera on cell migration, proliferation, and EMT marker expression, these models do not fully resemble the structural and biochemical complexity of the tumor microenvironment. Since the three-dimensional (3D) systems of cell culture better reproduce cell–cell and cell–matrix interactions and provides a more physiologically relevant system for investigating tumor behavior and the impact of circulating factors, during the research period carried out at the Instituto de Investigación Biomédica (INIBIC) in A Coruña (Spain), tumorspheres' culture models were developed for both tumor and normal cells. Cells were cultured in low-attachment flat-bottom plates, allowing spontaneous formation of three-dimensional (3D) aggregates driven by cell–cell interactions, rather than forced aggregation as occurs in conical (U-bottom) plates. As shown in **Figure 27**, efficient tumorsphere formation was achieved 8 days after seeding MDA-MB-231 cells. The resulting tumorspheres displayed a compact and rounded morphology and were homogeneous in size. This size uniformity was consistently observed both within and across individual wells, indicating a high degree of reproducibility and robustness of the tumorsphere generation protocol.

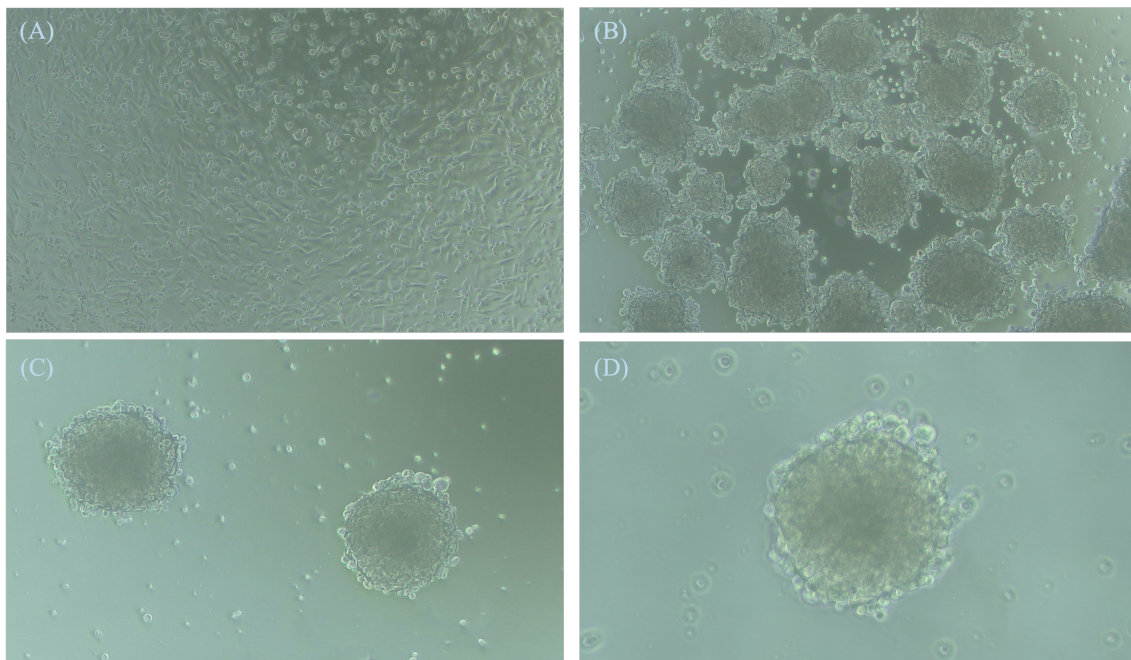


Figure 27. MDA-MB-231 cells grown in a monolayer (A) and tumorspheres at 40x (B), 100x (C), and 200x (D) magnification

Using this 3D cell model, quantitative PCR (qPCR) analysis was performed to assess the expression of stemness (*SOX2*) and proliferation (*MKI-67*) markers, as well as a panel of genes implicated in epithelial-to-mesenchymal transition, including *CDH1* (E-cadherin), *CDH2* (N-cadherin), *VIM* (Vimentin), *FNI* (Fibronectin), *TWIST1*, and *SNAIL* (Snail1). Gene expression levels in tumorspheres were compared to those of MDA-MB-231 cells cultured under conventional two-dimensional (2D) monolayer conditions, which served as the reference control. This analysis (**Figure 28**) showed that cell proliferation in the 3D model, as assessed by *MKI67* expression, was significantly lower than in the 2D model. In parallel, *VIM* expression was downregulated, suggesting that 3D cell aggregation is associated with morphological changes consistent with a less elongated phenotype. Conversely, the acquisition of the 3D structure was accompanied by a modest but non-significant increase in *CDH1* expression, indicative of enhanced cell–cell adhesion and structural compaction. Despite these epithelial-associated features, EMT-related pathways were activated in the 3D model, as evidenced by upregulation of *SNAIL* and *TWIST1*, along with a trend toward increased *CDH2* expression. At the same time, tumorspheres displayed increased stemness, reflected by *SOX2* upregulation and enhanced *FNI* expression, indicating increased extracellular matrix production and anchorage-independent growth.

Collectively, this transcriptional profile suggests that tumorspheres' formation does not correspond to a classical epithelial-to-mesenchymal transition, but rather reflects the establishment of a highly plastic cellular state. The coexistence of epithelial traits (*CDH1* upregulation, reduced *VIM* expression) with mesenchymal and stemness-associated features reinforces the emergence of a hybrid EMT phenotype, indicating that the 3D model captures a preparatory phase towards a more aggressive and adaptable tumor phenotype rather than a fixed epithelial or mesenchymal identity.

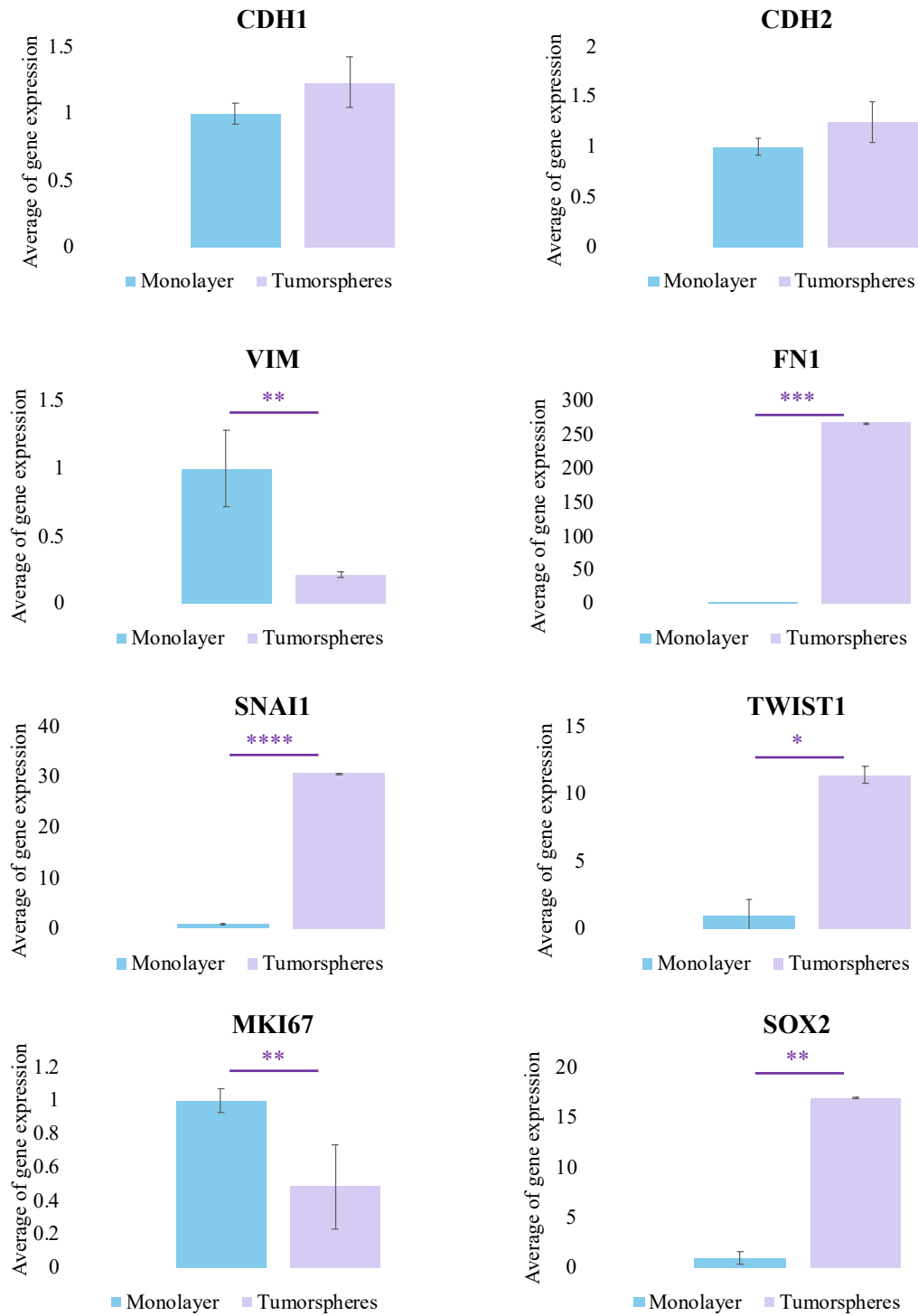


Figure 28. qPCR analysis conducted on MDA-MB-231 cells grown in a monolayer (blue) and as tumorspheres (violet). $p < 0.05$ was considered significant ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$, $p < 0.0001^{****}$)

To assess whether a 3D culture model could also be established using normal mammary epithelial cells, MCF10A cells were used to generate three-dimensional structures and to evaluate their behavior under 3D conditions. In this case, spheres' formation was much more challenging and characterized by the emergence of structures with heterogeneous morphologies.

As shown in **Figure 29**, MCF10A-derived 3D spheres exhibited limited size and morphological heterogeneity, reflecting slower growth kinetics and reduced long-term survival. These features are consistent with the non-transformed nature of MCF10A cells and further underscore the intrinsic differences between normal and malignant mammary cells in their ability to adapt to and sustain growth under 3D conditions.

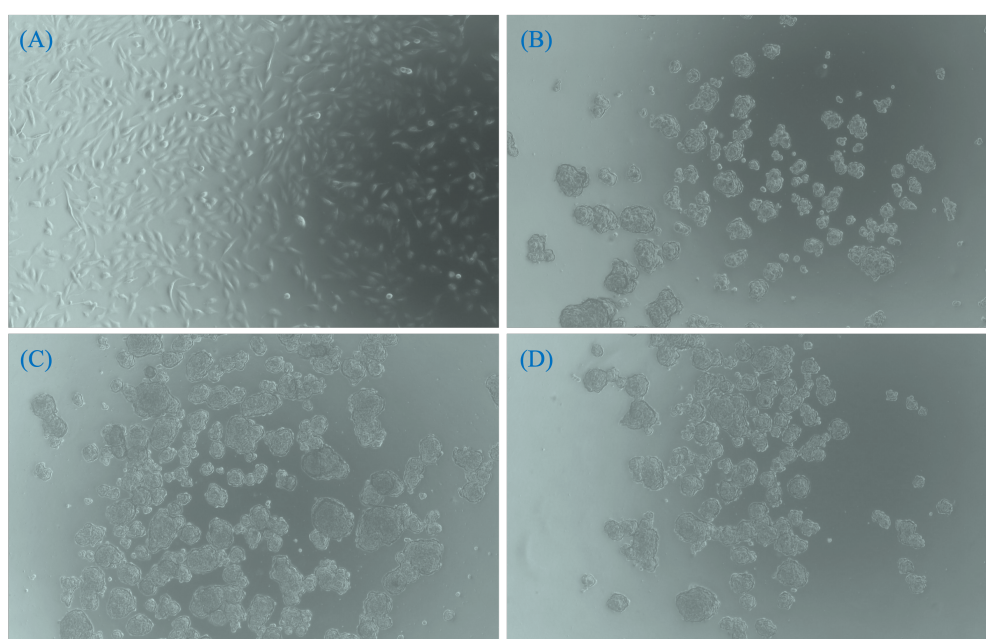


Figure 29. MCF10A cells grown in a monolayer (A) and 3D spheres at 40x magnification (B, C & D)

To further characterize this model, qPCR analyses were performed on MCF10A cells grown as a monolayer and as spheroids (**Figure 30**). 3D spheres displayed a significantly reduced proliferative activity compared to monolayer cultures, as indicated by a dramatic decrease in *MKI67* expression. Compared with MDA-MB-231 tumorspheres, MCF10A 3D spheres showed a similar *TWIST1* expression profile but a non-significant, less pronounced reduction in *VIM* expression compared to monolayer cultures. Conversely, *CDH1*, *CDH2*, *FNI*, and *SOX2* displayed opposite expression patterns, and *SNAIL* expression was not detectable in monolayer samples (Ct values > 40), further highlighting fundamental differences in the transcriptional programs activated in normal versus tumoral cells under 3D conditions.

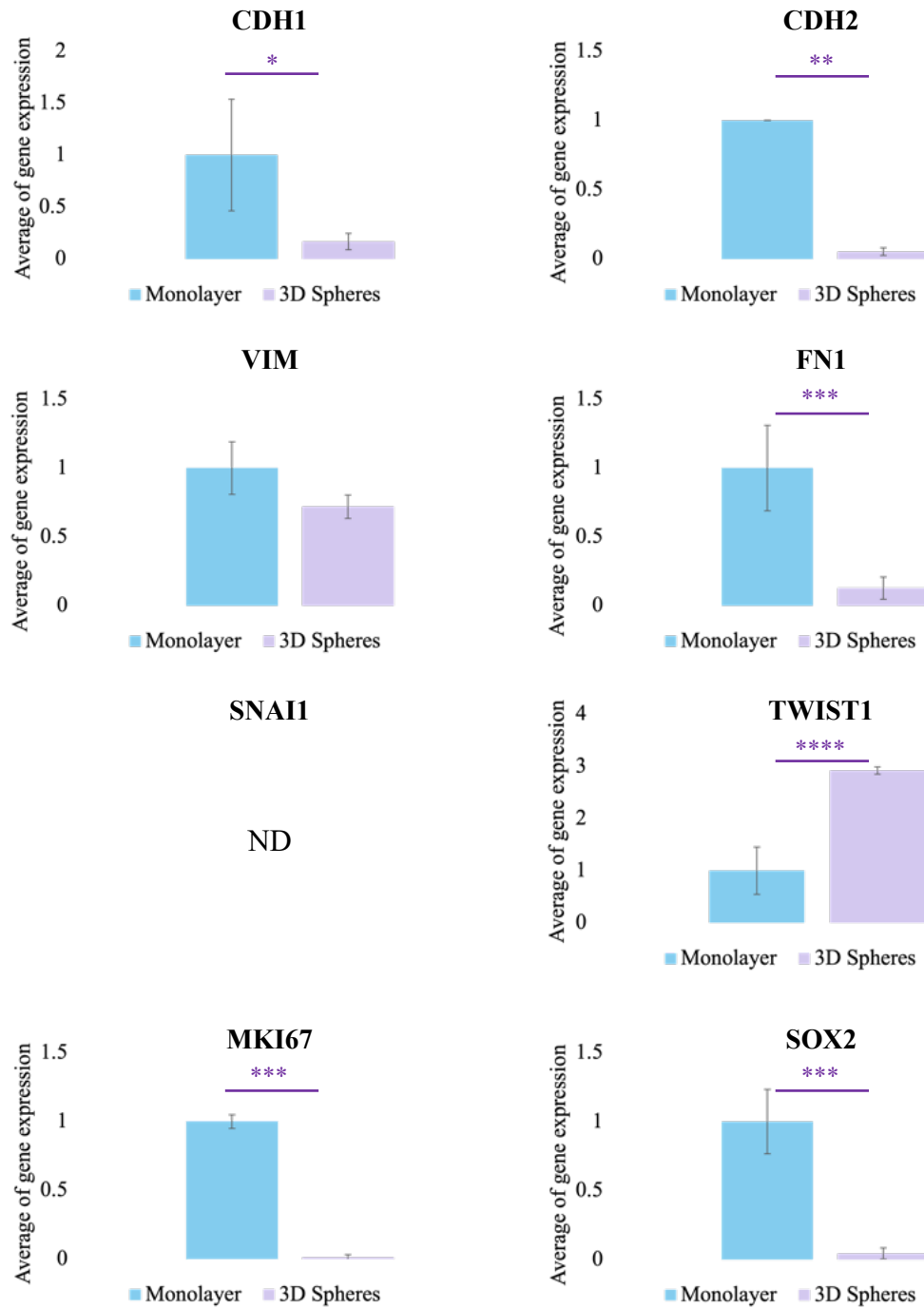


Figure 30. qPCR analysis conducted on MCF10A cells grown in a monolayer (blue) and as 3D spheres (violet). $p < 0.05$ was considered significant ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$, $p < 0.0001^{****}$)

MDA-MB-231 tumorspheres treatment with breast cancer and healthy serum samples

Although 3D spheres' formation was also explored in non-tumorigenic MCF10A cells, subsequent experiments assessing the effects of patient-derived sera were performed exclusively using MDA-MB-231 tumorspheres. This choice was based on the limited ability of MCF10A cells to sustain robust, homogeneous 3D growth and their reduced long-term survival under 3D conditions.

The MDA-MB-231 cells, both as monolayer and tumorspheres, were treated with 1% of primary breast cancer sera, previously used for the wound-healing assay (n=2: P6 and P10) and healthy donor sera (n=2: N8 and N30).

A direct comparison between the 2D and 3D culture systems revealed marked differences in their sensitivity to serum-induced effects. At the morphological level, monolayer cultures did not display appreciable differences following treatment with breast cancer versus healthy sera (**Figure 31A**). In contrast, the 3D system proved to be more sensitive: accordingly, tumorspheres exposed to healthy sera showed a partial loss of the organized, compact spherical architecture, adopting a more irregular, loosely aggregated morphology. Conversely, treatment with breast cancer sera supported the maintenance of tumorsphere integrity and roundness (**Figure 31B**). At the transcriptional level, the two culture systems exhibited distinct response patterns. In 2D monolayer cultures, breast cancer sera strongly upregulated *MKI67* and *CDH1* expression, and downregulated *FNI* and *CDH2*, even if not significantly. In 3D tumorspheres, the expression levels of these genes in MDA-MB-231 treated with primary breast cancer sera remained comparable to control conditions. In contrast, the downregulation of *MKI67*, *CDH1*, *CDH2*, and *FNI* induced by healthy sera was observed consistently in both 2D and 3D systems. *VIM* modulation was similar in both 2D and 3D systems: breast cancer sera did not consistently alter its expression, whereas healthy sera induced its upregulation. Notably, the modulation of EMT transcription factors *SNAIL* and *TWIST1* was more pronounced in the 3D system compared with 2D, indicating that these key regulators are particularly sensitive to serum-induced effects in a three-dimensional context (**Figure 32A-B**). Moreover, while differences between the control condition and primary breast cancer serum samples were not statistically significant for some genes, a clear and consistent divergence emerged between cells treated with primary breast cancer sera and those treated with healthy sera whenever expression values were detectable ($Ct < 40$), further underscoring the biological distinction between breast cancer-derived and healthy serum-mediated effects. Although these experiments were conducted with a limited number of breast cancer and healthy sera samples, they represent a proof of concept supporting the utility of 3D culture models for studying the impact of circulating factors on tumor cell behaviour.

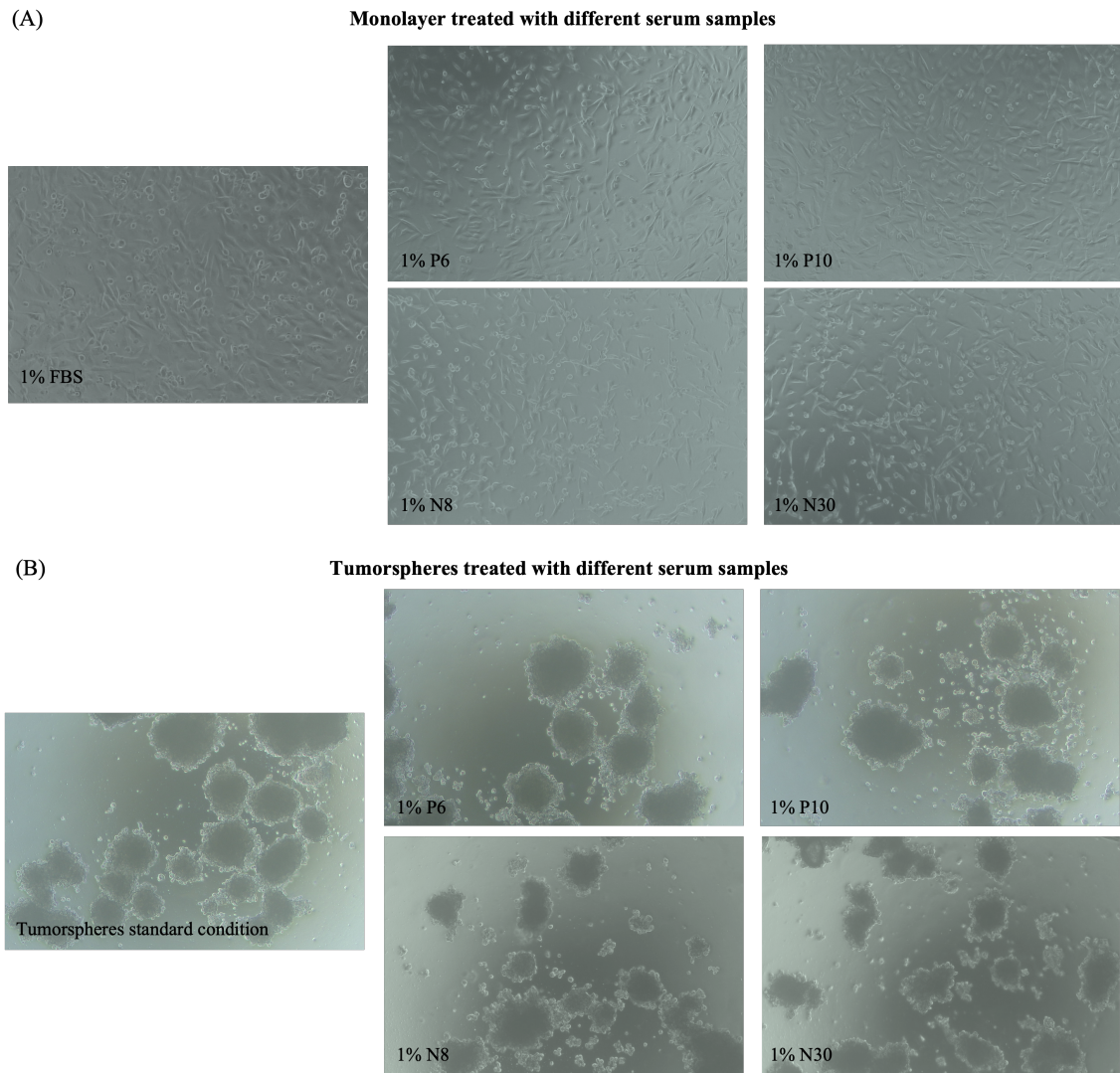
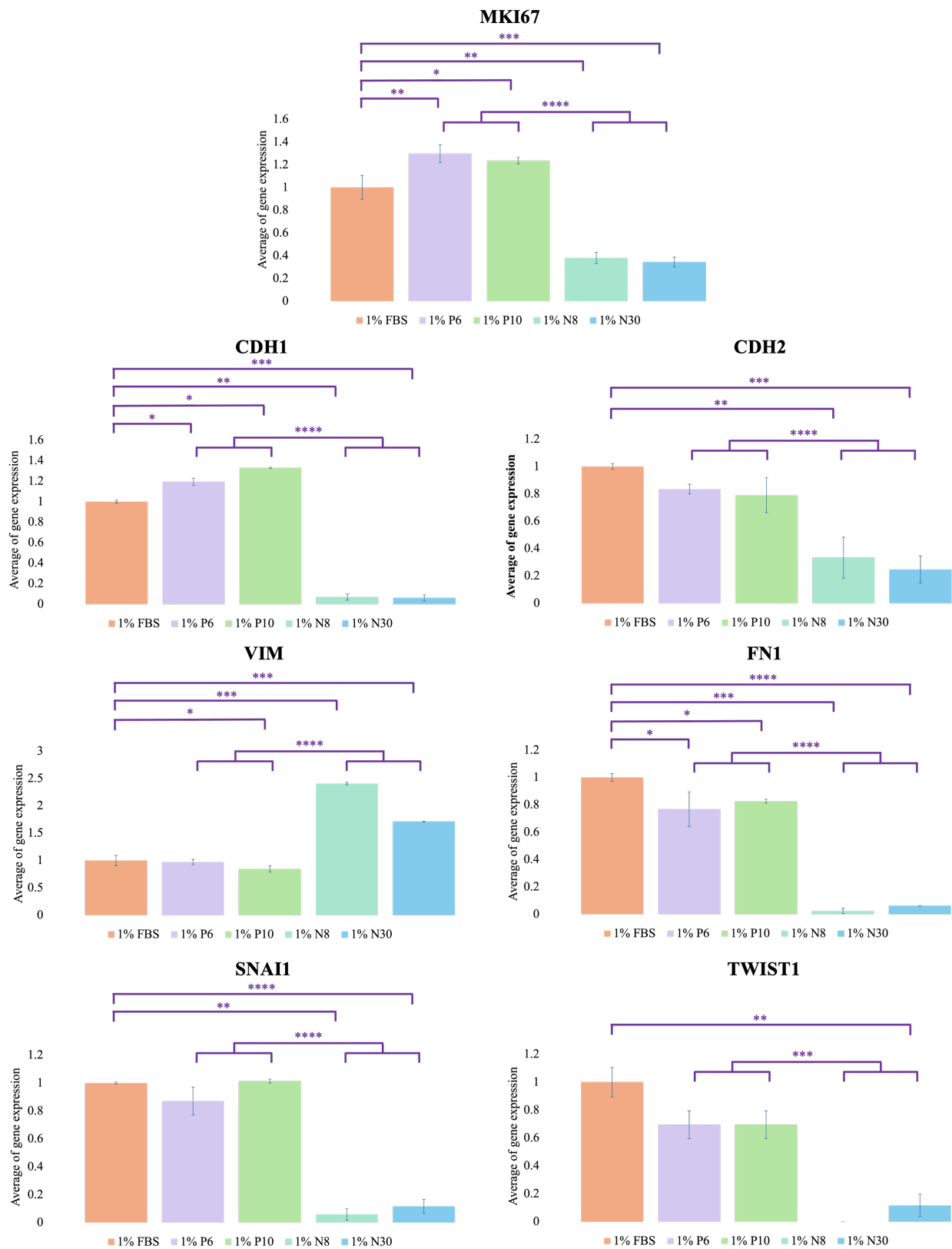


Figure 31. Morphological analysis at 40x magnification of MDA-MB-231 monolayer (A) and tumorspheres (B). Monolayer cells were treated with 1% of FBS (control) or primary breast cancer sera used for the wound-healing assay (P6 & P10), and sera from healthy non-pathological patients previously used (N8 & N30), whereas tumorsphere were maintained in 0% FBS (standard condition) or treated with BC (P6 & P10) and healthy sera (N8 & N30)

(A)

Monolayer treated with different serum samples



(B)

Tumorspheres treated with different serum samples

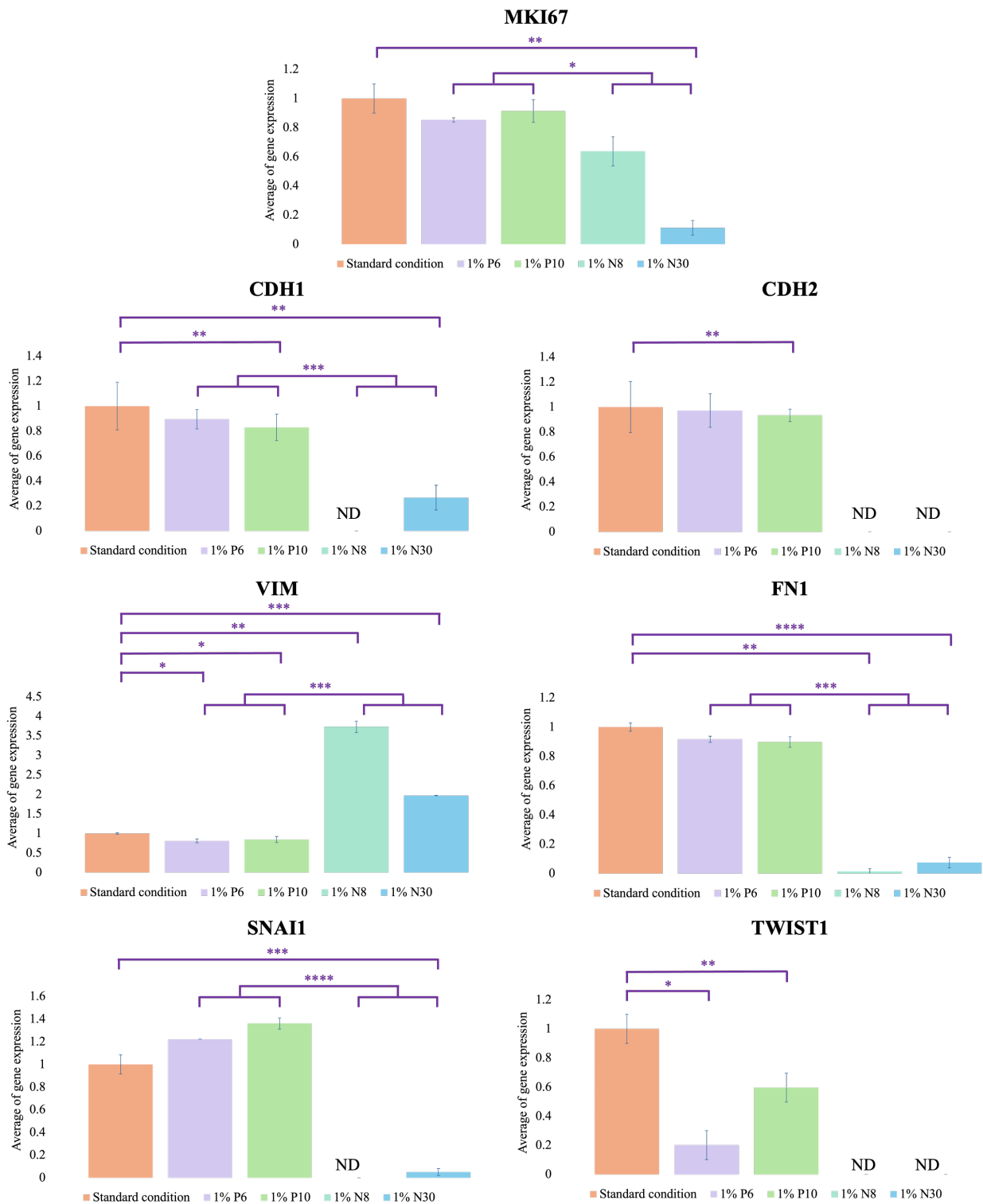


Figure 32. Quantitative PCR analysis of MDA-MB-231 monolayer (A) and tumorspheres (B). Monolayer cells were treated with 1% of FBS (control) or primary breast cancer sera used for the wound-healing assay (P6 & P10), and sera from healthy non-pathological patients previously used (N8 & N30), whereas tumorsphere were maintained in 0% FBS (standard condition) or treated with BC (P6 & P10) and healthy sera (N8 & N30).

p-values were calculated for comparisons between the standard condition and each treatment, and between the tumor sera group and the healthy sera group. $p < 0.05$ was considered significant ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$, $p < 0.0001^{****}$)

Notably, both the tissue- and serum-based results underscore that EMT is a complex, multifaceted process that cannot be fully captured by the expression of a few selected markers. To obtain a more comprehensive understanding of the EMT landscape in breast cancer, analyses integrating large datasets are needed. For this reason, a bioinformatic approach was considered appropriate to dissect the complexity and heterogeneity of EMT programs in a broader context.

***In silico* selection, diagnostic, and prognostic significance of EMT-breast cancer genes**

The EMTome database was interrogated to obtain a set of genes specifically associated with EMT in BC, based on the 14 studies annotated in the platform (**Supplementary Table 2**).

To increase the robustness of the analysis, only genes reported in at least two independent studies were considered. Based on this selection criterion, a total of 144 breast EMT-related genes were identified (**Supplementary Table 3**). This gene set was subsequently employed to investigate its diagnostic and prognostic relevance in breast cancer.

Gene expression differences between BC tissues and matched normal samples were first assessed using the UALCAN database. Of the 144 genes analyzed, 117 showed significant differential expression in tumor tissues compared to normal counterparts. Specifically, 57 genes were significantly upregulated (highlighted in red in **Table 2**), whereas 60 genes were downregulated (highlighted in black in **Table 2**) in breast cancer, indicating that an interplay between gene activation and repression characterizes not only breast cancer but also EMT.

Comparison of gene expression (normal vs BC) by using UALCAN							
Genes upregulated in BC	Statistical significance	Genes upregulated in BC	Statistical significance	Genes downregulated in BC	Statistical significance	Genes downregulated in BC	Statistical significance
ABCA12	$6,03 \times 10^{-5}$	KRT18	$1,62 \times 10^{-12}$	ABCC4	$1,06 \times 10^{-3}$	KRT17	$5,55 \times 10^{-16}$
ARTN	$1,62 \times 10^{-12}$	LAD1	$1,62 \times 10^{-12}$	ABLIM1	$1,62 \times 10^{-12}$	KRT5	$<1 \times 10^{-12}$
CD24	$1,62 \times 10^{-12}$	LRRC1	$1,11 \times 10^{-16}$	ACSL4	$1,62 \times 10^{-12}$	LAMA3	$1,62 \times 10^{-12}$
CDH1	$9,56 \times 10^{-8}$	MAL2	$1,62 \times 10^{-12}$	ADAM9	$1,27 \times 10^{-8}$	LIFR	$<1 \times 10^{-12}$
CDH2	$3,45 \times 10^{-8}$	MGAT5B	$1,11 \times 10^{-16}$	ALDH1A1	$<1 \times 10^{-12}$	MCAM	$<1 \times 10^{-12}$
CKMT1A	$<1 \times 10^{-12}$	MLPH	$1,62 \times 10^{-12}$	ALDH1A3	$1,62 \times 10^{-12}$	NR2F1	$7,51 \times 10^{-9}$
CKMT1B	$1,62 \times 10^{-12}$	MMP3	$<1 \times 10^{-12}$	ANXA8	$3,67 \times 10^{-6}$	NRP1	$<1 \times 10^{-12}$
CLDN3	$<1 \times 10^{-12}$	MMP9	$2,09 \times 10^{-5}$	AXL	$3,33 \times 10^{-16}$	PMP22	$1,11 \times 10^{-16}$
CLDN4	$2,92 \times 10^{-8}$	MST1R	$<1 \times 10^{-12}$	CD44	$9,79 \times 10^{-5}$	PRKCH	$<1 \times 10^{-12}$
CLDN7	$1,62 \times 10^{-12}$	OCLN	$1,62 \times 10^{-12}$	CDH3	$1,10 \times 10^{-3}$	PRRX1	4×10^{-2}
COL1A1	$1,62 \times 10^{-12}$	PKP3	$1,62 \times 10^{-12}$	COL17A1	$<1 \times 10^{-12}$	PTX3	$1,81 \times 10^{-8}$
COL1A2	$1,62 \times 10^{-12}$	PLIN3	$<1 \times 10^{-12}$	DCBLD2	$<1 \times 10^{-12}$	RGL1	$<1 \times 10^{-12}$
COL3A1	$<1 \times 10^{-12}$	PLP2	$1,62 \times 10^{-12}$	DCN	$1,62 \times 10^{-12}$	S100A2	$2,82 \times 10^{-3}$
COL5A1	$1,62 \times 10^{-12}$	POSTN	$1,62 \times 10^{-12}$	DNAJB4	$1,62 \times 10^{-12}$	S100A8	$1,31 \times 10^{-6}$
COL5A2	$1,62 \times 10^{-12}$	RAB25	$<1 \times 10^{-12}$	EGFR	$2,22 \times 10^{-16}$	SAA1	$5,55 \times 10^{-16}$
COL6A1	$<1 \times 10^{-12}$	S100A14	$<1 \times 10^{-12}$	ELK3	$1,11 \times 10^{-16}$	SEMA5A	$1,62 \times 10^{-12}$
CORO1A	$1,62 \times 10^{-12}$	SAMD9	$<1 \times 10^{-12}$	FBLN5	$1,62 \times 10^{-12}$	SERPINB2	$1,03 \times 10^{-4}$
DSP	$6,9 \times 10^{-10}$	SERPINE1	$8,56 \times 10^{-11}$	FGFBP1	$2,13 \times 10^{-5}$	SNAI2	$1,74 \times 10^{-12}$
ECM1	$1,62 \times 10^{-12}$	SPINT2	$1,62 \times 10^{-12}$	GJB3	$9,02 \times 10^{-11}$	SOX10	$<1 \times 10^{-12}$
EPCAM	$1,62 \times 10^{-12}$	SPRR1B	$1,26 \times 10^{-3}$	GNG11	$<1 \times 10^{-12}$	TBX3	$5,36 \times 10^{-4}$
ERBB3	$<1 \times 10^{-12}$	ST14	$<1 \times 10^{-12}$	IL1B	$3,04 \times 10^{-3}$	TFPI	$<1 \times 10^{-12}$
ESR1	$<1 \times 10^{-12}$	SYK	$2,6 \times 10^{-6}$	IL4R	$1,00 \times 10^{-9}$	TGFB111	$<1 \times 10^{-12}$
ESRP1	$<1 \times 10^{-12}$	TCF3	$1,62 \times 10^{-12}$	ITGA6	$<1 \times 10^{-12}$	THBD	$1,62 \times 10^{-12}$
ESRP2	$1,62 \times 10^{-12}$	TGFB1	$1,62 \times 10^{-12}$	ITGB1	$1,39 \times 10^{-13}$	TP63	$<1 \times 10^{-12}$
FN1	$<1 \times 10^{-12}$	THY1	$1,62 \times 10^{-12}$	JAG1	$3,33 \times 10^{-16}$	TWIST1	$7,72 \times 10^{-12}$
FXYP3	$1,62 \times 10^{-12}$	TSPAN1	$1,62 \times 10^{-12}$	KLF10	$<1 \times 10^{-12}$	TWIST2	$<1 \times 10^{-12}$
GREM1	$2,09 \times 10^{-9}$	VCAN	$<1 \times 10^{-12}$	KLK5	$2,34 \times 10^{-8}$	VIM	$1,62 \times 10^{-12}$
GRHL2	$1,62 \times 10^{-12}$			KRT14	$1,62 \times 10^{-12}$	WNT5A	$1,8 \times 10^{-2}$
ITGA5	$2,42 \times 10^{-4}$			KRT15	$<1 \times 10^{-12}$	ZEB1	$1,62 \times 10^{-12}$
ITGBL1	$3,33 \times 10^{-16}$			KRT16	$8,79 \times 10^{-8}$	ZEB2	$<1 \times 10^{-12}$

Table 2. Genes significantly upregulated (red) and downregulated (black) in BC vs healthy patients, analyzed in the UALCAN database, and sorted by protein name

The prognostic value of EMTome-derived genes was evaluated in BC using the Kaplan-Meier Plotter (KM Plotter) database. This platform integrates gene expression profiles with patient survival data, evaluated as RFS (Relapse-Free Survival, n=4929 patients), OS (Overall Survival, n=1879 patients), and DMFS (Distant Metastasis-Free Survival, n=2765 patients). For each gene, by selecting the best cut-off mode, the software stratifies patients into high- and low-expression groups based on the most significant probe expression value. Survival curves were generated for each comparison, and statistical significance was assessed using the log-rank test (**Figure 33**); p-values < 0.05 were considered significant.

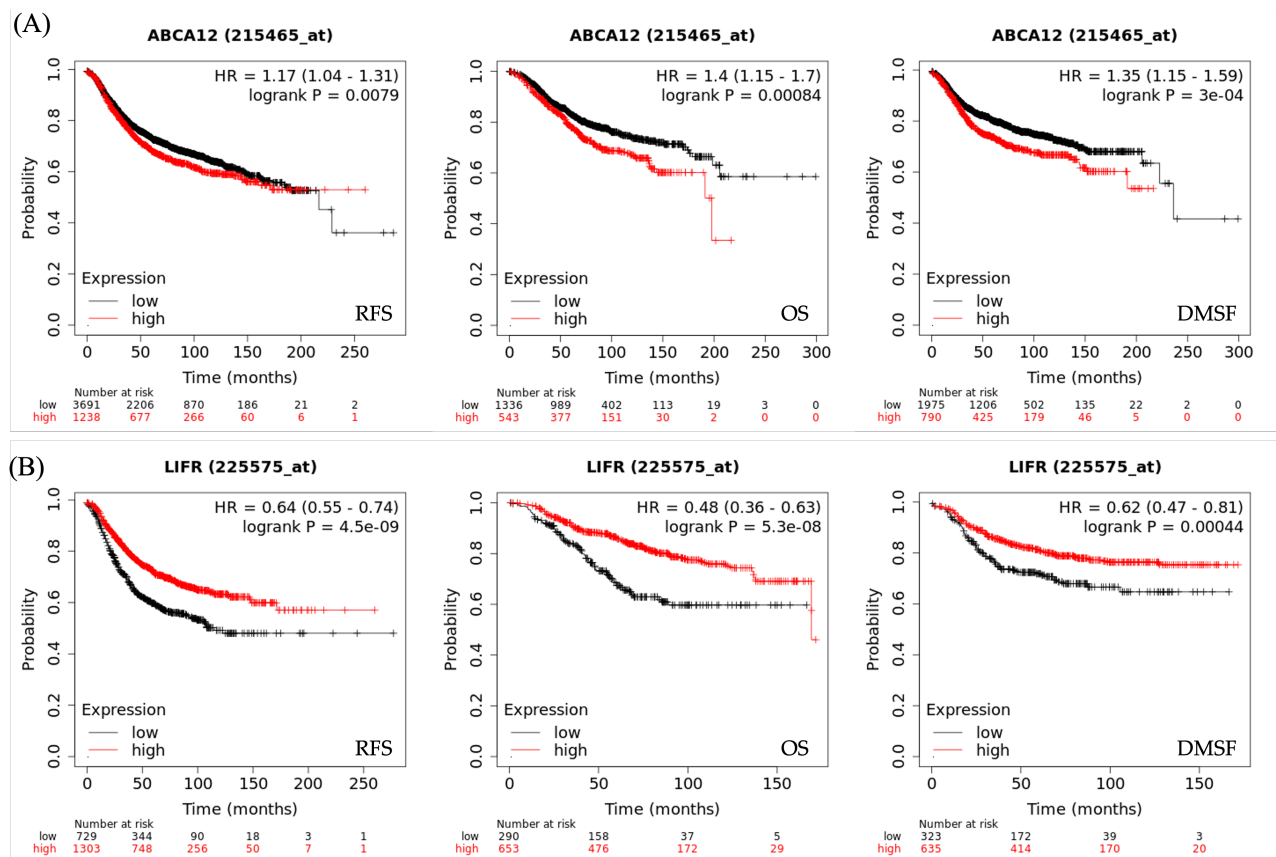


Figure 33. Example of a significant association between survival outcomes and better or worse prognosis. Expression levels of ABCA12 (e.g.) with all the survival outcomes (evaluated as RFS, OS, and DMFS) associated with worse prognosis (A). Expression levels of LIFR (e.g.) with all the survival outcomes (evaluated as RFS, OS, and DMFS) associated with better prognosis (B)

For this analysis, genes were defined as prognostically relevant only when their expression levels showed significant and coherent associations across all three survival endpoints (RFS, OS, and DMFS). Based on this criterion, elevated expression was associated with an unfavorable prognosis for 32 genes, whereas higher expression correlated with improved clinical outcomes for 28 genes (**Table 3**).

Survival analysis by KM plotter							
Genes whose high expression correlates with worse survival probability							
Gene	DMFS	OS	RFS	Gene	DMFS	OS	RFS
ABCA12	3 x 10 ⁻⁴	8 x 10 ⁻⁴	7,9 x 10 ⁻³	KRT16	9,8 x 10 ⁻⁹	1,5 x 10 ⁻⁵	9 x 10 ⁻¹⁵
ADAM9	1,3 x 10 ⁻⁷	3,8 x 10 ⁻⁵	4,6 x 10 ⁻¹³	LAD1	4,4 x 10 ⁻¹¹	2,4 x 10 ⁻⁴	1,4 x 10 ⁻⁸
ARTN	7,5 x 10 ⁻³	3,8 x 10 ⁻²	3,1 x 10 ⁻³	LAMA3	4,9 x 10 ⁻²	1,8 x 10 ⁻²	7,1 x 10 ⁻³
CD24	1,4 x 10 ⁻⁷	9,2 x 10 ⁻⁷	4 x 10 ⁻¹²	LTBP1	2 x 10 ⁻⁷	1,4 x 10 ⁻²	1,6 x 10 ⁻⁶
CDH2	1,2 x 10 ⁻⁴	8,3 x 10 ⁻⁶	4,2 x 10 ⁻⁷	MGAT5B	1,2 x 10 ⁻²	1,2 x 10 ⁻²	2,1 x 10 ⁻⁵
CKMT1A	2,1 x 10 ⁻¹⁶	3,6 x 10 ⁻⁸	7,8 x 10 ⁻¹⁴	MT2A	3,8 x 10 ⁻⁵	8,2 x 10 ⁻⁵	8,5 x 10 ⁻⁷
CKMT1B	2,1 x 10 ⁻¹⁶	3,6 x 10 ⁻⁸	7,8 x 10 ⁻¹⁴	NDRG1	4,6 x 10 ⁻¹¹	3,4 x 10 ⁻⁷	< 10 ⁻¹⁶
CLDN1	9,7 x 10 ⁻⁵	2,1 x 10 ⁻²	4,5 x 10 ⁻⁵	PLP2	2,7 x 10 ⁻⁴	8 x 10 ⁻³	3 x 10 ⁻¹¹
CLDN3	2 x 10 ⁻²	7,4 x 10 ⁻⁴	6,4 x 10 ⁻⁵	S100A8	6,9 x 10 ⁻⁹	6,8 x 10 ⁻⁶	1,3 x 10 ⁻¹²
CLDN4	9,5 x 10 ⁻³	3 x 10 ⁻³	1,2 x 10 ⁻²	SERPINE1	4,1 x 10 ⁻³	2,5 x 10 ⁻²	1,7 x 10 ⁻⁵
CLDN7	5,3 x 10 ⁻⁶	2,1 x 10 ⁻⁴	7,3 x 10 ⁻³	SLPI	1,4 x 10 ⁻⁶	4,5 x 10 ⁻⁴	8,3 x 10 ⁻⁶
EPCAM	4,5 x 10 ⁻⁶	2,1 x 10 ⁻⁴	< 10 ⁻¹⁶	SNAI1	1,8 x 10 ⁻⁷	1,6 x 10 ⁻⁴	7,4 x 10 ⁻⁴
ESRP1	3,9 x 10 ⁻⁷	3,7 x 10 ⁻⁵	8 x 10 ⁻¹⁴	ST14	1,6 x 10 ⁻⁵	1,5 x 10 ⁻⁶	4,6 x 10 ⁻⁹
ESRP2	5,4 x 10 ⁻⁴	4,5 x 10 ⁻⁵	3,4 x 10 ⁻⁴	TCF3	1,5 x 10 ⁻⁴	1,8 x 10 ⁻³	6,6 x 10 ⁻⁶
FN1	9,7 x 10 ⁻⁴	2,9 x 10 ⁻²	5 x 10 ⁻³	TGFB1	3,6 x 10 ⁻²	4,6 x 10 ⁻²	1,6 x 10 ⁻²
GREM1	4,6 x 10 ⁻⁷	2,4 x 10 ⁻³	5,6 x 10 ⁻¹¹	THY1	1,8 x 10 ⁻³	5,4 x 10 ⁻³	3,7 x 10 ⁻³
ITGB1	1 x 10 ⁻³	5,5 x 10 ⁻³	1,1 x 10 ⁻⁹				

Genes whose high expression correlates with better survival probability							
Gene	DMFS	OS	RFS	Gene	DMFS	OS	RFS
ALDH1A1	1,1 x 10 ⁻⁶	5 x 10 ⁻⁸	8,2 x 10 ⁻¹⁰	KCNMA1	8,2 x 10 ⁻⁵	4,5 x 10 ⁻⁵	1,7 x 10 ⁻⁸
AXL	3,8 x 10 ⁻²	1,4 x 10 ⁻²	1,3 x 10 ⁻⁴	KRT14	1,2 x 10 ⁻⁴	2,8 x 10 ⁻³	6,8 x 10 ⁻⁷
CD44	3,6 x 10 ⁻⁸	7,9 x 10 ⁻⁵	3 x 10 ⁻¹³	LIFR	4,4 x 10 ⁻⁴	5,3 x 10 ⁻⁸	4,5 x 10 ⁻⁹
CDH1	3,2 x 10 ⁻²	4 x 10 ⁻²	3,5 x 10 ⁻⁶	MLPH	2,2 x 10 ⁻⁸	3,9 x 10 ⁻³	< 10 ⁻¹⁶
COL17A1	2,5 x 10 ⁻³	7,8 x 10 ⁻⁴	8 x 10 ⁻¹⁶	NR2F1	2,2 x 10 ⁻²	1,6 x 10 ⁻²	4,2 x 10 ⁻²
COL6A1	3,3 x 10 ⁻²	3,6 x 10 ⁻³	1,1 x 10 ⁻⁴	PLAT	5,9 x 10 ⁻¹⁰	2,1 x 10 ⁻⁴	1,1 x 10 ⁻¹¹
CORO1A	1,7 x 10 ⁻³	3,1 x 10 ⁻⁴	2,3 x 10 ⁻³	PRKCH	1,4 x 10 ⁻³	8,3 x 10 ⁻³	1,6 x 10 ⁻⁹
DCN	8,7 x 10 ⁻⁵	3,4 x 10 ⁻³	1,7 x 10 ⁻⁴	S100A14	2,9 x 10 ⁻²	6 x 10 ⁻³	6,2 x 10 ⁻³
ERBB3	2,6 x 10 ⁻³	5,8 x 10 ⁻⁴	7,9 x 10 ⁻⁵	SAA1	1,8 x 10 ⁻⁵	4,2 x 10 ⁻³	6,6 x 10 ⁻⁵
ESR1	< 10 ⁻¹⁶	2,2 x 10 ⁻⁷	< 10 ⁻¹⁶	SEMA5A	1,9 x 10 ⁻²	1,3 x 10 ⁻²	5,6 x 10 ⁻⁶
FBLN5	1,1 x 10 ⁻²	5,3 x 10 ⁻⁴	5,9 x 10 ⁻⁷	SERPINB1	1,5 x 10 ⁻⁴	1,4 x 10 ⁻³	< 10 ⁻¹⁶
FGFR2	1,8 x 10 ⁻¹¹	1,2 x 10 ⁻⁸	3,1 x 10 ⁻¹¹	TBX3	1,8 x 10 ⁻⁴	5,7 x 10 ⁻⁴	3,1 x 10 ⁻¹⁰
FST	4,4 x 10 ⁻²	1,8 x 10 ⁻²	5 x 10 ⁻⁷	TFPI	4,3 x 10 ⁻²	5,5 x 10 ⁻⁵	5,4 x 10 ⁻³
ITGBL1	7,6 x 10 ⁻³	5,2 x 10 ⁻³	4 x 10 ⁻⁷	TP63	3,7 x 10 ⁻²	2,4 x 10 ⁻³	3,5 x 10 ⁻¹⁰

Table 3. List of genes coherently associated with all survival (DMFS, OS, and RFS). The red or black colors indicate the p-value

***In silico* biological connectivity between the EMT-selected genes**

Interpreting the obtained results to correlate genes with specific pathways is a challenging task. To address this aspect, genes showing significant expression changes in both the KM Plotter and UALCAN databases were further investigated using the STRING database to determine whether they are involved in specific biological pathways. In STRING, predicted gene relationships are visualized as interaction networks, with proteins represented as nodes and their interactions as edges. These networks may then be explored to check the underlying enriched pathways.

When all significant genes identified by UALCAN and KM Plotter were considered (n=129 genes) (**Figure 34A**), pathway enrichment analysis highlighted significantly enriched pathways, including ECM proteoglycans, with a particular focus on Syndecan, ECM organization and remodeling, and cell-cell/cell-matrix interactions. Collectively, these findings suggest that the selected genes are part of a network that could regulate or enable the transition from an epithelial to a mesenchymal phenotype, as well as its reverse process.

To further refine the analysis, the gene set was stratified according to concordant patterns of upregulation or downregulation observed in both UALCAN and KM Plotter, obtaining two distinct gene groups with complementary biological implications. The first group included genes upregulated in BC tissues and those whose elevated expression correlated with poorer clinical outcomes (n=67). Enrichment analysis of this subset confirmed a strong association with ECM proteoglycans, including Syndecan, and ECM remodeling pathways, underscoring their role in promoting cellular migration and invasion during EMT (**Figure 34B**).

The second group consisted of genes downregulated in BC tissues whose reduced expression was associated with a better prognosis (n=73). For this subset, pathway enrichment analysis (**Figure 34C**) revealed significant associations with Interleukin (IL)-4 and IL-13 signaling, tyrosine kinase receptor-mediated signaling, and L1 receptor signaling, as well as regulation of cell junctions and hemidesmosomes organization. Downregulation of these genes may lead to a more stable epithelial phenotype, thereby limiting EMT initiation and contributing to more favorable clinical outcomes.

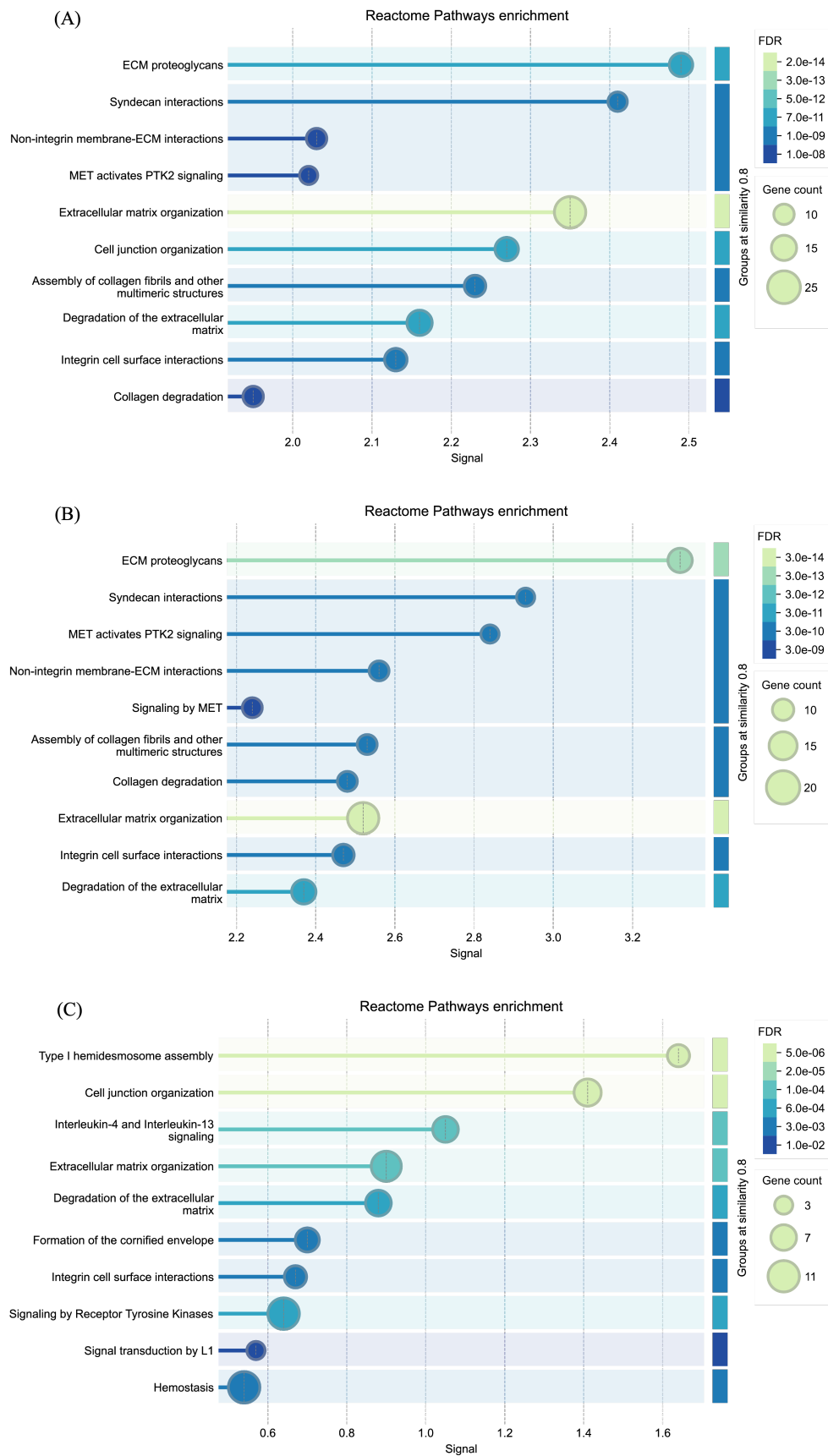


Figure 34. Reactome pathways enrichment analysis obtained by the STRING database using: Genes found differentially expressed by UALCAN and significantly associated with prognosis by KM Plotter analysis ($n = 129$) (A); Genes upregulated in BC compared to normal tissues and genes whose elevated expression associated with worse prognosis ($n = 67$) (B); Genes downregulated in BC compared to normal tissues and genes whose reduced expression associated with a better prognosis ($n = 73$ genes) (C)

To derive the most stringent BC-specific EMT gene signature, only genes demonstrating both diagnostic relevance in UALCAN and prognostic significance in the KM Plotter were considered. This additional level of filtering was applied to enrich for EMT-related genes with robust and consistent relevance across independent datasets and analytical platforms, ultimately improving the ability to identify more homogeneous patient subgroups.

A concordance between UALCAN and KM Plotter analyses was observed for 37 genes (**Figure 35**). Among these, 22 genes were upregulated in breast cancer tissues compared to normal counterparts, and their higher expression was associated with poorer prognosis. This subset represents potential markers that are not only biologically relevant but also informative for disease progression and outcome prediction. Conversely, 15 genes were downregulated in tumor tissues, and their downregulation was associated with improved prognosis, suggesting a possible protective role when these genes are suppressed in breast cancer.

For instance, *COL17A1* is more expressed in normal breast tissue and has been associated with the inhibition of cell migration and invasion; accordingly, elevated *COL17A1* expression in breast cancer patients correlates with better clinical outcomes (Yodsurang et al., 2017). Similarly, reduced expression of *LIFR* has been linked to breast cancer progression, with lower protein levels observed in advanced-stage disease (Real et al., 2019). Analogously, *FBLN5* has been reported to exert anti-tumor effects in breast cancer cells (Mohamedi et al., 2019).

Interestingly, among these 37 genes, several are well-established regulators of stemness, suggesting that heterogeneous signals from the tumor microenvironment may sustain the cancer stem cell population and contribute to cellular plasticity and hybrid EMT states (Morel & Ouzounova, 2025). Key examples include TGF- β , a central regulator of both stemness and EMT (Bellomo et al., 2016), whose signaling, in cooperation with ECM components like fibronectin (*FNI*), may support cancer stem cell self-renewal by inducing ECM remodeling and reinforcing stemness-related signaling networks.

Moreover, several of the 37 identified genes have been widely investigated as potential markers for CSCs, including *AXL* (Asiedu et al., 2014; Pan et al., 2025), *CD44*, *CD24*, *ALDH1A1* (Ansari et al., 2024), *EPCAM* (Hiraga et al., 2016), and claudins, which are well-established biomarkers of breast cancer stem cells (Hsu et al., 2020; Lu & Kang, 2019). Beyond their role as markers, claudins have also been implicated in EMT, metastatic progression, CSC enrichment, and therapy resistance, underscoring their contribution to tumor plasticity (Gowrikumar et al., 2019).

Furthermore, other genes identified have been implicated in regulating CSC behavior, either by enhancing their proliferative capacity, as reported for *PRKCH* (Kozawa et al., 2021), or by influencing their differentiation, as observed for *ST14* (X. Yan et al., 2016). *TCF3*, also known as *TCF7L1* and belonging to the TCF/LEF family of transcription factors, plays a central role in controlling stem cell identity, exerting context-dependent effects that can favor either self-renewal or differentiation (Slyper et al., 2012). Conversely, the splicing regulator *ESRP1* has been shown to inhibit CD44s-dependent CSC activity and displays an inverse association with CSC-related gene signatures (Zhang et al., 2019), while silencing of *ESRP2* reduces breast cancer cell proliferation (He et al., 2024). In addition, leukemia inhibitory factor (LIF) negatively regulates CSC tumorigenic potential by activating Hippo signaling kinases (Seeneevassen et al., 2020).

Among secreted factors, both SAA1 and GREM1 are predominantly produced by cancer stem cells (Wang et al., 2023; K. Yan et al., 2014). Notably, GREM1 also functions as a TGF- β antagonist and acts as a ligand for VEGFR2, highlighting its multifaceted role in CSC-associated signaling pathways (Zhu et al., 2023).

Moreover, CSC-associated stemness is tightly linked to epithelial–mesenchymal plasticity and migratory capacity. Epithelial cancer cells undergoing EMT retain a dynamic cell–cell adhesion system that enables collective migration. In this context, CDH2- and PLP2-mediated adhesion are essential for collective migration in three-dimensional settings, the latter acting through ZO-1–dependent cytoskeletal remodeling (Kim et al., 2024; Ghosh et al., 2021). Tumor cell motility associated with plastic conversion toward a stem cell–like phenotype is further supported by SERPINE1 in response to TGF- β 1 and EGF, while SEMA5A promotes invasiveness and metastasis via ERK activation and induction of angiogenic factors (Sadanandam et al., 2012).

Furthermore, mechanotransduction also contributes to CSC invasion, as MGAT5-driven N-glycan branching regulates stiffness-induced invasion in CSCs (Marhuenda et al., 2021), whereas decorin (*DCN*) counteracts invasion and metastasis in inflammatory breast cancer (Hu et al., 2021). In addition, several surface-associated proteins identified here are linked to both stemness and migration, including Thy-1 (CD90), a GPI-anchored protein involved in adhesion, differentiation, and migration in mesenchymal stem cells (Yang et al., 2020), and *LADI*, an ankyrin family member implicated in cell motility (Jiang et al., 2023).

Finally, therapy resistance emerges as a recurrent feature of CSC-associated genes. Artemin (*ARTN*) promotes resistance to trastuzumab, chemotherapy, and radiotherapy by inducing CSC-like traits in breast cancer cells (Ding et al., 2014; Banerjee et al., 2012), while the lipid transporter ABCA12

sustains CSC survival and self-renewal by enabling evasion of chemotherapy-induced cell death (J. Cui et al., 2023).

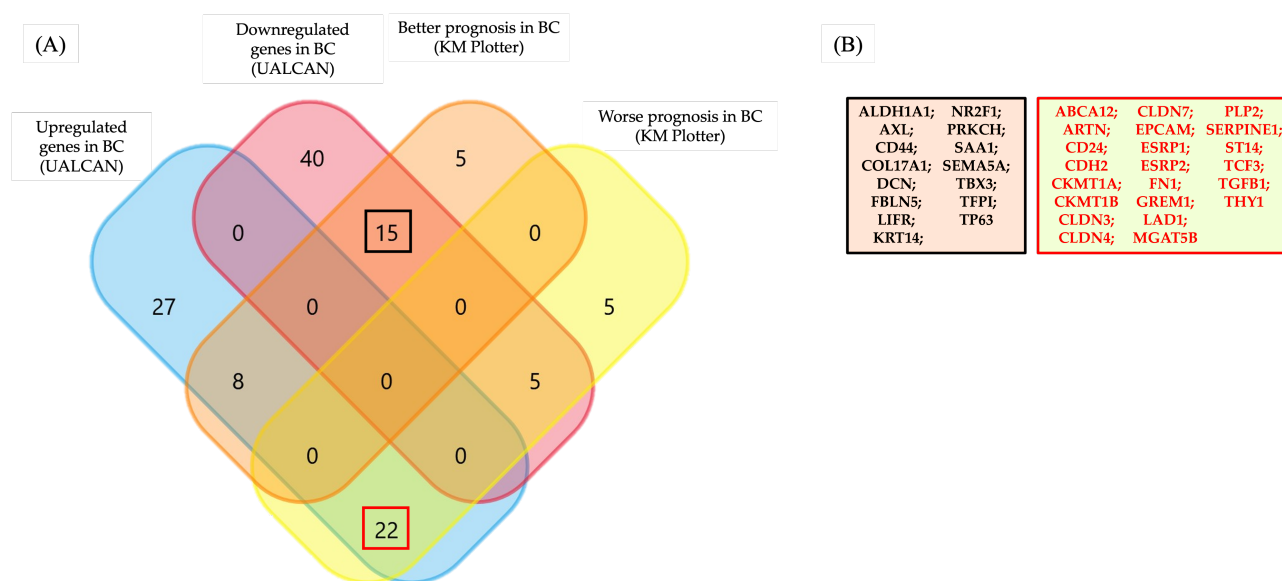


Figure 35. Venn diagram of gene surveys from UALCAN and Kaplan-Meier Plotter (A). List of 37 genes, downregulated (black) and overexpressed (red), whose expression was found to be significant in both Kaplan-Meier plotter and UALCAN (B)

Finally, to further explore the clinical relevance of these findings, their associations with current clinical-pathological parameters were investigated using the bcGeneXMiner and Gene expression-based Outcome for Breast Cancer Online (GOBO) databases (**Table 4**). The analysis included the immunohistochemical expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), lymph node (N) metastasis status, and tumor grading (G1-G3).

In line with the proposed hypothesis, the 22 genes linked to unfavorable prognosis (highlighted in red in **Table 4**) were significantly associated with aggressive tumor features, including ER-/PR-, HER2+, lymph node positivity, and high tumor grade (G3). These parameters are widely recognized as markers of poor clinical outcome in breast cancer. Conversely, the 15 genes associated with favorable prognosis (highlighted in black in **Table 4**) showed the opposite pattern, correlating with ER+/PR+, HER2-, node-negative status, and low-grade tumors (G1), which are generally indicative of less aggressive disease.

Overall, these findings underscore the relevance of the identified gene signature as a robust indicator of tumor aggressiveness and a valuable tool for stratifying breast cancer patients based on molecular and clinical risk profiles. At the same time, they highlight the profound heterogeneity of both breast cancer and the EMT process, emphasizing that EMT is governed by highly regulated and context-

dependent molecular programs that a limited set of conventional markers cannot fully capture. Taken together, these results therefore support the need for integrative and multi-level approaches to more accurately characterize EMT dynamics in breast cancer.

Gene name	ER+/PR+	ER-/PR-	HER2+	HER2-	N+	N-	G1	G2	G3
ABCA12	-	-	< 0.0001***		0.0002***			0.00324**	
ARTN	< 0.0001***			< 0.0001***	-	-			0.08766
CD24		< 0.0001***	< 0.0001***		0.0009***				< 0.0001***
CDH2		< 0.0001***	< 0.0001***		-	-			0.0001***
CKMT1A	/	/	< 0.0001***		-	-	/	/	/
CKMT1B		< 0.0001***	< 0.0001***		0.0017**		/	/	/
CLDN3	/	/	0.0040**		< 0.0001***				0.0842
CLDN4		< 0.0001***	0.0006***		0.0002***				< 0.0001***
CLDN7	< 0.0001***		-	-	< 0.0001***			< 0.0001***	
EPCAM		< 0.0001***	0.0004***		0.0073**				< 0.0001***
ESRP1		< 0.0001***	< 0.0001***		< 0.0001***				< 0.0001***
ESRP2	< 0.05*		< 0.0001***		< 0.0001***			< 0.0001***	
FN1	-	-	< 0.0001***		0.0022**		0.00321**		
GREM1		< 0.0001***	< 0.0001***		< 0.0001***			0.02523*	
LAD1		< 0.0001***	< 0.0001***		-	-			< 0.0001***
MGAT5B		< 0.0001***	0.0020**		0.0385*		/	/	/
PLP2		< 0.0001***	< 0.0001***		< 0.0001***				0.55961
SERPINE1		< 0.05*	-	-	< 0.0001***			0.00007***	
ST14		< 0.0001***	< 0.0001***			0.0095**			0.0002***
TCF3		< 0.0001***	< 0.0001***		-	-	0.33625		
TGFB1	/	/	-	-	-	-	0.00033***		
THY1	-	-	< 0.0001***		-	-	< 0.0001***		

Gene name	ER+/PR+	ER-/PR-	HER2+	HER2-	N+	N-	G1	G2	G3
ALDH1A1	< 0.01*		0.0164*		0.0103*		0.00292**		
AXL	< 0.0001***		-	-	-	-	< 0.0001***		
CD44		< 0.0001***		< 0.0001***		< 0.0001***	< 0.0001***		
COL17A1	< 0.0001***		-	-		< 0.0001***	< 0.0001***		
DCN	< 0.0001***		-	-	-	-	< 0.0001***		
FBLN5		< 0.0001***	-	-	-	-	< 0.0001***		
KRT14		< 0.0001***		< 0.0001***	-	-	0.00002***		
LIFR	< 0.0001***			< 0.0001***		0.0037**	0.00836**		
NR2F1	< 0.0001***		< 0.0001***		0.0023**		< 0.0001***		
PRKCH		< 0.0001***	< 0.0001***		0.0006***		< 0.0001***		
SAA1		< 0.0001***		< 0.0001***		0.0015**	/	/	/
SEMA5A	< 0.0001***		-	-		0.0372*	< 0.0001***		
TBX3	< 0.0001***			< 0.0001***		0.0113*	< 0.0001***		
TFPI		< 0.0001***	< 0.0001***		0.0074**		< 0.0001***		
TP63	< 0.0001***		-	-	-	-	< 0.0001***		

Table 4. Relationship between gene expression of EMT-BC genes and clinical-pathological parameters, including estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth receptor 2 (HER) status, lymph node (N) involvement, and grading (G1/G2/G3). The genes associated with poor and good prognosis, as determined by KM Plotter analysis, are indicated in red and in black, respectively. Data were retrieved for each gene using bcGeneXMiner, and the grading was assessed using the GOBO database. p < 0.05 was considered significant (p < 0.05*, p < 0.01**, p < 0.001***)

Gelatinolytic activities of MMP-2 and MMP-9 in breast cancer patients

Recent findings have implicated matrix metalloproteinases (MMPs) as key promoters and mediators of both developmental and pathological epithelial-to-mesenchymal transition (EMT) processes in the breast. In the context of cancer progression, MMPs contribute to EMT by directly inducing phenotypic changes in epithelial cells when enriched in the tumor microenvironment, by being upregulated in cancer cells that have undergone EMT and thereby facilitating invasion and metastasis, and by promoting the generation of activated stromal-like cells that further sustain tumor progression through continued MMPs production. Despite this evidence, a direct association between MMPs activity and EMT in human breast tissues remains largely unestablished. Based on previously published data demonstrating marked heterogeneity in the gelatinolytic activity of MMP-2 and MMP-9 in breast cancer tissues, as well as the presence of their activated forms predominantly in tumor samples (Di Cara et al., 2018; Cancemi et al., 2019) the next step was to determine whether the EMT phenotype of breast tumors, defined in the patient cohort by western blot analysis, is directly associated with gelatinases expression and activation. Specifically, the relationship between EMT marker expression and the gelatinolytic activity of MMP-2 and MMP-9 was assessed by gelatin zymography, to provide experimental evidence for a functional link between EMT and MMPs-driven extracellular matrix remodeling in breast cancer.

As shown in **Figure 36**, gelatinolytic activity was detected in almost all breast cancer tissues analyzed, although some variations in the presence and intensity of the zymographic bands were observed. The activity levels of the collagenolytic bands within the group, normalized to β -Actin levels, were compared with the expression levels of EMT markers to identify possible cross-correlations.

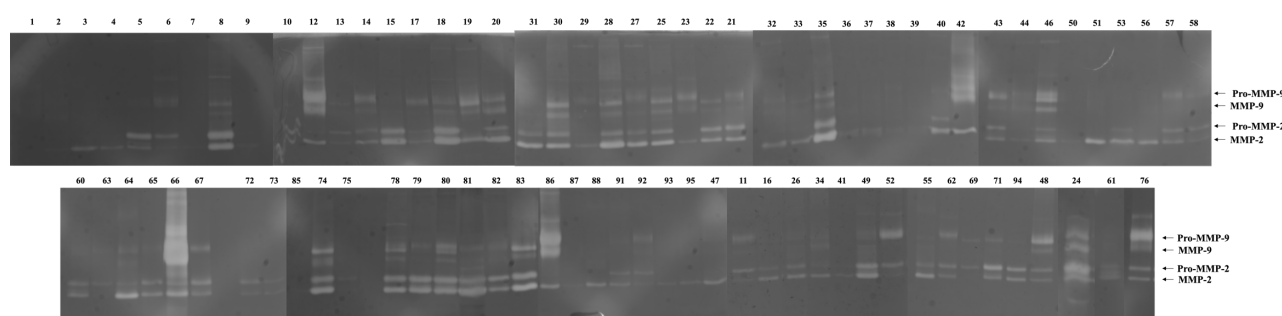


Figure 36. Gelatine zymography performed on tissue extracts from 86 BC patients, previously characterized for EMT marker expression by western blot. Distinct and heterogeneous lytic bands corresponding to both the latent and active forms of MMP-2 and MMP-9 were observed across the patients' cohort

The results showed a weak linear correlation between pro-MMP-2 and pro-MMP-9, a significant correlation between the pro-enzymatic forms and their respective activated forms, and a moderate correlation between the two activated enzymes (**Table 5**), as previously reported (Di Cara et al., 2018). Regarding EMT markers, only weak correlations were observed between MMP-2 and E-cadherin, CK-18, and α -SMA, whereas pro-MMP-2 showed weak correlations with Vimentin and CK-18. Finally, pro-MMP-9 was weakly correlated only with CK-18. Overall, the data point to multiple pathways that may be involved in the crosstalk between the microenvironment, specifically MMPs expression, and EMT.

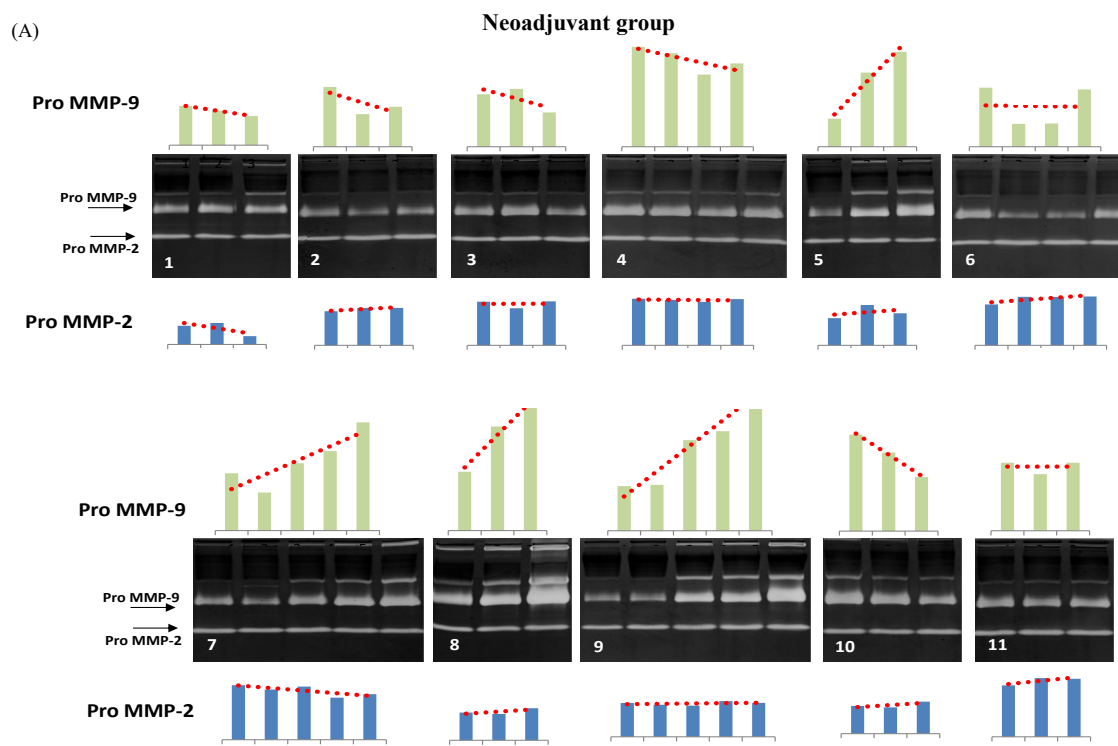
	pro-MMP-9	MMP-9	pro-MMP-2	MMP-2
pro-MMP-9		$r = 0,5642135^*$	$r = 0,3062307^{**}$	
MMP-9	$r = 0,5642135^*$		$r = 0,2220321^*$	$r = 0,2756466^{**}$
pro-MMP-2	$r = 0,3062307^{**}$	$r = 0,2220321^*$		$r = 0,6590134^*$
MMP-2		$r = 0,2756466^{**}$	$r = 0,6590134^*$	
E-cadherin				$r = 0,3387393^{**}$
Vimentin			$r = 0,2138676^*$	
CK-18	$r = 0,2133239^*$		$r = 0,2429915^*$	$r = 0,2674085^{**}$
α -SMA				$r = 0,2126342^*$

Table 5. Pearson correlation of MMPs and EMT markers.
r indicates the correlation coefficient. $p < 0.05$ was considered significant ($p < 0.05^*$, $p < 0.01^{**}$)

Assessment of metalloproteases levels in breast cancer sera and correlation with response to therapy

Based on the previous results, which suggest a possible association between MMPs and EMT, and considering the relative simplicity of serum-based analyses, the last objective of this thesis was to assess whether MMPs expression could, to some extent, be indicative of therapeutic response in patients receiving neoadjuvant treatment (n= 11) or treated following a diagnosis of metastatic carcinoma (n= 10).

Figures 37A and **37B** show the results obtained for the analyzed patient groups. Quantitative analyses are reported above and below each zymogram respectively, and the red line indicates the trend of expression across the different sampling time points.



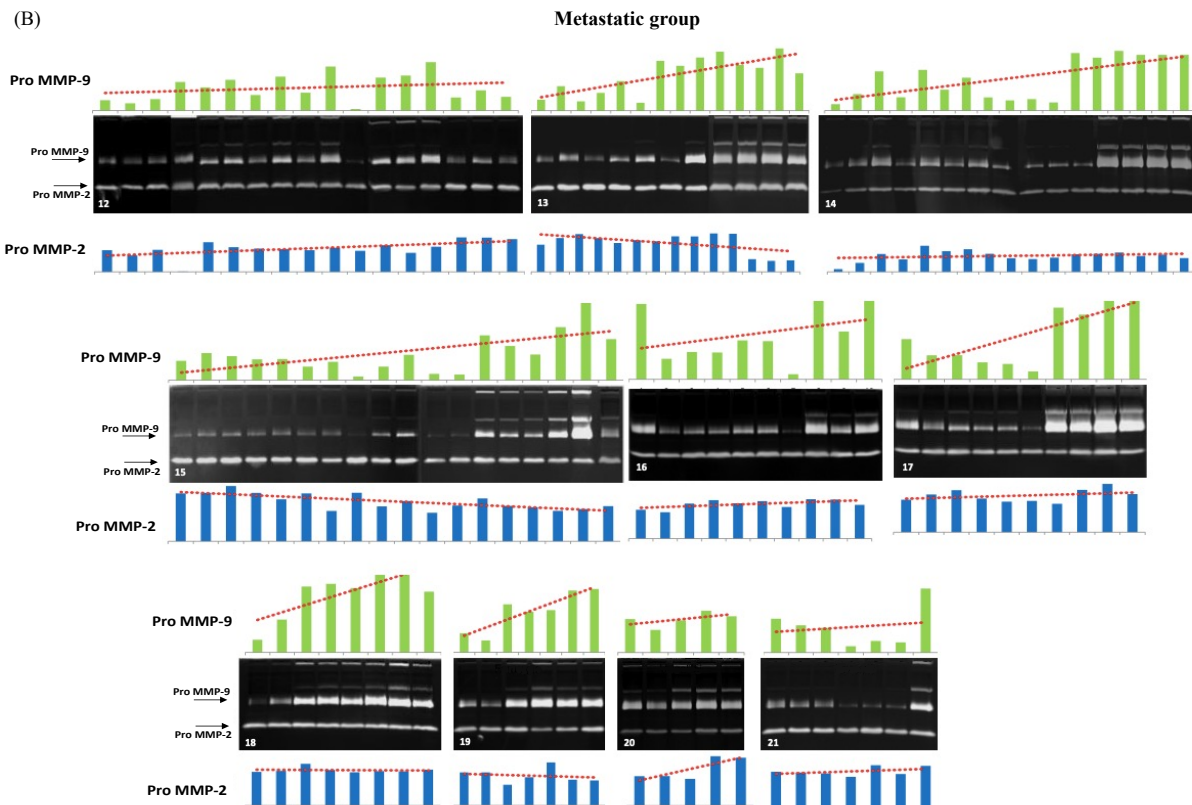


Figure 37. Zymograms of the “neoadjuvant” group (A) and the “metastatic” group (B). Each lane represents a treatment session, arranged in the chronological order of sample collection. Histograms above (pro-MMP-9) and below (pro-MMP-2) each zymogram show the quantitative analysis of the lytic bands, with the red line indicating the trend.

Interestingly, the modulation of enzymatic activity during therapy is more pronounced for pro-MMP-9 than for pro-MMP-2 across the selected groups.

To evaluate potential correlations between changes in MMPs activity and response to neoadjuvant and adjuvant treatment, variations in pro-MMP-9 and pro-MMP-2 activity between the first and last available samples were monitored. For this purpose, zymographic assays were repeated in parallel and in triplicate, using only the first and last available samples (**Figure 38**).

Patients in the “neoadjuvant” group were subsequently classified as responders or non-responders based on the percentage of tumor regression observed at the end of the treatment. Specifically, patients showing a regression of $\geq 50\%$ were classified as responders, while those with a regression of $< 50\%$ were classified as non-responders.

On the other hand, metastatic patients undergoing adjuvant treatment were instead classified according to disease evolution into stable disease (S) and progression (P).

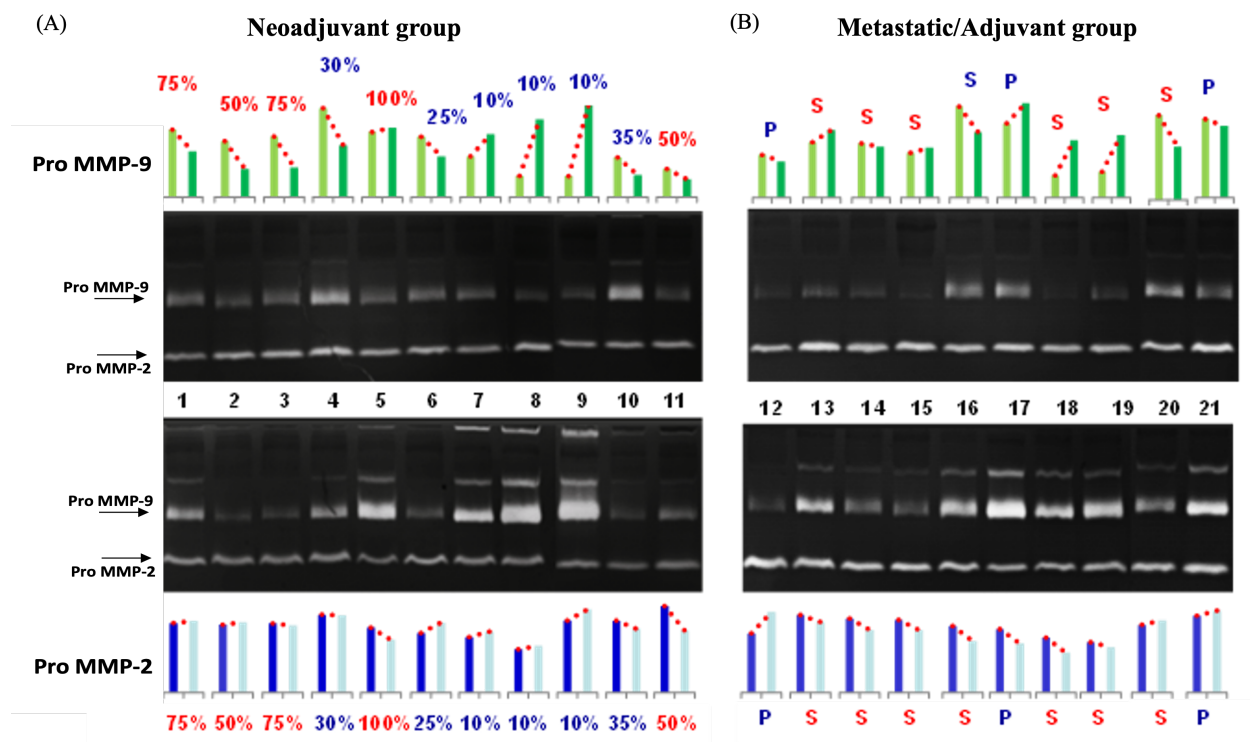


Figure 38. Zymograms of the “neoadjuvant group” (A) and “metastatic/adjuvant” group (B). For each patient, the top and bottom zymograms correspond to the first and last samples, respectively. Histograms above (pro-MMP-9) and below (pro-MMP-2) each zymogram show the quantitative analysis of the lytic bands, with the red line representing the trend. Therapy response is indicated as the percentage of tumor mass regression in neoadjuvant patients and as stable disease (S) or disease progression (P) in adjuvant patients

The percentage variation in the activity of the two gelatinases was calculated for both patients’ groups, and statistical significance was evaluated using a t-test. As shown in **Figure 39**, the percentage variation in circulating pro-MMP-2 was significantly lower in patients who responded to neoadjuvant treatment and in those with stable disease during adjuvant therapy. Conversely, patients with disease progression showed a higher increase in pro-MMP-2 levels. These findings suggest that tumor regression was associated with reduced or relatively stable circulating pro-MMP-2 levels, whereas disease progression was associated with increased pro-MMP-2 levels.

Conversely, pro-MMP-9 in the neoadjuvant group classified as non-responders showed a marked percentage variation, although this difference did not reach significance, likely due to the high variability observed in the dataset.

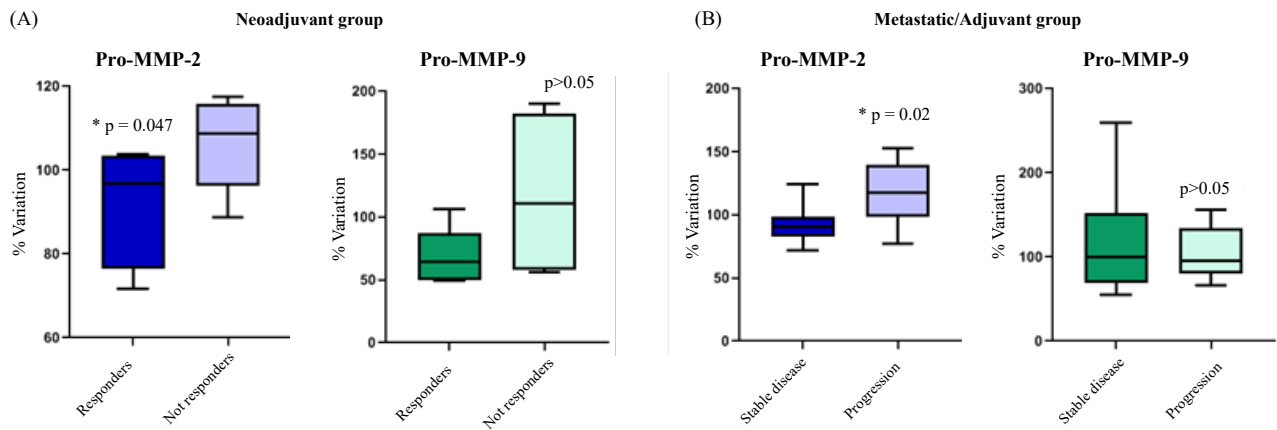


Figure 39. Box plots showing the percentage change in pro-MMP-2 and pro-MMP-9 between the first and last available samples for each patient in the “neoadjuvant” group (A) and the “metastatic/adjuvant” group (B). “Neoadjuvant” patients were classified as responders (>50% tumor regression) or non-responders (<50% regression) at the end of therapy. “Adjuvant” patients were categorized as having stable disease or progression based on clinical outcome. Statistical significance was assessed using the t-test ($p < 0.05$)

The observed significance of pro-MMP-2, in contrast to the non-significant changes in pro-MMP-9, highlights the utility of including additional functional markers in patient sera analyses. Moreover, this divergence between the two gelatinases underscores the complex and heterogeneous regulation of EMT in breast cancer: rather than following a rigid program, EMT appears to reflect a spectrum of molecular states that depends on the tumor microenvironment and specific circulating factors. Therefore, these findings reinforce the idea that EMT should be viewed as a dynamic, context-dependent process, in which distinct markers may contribute differently to functional phenotypes such as cell migration and invasion.

Supplementary Tables

Supplementary Table 1. Clinicopathological characteristics of breast cancer patients analyzed in this study

Patient ID	Age	T	N	Grading	HER2	ER%	PGR%	Ki-67%
1	67	1	0	2	2	95	10	30
2	65	2	0	3	0	95	30	70
3	64	2	1	2	1	85	5	17
4	63	1	1	1	0	60	70	5
5	42	1	1	2	0	40	60	45
6	61	1	-	3	0	50	40	-
7	63	2	1	3	1	95	90	50
8	47	1	0	2	1	80	60	20
9	63	2	1	2	0	90	65	14
10	67	1	0	3	1	95	20	40
11	41	2	1	3	2	80	5	60
12	58	2	1	3	0	0	0	85
13	56	2	1	3	2	0	0	40
14	60	1	0	3	1	0	0	60
15	51	1	1	1	1	95	95	7
16	58	4	1	3	3	0	0	13
17	56	1	0	3	0	0	0	70
18	52	2	1	1	1	65	40	12
19	43	2	0	2	0	80	30	35
20	65	1	1	2	1	95	5	25
21	66	2	1	3	3	0	0	35
22	64	1	0	2	1	0	0	10
23	61	2	0	3	0	3	0	61
24	60	2	1	3	3	70	60	20
25	53	2	0	3	0	0	0	60
26	75	2	1	1	2	95	80	15
27	54	1	0	3	2	0	0	85
28	44	2	1	3	3	70	60	45
29	48	2	0	3	1	85	85	60
30	45	2	1	3	0	95	95	60
31	75	1	0	3	1	95	40	32
32	41	2	0	2	2	75	65	85
33	67	2	0	2	1	95	80	20
34	49	2	1	3	3	65	85	45
35	75	2	1	2	0	95	90	22
36	48	1	0	2	2	70	60	18
37	37	3	1	3	0	80	0	17
38	55	2	1	3	1	90	0	15
39	64	2	1	2	1	80	70	9
40	57	2	0	2	3	70	70	25
41	37	2	1	2	0	95	95	25
42	67	2	1	3	0	0	0	25

43	64	2	1	3	3	0	5	50
44	72	3	1	3	0	0	0	90
45	44	2	1	3	0	70	80	40
46	31	1	0	3	0	10	0	40
47	57	1	0	3	1	0	0	28
48	45	2	0	3	0	0	0	60
49	67	2	1	3	1	95	95	36
50	61	1	1	2	0	95	80	25
51	56	2	1	2	2	95	90	15
52	64	3	0	3	0	0	0	0
53	57	2	1	3	0	95	60	70
54	83	3	1	3	-	15	20	-
55	65	2	0	1	1	95	95	22
56	47	1	0	2	0	70	75	2
57	54	2	0	3	0	80	40	-
58	47	1	1	2	3	5	5	-
59	64	2	1	2	1	85	5	17
60	70	2	0	2	-	10	5	-
61	72	-	-	3	3	0	0	-
62	64	1	0	2	2	5	15	-
63	76	2	-	3	0	0	0	-
64	51	2	1	3	2	95	75	28
65	77	1	-	2	-	5	10	-
67		4	0	2	2	5	5	-
68	80	2	1	3	0	80	90	-
69	73	-	0	-	-	-	-	-
70	-	2	1	-	2	0	0	5
71	64	2	1	3	3	60	0	-
73	51	-	0	3	3	0	0	60
74	38	2	0	3	3	0	0	55
75	53	1	1	3	0	75	20	30
76	60	-	1	3	1	70	80	10
77	52	2	1	3	0	5	0	50
78	36	2	1	3	3	5	0	42
79	46	2	0	3	1	50	70	20
80	37	1	1	2	2	70	70	25
81	59	2	1	3	0	85	25	40
82	60	2	0	2	0	85	5	20
83	52	3	0	3	1	80	40	60
84	75	4	1	3	0	60	0	5
87	66	2	0	2	1	90	80	11
88	78	3	1	3	1	0	0	28
89	56	1	0	3	3	75	5	40
92	44	1	1	3	3	30	10	45
94	56	1	1	3	3	80	5	30
95	66	2	1	2	1	90	85	20

Supplementary Table 2. List of 14 selected studies from EMTome used to generate the EMT signature specifically associated with breast cancer

	Signature	Pubmed Identification	Description	Source	Cancer type	Genes/markers
1	Alsuliman et al.2015	PMID: 26245467	17 EMT signature genes	Microarray	Breast Cancer	17
2	Berardi et al.2015	PMID: 26044847	ATRA treatment modulated EMT signature	qPCR	Breast cancer	27
3	Cheng et al.2014	PMID: 25060555	A signature of EM plasticity and stromal activation in primary tumor	Microarray	Breast Cancer	51
4	Choi et al.2010	PMID: 20215510	A cell line-derived EMT signature	Microarray	Breast Cancer	200
5	Creighton et al.2009	PMID: 19666588	EMT profile in CD44(+)/CD24(-/low)-MS and claudin-low signatures	Microarray	Breast Cancer	15
6	Cursons et al.2015	PMID: 25975820	EGF- and/or hypoxia-induced EMT (MDA-MB-468, PMC42-ET, PMC42-LA)	RNAseq	Breast cancer	206
7	Grosse-Wilde et al.2015	PMID: 26020648	EM expression signature and stemness	Microarray	Breast Cancer	60
8	Iseri et al.2011	PMID: 21177063	Multidrug resistant MCF-7 associated EMT	Microarray	Breast Cancer	15
9	Javaid et al.2015	PMID: 25678598	26-gene EMT RNA signature	shRNA library screen	Breast Cancer	26
10	Jordan et al.2013	PMID: 23716599	EMT gene signature shared between epithelial stem cells developmentally entering EMT and Claudin-low breast cancer cells	Microarray	Breast Cancer	137
11	Remšík et al.2018	PMID: 29462126	EMT associated 10-molecule surface signature	Antibody-based cell surface screening and flow cytometry	Breast Cancer	10
12	Soundararajan et al.2015	PMID: 26123483	EMT-/stem-cell markers	DMFS- and RFS analyses	Breast Cancer	39
13	Taube et al.2010	PMID: 20713713	Core EMT signature	Microarray	Breast Cancer	247
14	Thompson et al.2020	PMID: 31683225	EMT signature to immune checkpoint blockade in lung cancer	Literature	Breast Cancer	12

Supplementary Table 3. List of 144 genes selected by the EMTome database

Number	Gene Symbol	Gene name	Derived from EMTOME
1	<u>ABCA12</u>	ATP binding cassette subfamily A member 12	4-13
2	<u>ABCC4</u>	ATP binding cassette subfamily C member 4	4-10
3	<u>ABLIM1</u>	Actin binding LIM protein 1	6-13
4	<u>ACKR3</u>	Atypical chemokine receptor 3	3-10
5	<u>ACSL4</u>	Acyl-CoA synthetase long chain family member 4	4-10
6	<u>ADAM9</u>	ADAM metalloproteinase domain 9	3-6
7	<u>ALDH1A1</u>	Aldehyde dehydrogenase 1 family member A1	6-12
8	<u>ALDH1A3</u>	Aldehyde dehydrogenase 1 family member A3	7-13
9	<u>ANXA8</u>	Annexin A8	7-13
10	<u>ARTN</u>	Artemin	6-13
11	<u>AXL</u>	AXL receptor tyrosine kinase	6-10
12	<u>C1orf116</u>	Chromosome 1 open reading frame 116	6-13
13	<u>CCN2</u>	Cellular communication network factor 2	9-13
14	<u>CD24</u>	CD24 molecule	4-7-11
15	<u>CD44</u>	CD44 molecule (Indian blood group)	11-12
16	<u>CDH1</u>	Cadherin 1	1-4-5-7-8-9-12-13-14
17	<u>CDH2</u>	Cadherin 2	5-8-9-10-12-13
18	<u>CDH3</u>	Cadherin 3	13-14
19	<u>CHN1</u>	Chimerin 1	4-13
20	<u>CKMT1A</u>	Creatine kinase, mitochondrial 1A	4-13
21	<u>CKMT1B</u>	Creatine kinase, mitochondrial 1B	4-7-13
22	<u>CLDN1</u>	Claudin 1	8-13
23	<u>CLDN3</u>	Claudin 3	1-4-8
24	<u>CLDN4</u>	Claudin 4	1-4-8-9-14
25	<u>CLDN7</u>	Claudin 7	1-4-8
26	<u>COL17A1</u>	Collagen type XVII alpha 1 chain	6-7-13
27	<u>COL1A1</u>	Collagen type I alpha 1 chain	3-7
28	<u>COL1A2</u>	Collagen type I alpha 2 chain	2-3-13
29	<u>COL3A1</u>	Collagen type III alpha 1 chain	3-13
30	<u>COL5A1</u>	Collagen type V alpha 1 chain	3-10
31	<u>COL5A2</u>	Collagen type V alpha 2 chain	7-13
32	<u>COL6A1</u>	Collagen type VI alpha 1 chain	7-10
33	<u>CORO1A</u>	Coronin 1A	10-13
34	<u>DCBLD2</u>	Discoidin, CUB and LCCL domain containing 2	4-6
35	<u>DCN</u>	Decorin	7-13
36	<u>DNAJB4</u>	DnaJ heat shock protein family (Hsp40) member B4	4-13
37	<u>DSP</u>	Desmoplakin	1-5
38	<u>ECM1</u>	Extracellular matrix protein 1	6-10-13
39	<u>EGFR</u>	Epidermal growth factor receptor	2-8-10
40	<u>ELK3</u>	ETS transcription factor ELK3	4-6-10
41	<u>EPCAM</u>	Epithelial cell adhesion molecule	4-7-13-14
42	<u>ERBB3</u>	Erb-b2 receptor tyrosine kinase 3	4-12
43	<u>ESR1</u>	Estrogen receptor 1	8-12
44	<u>ESRP1</u>	Epithelial splicing regulatory protein 1	4-13

45	<u>ESRP2</u>	Epithelial splicing regulatory protein 2	4-13
46	<u>FBLN5</u>	Fibulin 5	7-13
47	<u>FGFBP1</u>	Fibroblast growth factor binding protein 1	7-13
48	<u>FGFR2</u>	Fibroblast growth factor receptor 2	6-13
49	<u>FN1</u>	Fibronectin 1	1-2-5-12
50	<u>FOXC2</u>	Forkhead box C2	2-5-12
51	<u>FST</u>	Follistatin	7-13
52	<u>FXYD3</u>	FXYD domain containing ion transport regulator 3	4-7
53	<u>GJB3</u>	Gap junction protein beta 3	6-9-13
54	<u>GNG11</u>	G protein subunit gamma 11	7-10
55	<u>GREM1</u>	Gremlin 1, DAN family BMP antagonist	7-13
56	<u>GRHL2</u>	Grainyhead like transcription factor 2	4-13
57	<u>GSC</u>	Goosecoid homeobox	5-12
58	<u>IL1B</u>	Interleukin 1 beta	7-13
59	<u>IL4R</u>	Interleukin 4 receptor	6-13
60	<u>ITGA5</u>	Integrin subunit alpha 5	2-3
61	<u>ITGA6</u>	Integrin subunit alpha 6	6-11
62	<u>ITGB1</u>	Integrin subunit beta 1	2-11
63	<u>ITGBL1</u>	Integrin subunit beta like 1	6-7
64	<u>JAG1</u>	Jagged canonical Notch ligand 1	2-3
65	<u>KCNMA1</u>	Potassium calcium-activated channel subfamily M alpha 1	6-7
66	<u>KLF10</u>	Kruppel like factor 10	6-12
67	<u>KLK10</u>	Kallikrein related peptidase 10	7-13
68	<u>KLK5</u>	Kallikrein related peptidase 5	7-13
69	<u>KLK8</u>	Kallikrein related peptidase 8	7-13
70	<u>KRT14</u>	Keratin 14	7-13
71	<u>KRT15</u>	Keratin 15	6-13
72	<u>KRT16</u>	Keratin 16	6-13
73	<u>KRT17</u>	Keratin 17	7-13
74	<u>KRT18</u>	Keratin 18	9-12-13
75	<u>KRT5</u>	Keratin 5	7-12
76	<u>KRT6B</u>	Keratin 6B	7-13
77	<u>LAD1</u>	Ladinin 1	4-7-13
78	<u>LAMA3</u>	Laminin subunit alpha 3	6-7
79	<u>LIFR</u>	LIF receptor subunit alpha	4-10
80	<u>LRRC1</u>	Leucine rich repeat containing 1	4-13
81	<u>LTBP1</u>	Latent transforming growth factor beta binding protein 1	7-13
82	<u>MAL2</u>	Mal, T cell differentiation protein 2 (gene/pseudogene)	4-14
83	<u>MCAM</u>	Melanoma cell adhesion molecule	4-10
84	<u>MGAT5B</u>	Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase B	4-6
85	<u>MLPH</u>	Melanophilin	4-13
86	<u>MMP2</u>	Matrix metalloproteinase 2	1-2-5-13-14
87	<u>MMP3</u>	Matrix metalloproteinase 3	1-2-5
88	<u>MMP9</u>	Matrix metalloproteinase 9	2-5-10
89	<u>MST1R</u>	Macrophage stimulating 1 receptor	2-13
90	<u>MT2A</u>	Metallothionein 2A	6-10

91	<u>NDRG1</u>	N-myc downstream regulated 1	10-13
92	<u>NECTIN3</u>	Nectin cell adhesion molecule 3	10-13
93	<u>NR2F1</u>	Nuclear receptor subfamily 2 group F member 1	7-13
94	<u>NREP</u>	Neuronal regeneration related protein	7-13
95	<u>NRP1</u>	Neuropilin 1	6-13
96	<u>OCLN</u>	Occludin	4-8-9
97	<u>PCOLCE</u>	Procollagen C-endopeptidase enhancer	7-13
98	<u>PKP3</u>	Plakophilin 3	4-9
99	<u>PLAT</u>	Plasminogen activator, tissue type	6-10
100	<u>PLIN3</u>	Perilipin 3	3-6
101	<u>PLP2</u>	Proteolipid protein 2	6-10
102	<u>PLPP3</u>	Phospholipid phosphatase 3	10-13
103	<u>PMP22</u>	Peripheral myelin protein 22	10-13
104	<u>POSTN</u>	Periostin	3-13
105	<u>PRKCH</u>	Protein kinase C eta	4-13
106	<u>PRRX1</u>	Paired related homeobox 1	12-13
107	<u>PTX3</u>	Pentraxin 3	7-13
108	<u>RAB25</u>	RAB25, member RAS oncogene family	4-13
109	<u>RGL1</u>	Ral guanine nucleotide dissociation stimulator like 1	10-13
110	<u>S100A14</u>	S100 calcium binding protein A14	4-7-13
111	<u>S100A2</u>	S100 calcium binding protein A2	6-9
112	<u>S100A8</u>	S100 calcium binding protein A8	7-13
113	<u>SAA1</u>	Serum amyloid A1	7-13
114	<u>SAMD9</u>	Sterile alpha motif domain containing 9	4-6
115	<u>SEMA5A</u>	Semaphorin 5A	3-13
116	<u>SERPINB1</u>	Serpin family B member 1	6-10-13
117	<u>SERPINB2</u>	Serpin family B member 2	6-13
118	<u>SERPINE1</u>	Serpin family E member 1	6-9
119	<u>SLPI</u>	Secretory leukocyte peptidase inhibitor	7-13
120	<u>SNAI1</u>	Snail family transcriptional repressor 1	1-5-9-12
121	<u>SNAI2</u>	Snail family transcriptional repressor 2	1-3-5-8-9-12-14
122	<u>SOX10</u>	SRY-box transcription factor 10	1-5
123	<u>SPINT2</u>	Serine peptidase inhibitor, Kunitz type 2	4-7-13
124	<u>SPRR1B</u>	Small proline rich protein 1B	7-13
125	<u>ST14</u>	Suppression of tumorigenicity 14	4-13-14
126	<u>SYK</u>	Spleen associated tyrosine kinase	10-13
127	<u>TBX3</u>	T-box transcription factor 3	10-13
128	<u>TCF3</u>	Transcription factor 3	9-12
129	<u>TFPI</u>	Tissue factor pathway inhibitor	7-10
130	<u>TGFB1</u>	Transforming growth factor beta 1	2-12
131	<u>TGFB1I1</u>	Transforming growth factor beta 1 induced transcript 1	3-13
132	<u>THBD</u>	Thrombomodulin	10-13
133	<u>THY1</u>	Thy-1 cell surface antigen	3-10-12-13
134	<u>TNC</u>	Tenascin C	3-6
135	<u>TP63</u>	Tumor protein p63	7-13
136	<u>TSPAN1</u>	Tetraspanin 1	4-6-13
137	<u>TWIST1</u>	Twist family bHLH transcription factor 1	1-2-3-5-9-12

138	<u>TWIST2</u>	Twist family bHLH transcription factor 2	1-12
139	<u>VCAN</u>	Versican	2-3
140	<u>VIM</u>	Vimentin	1-2-5-6-8-9-10-12-13-14
141	<u>WNT5A</u>	Wnt family member 5A	2-7-13
142	<u>XDH</u>	Xanthine dehydrogenase	6-13
143	<u>ZEB1</u>	Zinc finger E-box binding homeobox 1	1-9-12-13
144	<u>ZEB2</u>	Zinc finger E-box binding homeobox 2	1-9-12-14

CONCLUSIONS

This PhD thesis aimed to offer a new vision of the epithelial-to-mesenchymal transition (EMT) in the context of a complex, high heterogeneous disease as the breast cancer (BC). With this idea, a multi-level approach including protein analysis (western blot, zymography), gene expression (qPCR), cell-based assays (MTT, wound healing), and *in silico* studies, was conducted.

Overall, the results demonstrate that EMT is a tightly regulated and multifaceted process. Indeed, rather than a binary transition between epithelial and mesenchymal phenotypes, EMT emerges as a highly dynamic continuum comprising epithelial, hybrid, and mesenchymal states.

In this work the expression of key epithelial and mesenchymal markers was first investigated in breast cancer (BC) tissues, revealing marked intra- and inter-patient heterogeneity. Unexpected positive correlations between epithelial (E-cadherin, Cytokeratin-18) and mesenchymal (Vimentin, α -SMA) markers were observed, supporting the existence of regulated hybrid EMT states rather than a strict binary epithelial or mesenchymal phenotype. This notion was further reinforced by analyses of paired tumoral and adjacent normal tissues, in which EMT-related markers were often expressed at levels comparable to or even higher in non-tumoral samples. Together, these findings highlight the complexity of EMT regulation in breast cancer and suggest that EMT-related features are not exclusively restricted to malignant epithelial compartments.

Consistent with this interpretation, correlations between mesenchymal markers and components of the tumor microenvironment (TME), assessed using the TIMER database, indicated significant associations between EMT features and stromal and immune elements. These results support the idea that microenvironmental cues strongly influence EMT dynamics and are deeply embedded within tissue-level interactions rather than being solely tumor cell-intrinsic programs.

To further explore the relationship between EMT markers and tumor aggressiveness, the analysis was extended to patient-derived sera.

Breast cancer sera, derived from both primary and metastatic patients, consistently promoted proliferation in both malignant (MDA-MB-231) and non-transformed (MCF10A) mammary epithelial cells. In contrast, sera from healthy donors exerted a suppressive effect. These data indicate that BC sera can effectively carry systemic signals capable of inducing pro-growth or pro-survival phenotypes.

In addition to proliferation, breast cancer sera significantly enhanced the migratory capacity of MDA-MB-231 cells, evaluated with a wound healing assay, while healthy sera consistently reduced wound closure. Despite this increased migratory behavior, western blot analyses revealed high variability

and positive correlations between epithelial and mesenchymal markers, without a direct association between Vimentin expression and migration. This dissociation further underscores that functional EMT-related behaviors cannot be attributed solely to the expression of individual canonical markers.

Moreover, recognizing the limitations of two-dimensional culture systems for modeling tumor complexity, EMT regulation was subsequently explored in three-dimensional (3D) tumorsphere cultures. Compared with 2D monolayers, tumorspheres displayed reduced proliferation (*MKI67* downregulation) and *VIM* expression, alongside a modest but non-significant increase in *CDH1*, suggesting enhanced cell–cell interactions and structural compaction. Notably, these epithelial-associated features coexisted with the activation of EMT-related transcription factors (*SNAIL* and *TWIST1*) along with a trend toward increased *CDH2* expression, increased stemness (*SOX2* upregulation), and elevated extracellular matrix-related signals (*FNI* upregulation). This transcriptional profile indicates that tumorsphere formation reflects a highly plastic, hybrid cellular state rather than a classical EMT program.

When exposed to patient-derived sera, the 3D system proved to be more sensitive than 2D cultures, both morphologically and transcriptionally. Indeed, if healthy sera disrupted tumorspheres' integrity and induced consistent downregulation of proliferation- and EMT-related genes, breast cancer sera supported tumorspheres' architecture and maintained gene expression levels close to control conditions.

Significantly, although statistical significance was not achieved for all genes when primary breast cancer sera were compared with control conditions, the overall expression pattern consistently differed from that observed in cells treated with healthy sera. Indeed, cells exposed to breast cancer-derived sera showed a significant deviation from the healthy serum condition, highlighting a distinct, biologically relevant serum-driven effect.

Given the multifaceted nature of EMT as revealed by tissue- and serum-based analyses, a bioinformatic approach was employed to capture EMT complexity at a broader scale. By integrating EMTome, UALCAN, and Kaplan–Meier Plotter databases, a stringent breast cancer-specific EMT gene signature was identified. Pathway enrichment analysis revealed a strong involvement of extracellular matrix organization and transformation, and cell–cell/cell–matrix interactions, consistent with a model in which EMT is closely linked to microenvironmental remodeling.

To define a highly stringent breast cancer-specific EMT gene signature, the analysis was restricted to genes showing both diagnostic relevance in the UALCAN database and prognostic significance in the Kaplan–Meier Plotter. Application of this dual-criterion approach resulted in the identification of

37 concordant genes across the two platforms. Notably, several of these genes are known regulators of stemness-related programs, supporting the hypothesis that microenvironment-derived and heterogeneous signals may contribute to the maintenance of cancer stem cell traits and promote cellular plasticity, including the emergence of hybrid EMT states.

Furthermore, clinical relevance was explored through analyses using bcGeneXMiner and GOBO databases, which demonstrated that genes associated with poor prognosis correlated with aggressive clinicopathological parameters. In contrast, genes linked to favorable outcomes showed the opposite trend. These associations confirm that EMT-related transcriptional programs are tightly connected to breast cancer aggressiveness and clinical behavior.

Finally, given the central role of ECM remodeling, functional analyses of metalloprotease activity revealed heterogeneous but widespread gelatinolytic activity in breast cancer tissues, with complex correlations with several EMT markers. Notably, when zymographic analysis was conducted using BC sera, circulating pro-MMP-2 levels in patient sera emerged as a potential indicator of disease progression, decreasing or remaining stable in regressing tumors and increasing in progressing tumors, whereas pro-MMP-9 did not show similar associations. This divergence underscores the value of integrating functional biomarkers with expression-based analyses.

Overall, the findings of this study converge on the concept that EMT in breast cancer is a dynamic, context-dependent, and highly plastic process. Rather than representing a linear or uniform program, EMT encompasses a spectrum of hybrid states shaped by TME interactions and systemic circulating factors. Consequently, capturing EMT complexity requires integrative approaches that combine tissue, serum, functional assays, and large-scale bioinformatic analyses to better reflect its biological and clinical relevance.

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