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CYCLE XXXVI

HEPARANASE AS REGULATOR OF EPITHELIAL TO MESENCHYMAL TRANSITION (EMT) IN BREAST CANCER

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ABBREVIATIONS

2OSTs = 2-O-sulfotransferases

3OSTs = 3-O-sulfotransferases

6OSTs = 6-O-sulfotransferases

ADAMs = A disintegrin and metalloproteinases

ADAMTS = ADAM with thrombospondin motifs

E-CAD = E-Cadherin

ECM = extracellular matrix

EGF = epidermal growth factor

EGFR = epidermal growth factor receptor

EMT = epithelial-to-mesenchymal transition

ER = estrogen receptors

EXT1 and EXT2 = Exostosin-1 and -2

FGF-2 = fibroblast growth factor-2

FGFR-1 = fibroblast growth factor receptor-1

FN = Fibronectin

GAGs = glycosaminoglycans

GalNAc = N-acetylgalactosamine

GlcA = D-glucuronic acid

GLCE = glucuronic acid epimerase

GlcNAc = N-acetylglucosamine

GlcNS = N-sulfoglucosamine

GPI = glycosyl-phosphatidyl-inositol

HAT = histone acetyltransferase

HBD = hepan sulfate binding domain

HB-EGF = heparin-binding EGF

HDAC = Histone deacetylase

HER2 = human epidermal growth factor receptor 2

HGF = hepatocyte growth factor

HPSE = Heparanase-1

HS = heparan sulfate

HSPGs = Heparan sulfate proteoglycans

hTERT = human telomerase reverse transcriptase

IdoA = L-iduronic acid

IL-1β, -6, -8, -10 = interleukin-1 beta, -6, -8, -10

LOX = lysyl oxidases

LOXL = LOX-like proteins

LRP4 = low-density lipoprotein-like receptor 4

MMPs = metalloproteases

NDSTs = N-deacetylase and N-sulfotransferases

PDGF = platelet-derived growth factor

PGs = proteoglycans

PI3K/Akt = phosphatydil inositol 3 kinase/protein kinase B

PR = progesterone receptors

SDC-1 = syndecan-1

TGF-β = transforming growth factor-beta

TME = tumor microenvironment

TNBC = triple-negative breast cancer

TNF-α = tumor necrosis factor-alpha

VEGF = vascular endothelial growth factor

VIM = Vimentin

1. SUMMARY

In the last decade breast cancer (BC) has risen as one of the most prevalent tumors in the world among women, according to the World Health Organization (WHO). Noteworthy, BC patients do not die for the primary tumor mass per se, but for the widespread metastasis. A well-described cellular plasticity mechanism, the Epithelial to Mesenchymal Transition (EMT), is correlated with cancer progression and metastatic dissemination. Epithelial cells gradually lose their epithelial phenotype acquiring migration and invasion characteristics related to mesenchymal behaviour. A recent study pointed out that heparanase (HPSE), the only endo-β-glycosidase in mammals, is overexpressed not only in BC but also in all the solid tumors in human. Its enzymatic activity promotes the cleavage of Heparan Sulfate (HS) chains, actively participates in the remodelling of extracellular matrix (ECM) as well as allows the release of many HS-linked pro-tumoral molecules, such as TGF-β, bFGF, VEGF. These molecules induce a reciprocal crosstalk between cancer cells and the surrounding environment promoting inflammation and angiogenesis that sustain tumor growth and progression.

First, we characterize two breast cancer cell lines, MCF-7 and MDA-MB-231, notably for their different metastatic potential. We analysed the expression of fibronectin (FN) and vimentin (VIM) as mesenchymal- and E-Cadherin (E-CAD) as epithelial-marker. The differential expression of EMT markers confirmed the cell phenotypes but particularly that the MDA-MB-231 showed a two-fold increase in HPSE expression. In addition, the MCF-7 showed more E-Cad compared to MDA-MB-231, which on the other hand, expresses higher FN, VIM and especially HPSE. Moreover, the two cell lines respond differently at the treatment with FGF-2 and TGF-β, two well-known EMT activators. Gene and protein expression analyses of EMT-associated markers confirm that FGF-2 or TGF-β treatment increased the expression of Vim, FN and EMT-associated transcription factors SNAIl and SLUG, while E-Cad expression was reduced. HPSE inhibition with Roneparstat, a specific HPSE inhibitor, alone or in combination with the two EMT inducers, was able to interfere with the impairing of the EMT markers. In addition, HPSE overexpression increased the EMT marker in gene and protein expression in the case of the MCF-7 cell line, but not in MDA-MB-231. The

enzymatic inhibition with Roneparstat simulates the same slight reduction compared to the basal condition. Therefore, the downregulation of HPSE and the enzymatic inhibition upon the MDA-MB-231 seems to not affect the EMT markers expression both in gene and protein expression. Moreover, cell migration is higher in MCF-7 overexpressing cells compared to the control, while MDA-MB-231 showed no significant change in overexpressing or silenced cell lines. Roneparstat was able to reduce the cell migration not just at the basal level but also in overexpressing HPSE cell types.

The current literature highlights that HPSE can be involved in EMT regulation, both in tumor and non-tumor models, influencing different cellular pathways. Due to the correlation between HPSE levels and the EMT process, this enzyme could be an important regulatory factor for BC progression and metastases.

2. INTRODUCTION

Breast cancer is the most frequent malignant disease among women. Data from the World Health Organization (WHO) settle that with 2.3 million new cases worldwide (11.7% of all cancer cases), closely followed by lung (11.4%) and colorectal (10.0%), breast cancer became the most common cancer globally in 2021. In addition, breast cancer ranks first also in mortality: among 110 countries worldwide, 1 in 6 cancer deaths in women are caused by breast cancer (Sung et al., 2021). In these patients, the main cause of death is not the primary tumor but the high metastatic dissemination capacity at distant sites ('Effects of Chemotherapy and Hormonal Therapy for Early Breast Cancer on Recurrence and 15-Year Survival', 2005).

It is important to the underlying recent decrease in mortality rates both in North America and the European Union mostly attributable to early detection and efficient systemic therapies. Indeed, after the spreading of mammographic screening, between the 1980s and 1990s it was observed a rapid and uniform increase in breast cancer incidence rates, highlighting the power of such screening methods. In addition, the combination with a reduction in the use of menopausal hormone therapy led to an important drop in female breast cancer incidence during the early 2000s (Beverly & Volkar, 2021; Breen et al., 2011; Torre et al., 2017).

If it is real that the rates of metastasis and mortality in breast cancer patients have decreased as a result of early diagnosis by mammographic screening and the implementation of systemic adjuvant therapy, a specific and unique therapy is difficult to develop ('Effects of Chemotherapy and Hormonal Therapy for Early Breast Cancer on Recurrence and 15-Year Survival', 2005). Essentially, it depends on the heterogeneity of breast cancer, which shows different subtypes that exhibit different gene expression patterns and phenotypes (Sørlie et al., 2006). Owing to the relevance of this cancer among the population, massive breast cancer screening programs aimed at reducing mortality through early detection, while discovering effective treatments remain the great challenge.

The relevant issue for breast cancer treatment is related to its heterogeneity, consisting of different behaviours acquired by cells coming from the same anatomical organ structure, the terminal duct-lobular unit. Heterogeneity represents the primary challenge

in treating breast cancer (Polyak, 2011). Shedding light on the critical biomarkers that define carcinogenesis, drug resistance and responsiveness to specific treatment is a crucial point.

There are different classifications of breast carcinomas. From a histological point of view, based on morphological differences of the tumors, about 80% of all breast cancers will eventually belong to either one of the two major histopathological classes: invasive ductal carcinomas or invasive lobular carcinoma (Viale, 2012). This implies that tumors showing different biological and clinical profiles are grouped, resulting in minimalized prognostic and predictive capabilities and modest clinical utility. In 2000, Perou and colleagues introduced a new way of clustering breast tumors with a better predictive and prognostic value (Cadenas, 2012; Perou et al., 2000). The so-called "molecular subtypes of Perou" depend on gene expression profiles and the presence or absence of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2) (Cadenas, 2012; Perou et al., 2000). This classification identified five breast carcinoma subtypes: luminal A (ER+ and/or PR+; HER2-), luminal B (ER+ and/or PR+; HER2+), normal-like, basal-like (ER-, PR-, and HER2-) also called triple-negative breast cancer (TNBC), and HER2-enriched (ER-, PR-, and HER2+) (Cadenas, 2012; Eliyatkin et al., 2015; Perou et al., 2000) (**Fig. 1**).
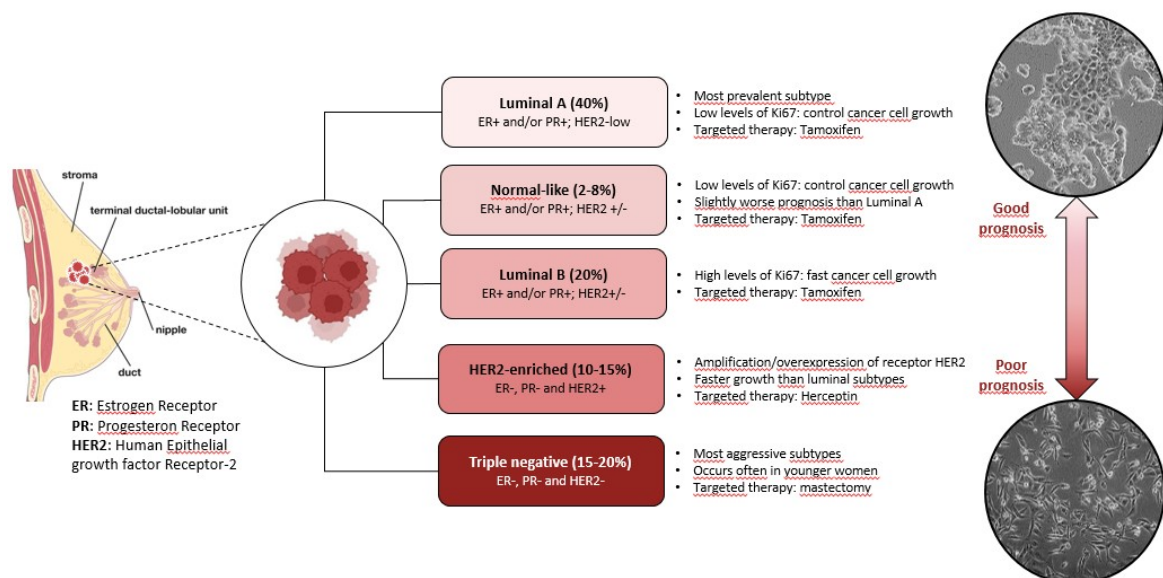

Fig. 1: Molecular subtypes of breast cancer and relative prognosis. The image shows the 5 different breast cancer subtypes based on the molecular classification that considers the presence or absence of receptors as prognostic markers. The two images represent the aspect of the luminal A (top) and the triple negative (bottom) breast cancer subtypes (Lazaratos, A. (2020) BioRender.com)

Due to its heterogeneity, it is difficult to develop a unique cure for breast cancer patients. For nonmetastatic breast cancer, surgery remains the primary treatment but different therapies are required considering the various molecular subtypes. The advent of chemotherapy and radiotherapy changed the therapy approach but only endocrine therapies upset the treatment of breast cancer patients. This approach, considered the gold standard therapy, is focused upon two main molecular receptors: the hormone receptors (estrogen receptor alpha (ERα)$^+$ and PR$^+$) and HER2$^+$ respectively treated, for example, with tamoxifen or trastuzumab (Waks & Winer, 2019).

Unfortunately, TNBC which makes up approximatively 15% of all breast tumors, due to the lack of any receptors, show an aggressive phenotype and a poorer outcome for patients. Their treatment mainly relies on cytotoxic chemotherapy, which however is inadequate and thus the identification of new molecular targets for TNBC is crucial for the development of more efficient targeted therapies (Waks & Winer, 2019).

The process of tumor formation and progression is particularly influenced by two factors: 1) genetic and epigenetic changes in the tumor cells (Gonzalez & Medici, 2014) and 2) the remodeling of tumor microenvironment (TME) composition through mutual and dynamic crosstalk between tumor mass and surrounding environment (P. Chen & Dey, 2022). TME include cancer cells, blood vessels, fibroblasts, immune/ inflammatory cells and soluble ligand molecules immersed in the extracellular matrix (ECM) (P. Chen & Dey, 2022).

The ECM composition and remodeling are two main aspects that influence directly or indirectly tumorigenesis and metastasis (Winkler et al., 2020).

2.1 The extracellular matrix composition

The ECM is a complex and dynamic 3D network of cross-linked glycoproteins that structurally organizes cells into tissues and organs in all multicellular organisms (Bich et al., 2019; Hynes, 2012; Karamanos et al., 2021) (**Fig. 2**). It is a fundamental component that coordinates not only cell growth, proliferation and migration but also tissue development, homeostasis and wound healing by modulating intracellular signaling pathways (Karamanos et al., 2021). In the last decade, the complexity of ECM composition and architecture has been deciphered thanks to proteomic and

transcriptomic approaches that have led to a greater understanding of the so-called "microsome", i.e. the ensemble of ECM proteins and associated factors (Naba, 2023). Each tissue is characterized by a specific composition of the matrisome that is generated in early embryonic stages and each cell type has a precise repertoire of ECM receptors that mediate cell-ECM interactions (Mecham, 2012). The matrisome is composed of "core" ECM proteins which include proteoglycans, collagens and multi-adhesive proteins, and matrisome-associated factors, such as secreted cytokines, growth factors and remodeling enzymes (Hynes & Naba, 2012; Mecham, 2012). The "core" proteins of the ECM are about 300 and are divided into 4 different groups as follows.
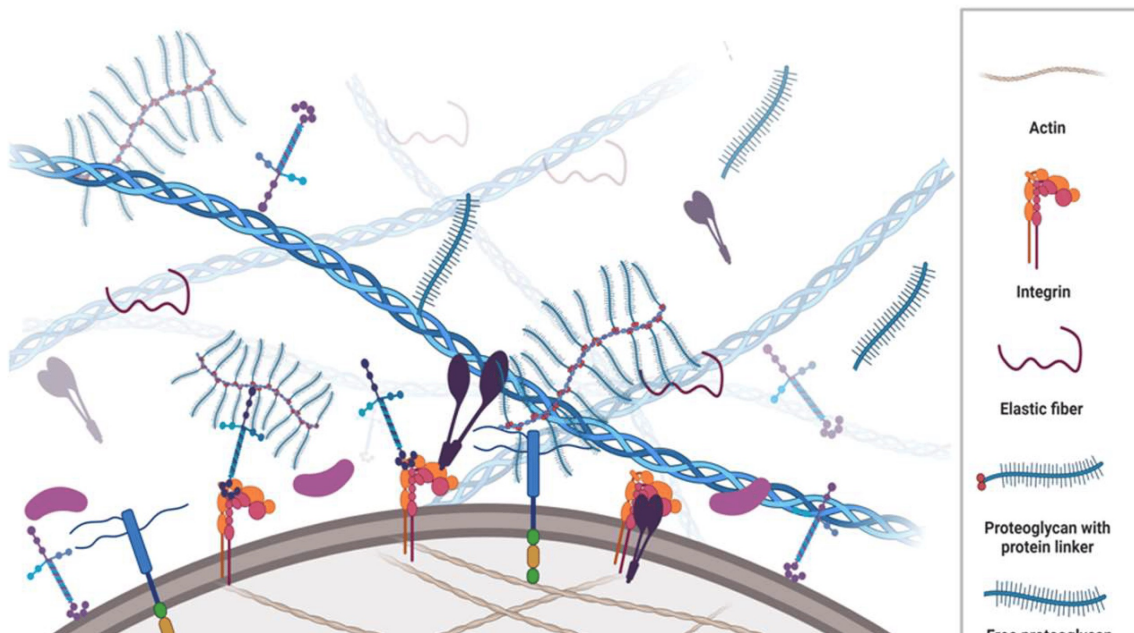
Fig. 2: Overview of ECM organization and component. The ECM is a complex 3D network structure comprised of multiple proteins such as collagens, fibronectin, proteoglycans, integrins, growth factors and metalloproteinases. The ECM influence many aspects that support homeostasis defined by specific structural, mechanical, and chemical properties. The image summarizes all the matrix proteins that compose the ECM and its role. The Fibronectin molecule is linked to a transmembranous integrin dimer, which is attached to a collagen molecule that creates a connection between the cytoskeleton and the ECM. In addition, laminin complexes are attached to integrins, glycoproteins, and glycolipids through the linker/anchor region (LG domain) on the membrane, creating a dynamic link between cells and the ECM. Syndecans associate with integrins, growth factor receptors, as well as other ECM glycoproteins and collagens. The extracellular domains of syndecans are important for cell–cell and cell–matrix interactions via the glycosaminoglycan sidechains. Image from (Raskov et al., 2023)

2.1.1 Collagens

Collagens are the most abundant proteins in mammals (approximately 30% of total protein mass) represented by 28 different collagen types. The collagen family is characterized by the presence of a collagenous triple-helix structure that confers mechanical strength but also shows some interruptions in the sequence that confers considerable plasticity and flexibility to these molecules. There are different classes of collagens structures in the ECM, including fibril-forming collagen (e.g., types I, II and III), network-forming collagens (e.g., type IV), transmembrane collagenous domains (e.g., type XIII) and atypical collagens (e.g., type VII), based on their supramolecular and hierarchical assembly. Interestingly, collagen supramolecular assembly plays a key role in regulating their biomechanical and biochemical properties, providing thermal stability, and elasticity and regulating the interaction with other ECM biomolecules (Ricard-Blum, 2011). Collagen biosynthesis has been extensively studied for fibril-forming collagens that are synthesized as procollagen molecules that interact in the lumen of the endoplasmic reticulum to form the right-handed helical conformation coil (Ricard-Blum, 2011). It is noteworthy that fibroblasts are the main cell type involved in collagen deposition, which reside in the interstitium and whose ability to synthesize ECM molecules is strictly related to environmental conditions (Plikus et al., 2021).

2.1.2 Proteoglycans

Proteoglycans (PGs) are high molecular mass glycoproteins composed of a specific core protein with one or more covalently linked polysaccharide chains. These long and unbranched polysaccharide chains, called glycosaminoglycan (GAG), consisting of repeating disaccharide units of a hexosamine (N-acetylglucosamine, GlcNAc, or N-acetylgalactosamine, GalNAc) and uronic acid (D-glucuronic GlcA or L-iduronic acid, IdoA) or galactose (-4 N-acetylglucosamine-β1,3-galactose-β1). The glycosidic linkage that connects the disaccharide units is type α $(1 \rightarrow 4)$ or β $(1 \rightarrow 4)$, while the inter-disaccharide bonds are always α $(1 \rightarrow 4)$ (Pomin & Mulloy, 2018). Upon macromolecular assembly in the Golgi apparatus, the disaccharide unit undergoes sulfation reactions in different positions conferring to GAGs an elevated negative charge. The high structural heterogeneity of proteoglycans is mainly given by: 1) the huge number of core proteins identified, 2) the number of GAGs chains attached to the

core protein and 3) the type of GAG which, according to its component sugars and pattern of modification, is categorized in chondroitin sulfate, dermatan sulfate, keratan sulfate and heparan sulfate/heparin. In addition, proteoglycans may contain different types of GAGs along the same core protein (hybrid proteoglycan) (Karamanos et al., 2018, 2021). Due to the high negative charge of GAGs, the major biological function of ECM proteoglycans provides tissue hydration and viscosity and favours nutrients, metabolites and hormones diffusion. According to their protein core, proteoglycans are classified into three main families: small leucine-rich proteoglycans (e.g., decorin and biglycan), modular proteoglycans (e.g., aggrecan and versican) and cell-surface proteoglycans (e.g., syndecan-4 and glypican-1) (Iozzo & Schaefer, 2015).

2.1.3 Adhesive glycoproteins

This is a broad group of ECM proteins which include about 200 different molecules in mammals. They are characterized by various types of multimodular primary structures which provide several roles, from regulating ECM assembly to promoting cell adhesion and controlling growth factors availability. The best-studied glycoproteins are laminins and fibronectin. (Hynes & Naba, 2012). The "matrisome-associated" factors include all the other secreted proteins that bind to, modify and degrade the ECM core protein. It is known that ECM works as a reservoir and sink of several growth factors (such as TGFβ, VEGF, BMP and Wnt ligands) and chemokines, being also involved in the formation of gradients which control embryo development and organ morphogenesis (Hynes & Naba, 2012; Karamanos et al., 2021).

2.1.4 Secreted enzyme

The last class of ECMs proteins is represented by the secreted enzymes, such as oxidases and proteases, that coordinate the post-translational modification of collagen fibrils and the glycoprotein network. For example, after production and deposition, collagens and elastin are cross-linked by disulfide bonds thanks to extracellular transglutaminases, lysyl oxidases (LOX) and LOX-like proteins (LOXL) (Vallet & Ricard-Blum, 2019). The ECM is a highly dynamic structure due to constant remodeling, that is important for tissue homeostasis during physiological as well as pathological conditions like cancer. Collagen, PGs and other ECM proteins are targets

for several metalloproteases (MMPs), A disintegrin and metalloproteinases (ADAMs) and ADAM with thrombospondin motifs (ADAMTS), proteases that specifically cleaves serine, cysteine and threonine residues, heparanase and sulphatases that facilitate their turnover or remodel ECM architecture (Bonnans et al., 2014). On the other hand, these proteolytic processes are also important because they promote the release of ECM-linked ligands but also small bioactive fragments of ECM components that play a role in physiological and pathological conditions (Bonnans et al., 2014; Vallet & Ricard-Blum, 2019; Winkler et al., 2020).

2.2 Heparan sulfate proteoglycan

Heparan sulfate proteoglycans (HSPGs) are a class of complex molecules found in the ECM and at the cell surface (Sarrazin et al., 2011). They play an important role in various physiological processes, especially in cell-cell communication, cell adhesion and the regulation of different cell signaling pathways. HSPGs, share a common structural feature with the proteoglycans family but they are characterized by the presence of highly sulfated GAG side chains (Vlodavsky et al., 2021). For this reason, the HSPGs show a strong negative charge that confers the capability to interact with a broad range of molecules, including growth factors, cytokines and other ligands. Indeed, they are essential for regulating the bioavailability, distribution and activity of different signaling molecules, influencing various cellular pathways (Hassan et al., 2021).

2.2.1 HSPGs biosynthesis and structure

The HSPGs constitute a heterogeneous group of molecules consisting of various types of proteins conjugate with heparan sulfate (HS) chains of variable length covalently linked. HS consists of the repetition of a disaccharide unit formed by a glucuronic acid (GlcA) or its iduronic acid epimer (IdoA) and by an N-acetyl glucosamine (GlcNAc) or N-sulfo glucosamine (GlcNS). The bond that joins the disaccharides is of type α (1 $\rightarrow$ 4) or β (1 $\rightarrow$ 4), while the inter-disaccharide bonds are always α (1 $\rightarrow$ 4) (Karamanos et al., 2021; Ravikumar et al., 2020; Sarrazin et al., 2011) **(Fig. 3)**. HSPGs assembly occurs in the endoplasmic reticulum and Golgi apparatus compartments were the synthetic machinery of HS is located. The polymerization starts from a tetrasaccharide linkage region: xylose, two galactose and o glucuronic acid, which is synthesized on

different serine residues in the core protein. Exostosin-1 and -2 (EXT1 and EXT2), two endoplasmic reticulum-resident type II transmembrane glycosyltransferase, are involved in the elongation of HS and then it is modified by sequential reactions of specific enzymes. The first modification is an N-deacetylation/N-sulfation of GlcNAc carried out of N-deacetylase and N-sulfotransferases (NDSTs) followed by C5-epimerization of GlcA to IdoA. At least, 2-O-sulfation of IdoA and 6-O-sulfation of GlcNAc occur. This step is a crucial point, especially in cancer signalling and progression because it is strictly related to the function of the HSPGs (Marques et al., 2021; Ravikumar et al., 2020) **(Fig. 3)**. In addition, depending on sulfation pattern, HS contains low sulfated domains and highly sulfated domains that is called heparin (Annaval et al., 2020; Multhaupt & Couchman, 2012). Beyond the sulfation, it is important to underlying the presence of non-sulfated regions of GlcA and GlcNAc, which define the type of molecule that can interact with the HSPGs (Lamanna et al., 2007; Ori, 2008).
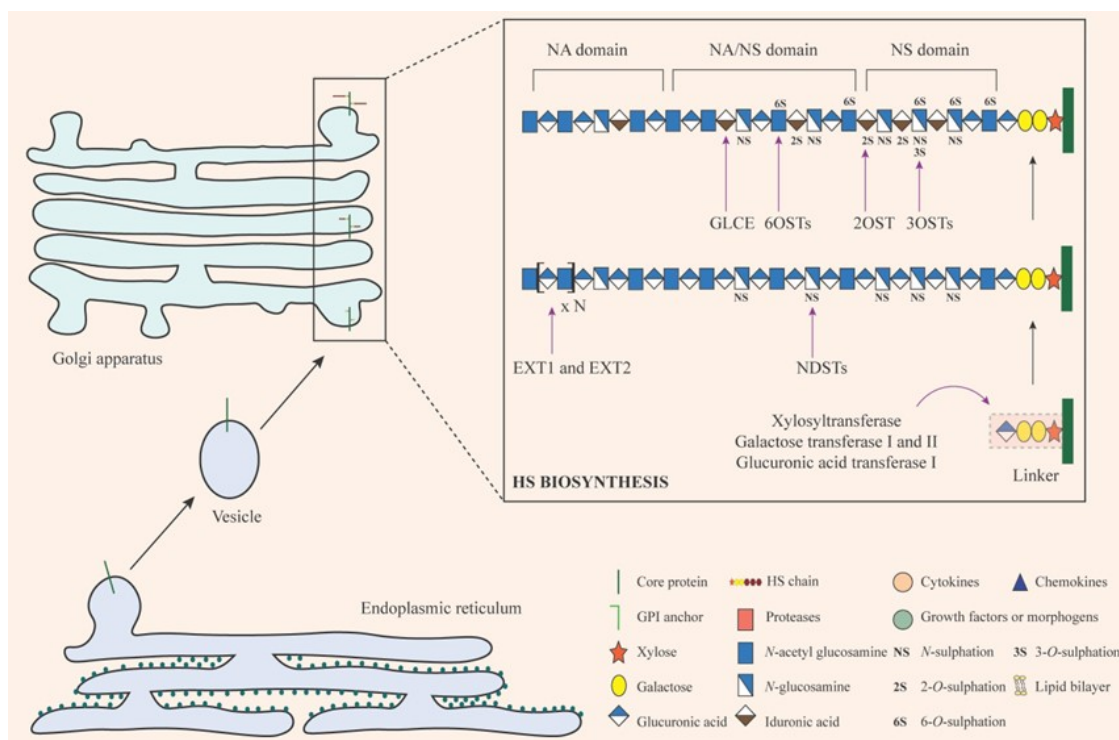
Fig. 3: HSPG proteoglycan biosynthesis. HS biosynthesis is a sequential process that occurs in the Golgi apparatus. The initiation process starts from the formation and attachment of a tetrasaccharide linker to a serine residue on the core protein. Following, EXT1 and EXT2 promote chain polymerization where *N*-acetylglucosamine and glucuronic acid residues are added in succession. Then, the HS chain is modified by a different of enzymes including the NDSTs, glucuronic acid epimerase (GLCE), 2-O-

sulfotransferases (2OSTs), 6-O-sulfotransferases (6OSTs), and 3-O-sulfotransferases (3OSTs). Selective sulfation and epimerization confer intricate structural nuances to the HS chain, at the basis of the various functional domains. Upon exit from the Golgi apparatus, HSPGs may be stored in secretory granules, transported to the plasma membrane or secreted into the ECM. Image from (Ravikumar et al., 2020)

2.2.2 Localization and function

Cells elaborate a relatively small set of HSPGs (~17) that fall into three groups according to their location: membrane HSPGs, such as syndecans and glycosylphosphatidylinositol-anchored proteoglycans (glypicans), the secreted extracellular matrix HSPGs (agrin, perlecan, type XVIII collagen), and the secretory vesicle proteoglycan, serglycin) (Annaval et al., 2020; Hassan et al., 2021) (**Fig. 4**).
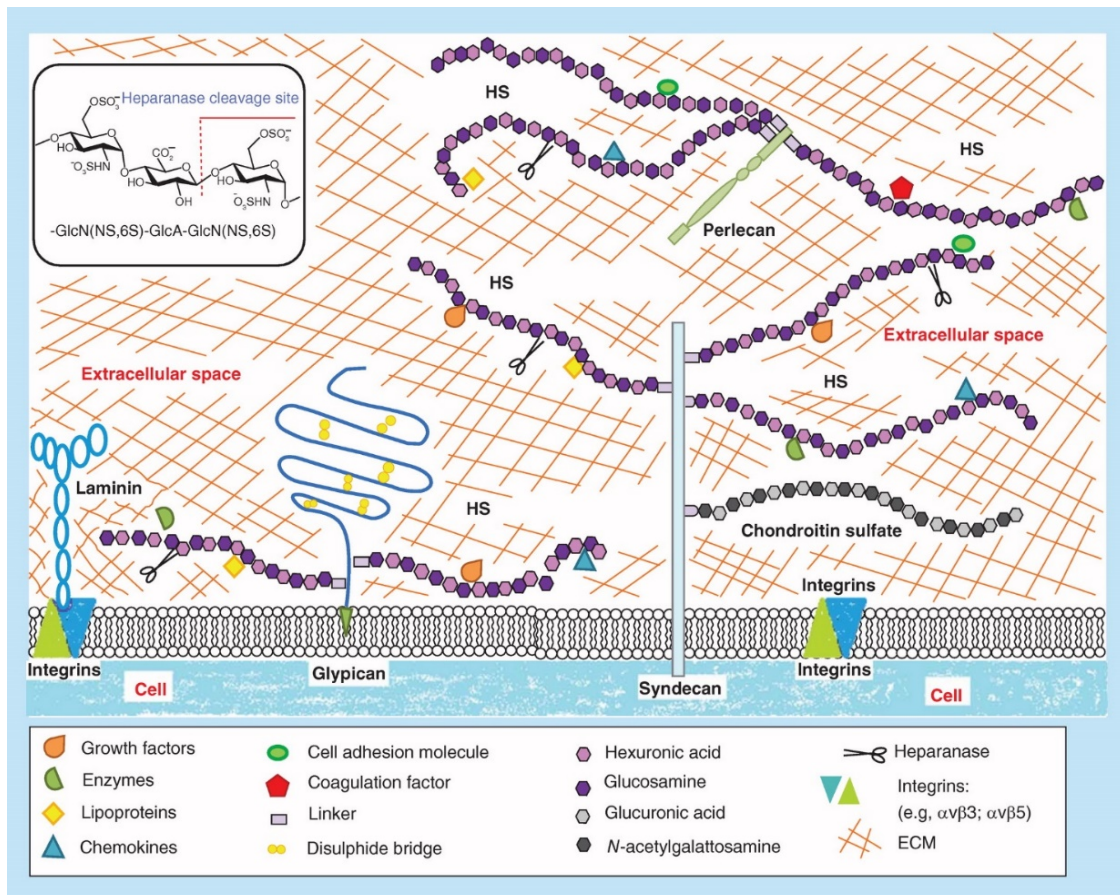

Fig. 4: Schematic representation of HSPG. Principal cell surface (syndecan and glypican) and pericellular (perlecan) heparan sulfate proteoglycans. In addition, due to their negative charge, HS can interact with several proteins including growth factors, chemokines, enzymes, lipoproteins and plasma proteins. HS chains are cleaved by heparanase enzyme. Image from (Rivara et al., 2016)

2.2.3 Pericellular HS-proteoglycans

Pericellular HSPGs are an important constituent of extracellular scaffold but also modulators of signalling pathways and morphogen gradients through the interaction with regulatory and signalling factors (Iozzo & Schaefer, 2015). This class of HSPG include perlecan, agrin and collagen XVIII. Perlecan is expressed at the basement membrane and contains a multi-domain protein core and three GAG chains (prevalently HS) at its N-terminus. Perlecan, beyond its structural role, is known to interact with different ECM components, growth factors and membrane proteins (integrins and tyrosine kinase receptors) and in this way, it can regulate several biological processes. It is important to point out that perlecan is involved in vascularization and tumor angiogenesis in which the HS chains function as a reservoir for a vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β) and fibroblast growth factor (FGF) family members (Farach-Carson et al., 2014; Gubbiotti et al., 2017). As perlecan, agrin is a multimodular HSPGs containing three HS chains present at the level of the basement membrane. Agrin is abundant in the synaptic region, which plays an important role as an organizer of the neuromuscular junction at postsynaptic membrane by the high affinity of its N-terminal to laminin in basal membrane and C-terminal domain to low-density lipoprotein-like receptor 4 (LRP4) in skeletal muscles (Daniels, 2012). Collagen XVIII is a ubiquitous component prevalently present at the basement membrane of vascular and endothelial cells. It is a structurally complex homotrimer organized in a triple helix and presents three HS chains and an endostatin domain located at the C-terminal (Heljasvaara et al., 2017). The last member is the testican, recently called SPOCK, a modular HSPGs with 2-5 HS chains associated at the C-terminal domain. Three members are part of this family and they are prevalently expressed in the central nervous system, principally involved in neuronal development (Iozzo & Schaefer, 2015).

2.2.4 Cell surface proteoglycans

Cell surface PGs are prevalently HSPGs, closely associated with cell membranes through a membrane-spanning core protein or a glycosyl-phosphatidyl-inositol (GPI) anchor. They show a different functions as adhesion molecules, endocytic receptors and as co-receptors thus controlling cell signaling, adhesion and motility. From the cell

surface proteoglycans, the two main families are represented by the syndecans and glypicans (Bishop et al., 2007). Syndecans comprise four group members called syndecan 1-4 (SDC 1-4). These are a transmembrane protein characterised by three domains: a small intracellular C-terminal domain, the transmembrane region and an N-terminal extracellular domain in which the HS chains are attached distally to plasma membrane. In addition, SDC-1 and -3 also contain proximal chondroitin sulfate chains. The transmembrane are very homologous sequence, while the ectodomain is the most variable region (Afratis et al., 2017; Hassan et al., 2021). Membrane HSPGs can activate receptors on the same cells *(in cis)* or on adjacent cells *(in trans)* promoting cell-cell cross-talk. A well-known example is represented by SDC-1 which promotes basic fibroblast growth factor-2 (FGF-2) binding and activation of FGF-2 receptor-1 (FGFR1). More specifically, released HS fragments bound to FGF-2 are potent activators of FGFR1 (Mohammadi et al., 2005). Glypicans are highly conserved protein characterized by a cysteine-rich extracellular protein core linked to the plasma membrane via a GPI anchor. This family comprises six glypicans 1-6, with different biological properties, related to cellular response of many growth factors and morphogens (Filmus, 2023). A peculiar member of the cell surface HSPG is the transforming growth factor (TGF-β) type III receptor, also known as betaglycan. It can bind simultaneously to TGF- β, promoting the binding to type II receptor by the protein core, and to FGF-2 by HS (Villarreal et al., 2016). The biological activity of membrane proteoglycans is post-translational modulated by the shedding of the HSPGs ectodomain. This process generates soluble HSPGs which relocalize HS-bound ligands that can act as autocrine or paracrine effectors. Syndecans are shed by MMPs that promote the release of the ectodomain while glypicans can be cleaved by a protease or a lipase, defining the release of the entire molecule. Shedding is known to contribute to the progression of several tumors by the generation of soluble bioactive syndecan-1 which, once released in the microenvironment, promotes angiogenesis and invasiveness (O. Jung et al., 2016).

2.2.5 Serglycin

Serglicyn is a peculiar protein because is the only intracellular member of PGs, localized primarily, but not exclusively, in secretory vesicles of hematopoietic and

endothelial cells. The name serglycin is derived from the multiple serine-glycine repeats that form the protein core. In addition, it is better characterized as a hybrid proteoglycan, containing both heparin and chondroitin sulfate chains and the nature of its GAG-associated chains differs among cells. In connective tissue mast cells, serglycin play an important role in the formation of secretory granules, because the highly anionic heparin mediates electrostatic interaction with proteases and inflammatory mediators, favouring their packaging (Kolset & Tveit, 2008). Degranulated mast cells after activation, release heparin that act as an anticoagulant factor, due its ability to interact with and activate antithrombin which, in turn, inhibits the coagulant factor thrombin. This interaction is mediated by a specific pentasaccharide sequence with a high affinity for antithrombin. Several commercially produced low molecular weight heparins (LMWHs) or chemically modified heparins are used as anticoagulant in clinic (Mulloy, 2019). Serglycin has long been regarded as the unique intracellular HSPGs. Recent data show that HS and HSPGs can migrate into the nucleus where they affect gene cell cycle and proliferation, but also transcription by the inhibition of histone acetyltransferase (HAT) and the destabilization of transcription machinery (Stewart & Sanderson, 2014).

2.3 HSPGs post translational modification

As mentioned before, ECM plays a multitude roles in tissue from physical to biochemical support. Cells are constantly rebuilding and the maintenance of this dynamic structure passes through well-regulate synthesis, degradation, reassembly and chemical modification. The ECM is constantly remodelled by a cohort of different degradative enzymes. Among these, a peculiar class of enzyme can be influenced by post-biosynthetic modifications in the composition of HSPGs thanks to the removal of specific sulfate groups. These enzymes are the sulfatases, that selectively remove the 6-O-sulfate groups from glucosamine in the HS chains and the only enzyme capable of inducing an intrachain cut of HS promoting the release of diffusible HS fragments, called heparanase. Together with shedding, the removal of specific sulfate groups by sulfatases and the cleavage of HS chains are other post-biosynthetic modifications of HSPGs that modify the capability of this versatile set of molecules.

2.3.1 Unique extracellular matrix enzyme: heparanase

Heparanase-1 (HPSE) was first isolated from the placenta and later from the platelets (Vlodavsky et al., 2023). It was cloning and characterised in the 1999s by different groups (Hulett et al., 1999; Kussie et al., 1999; Toyoshima & Nakajima, 1999; Vlodavsky et al., 1999) but the first was the group of I. Vlodavsky (Vlodavsky et al., 1999). This enzyme is the only endo-β-D-glucuronidase known in humans capable of selectively cutting the β-1,4-glycosidic bond between specific trisaccharide substrate (GlcNS/GlcNAc-GlcA-GlcNS) with a defined HS sulfation pattern in HS chains, generating 5-10 kDa of HS fragments (Peterson & Liu, 2013) (**Fig. 5**). The human HPSE gene is located on chromosome 4q21.3 and by alternative splicing express two mRNAs containing the same open reading frame (ORF) (Vlodavsky et al., 1999). In addition to HPSE, there is another protein with 40% similarity, HPSE-2 which has been described as having no glycosidase activity, while seems to act as an HPSE inhibitor (McKenzie et al., 2000).
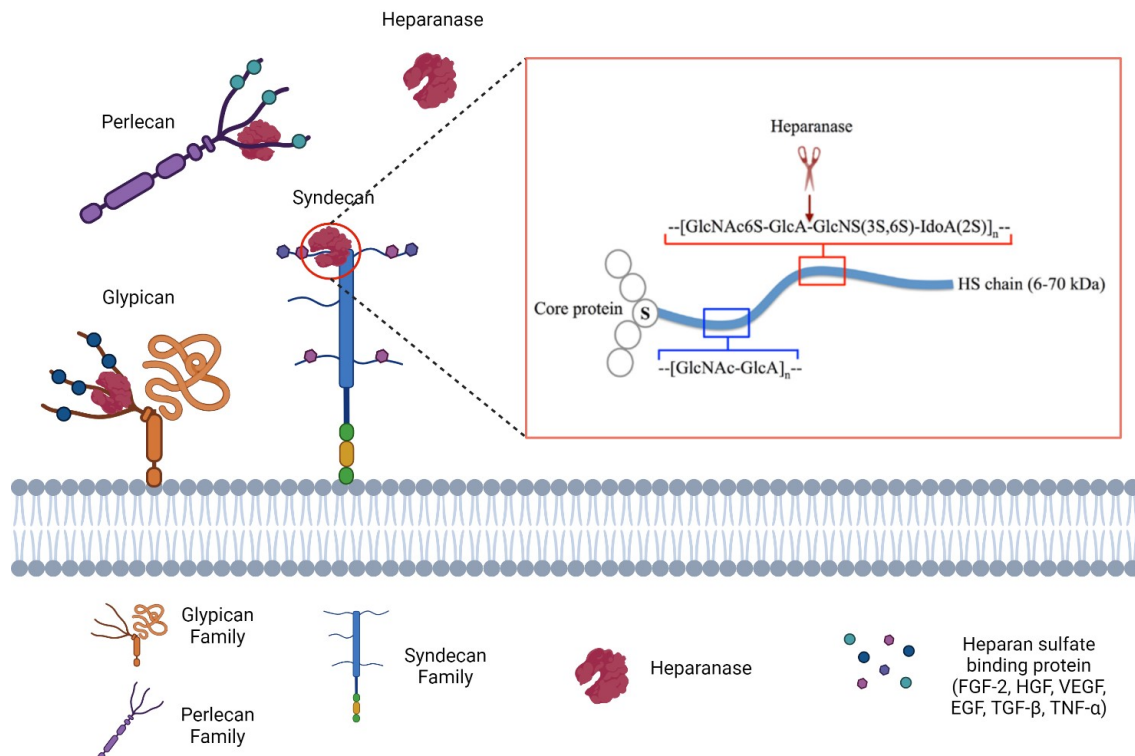
Fig. 5: **Heparanase enzymatic activity upon HS of HSPG**. HS chains (blue line) are linear polysaccharides composed of repeating disaccharide subunits of D-glucosamine and D-glucuronic acid (blue box). HS modifications include various degrees of O and N-sulfation and epimerization of D-glucuronic acid to D-iduronic acid (red box). HPSE endoglicosidic activity cleaves the highly sulfated HS chains producing HS fragments (5-10 kDa) (cleavage site indicated by red scissors and arrow). In addition, this activity promote the ECM remodeling and the release of many HS-linked molecule. Image created with BioRender.com and edited by (Simon Davis & Parish, 2013)

Heparanase is synthesized as a pre-proHpse of 543 aa precursor of 68 kDa and has a very complex pathway of maturation (**Fig. 6A**). The signal peptide at the N-terminal is removed before the translocation to the endoplasmic reticulum (ER) to generate the latent inactive pro-HPSE form of 65 kDa and subsequently processed in the Golgi apparatus. HPSE glycosylation at six predicted sites has been proven to be important for the transport across the endoplasmic reticulum and Golgi apparatus and its final secretion (Simizu et al., 2004). The precursor is then internalized in vesicles to be secreted into the extracellular space where it interacts with many membrane-bound proteins. The preferential substrate is the syndecan 1 (SDC-1), but it is also interacting with mannose 6-phosphate and low-density lipoprotein receptors (Levy-Adam et al., 2003; Simizu et al., 2004; Ben-Zaken et al., 2008) Due to the formation of a protein/substrate complex the pro-Hpse it is carried inside the cell by endocytosis, where the fusion of the late endosome with the lysosome occurring. The pro-HPSE is processed by the proteolytic activity of the enzyme Cathepsin-L that remove the 6 kDa linker peptide leading to the formation of two peptides (Abboud-Jarrous et al., 2008).

HPSE is a member of the glycosil hydrolases 79 (GH79) family and as the other family members requires a proton donor and a nucleophile residues for catalytic mechanism. Glu^{225} and Glu^{343}, both localized in the major subunit, has been identified as the two critical catalytic residues in HPSE (**Fig. 6A**). The N-terminal 8 kDa and the C-terminal 50 kDa by a noncovalent interaction, produce the active Hpse form. Once the crystalline structure of human HPSE-1 has been determined, it has been shown that this enzyme includes a $(\beta/\alpha)8$-TIM barrel domain and a β-sandwich domain of which the 8kDa subunit provides one β sheet of β-sandwich and the first β-α-β motif of $(\beta/\alpha)_8$ domain (L. Wu et al., 2015) (**Fig. 6B**). $(\beta/\alpha)_8$ domain contains two heparin/HS binding domains (HBDs): HBD1 comprises Lys^{158}-Asp^{162} at the N-terminus of the major subunit while

HBD2 (Gln^{270}-Lys^{280}) the fifth α-helix of TIM barrel (Levy-Adam et al., 2005). It is important to underlying that the C-terminal domain of the 50kDa subunit (413-Ile^{543} aminoacids) contributes to the remaining β-sandwich and is critical for protein secretion, enzymatic and non-enzymatic activity of HPSE (Fux et al., 2009). Tipically, the active enzyme is localized at the perinuclear acidic endosomal and lysosomal granules of fibroblast, neutrophils and tumor cells ready to be secreted but the fate of the enzyme is strictly depending by the cell signal (Shafat et al., 2006).
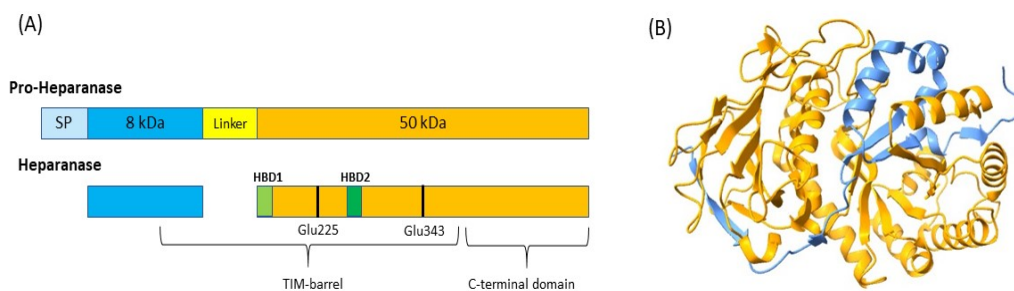

Fig. 6: Heparanase sequence and structure. In A is showed the diagram of human pro-heparanase and heparanase sequences, while B show the 3D crystallographic structure of human heparanase (PDB code 5E8M from the RSCB Protein Data Bank and modelled with UCSF Chimera software). In light blue is represented the signal peptide (SP); in blue the 8 kDa chain; in yellow the 6 kDa linker peptide; in orange the 50 kDa chain; in light green and green the HBD-1 and HBD-2 respectively. Black stick highlighted the two catalytic amino acids Glu residues. Image edited from (Rivara et al., 2016)

2.3.2 Different function of HPSE: enzymatic and non-enzymatic activities

As introduced in the previous paragraph, HPSE show a well-described and well-studied enzymatic activity, that can influence directly or indirectly the ECM environment, but also a non-enzymatic function, that is not completely understood. Several papers describe the enzymatic activity of this enzyme, but only a few numbers describe its non-enzymatic functions. According to its primary location in perinuclear acidic endosomal and lysosomal granules of fibroblast, neutrophils and tumor cells, the physiological role of HPSE is related to the degradation and the turnover of cell surface HSPGs (Goldshmidt, 2002; Shafat et al., 2006). Extracellular active HPSE, directly impacts not

only the architecture of basal membrane and ECM but also promotes the release and diffusion of HS-linked molecules, such as growth factors and cytokines, influencing cell motility, cell proliferation, angiogenesis and inflammation (Masola, Bellin, et al., 2018) (**Fig. 7**). Close to its well-known enzymatic role, an emerging role of HPSE as a non-enzymatic modulator of different functions came out. For example, by the interaction with not-yet identified cell membrane receptor(s), both proHPSE and mature HPSE activate signaling pathways and regulate gene expression associated with several cellular processes. Cell adhesion, migration and angiogenesis are stimulated by HPSE-induced pathways that mainly involve PI3K/Akt, Src and p38 MAPK. HPSE-mediated Akt activation requires RICTOR-mTOR and is promoted by integrins (Riaz et al., 2013). Efforts are now focused on the identification of the receptor(s) that mediates HPSE non-enzymatic functions. Consistently, it has been recently shown that the receptor that binds HPSE-inducing Akt phosphorylation is localized in lipid rafts (Ben-Zaken et al., 2007). In addition, HPSE localization is not restricted to intracellular vesicles. In response to proper stimuli, in which protein kinase A (PKA) and kinase C (PKC) pathways are involved, mature HPSE can be secreted by the exocytosis process (Shafat et al., 2006). Moreover, upon lysosome permeabilization and via interaction with the chaperon heat shock protein 90, active HPSE can translocate in the nucleus where it degrades nuclear HS and regulates gene expression (Nobuhisa et al., 2007; Stewart et al., 2015). HPSE regulates the expression of genes associated with glucose metabolism and inflammation in endothelial cells, differentiation in promyeloblast and tumorigenesis in melanoma cell lines. Interestingly, not only the mature HPSE but also latent proHPSE has been detected in the nucleus. The observation that exogenously added proHPSE can be translocated in the nucleus and converted into the mature enzyme has led to the hypothesis that HPSE processing may occur also in this compartment (Schubert et al., 2004). Considering the nuclear localization of HSPGs, it is not surprising that also HPSE has been discovered in the nucleus. It has been proposed two different modes of gene expression regulation by HPSE: the promotion of HAT activity by the cleavage of nuclear HS and through direct interaction with DNA (Purushothaman et al., 2011; Stewart et al., 2015; Y. Yang et al., 2015). HPSE was found to colocalize at the nuclear level with SDC-1, its preferential surface receptors (Zong et al., 2009). Furthermore, the presence of SDC-1 in the nucleus was found

regulated by the enzymatic activity of HPSE, an effect that has been correlated to the transcription of genes such as MMP-9, VEGF and hepatocyte growth factor (HGF), driving an aggressive phenotype (Purushothaman et al., 2011). Curiously, shed SDC-1 lacks the consensus sequence for nuclear localization present in the full-length molecule, so HS-ligands are likely to provide the nuclear signal to the shed HSPG. Shed SDC-1 harbouring intact HS chains was demonstrated to form a complex with HGF and shuttled to the nucleus of myeloma cells (Stewart et al., 2015).
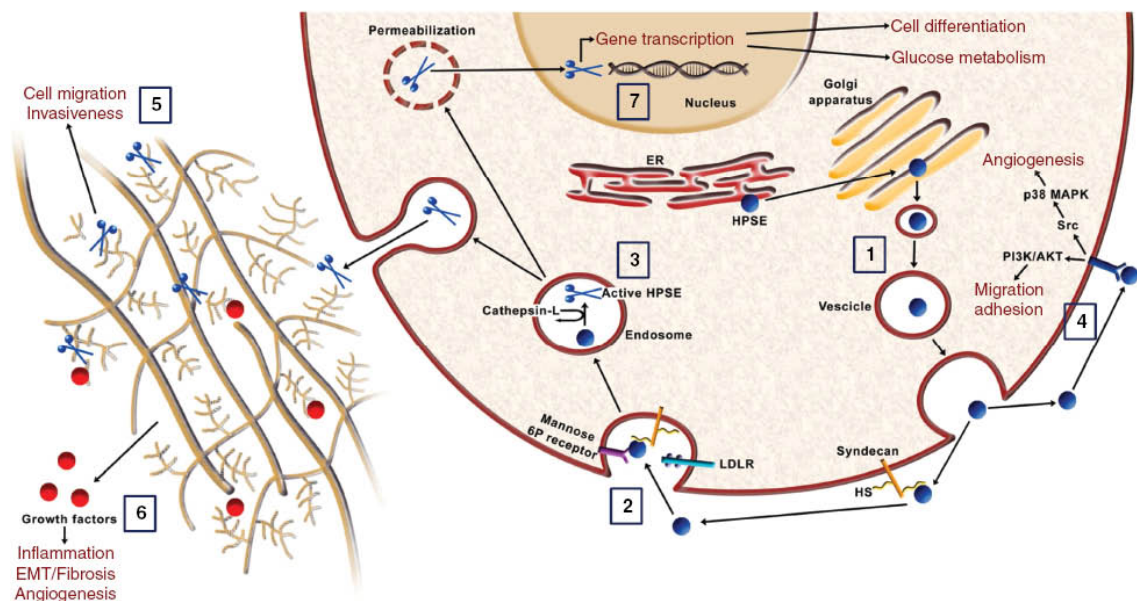

Figure 7: Schematic overview of HPSE production, maturation and function. (1) proHPSE secretion in the ECM. (2) proHPSE/substrate complex formation induce endocytosis. (3) proHPSE maturation promoted in the endolysosome by Cathepsin L (4) HPSE modulates different signalling pathways (5) mature HPSE is secreted in the ECM to carries out its enzymatic activity (6) HPSE induces the release of many HS-linked molecules (7) rather to being secreted in ECM, HPSE can migrate to the nucleus to influence gene expression. Image from (Masola, Bellin, et al., 2018).

2.3.3 Heparanase in physiology

Cellular HPSE expression is tightly regulated to prevent uncontrolled HS cleavage and adverse biological processes. Under physiological conditions, the HPSE promoter is silenced by methylation (Shteper et al., 2003) and the activity of the wild-type transcription factor p53 reduces its expression in most tissues (Baraz et al., 2006). Indeed, HPSE is expressed ubiquitously at a low level in all tissues except for immune cells, such as mast cells and leukocytes, platelets, keratinocytes, heart muscle,

endothelial cells and placental trophoblasts (https://www.genecards.org/cgi-bin/carddisp.pl?gene=HPSE#expression). During normal cellular processes, HPSE expression can be upregulated in response to an immune cell activation or a viral infection by the action of NF-kB (Agelidis et al., 2017; Secchi et al., 2017). HPSE is tightly regulated not only considering its gene expression but also in the secretion, internalization and activation of the enzyme. For example, sequence analysis predicted six-potential N-linked glycosylation sites and the glycosylation pattern is essential for HPSE secretion but not for its enzymatic function (Simizu et al., 2004). The activation of the enzyme is strictly related to the pH: basically, at the neutral pH as at cytoplasm and the ECM environment, HPSE is virtually inactive. When the pH becomes acidic, with an optimal enzymatic activity comprised from 5 to 6, such as an inflammatory process or tumor progression, the HPSE is then activated (Vlodavsky & Friedmann, 2001; Nagarajan & Vetrivel, 2018). Enzymatic remodelling of HS is a crucial point in all the physiological processes that require cell movement and growth factor bioavailability. HPSE action is involved in all these events, such as embryo development, hair growth, wound healing and angiogenesis (Nasser, 2008). One of the most studied effects of HPSE regarding angiogenesis. In wound healing, HPSE stimulates angiogenesis and keratinocyte migration drives tissue reorganization and repair (Zcharia et al., 2005). In addition, HPSE derived from degranulated platelets and immune cells facilitates the interaction of inflammatory cells with the subendothelial membranes and their extravasation as well as blood coagulation (Nadir, 2014).

2.3.4 HPSE in disease

Through HS degradation and non-catalytic mechanisms, HPSE is strongly implicated in several pathological conditions. Up-regulation of HPSE has been demonstrated in tumors, and inflammatory and degenerative diseases (Secchi et al., 2015). Up to today, several works have demonstrated that exist many HPSE regulators and in turn, HPSE can activate several downstream targets (Mayfosh et al., 2021). Here, we will focus our attention on cancer and the tightly interconnected inflammation.

2.3.5 HPSE in inflammation

Inflammation is a defence response to tissue damage which implies the recruitment of circulating immune cells to the site of injury. The HS plays an important role in inflammation and is known to control inflammatory responses at multiple levels, including sequestration of cytokines/chemokines in the ECM (Xie & Li, 2019) and modulation of leukocyte interactions with endothelium and ECM (Higashi et al., 2020; Masola, Greco, et al., 2022b), and initiation of the innate immune response through interactions with toll-like receptors 4 (TLR4) (Goodall et al., 2014; Elkin, 2020). Related to its capability to cleavage the HS, HPSE affect many aspects of inflammation, such as the regulation of pro-inflammatory immune cell activation and migration, establishing acute and chronic inflammation, regulating the secretion of cytokines and chemokines anchored within the ECM and promoting lymphangiogenesis (Masola, Bellin, et al., 2018). More in general, considering the multitude of pro-inflammatory chemokines bound to HS, the function of HPSE produces a gradient of cytokines such as interleukin (IL)-1β, IL-6, IL-8, IL-10 and TNF-α, stimulating the recruitment rolling process and extravasation of leukocytes (Collins & Troeberg, 2018; Xie & Li, 2019). Leukocyte migration through the vessel wall is also defined by adhesive interactions with HS and other cell surface molecules (i.e. selectin and integrin) of endothelial cells, leading to cell arrest, adhesion strengthening and crawling into sites of inflammation (Higashi et al., 2020; Masola, Greco, et al., 2022b). So, HPSE activity is implicated in many functions of different innate immune cells such as neutrophils, macrophages and DCs that mediate both acute and chronic inflammatory responses. HPSE expression has been shown to increase pancreatic cytokine (TNF-α, IL-6, etc.) and signalling molecule (phospho-STAT3) activity, along with enhanced oedema and inflammation marked by neutrophil infiltration, which ultimately led to acute pancreatitis (Khamaysi et al., 2017; Hamo-Giladi et al., 2023). HPSE is secreted by neutrophils, activated T-lymphocytes, platelets and also vascular endothelial cells, which promotes the extravasation of immune cells through the remodelling and the consequent increased permeability of subendothelial basement membrane (Goldberg et al., 2013; Masola, Greco, et al., 2022b). For example, a study conducted on a mouse model of sepsis-associated inflammatory lung disease and HPSE null mice suggests that the activity of HPSE

favours neutrophil infiltration in pulmonary microvascular endothelial cells (Schmidt et al., 2012). HPSE expression correlates with macrophage activation by TNF-α in renal tissue enhancing chronic inflammation associated with diabetic nephropathy (Goldberg et al., 2014). In addition, HPSE participates in macrophage activation mediated by the interaction of soluble HS fragments produced by enzymatic activity with the toll-like receptor (TLRs) (Goodall et al., 2014; Elkin, 2020).

Recently, it has been demonstrated that after ischemia/reperfusion (I/R) injury in the kidney, HS fragments released by HPSE activate TLRs of macrophage and proximal tubular epithelial cells. This defines a pro-inflammatory cytokines gradient which attracts and activates macrophages (Masola, Zaza, et al., 2018). Moreover, the presence of HPSE sustains the polarization of infiltrated macrophages towards an M1 pro-inflammatory phenotype (Masola, Zaza, et al., 2018). Once again, by a genetic approach, it has been demonstrated that mice lacking HPSE generate macrophages expressing lower levels of cytokines (e.g. TNFα, IL-1β, IL-6 and IL-10), impaired phagocytic activity and reduced infiltrative capacity (Waterman et al., 2007). HPSE has been implicated in several inflammation-driven cancers (see "Inflammation in cancer").

2.4 HPSE influences the hallmark of cancer

From the first characterization by Vlodavsky (see Chapter 3), HPSE attracted more and more attention for its potential role in cancer. It consolidated the idea that this enzyme is overexpressed in all human cancers and correlates with poor prognosis for patients. HPSE could be considered a "paramount" enzyme due to its heterogeneous activity. In fact, HPSE influences directly or indirectly all the "hallmarks" of cancer acting to the ECM in the TME, adding a plasticity factor that supports tumor growth, progression and metastasis. Its enzymatic activity leads to ECM remodelling and increasing the release of HS-linked molecules (**Fig. 8**). In addition, the less-described non-enzymatic function, adds another step of complexity (Jayatilleke & Hulett, 2020; Masola, Greco, et al., 2022a).
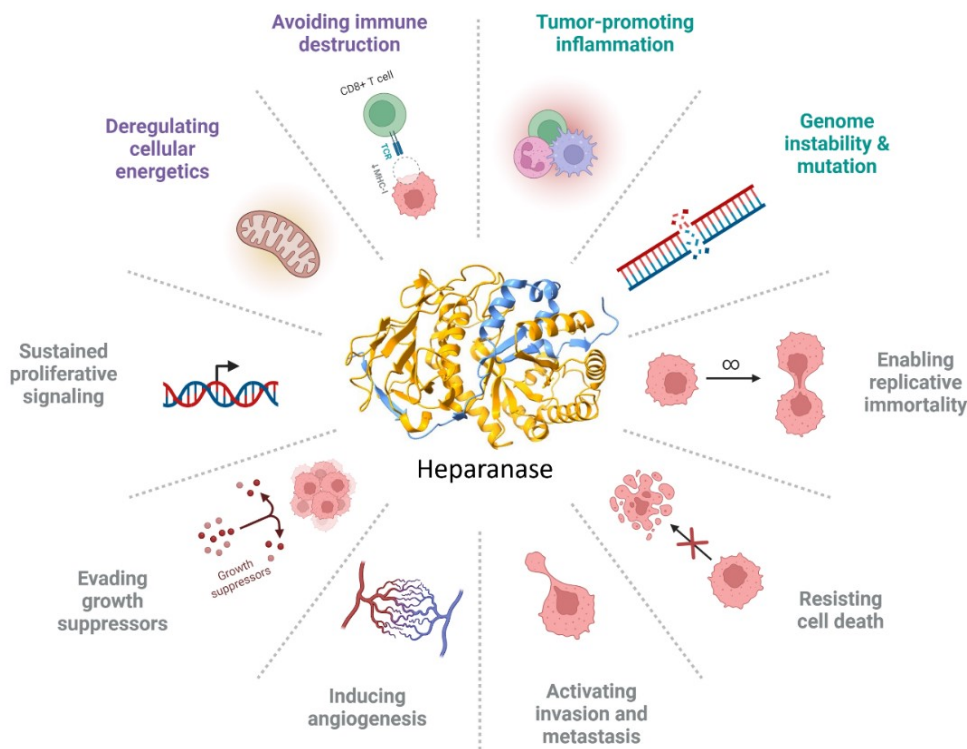
Fig. 8: HPSE influences the hallmark of cancer. The image summarizes all the hallmarks of cancer and the different colours represent: Grey: original hallmarks; Purple: enabling factors; Green: emerging hallmarks. Image created by Biorender.com

2.4.1 HPSE influences oncogenic, proliferative and growth signal

HPSE role in cancer is mainly due to its capability to modify the cellular response at different levels, affecting oncogenic, proliferative and growth signals. It is also important to point out that some mutations that correlate with cancer formation and progression can influence HPSE expression. One of the most important HPSE abilities is to influence the activity of different oncogenes such as BRAF, c-Myc and RAS, promoting the development and progression of cancer. For example, the product of mutant BRAF oncogenes, the B-Raf kinase, can activate the HPSE promoter inducing the upregulation of HPSE. This data is supported by an in vitro experiment in which HPSE gene expression was increased in HEK293 cells transiently transfected with mutated RAS gene (Rao et al., 2010). In addition, was found that the overexpression of HPSE is coincident with mutated RAS gene expression and correlates with breast and skin cancer growth in vivo (Boyango et al., 2014). Another example, the human telomerase reverse transcriptase (hTERT) plays a key role in maintaining telomere

length in many cancers and it has been associated with Myc and HPSE expression in gastric cancer. Interestingly, the hTERT promotes an indirect control of HPSE promoted by the formation of an hTERT/c-Myc complex which binds and activates the HPSE promoter, driving to gastric cancer cell invasion and metastasis (B. Tang et al., 2016). In addition, HPSE/HSPG axis is thought to be associated with Myc oncogenic signaling in medulloblastoma (Ridgway et al., 2011).

In cancer, cell proliferation is a deregulated process. The different signal factors are unbalanced and this situation drives the tumor progression. Indeed, early studies proposed that HS binding promotes growth factor stabilization and protection from degradation, but also as a storage reservoir. In addition, individual HSPGs appear to play distinct roles in specific cancers (Gallagher, 2015; Vallet et al., 2022). HPSE contributes to the impairing of growth factors homeostasis by the increase of their bioavailability. The enzymatic cleavage of the HS chains of HSPG, promotes the release of HS-linked molecules such as FGF, HGF and VEGF from the ECM, influencing different signalling pathways in both tumor and stromal cells within the TME (Vlodavsky et al., 2016; Masola et al., 2020) (**Fig. 9A**).

One of the growth factors family that is most affected is the fibroblast growth factors (FGFs) the interplay between HSPGs and the FGF/FGFR axis is currently the best characterized in tumor. All FGFs exhibit a globular β-trefoil domain with an HS binding site on its surface that facilitates the growth factor sequestration. The activation of the FGF signal strictly depends on the binding to HS, a crucial step for dimerization and activation of FGFR mediated by the formation of an FGF-FGFR-HS ternary complex (Goetz & Mohammadi, 2013). In addition, HS sulfation and epimerization, which are regulated in a tissue-specific way, contribute to the fine-tuning of the FGF/FGFR system in different biological contexts. For example, 2-O-sulfated L-iduronate and N-sulfated D-glucosamine have been demonstrated to be essential for the interaction between FGF2 and HS, and 6-O-sulfate groups have been reported to be required for the mitogenic activity of the complex. FGF1 requires 2-O-, N- and 6-O-sulfate groups for optimal HS binding and transduction of the mitogenic signal (Mohammadi et al., 2005). It has been shown that the overexpression of HPSE in human tumors is associated with enhanced 6-O-sulfation of HS, thus promoting the formation of a ternary complex of HS with FGF and FGFR (Escobar Galvis, M., et al 2007). HS fragments derived from

SDC-1 degradation by HPSE were shown to potentiate the FGF2 mitogenicity, which could further contribute to modulating the growth factor activity (Kato et al., 1998). Interestingly, FGF-2, SDC-1 and HPSE but not FGFR colocalize in the nucleus of mesenchymal tumor cells (Zong et al., 2009). Nuclear translocation of SDC1 also allows the shuttling of HS binding growth factors including FGF2 and HGF (Stewart et al., 2015).

Hepatocyte growth factor (HGF) is an additional HS binding growth factor that is affected by the action of HPSE. The action of HGF is mediated via its binding to the proto-oncogenic c-met receptor (Ma et al., 2003). It was found that HPSE exerts two different roles upon HGF by studies conducted in vitro and in vivo myeloma models. High serum levels of HPSE, shed SDC-1 and HGF are associated with poor prognosis, suggesting functional cooperation (Alexandrakis et al., 2003). The transcript and protein levels of HGF were upregulated by HPSE and the HGF produced and secreted by the tumor cells, binds to shed SDC-1 induced by HPSE, which enhance HGF signaling via the c-Met receptor. It is important to understand that the influences of HPSE upon HGF synthesis are independent of its enzymatic activity which is correlated to the bioactivity of HGF mediated by shed SDC-1 (Ramani et al., 2011). It has also been shown that HGF activates the PI3K/Akt and NF-kB signalling to promote the HPSE expression in gastric cancer cells that is correlates with poor prognosis for patients (Hao et al., 2015).

HPSE is strictly related to angiogenesis, vascular permeability, and lymphangiogenesis in cancer, activity mediated by the modulation of VEGF. Overexpression of HPSE promotes an increase in VEGF mRNA and protein levels in different cancer cell models. This interplay is mediated by an elevated p38 phosphorylation and the activation of Src (Zetser et al., 2006) (see below "HPSE in inflammation").

The epidermal growth factor (EGF) receptors (EGFRs) is a tyrosine kinase receptor strictly correlated to cell proliferation, that is commonly deregulated in numerous cancers. Overexpression of EGFR is associated with an over-activation of different downstream pro-oncogenic signalling pathways that drive uncontrolled proliferation (Wee & Wang, 2017). One member belonging to the EGF, the heparin-binding EGF (HB-EGF), is a potent activator of EGFR with a high affinity to HS (Normanno et al., 2006). It was found that high expression of HPSE induces the activation of HB-EGF for differentiation and lymph node metastasis in pancreatic ductal adenocarcinoma patients

(Hoffmann et al., 2008). HPSE thanks to the induction of SDC-1 shedding, activate indirectly the EGFR signalling. The soluble SDC-1 can bind the HB-EGF by its intact HS chains and activate the EGFR downstream pathways to promote chemotherapy resistance in colorectal cancer (X. Wang et al., 2014). On the other hand, EGF induces the nucleolar localisation of HPSE that promotes the modulation of the DNA topoisomerase-I enhanced cell proliferation (L. Zhang et al., 2010).

TGF-β exerts a paradoxical role in cancer cell proliferation, moving from a tumor suppressor in benign and early-stage tumors to a potent tumour-promoting factor in the advanced stages of cancer (Principe et al., 2014). It is noteworthy that TGF-β interacts with HS which regulates its bioavailability, pointing out the important role of the betaglycan receptor (Troilo et al., 2016; Villarreal et al., 2016). In non-tumorigenic model, was demonstrated how HPSE upregulation and activity coordinate TGF-β activity driving EMT (Masola et al., 2014). These data suggest a potential link between HPSE and TGF-β also in cancer.
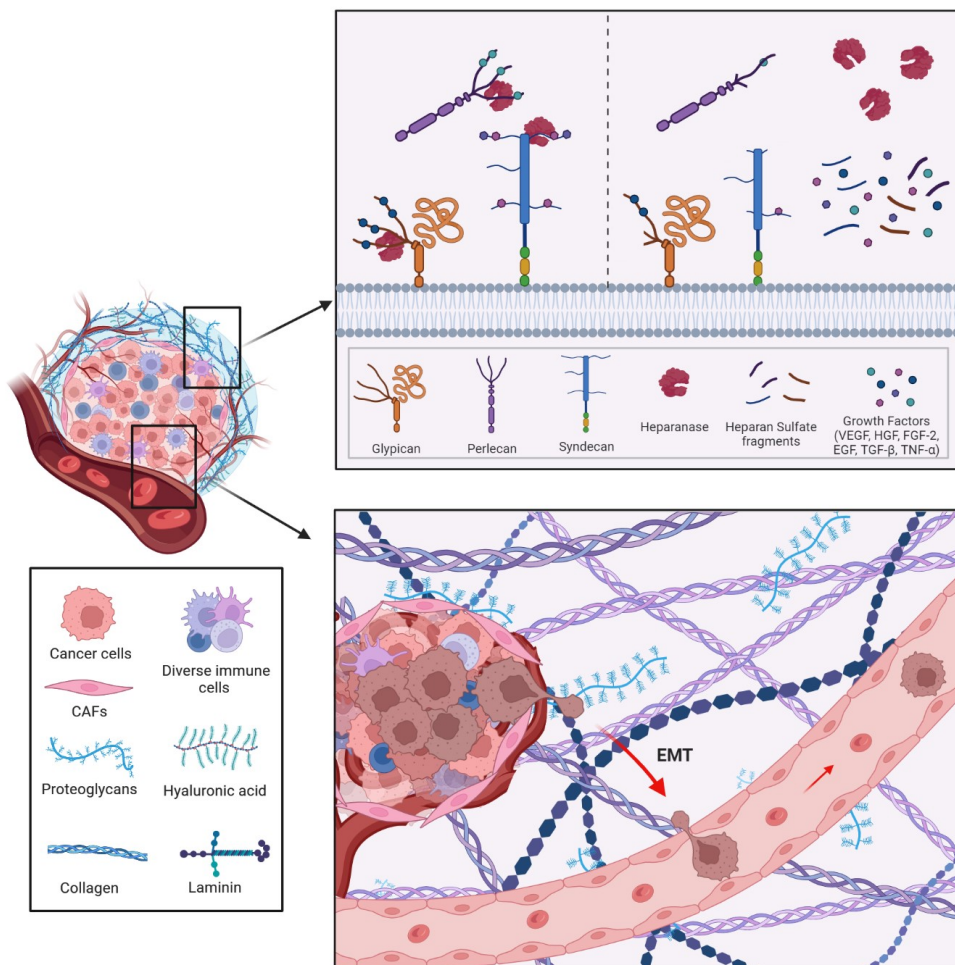
Fig. 9: Main functions of HPSE in cancer. Heparanase is able to modify directly the surrounding environment (by enzymatic activity) and indirectly because promotes the release of many HS-linked molecules, such as growth factors, cytokines and enzymes that sustain angiogenesis and inflammation (**A**). HPSE facilitate tumor cell migration, penetration of the basement membrane and metastatic dissemination (**B**). Image created by Biorender.com

2.4.2 Angiogenesis

Angiogenesis and lymphangiogenesis are two main mechanisms that potentiate the cancer vascular network. They are as important as the proliferation or metastatic spread, basically due to an adequate supply of oxygen and nutrients for the cells that compose the tumor mass. Angiogenesis and angiogenic associated factors are strictly associated with tumor aggressiveness (Nishida et al., 2006). HPSE is an active player involved in various aspects of neo-angiogenesis, as HSPGs are structural components of the endothelial glycocalyx of capillaries. The cleavage of HS chains promoted by HPSE, contributes significantly to tumor angiogenesis, enabling endothelial cells to proliferate and migrate in response to angiogenic stimuli (Masola, Greco, et al., 2022b). HPSE activity determines the release and diffusion of VEGF and FGF, two HS-binding protein that has been showed to be a potent regulator of angiogenesis in cancer (Marchetti et al., 2006; Zetser et al., 2006; G. Liu et al., 2021). A study conducted on primary breast tumors, suggests that the overexpression and activity of HPSE in the TME induces the activation of VEGF and FGF signalling pathways promoting tumor angiogenesis. MCF-7 human breast cancer cells that actively express HPSE exhibit higher angiogenesis in vivo which is also correlated with large tumor size (Cohen et al., 2006). A positive correlation between HPSE levels and angiogenesis was found in a histological analysis of human colorectal cancers (Sato et al., 2004). In addition, a recent work by Jayatilleke et al. demonstrates that in the HPSE-knock-out murine mammary carcinoma model (MMTV-PyMT) the angiogenesis potential was dramatically decreased on the mammary gland (Jayatilleke et al., 2023).

The mechanism of HPSE action was unveiled in multiple myeloma. In these cells, the trimming of HS chains on SDC-1 produced by HPSE facilitates the cleavage by the MMP-9 (whose expression is correlated with high HPSE). Shedding of SDC-1 exposes a latent domain that promotes the interaction of the VEGFR2 with $\alpha 4\beta 1$ integrin leading

to the activation of the VEGF-2 receptor both on myeloma and endothelial cells. This mechanism promotes at the same time angiogenesis, invasion and metastasis driving myeloma progression (O. Jung et al., 2016). Another HPSE-induced mechanism of angiogenesis proposed that HPSE activates the cyclooxygenase-2 (Cox-2)/HIF1-α pathway (Naomoto et al., 2007). Indeed, in cervical cancer, it was found that HPSE in response to radiation induces not only the increase of HIF-1 but also of VEGF and FGF, promoting both radiation resistance and angiogenesis (Li et al., 2017).

2.4.3 Invasion and metastasis

As mentioned in the introduction, the death of breast cancer patients is caused mainly by metastasis. Despite significant advances in the diagnosis and treatment of cancer, metastasis is also associated with more than 90% of all cancer-related deaths (Steeg, 2006). At the basis of the metastasis, there is a well-known and well-described cellular process, the epithelial–to–mesenchymal transition (EMT) (**Fig. 9B**). This is a cellular process in which epithelial cells ruined cell-cell junction, apical-basal polarity and basal membrane interaction acquiring fibroblast-like morphology, as well as increased migratory capacity and often invasive properties. EMT is defined as the results of interaction between cells and the surrounding microenvironment, which triggers the changes in gene expression and post-translational regulation mechanisms promoting phenotype switching (J. Yang et al., 2020). An important element for the invasive and metastatic capacities of tumor cells is due to the overexpression of ECM degradative enzymes, that impair the ECM homeostasis increasing the degradation potential. The collective expression of degrative enzymes, such as MMPs, ADAMs and ADAMTS, plasminogen activation system components, cathepsins and HPSE in the tumor microenvironment enables invading cells to migrate inside of ECM and then to disseminate into the circulation (Piperigkou et al., 2021).

Several studies have demonstrated how HPSE overexpression is correlated with an increase in metastatic potential in various tumor. Immunohistochemical analysis of tumor samples of patients shed light on how the areas of tumor invasiveness were attributable to HPSE positivity compared with no detectable signal level in healthy adjacent tissue. On the other hand, studies conducted upon inhibition of HPSE by gene silencing or specific inhibitors, demonstrate a reduction in the invasive capacity and

metastasis of different tumor cell lines both in vitro and in vivo. Some of HPSE functions as promoters of metastasis and invasion in different tumor are reported in **Table 1**.

Table 1: HPSE expression is involved in invasion and metastasis in various tumor

Tumor type	Observation	Reference
Breast cancer	Analyses of fifty-one primary breast tumors showed that HPSE expression is significantly correlated with sentinel node metastasis. HPSE-positive tumors (90%) showed a reduced level of HS deposition	(Maxhimer et al., 2002)
Breast cancer	Serum MMP-9 and HPSE are higher in breast cancer patients and correlate with histology grade, lymph node status and lymphovascular invasion	(D. Tang et al., 2014)
Breast Cancer	miR-1258 suppressed breast cancer brain metastasis in vitro by inhibiting the expression and activity of HPSE, targeting the HPSE 3′-untranslated region	(L. Zhang et al., 2011)
Cervical cancer	HPSE expression in cervical cancer patients correlates with tumor size and clinical stage by Immunohistochemistry analyses. HPSE-overexpressing cervical cancer cells increased proliferation in vitro and tumor growth in vivo	(Zeng et al., 2013)
Colorectal cancer	Knockdown HPSE in different colorectal cancer cell lines inhibited invasion and liver metastasis in vitro and in vivo. Rna-seq showed alteration in invasion and metastasis-related genes	(X. Liu et al., 2019)
Gastric cancer	HPSE mRNA expression significantly correlates in late-stage, large size, lymph nodal and metastasis by in-situ hybridization of primary gastric carcinomas	(W. Tang et al., 2002)
Gastric cancer	miR-299-3p targets the 3'-UTR of HPSE mRNA regulating its expression. Similarly, the miR-1258 demonstrated a reduction of HPSE protein and gene expression reducing invasion and metastasis in gastric cancer cells in vitro	(Shi et al., 2017; X. Zhou et al., 2019)

Head and neck squamous cell carcinomas (HNSCC)	In situ hybridization shows that HPSE expression is associated with lymph node metastasis in HNSCC biopsies. In addition, in vitro and in vivo confirmed that HPSE was correlated with prolonged disease-free survival and overall survival	(Beckhove et al., 2005)
Hepatocellular cancer	High-expressed HPSE-induced necroptosis of the adjacent microvascular endothelial cells (MVECs) activating the HPSE/SDC-1/TNF-α axis and p38 MAPK pathway that promote intrahepatic metastasis	(X. Chen et al., 2021)
Melanoma	Immunohistochemistry analyses demonstrated that high levels of HPSE were associated to late-stage melanoma patients	(Vornicova et al., 2016)
Multiple myeloma	HPSE drive multiple myeloma metastasis and progression enhancing Fibronectin and Vimentin partially due to the activation of the ERK pathway in vitro and in vivo	(Li et al., 2016)
Ovarian cancer	Elevated serum Cathepsin L, HPSE, and MMP-9 levels are correlated with malignant invasion and progression in ovarian cancer	(W. Zhang et al., 2011)
Pancreatic cancer	Cultured pancreatic cancer cells transfected with HPSE full-length construct displayed enhanced invasiveness in an invasion chamber assay. HPSE expression in pancreatic cancer by using in situ hybridization correlated with any clinicopathologic parameters. Log-rank test of the Kaplan-Meier survival curves revealed that HPSE expression in early-stage tumors was associated with decreased survival.	(Koliopanos et al., 2001; Kim, 2002)
Prostate cancer	I*n situ* hybridization demonstrated that HPSE mRNA expression in prostate carcinomas was significantly correlated with tumor differentiation and tumor stage	(Stadlmann et al., 2003)
Prostate cancer	In vitro experiments showed that HPSE expression influences EMT and stemness marker expression in two different prostate cancer cell lines	(Masola, Franchi, et al., 2022)

2.4.4 HPSE in cancer Inflammation

The tumor microenvironment is characterized by chronic inflammation and cancers have been described as "wounds that never heal" (Singel & Segal, 2016). Numerous immune cells are intimately involved with the TME, some for eliminating tumours but others to promote tumour growth and progression. Immune cells in the tumour mass establish a cross-talk with tumour cells undergoing a phenotype switch to become tumor-supporting cells (Dehne et al., 2017; Marzagalli et al., 2019). Macrophages form a significant portion of tumor mass and for example in breast cancer are considered a prognostic marker (Medrek et al., 2012).

As mentioned before, HPSE can influence many aspects of immune cells at multiple levels (see "HPSE in inflammation"). Recent findings have pointed to HPSE as an important link between inflammation and inflammation-associated cancer (Meirovitz et al., 2013).

For example, HPSE was identified as the driver in the transition of Barrett's oesophagus to oesophageal adenocarcinoma. Immunohistochemical analysis evidenced a progressively increasing of HPSE from normal oesophagus to high-grade Barrett's oesophagus carcinoma (Brun et al., 2009).

A study conducted on a mouse model of acute and chronic colitis demonstrated that epithelial-derived HPSE modulates and sustains the chronic activation of inflammatory macrophages which induce epithelial colon cells to express and release HPSE (via TNF-α) that is subsequently activated by cathepsin L secretion. This chronic inflammatory circuit creates a tumor-promoting microenvironment that facilitates the invasion of colon cells promoting colorectal cancer progression (Menzel et al., 2006; Lerner et al., 2011).

Studies on chronic gastritis induced by Helicobacter pylori (considered the major risk factor for gastric cancer) have suggested that HPSE is upregulated and this is involved in the early stages of gastric cancer. Similarly, to colorectal cancer, the role of HPSE is mainly associated with the recruitment of macrophages generating a vicious cycle (driven by NF-kB and p38-MAPK signalling pathway) that sustains chronic inflammation supporting the development and progression of gastric cancer (L. Tang et al., 2021). Chronic inflammation of the liver often caused by viral hepatitis is considered a risk for the development of hepatocellular carcinoma (HCC). Higher levels

of HPSE in patients with hepatitis-C-related HCC are positively correlated to promote angiogenesis and facilitate invasion contributing to tumor progression (El-Assal et al., 2001).

HPSE is not only able to drive cancer growth and progression but could support immune evasion, an emerging role associated with the tumor-associated macrophages (TAMs). Noteworthy, HPSE plays an important role in the activation and function of macrophages, which in turn protect tumor in two different ways: 1) by the expression of the human leukocyte antigen (HLA), that in turn avoids the activation of NK cells and some T cells; 2) by the releasing of chemokines recruits T regulatory cells that inhibit the activity of CD4+ and CD8+ T cells (Mantovani et al., 2022).

Due to its implication in different aspects of immune cell functions, it is important to underlying also a positive role in cancer therapy. Recently, Caruana et al. discovered that HPSE plays a relevant role in chimeric antigen receptor (CAR)-T lymphocyte therapy. Interestingly, data suggest that HPSE expression in long-term ex vivo-expanded T cells expressing tumor-specific CAR ameliorates their capacity to degrade the ECM improving CAR-T antitumor activity (Caruana et al., 2015).

2.4.5 HPSE role in cell death evasion

Evading cell death is considered one of the hallmarks of cancer. Indeed, during the progression, cancer cells have to acquire the capability to escape apoptosis, the physiologically programmed cell death. This happens by the inhibition of apoptosis promoted by the up-regulation of anti-apoptotic or by the deregulation of pro-apoptotic signals (Fernald & Kurokawa, 2013). In this context, HPSE plays an anti-apoptotic role both for enzymatic and non-enzymatic activity. In breast cancer, the release of FGF promoted by HPSE was correlated with apoptosis inhibition and prolonged tumor survival (Cohen et al., 2006). In addition, an RNA-seq experiment in HPSE-overexpressing MCF-7 cells identified the regulation of apoptosis as one of the potential pathways associated with cell viability after 5-fluorouracil treatment (Zahavi et al., 2021). Additionally, HPSE non-enzymatic activity was shown to inhibit apoptosis induced by oxidative stress and growth factor starvation via PI3K/Akt activation (Riaz et al., 2013).

Another well-described cellular mechanism that directly contributes to cancer cell survival and leads also to chemoresistance is autophagy. This evolutionary conserved catabolic pathway contributes to cellular homeostasis by the degradation of damaged cellular components (Mulcahy Levy & Thorburn, 2020). Recent data suggests that the expression of HPSE promotes autophagy through the reduction of mTOR1, the key regulator of autophagy, inducing tumor growth and chemoresistance by promoting (Shteingauz et al., 2015). In addition, new evidence has pointed out that both active and inactive HPSE modulates TFEB-mediated autophagy in gastric cancer cells (M. Yang et al., 2022).

In addition, it was demonstrated that highly expressed HPSE HCC cells mediated necroptosis in adjacent microvascular endothelial cells (MVECs) activating the trans-endothelial migration that involved a HPSE/SDC-1/TNF-α axis and p38 MAPK pathway. HPSE knockdowns reversed necroptosis and decreased TNF-α expression level, while HPSE over-expression increased SDC-1 and TNF-α expression and aggravated necroptosis (X. Chen et al., 2021).

2.4.6 Energy metabolism reprogramming: an emerging role

In cancer, support for rapid proliferation requires a high energy demand. The Warburg effect is the metabolic phenomenon of cancer cells which preferentially produce energy by glycolysis instead of providing energy through oxidative phosphorylation in the mitochondria (Liberti & Locasale, 2016).

Also, the autophagy in cancer is induced by starvation and stress to bear the additional metabolic demand (Levy et al., 2017). In lung adenocarcinoma, the PI3K/AKT/mTOR signaling pathway has been shown to promote glycolysis and the Warburg effect (Makinoshima et al., 2015).

So, although HPSE has no direct effect on the Warburg effect, it could control indirectly the energy reprogramming, for example by the activation of PI3K-related signaling (Riaz et al., 2013). It is important to emphasize that the HPSE expression was also correlated to glycemia. For example, in patients with type 2 diabetes, the levels of HPSE in urine were correlated with high glucose, suggesting a correlation between glucose levels and HPSE expression and secretion. Interestingly, in transgenic mice overexpressing HPSE, it contributes to glucose-stimulated insulin secretion and reduces

the severity of chemically-induced diabetes (D. Zhang et al., 2017). In addition, inhibition of HPSE minimizes the glucose-induced EMT in mesothelial cells (Masola et al., 2017). Moreover, HPSE inhibition significantly reduces blood pressure, but also serum glucose levels and oxidative stress in apolipoprotein E knockout mice (Hamoud et al., 2017).

2.5. HPSE inhibition

Heparanase had a long history and attracted more interest starting from the first description as a potential regulator in cancer metastasis in 1983 (Vlodavsky et al., 1983). Following its functional characterization in the 1999s (see the chapter "HPSE: a unique enzyme"), the first works have begun to search for potential new inhibitors of this enzyme. Indeed, related to its enzymatic activity due to its capacity to recognize and cut specifically a β 1,4 glycosidic bond, making it a good and interesting therapeutic target. Of course, there exist different ways to reach this point and different approaches were developed from natural components up to sophisticated and functionalized molecules (Rivara et al., 2016). Here, only an overview of the different options will be listed, with an extensive explanation of HS mimetics compounds that are currently under clinical trial investigation: SST0001, M-402, PI-88 and PG545 (**Fig.11**).

2.5.1 From heparin to its derivates HS-mimetics molecules

Heparin is a well-known sulfated polysaccharide largely used as an anticoagulant and antithrombotic drug. Due to its strict homology with HS, acts as an analogue of the natural substrate of HPSE competing for the HSBDs and it is commonly considered a potent inhibitor of HPSE (Casu et al., 2015). Heparin, unfractionated heparin (UFH) and low molecular weight heparin (LMWH) were reported to exert a beneficial effect on cancer, affecting the proliferation, adhesion, angiogenesis, migration and invasion of cancer cells via multiple mechanisms (Ma et al., 2020). Unfortunately, due to its capacity to bind different HS-binding proteins (for example FGF-2), the increasing off-target and "side effect" as anticoagulant activity, making the use of heparin as a specific HPSE inhibitor complicated (Xu & Esko, 2014). The antithrombin binding region important for the anticoagulant and antithrombotic activity of HS is constituted by a specific pentasacharride sequence of N-acetyl-D-glucuronic acid β1→4 D-glucosamine

N,3-O,6-O trisulfate α1→4 L-iduronic acid 2-O-sulfate α1→4 D-glucosamine N,6-O-disulfate (**Fig. 10**). Starting from the heparin structure, the researchers have been starting to design new heparin-like molecules useful for cancer therapy. The purpose of this new class of synthesized molecules, called "heparin mimetics" was to increase the binding affinity to the HPSE and reduce the incoming side effects (Xu & Esko, 2014).
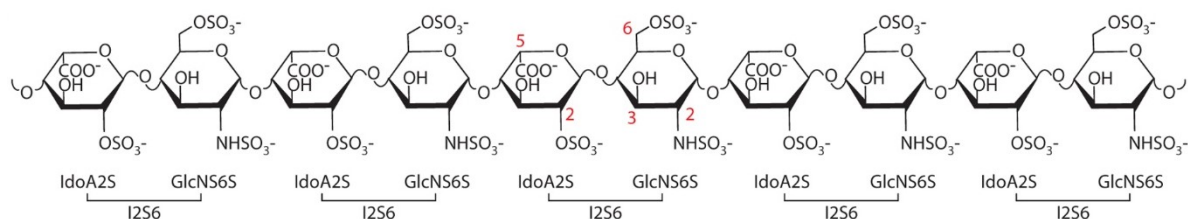

Fig. 10: Structure of heparan sulfate. Chemical structure of a heparin-derived decasaccharide. The red number indicates the carbon modification sites. GlcNS, N-sulfoglucosamine; IdoA, l-iduronic acid. pentasaccharide of heparin-antithrombin binding sequence. Image from (Xu & Esko, 2014)

2.5.2 Glycol-split heparins: SST0001 and M-402

Roneparstat also known as SST0001 (Sigma-Tau Switzerland S.A) is a high molecular-weight heparin (from 15-25 kDa), chemically modified to obtain a fully *N-desulphated/N-reacetylated*, glycol split molecule (Naggi et al., 2005) (**Fig. 11-A**). This compound is a potent HPSE inhibitor with reduced anticoagulant activity and is currently in phase I of clinical trials for the treatment of advanced multiple myeloma in combination with dexamethasone (Ritchie et al., 2011; Galli et al., 2015; Pala et al., 2016). The idea was to combine different chemical modifications found in previous studies to functionalize an efficient molecule with lower or absent off-target effect. In general, HPSE inhibition is dependent on the O-sulfation degree of glucosamine residues compared to the sulfation in a specific position. Indeed, 2-O desulfated derivatives were shown to retain the inhibitory capacity, whereas *N-desulfation/N-reacetylation* of glucosamines reduced the inhibitory activity of the molecules, suggesting that at least one NSO_3 group per disaccharide unit is involved in interaction with the enzyme. The glycol-split is the result of a controlled periodate oxidation/borohydride reduction of the C2-C3 glycol bond of non-sulfated uronic acid residues that hamper the anticoagulant activity. It is interesting to understand that this

molecule is not a substrate for HPSE, but the high conformational freedom acquired by the split portions favours the accommodation of the polysaccharide chains at the level of the HPSE binding site. Moreover, O-desulfates, *N*-acetyl and glycol-split heparin do not bind FGF-2, preventing the FGF-2 release in the ECM environment and inhibiting the activation of angiogenesis (Naggi et al., 2005). Dose-inhibition curves studies confirmed the high inhibitory potential of Roneparstat (IC$_{50}$ $\approx$ 3 nM) and pointed out the hypothesis of multiple protein-ligand interactions depending on its concentrations (Pala et al., 2016).

HPSE-inhibition by Roneparstat both in vivo and in vitro is reported to reduce myeloma growth induced by modification in tumor as downregulation of HGF, VEGF and MMP-9 expression that suppress angiogenesis. In vitro analysis conducted on the molecular size of SDC-1 demonstrated a reduction in HPSE-mediated degradation, confirming the inhibitory activity of this compound. In addition, the use of Roneparstat reduced human myeloma tumor cell growth in SCID mice acting on dual targeting of the tumor mass and its microenvironment (Ritchie et al., 2011).

Roneparstat was shown to be active also in other tumor models to modulate cancer resistance to chemical or physical stress. It demonstrated a significant increase in HPSE expression after high-dose of melphalan chemotherapy in myeloma patients. The combinatory treatment with melphalan sustained with Roneparstat after the injection with HPSE-high cells in mice, reduced tumor relapse (Ramani et al., 2016). The combinatory therapy with Roneparstat to counteract the cytoprotective role due to high HPSE levels after stress induction was demonstrated also to reduce lapatinib-resistant breast cancer metastases to the brain (L. Zhang et al., 2015) or reduce the invasiveness induced by radiation in pancreatic cancer (Meirovitz et al., 2011). In addition, Roneparstat treatment improves the response of HDAC inhibitor therapies to inhibit the invasiveness of synovial sarcoma (Lanzi et al., 2021).

Another compound based on the same technology as Roneparstat is the necuparanib or M402 (Momenta Pharmaceuticals, USA). This is semisynthetic low-molecular weight glycol-split heparin (5-8 kDa), obtained by controlled depolymerization of unfractionated heparin with nitrous acid, followed by sequential periodate oxidation and borohydride reduction (**Fig. 11-B**). The M-402 inhibition activity on HPSE (IC$_{50}$ $\approx$ 800 nM). show reduced anticoagulant activity and preserved or improved the binding

properties of HS to multiple targets associated with tumor growth (H. Zhou et al., 2011). M-402 is currently in phase I/II for the treatment of pancreatic cancer (in combination with nabpaclitaxel and gemcitabine) (O'Reilly et al., 2017).
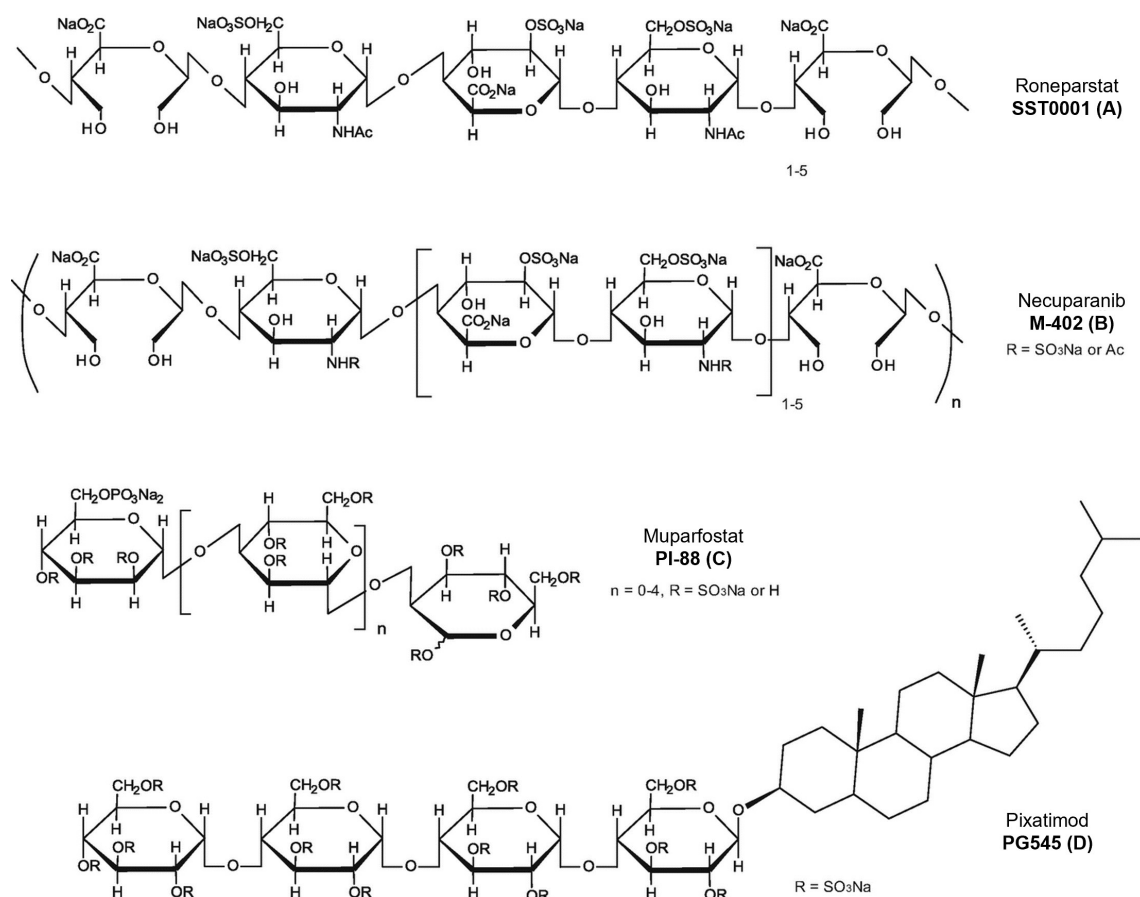

Fig. 11: Molecular structures of HPSE inhibitors. The structure of HPSE inhibitors that are currently under clinical trials investigation. Image edited from (Rivara et al., 2016)

2.5.3 PI-88

PI-88, also known as muparfostat (Progen Pharmaceuticals, Australia), is a mixture of highly sulfated, monophosphorylated mannose oligosaccharides produced by the extracellular phosphomannan of the yeast species *Pichia holstii* NRRLY-2448 (Parish et al., 1999; Ferro et al., 2001) (**Fig. 11-C**). PI-88 is the most extensively studied HS-mimetic candidate for different clinical trials due to its antiangiogenic and

antimetastatic activity principally related to HPSE inhibition (IC$_{50}$ $\approx$ 8 nM), hampering the interactions from FGF-1, FGF-2 and VEGF to their receptors (Joyce et al., 2005; Basche et al., 2006; Lewis et al., 2008). For its potent anticancer activity, PI-88 was evaluated in a Phase III clinical trial in post-resection hepatocellular carcinoma (HCC). Despite the encouraging effect, it was stopped at interim analysis because it did not meet the primary endpoint (disease-free survival) (P.-J. Chen et al., 2017).

2.5.4 PG545

Due to the potentiality of PI-88, a new class of compounds called the PG500 series has been developed to improve the inhibition of HPSE and PG545 was the best candidate as a novel drug for cancer treatment. PG545 (Progen, Pharmaceuticals, Australia), also called Pixatimod (IC$_{50}$ $\approx$ 10 nM), is a synthetic fully sulphated oligosaccharide attached to a lipophilic moiety, which improves have been optimized for drug development and reduced anticoagulant activity (Dredge et al., 2010) (**Fig. 11-D**). This compound is a potent HPSE inhibitor working as an antagonist substrate hampering the binding to VEGF, FGF-1 and FGF-2 reducing angiogenesis and metastasis in various preclinical models including for example breast, pancreatic and ovarian (Dredge et al., 2011; Hammond et al., 2012; Ostapoff et al., 2013; Winterhoff et al., 2015). As for the other HS inhibitor, PG545 shows synergistic activity with different chemotherapy drugs. In ovarian cancer, alone it was demonstrated to inhibit growth factor-mediated cell migration by reducing HB-EGF-induced phosphorylation of ERK, AKT, and EGFR in vitro. The pre-treatment with PG545 following paclitaxel and cisplatin treatment showed synergistic effects and significantly improved overall survival (Winterhoff et al., 2015). In addition, PG545 showed synergistic activity in combination with gemcitabine to alter the Wnt/β-catenin pathway to decrease proliferation in pancreatic cancer (D.-B. Jung et al., 2019). PG545 attracted more interest because it was found that in a mouse lymphoma model, it was able to activate natural killer (NK) cells, which in turn required the involvement of the Toll-like receptor 9/MyD88 pathway (Brennan et al., 2015). PG545 also inhibited tumor xenograft growth and is currently undergoing investigation in Phase I study in patients with solid tumors. It is important to point out that although some adverse effects were observed in the Phase I trials, no dose-limiting toxicity was found (Dredge et al., 2018).

2.5.5 Different approaches for HPSE-inhibition

It is real that the principal inhibition approach is the use of HS-mimetics inhibitor but also other approaches were evaluated to attenuate HPSE expression in cancer. One of these approaches is represented by the class of small molecule inhibitors. Suramin, a synthetic polysulfonated naphtylurea, was one of the first molecules studied with a potent HPSE inhibition ($IC_{50} = 46$ µM), but the high toxicity blocked its potential use in clinical trials. Some suramin analogues with higher HPSE inhibition and lower toxicity were prepared. A new series of small molecule HPSE inhibitors via in vitro and in silico screenings are under investigation for their drug-like characteristics (Y. Zhang & Cui, 2023).

Anti-HPSE antibody was also investigated and two potential peptides blocking the enzymatic activity with a promising result have been reported. Both the antibodies (with different epitopes) inhibited the lymphoma growth in xenograft models and a further HPSE inhibition was detected when used in combination. The combinatory activity of the two antibodies significantly reduced metastasis and invasion in lymphoma murine models. Interestingly, these antibodies seem to not affect the viability of tumor cells but act only in the TME. There is no data about the inhibitory activity of this antibody upon the non-enzymatic activity of HPSE (Weissmann et al., 2016). Nucleic acid inhibitors such as polydeoxyribonucleotide defibrotide have been evaluated as HPSE inhibitors. This oligonucleotide was demonstrated to suppress transcript, protein levels and also enzymatic activity of human HPSE in both treated or constitutively expressing HPSE myeloma cells in vitro (Mitsiades et al., 2009). Recently, some researchers have begun to evaluate HPSE inhibition through vaccination. Two main strategies have been considered; 1) a prophylactic immunization by the transfection with a plasmid DNA encoding the HPSE gene (Fu et al., 2012); 2) the use of multiple antigenic peptides to stimulate the dendritic cells to induce immune response (X.-D. Tang et al., 2012). In both the case studied there was a significant reduction in melanoma growth and metastasis (Fu et al., 2012; X.-D. Tang et al., 2012).

3. AIM OF THE WORK

Breast cancer is the most frequent malignant disease among women according to the World Health Organization. In 2021 it became the first cancer globally, accounting for 12% of all new annual cancer cases worldwide. In these patients, the main cause of death is not related to the primary tumor mass, but it is correlated to the widespread metastases.

The metastatic process is a key point of malignant tumor, because sets the basis for the colonization of other vital organs at distant sites. At the basis of metastases has been found the epithelial to mesenchymal transition (EMT), an evolutionary conserved developmental program and one of the best-described examples of cellular plasticity. This mechanism confers at the cancer cells the metastatic properties enhancing mobility, invasion, and progression.

A study conducted on fifty-one primary breast tumors shed light that HPSE was overexpressed in breast cancer. The current literature pointed out that HPSE is overexpressed in all human tumors (for example colon, liver, pancreas, prostate, head and neck). In addition, several studies have shown that HPSE overexpression can induce EMT, a common event found in different disease settings such for example multiple myeloma and renal fibrosis.
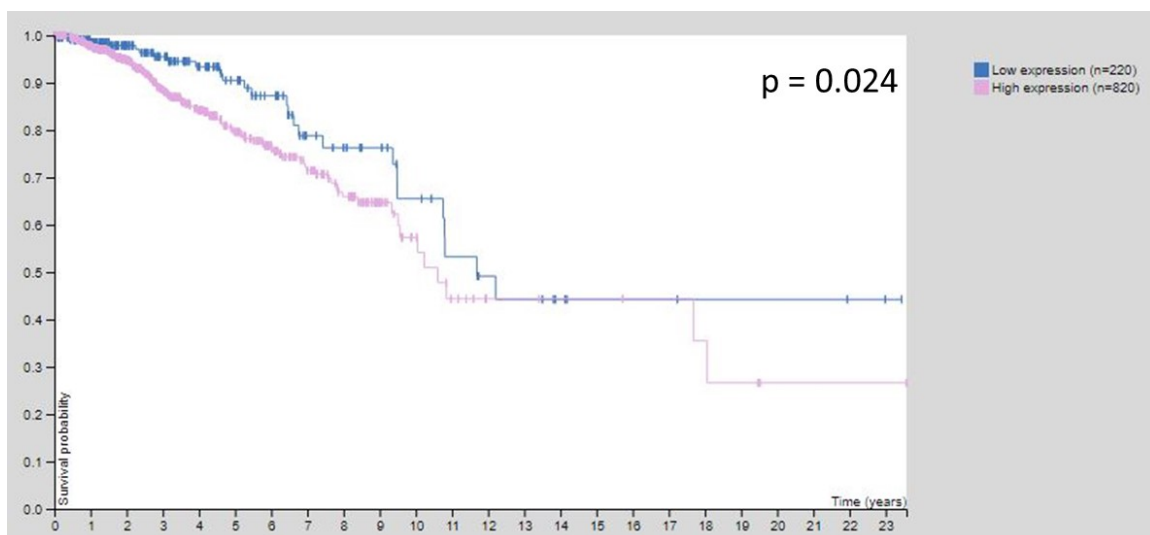
Fig. 12: Survival analysis. The Kaplan-Meier curve shows the survival of low (n = 220, blue line) and high (n = 820, purple line) HPSE levels in breast cancer patients with stage III and IV malignancies. The curve comparison with the log-rank (Mantel-Cox) test revealed statistically significant differences (p-Value = 0.024). Image obtained from https://www.proteinatlas.org/ENSG00000173083-HPSE/pathology/breast+cancer

It is noteworthy that in breast cancer cell models a key regulator of EMT is FGF-2 and its receptor but also that HPSE enzymatic activity modulates the activation of the FGF-2 signalling pathway. A different pleiotropic cytokine, the TGF-β1, is also known to modulate the EMT process in breast cancer. In addition, it has been proved that in renal fibrosis HPSE is a regulator of EMT induced by FGF-2 and TGF-β in renal tubular cells.

Starting from this evidence in which HPSE is overexpressed in tumor models and can modulate EMT in several pathological conditions, we plan to develop this PhD project to investigate the role of HPSE in the regulation of the EMT process in breast cancer. We considered using two different breast cancer cell lines, the MCF-7, non-metastatic epithelial-like and the MDA-MB-231, the triple negative breast cancer characterized by high metastatic potential. So, to understand how the level of HPSE influences the cell type behavior, our approach was based on an enzymatical inhibition of the HPSE protein by a specific inhibitor. In addition, a gene editing approach based on the knockout, silencing and overexpressing of HPSE cell lines was used.

4. MATERIAL AND METHODS

4.1 Cell Cultures

MCF-7 (low metastatic, ER-positive) and MDA-MB 231 (high metastatic, triple negative) breast cancer cell lines obtained by American Type Culture Collection (ATCC), were cultured in Dulbecco's Modified Eagle Medium-High Glucose (DMEM-HG) (#D6429, Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS) (#yourSIAL-FBS-SA, yourSIAL), penicillin (100 U/mL) and streptomycin (100 µg/mL) (#15140-122, ThermoFisher Scientific). Cells were harvested in a humidified 95% air 5% CO_2 incubator at 37 °C. Cells medium was replaced with a fresh one or divided with Trypsin-EDTA solution (#T4049, Sigma-Aldrich) every 2-3 days. The cells were maintained in culture and used at appropriate passages for each experiment.

4.2 Cell treatment

To analyze whether Roneparstat (Sigma-Tau Research Switzerland), FGF-2 (#F3685, Sigma-Aldrich) and TGF-β1 (#GF346, Sigma-Aldrich) were able to regulate the expression of EMT markers, MCF-7 were seeded in a 6-well plate in the presence of complete DMEM-HG medium at the confluence of $5*10^5$ cells/well, while MDA-MB-231 were seeded at the same condition but at the confluence of $3,5*10^5$ cells/well. After 24 hours the medium was replaced and the treatment was carried out according to the following scheme for both cell lines:

- control wells (CTR) the cells were incubated in DMEM-HG 1% FBS

- one well treated with Roneparstat at the final concentration of 200 ug/ml in DMEM-HG 1% FBS

- one well treated with FGF-2 at the final concentration of 20 ng/ml in DMEM-HG 1% FBS

- one well co-treated* with Roneparstat (200 ug/ml) and FGF-2 (20 ng/ml) in DMEM-HG 1% FBS

- one well treated with TGF-β at the final concentration of 20 ng/ml in DMEM-HG 1% FBS

- one well co-treated* with Roneparstat (200 ug/ml) and TGF-β (20 ng/ml) in DMEM-HG 1% FBS

* = For the co-treatment, the cells were treated before with Roneparstat and after 2 hours was added the respective growth factor

For gene expression analysis cells were treated for 6, 12 and 24 hours, while for protein expression cells were treated for 24 and 48 hours.

4.3 Kill curve for antibiotic selection

The kill curve for antibiotic selection was performed to determine the optimal antibiotic concentration useful to kill all the cells after 2-7 days. MCF-7 and MDA-MB-231 were seeded in a 12-well plate at an initial concentration of $2,5 \times 10^5$ cells/well and cultured in complete DMEM-HG. The following day, at the confluence of approx. 80%, the medium was replaced with fresh complete DMEM-HG supplemented with a range of antibiotics concentrations (see figure). The range tested for G418 (#5.09227.0001, Merck Millipore) was 0.1 – 2.0 mg/mL, while for Puromycin (#ant-pr, Invivogen) was 0.1 – 5 ug/mL. For the selection, the media was replaced every 48 hours with a fresh Selection media for up to a week, checking every day the confluence of the cell at the microscope. The optimal concentration was determined as the lowest antibiotic concentration at which all cells were dead after the selection period.

4.4 Stable cell lines generation

MCF-7 and MDA-MB-231 cells were plated onto 6-well plates and cultured for 24 hours at a final confluence of 70%. The next day, cells were transfected using Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific) with 2,5 μg of plasmid DNA-true ORF-HPSE (#TR307138, Origene) with G418 resistance or shRNA vector (#RC208556, Origene) with Puromycin resistance. 48 hours after transfection, MCF-7 were selected respectively with 1,4 mg/mL of G418 and 1 μg/mL of puromycin, while MDA-MB-231 with 1,2 mg/mL of G418 and 0,8 μg/mL of puromycin. One point

for transfection and antibiotic as experimental control was also included. After one week of selection, single colonies were picked up and seeded into 48-well plates and at the confluence, cells were expanded in a gradually larger new. Single clones obtained were then screened for their HPSE expression levels, via RT-qPCR (see "RNA extraction and gene expression analysis" for details) and by Western Blot (see "Western immunoblot analysis")

4.5 Crispr-Cas9 gene editing

To create stable knock-out cell lines, we used CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas9 system. This approach allows the production of specific gene-editing essentially through two main components: a guide RNA (gRNA) targeted to the sequence of interest and the bacterially-derived nuclease Cas9. For successful binding of the CRISPR-complex to the DNA the presence of a protospacer adjacent motif (PAM) sequence downstream of the target side is necessary. The system produces a double-strand break that is repaired by the native cellular DNA repair machinery. Today different approaches of CRISPR/Cas9 are commercially available and we have chosen to use the Ribonucleoprotein (RNP) complex.

The concept of this approach is that the synthetic gRNA is coupled with Cas9 conjugated with GFP protein as a reporter (Cas9-GFP protein, #CAS9GFPPRO, Sigma-Aldrich). Thus, we used three different guide RNA with different exon targets of HPSE gene (**Table 2**)

Table 2: HPSE guide sequence (Sigma-Aldrich) for CRISPR

Oligo name	Sequence	Corresponding DNA sequence	DNA localization
Guide A – ID: HSPD0000065339	5' – GUCCUGUGCUUGCGCAGGU	5'– GTCCTGTGCTTGCGCAGGT	5464 - 5482 (Exon 2)
Guide B – ID: HSPD0000065342	5' – GUGGAGGAGAAGUUACGGU	5' – GTGGAGGAGAAGTTACGGT	20717– 20735 (Exon 4)
Guide C – ID: HSPD0000065340	5' – UCUUAGCCGUCUUUCUUCG	5' – TCTTAGCCGTCTTTCTTCG	29404– 29422 (Exon 6)

MCF-7 and MDA-MB-231 have been cultured at standard conditions, at 37 °C and 5 % of CO_2 in a 10 cm^2 well. Each cell line, at a confluence of 70-80%, was transfected separately with the different guides plus control with only Cas9-GFP, using the liposome system X-TremeGENE 360 Transfection Reagent (#08724105001, Roche) at the concentrations indicated by the manufacturer's instructions. After 24 hours, cells were selected by fluorescence-activated cell sorting (FACS) for the GFP signal. The single cells obtained by sorting were plated in 96-multiwell plates. The cells were expanded in a gradually larger new well until the 6-well passage was reached. At this stage, at the right confluence, the RNA was extracted and then analysed the expression level of HPSE mRNA by RT-qPCR. To better understand the putative mRNA modification, we used a specific primer designed upstream and downstream of the sequence of gRNA (the sequences used are in **Table 3**). For the clones that presented any reduction of HPSE gene expression, we quantified HPSE protein levels via Western immunoblot and then the sample with a reduction in protein level was sequenced by Sanger (see "RNA extraction and gene analysis" and "Western immunoblot analyses for more detail)

Table 3: primer used for real time-PCR on mRNA obtained from clones transfected by different each gRNA

Oligo Name	Sequence 5' – 3'
Guide A - HPSE	F: AGGGATGCAGAAGAGGAGTG
	R: ACTAAGAGCCCAGATGCCAC
Guide B - HPSE	F: CCTCAAGTGATTCGCCCATC
	R: GACTTGCTAGATTGGTGCCC
Guide C - HPSE	F: GCCATAGCGCCAGACCTG
	R: TGGGAGGTCGAAGTTGCAG

4.6 Cell Viability Assay

To study cell viability upon Roneparstat, FGF-2 and TGF-β treatment, the XTT Reagent (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) and the Electron Coupling Reagent of CyQUANT XTT cell viability assay (#X12223, ThermoFisher Scientific) was used. Briefly, MCF-7 and MDA-MB-231 were seeded

with an initial concentration of $2*10^3$ cells/well in 3 different 96-well plates (one plate per day). The next day the cells were treated using Roneparstat, FGF-2 and TGF-β respectively at the concentration of 200 μg/ml, 20 ng/ml and 20 ng/ml. The following day, the XTT cell proliferation assay was used at the concentrations indicated by the manufacturer's instructions. After 2-4 hours of incubation, the absorbance of the plate was read at 450 nm (XTT specific absorbance) and 660 nm (used to eliminate background signal for all non-specific absorbance). The proliferation rate was tested at 24, 48 and 72 hours.

4.7 Wound healing assay

A wound healing assay (also known as scratch assay) was performed to determine 2D cell migration in vitro. Cells were plated in a 6-multiwell plate. The next day, at a confluence of 70-80%, using a sterile 200 μL pipet tip, a scratch was made in the monolayer cells. Then, the medium was replaced with fresh DMEM-HG supplemented with 1% FBS, Penicillin (100 U/mL) and Streptomycin (100 μg/mL) to minimize the proliferation component and favor cell migration. HPSE-inhibitor Roneparstat was added at the concentration of 200 μg/mL. After 2 hours, FGF-2 and TGF-β were added at the concentration of 20 ng/mL. Pictures were taken at time 0 immediately after the scratch was made on the monolayer cells, as well as at the 24- and 48-hour time points. Migration was quantified as the difference in migration area between the corresponding time point (T24 or T48) and T0 by using the plug-in function of ImageJ - Wound_Healing_Size_Tool (Suarez-Arnedo et al., 2020). Results are presented as a percentage of wound closure (% of area migration).

4.8 RNA extraction and gene expression analysis

Total RNA was extracted from cells by TRIzol™ Reagent (#15596026, Invitrogen), an RNA isolation kit designed to isolate high-quality total RNA to maintain its integrity. RNA sample yield and purity were quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Life Technologies). 500 ng of total RNA were mixed with 0.5μl dNTPs Mix (#D7295, Sigma-Aldrich), 0.5μl Random Nonamers primers (#R7647, Sigma-Aldrich) and Ambion™ Nuclease-Free Water (#AM9938, Thermo Fischer Scientific) up to a 6μl final volume.

Then, to denature RNA and to allow the primers annealing, the mix was incubated for 10 minutes at 70 °C and immediately cooled on ice. Then, for each sample the following components were added: 0.5μl of Moloney - Murine Leukemia Virus Reverse Transcriptase (M-MLV Reverse Transcriptase #M1427-40KU, Sigma-Aldrich), 1μl 10X M-MLV Reverse Transcriptase Buffer (#B8559-1VL, Sigma-Aldrich) and 2.5μl Ambion™ Nuclease-Free Water. The final 10μl reaction mix was incubated at room temperature for 10 minutes, at 37 °C for 50 minutes, and at 85°C for 10 minutes. Amplification cycling was performed using a DNA Thermal Cycler (Perkin Elmer Cetus). The cDNA obtained was diluted with Ambion™ Nuclease-Free Water at a final concentration of 4 ng/μL and stored at -20°C until use.

Then, the cDNA was analyzed by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) on a StepOne™ Real-Time PCR System (Thermo Fisher Scientific, Life technologies). Each reaction mix was composed: of 5 μL of SensiFAST SYBR Hi-ROX (#BIO-92020, Meridian Biosciences®), 0,8 μLx2 of forward and reverse (final concentration of 5 μM), 2 μL of cDNA template and Ambion™ Nuclease-Free Water for a final volume of 10 μL. The forward and reverse primer sequences for each were reported in **Table 4**.

Table 4: primers used for real-time PCR on cell extract

Gene	Primer Sequence	Product lenght
E-Cadherin (E-Cad)	F: TTCTGCTGCTCTTGCTGTTT, R: TGGCTCAAGTCAAAGTCCTG;	142
Fibronectin (FN)	F: GTGTGTTGGGAATGGTCGTG, R-GACGCTTGTGGAATGTGTCG;	113
GAPDH	F: ACACCCACTCCTCCACCTTT R: TCCACCACCCTGTTGCTGTA;	112
HPSE	F: ATTTGAATGGACGGACTGC R: GTTTCTCCTAACCAGACCTTC;	136
SDC-1	F: GAAGATCAAGATGGCTCTGGG R: GTTCTGGAGACGTGGGAATAG;	145
SLUG	F: GTTCGTAAAGGAGCCGGGTGA, R: ACACGGCGGTCCCTACAGCAT;	111
SNAI1	F: AGTTTACCTTCCAGCAGCCCTAC, R: AGCCTTTCCCACTGTCCTCATC;	116

| Vimentin (VIM) | F: AAAACACCCTGCAATCTTTCAGA, | 75 |
| | R: CACTTTGCGTTCAAGGTCAAGAC; | |

Each reaction mix was processed using the following PCR conditions: polymerase activation at 95°C for 2 min followed by 40 cycles of 95°C for 15 sec (denaturation) and 60-62°C for 30 sec (combined annealing/extension). The presence of non-specific amplification and primer dimerization products was excluded by melting curve analysis. The expression of target genes was normalized to the expression levels of the GAPDH reference gene and relative normalized expression was calculated based on the ΔΔCt method.

4.9 Western immunoblot analysis

For protein expression analysis cells were washed with ice-cold PBS (#D5652, Sigma-Aldrich) and were lysed in RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% Sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100) with cOmplete™ Mini Protease Inhibitor Cocktails (#11836153001, Roche). Lysates were collected using a cell scraper, transferred in a 1,5 mL tube maintained in ice and then centrifuged for 30 minutes at 12000g at 4°C. Supernatants were collected and transferred into a new tube. Protein concentration was measured by Pierce™ BCA Protein Assay kit (#23227, Thermo Fisher Scientific) according to the manufacturer's instructions and frozen at -80°C until use. Equal amounts of protein samples were prepared in reducing Laemmli loading buffer 4X (0,25 M Tris-HCl pH 6.8, 0,28 M SDS, 40% glycerol, 20% β-mercaptoethanol, 1% Bromophenol blue) and denatured for 10 min at 100 °C. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10-12% acrylamide gel at 50 mA in running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS), followed by electro-transfer to a 0.45μm nitrocellulose membrane at 350 mA in transfer buffer (25mM Tris, 190mM glycine, 20% methanol) for 1 hours. Membranes were blocked with 3% bovine serum albumin (#10735086001, Sigma-Aldrich) in Tris-buffered saline with 0.1% Tween-20 for 2 hours and incubated overnight with primary antibody in antibody buffer (1.5% in Tris-buffered saline with 0.1% Tween-20). Subsequently, the membranes were washed 3 times for 10 minutes each with Tris-buffered saline with 0.1% Tween-20 followed by incubation with anti-

horseradish peroxidase-conjugated specific secondary antibodies for 90 minutes at room temperature. Primary and secondary antibody dilution are reported in **Table 5**. After 3 washes of 10 minutes with Tris-buffered saline with 0.1% Tween-20, the antibody/substrate complex was visualized by chemiluminescence using a home-made ECL and signal detection was performed using NineAlliance software (Uvitec, Cambridge). For densitometry analysis, ImageJ software was used. β-Actin or GAPDH were used as the protein loading control.

Table 5: Primary and secondary antibodies used for Western Blot on cell extract

	Target	Company	Host species	Dilution
Primary antibody	Fibronectin	Santa Cruz (sc-9068)	Rabbit	1:1000
	E-Cadherin	GeneTex (GTX-100443)	Rabbit	1:1000
	Vimentin	Santa Cruz (sc-7557)	Goat	1:1000
	HPSE	HP/M17	Mouse	1:1000
	β-Actin	Santa Cruz (sc-1616)	Goat	1:2000
	GAPDH	Santa Cruz (sc-47724)	Mouse	1:2000
Secondary antibody	anti-rabbit IgG HRP	Santa Cruz	goat	1:1000
	anti-mouse IgG HRP	Santa Cruz	goat	1:1000
	anti-goat IgG HRP	Santa Cruz	donkey	1:1000

4.10 Statistical analysis

All experiments were repeated at least three times in duplicates, if not differently indicated. Data were reported as means ± s.e.m. (standard error of the mean). Statistical analysis of data for comparison between two distributions was performed with the unpaired two-tailed t-test (GraphPad Prism). For experiments with more than two conditions, data were tested employing the one-way or two-way Analysis of Variances (ANOVA) test (GraphPad Prism). p-values < 0.05 were considered statistically significant.

5. RESULTS

5.1 Breast cancer cell lines characterization for EMT markers and HPSE expression

To investigate the role of HPSE in breast cancer, we choose two breast cancer cell lines expressing different prognostic markers and characterized by dissimilar metastatic potential. MCF-7 is catalogued as a luminal-A cell type (ER^+ and/or PR^+, HER2 low) with a marked epithelial phenotype exhibiting a cobblestone morphology. Otherwise, MDA-MB-231 belonging to the triple negative subgroup (ER^-, PR^-, $HER2^-$) show a mesenchymal-like phenotype appearing as elongated and spindle-shaped cells. Starting from this evidence, we decide to characterize EMT markers and HPSE expression. We choose Fibronectin (FN) and Vimentin (VIM) as mesenchymal markers, while E-Cadherin (E-CAD) as epithelial marker. The results showed that MCF-7 express higher gene and protein levels of E-CAD than MDA-MB-231. On the other hand, MDA-MB-231 expresses more FN and VIM compared to the MCF-7. In addition, MDA-MB-231 show two-fold higher levels of HPSE both at gene and protein expression compared to MCF-7 (**Fig. 13**)
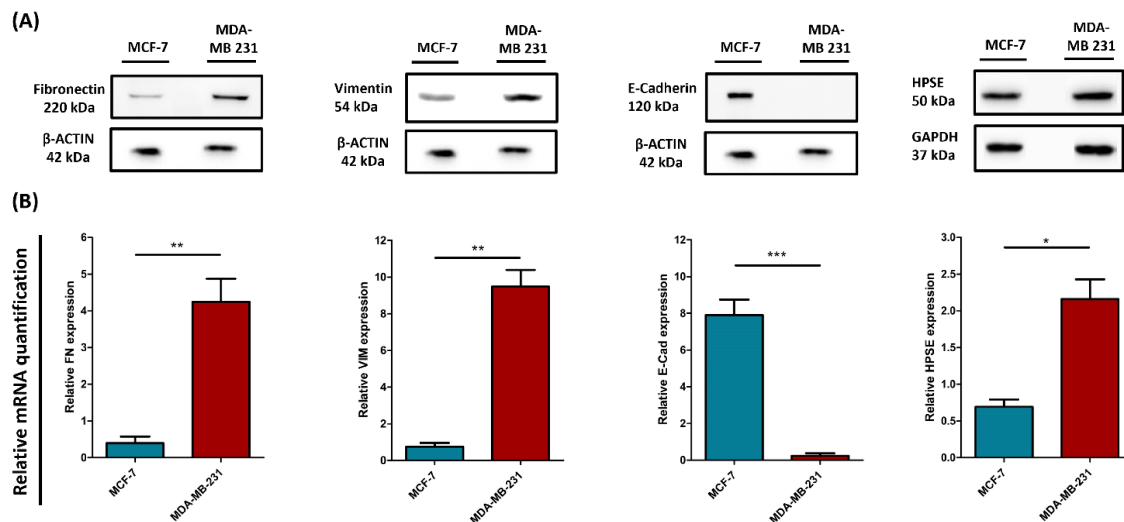
Figure 13 - EMT-related markers and HPSE expression in MCF-7 and MDA-MB-231 cell lines. The panel (**A**) shows the differences in protein expression evaluated by Western blot, while the graphs in panel (**B**) show the relative mRNA expression analysed by real-time PCR of Fibronectin, Vimentin, E-Cadherin and Heparanase in the two cell lines. Bars represent the mean ± standard deviation (SD); n = 6. * p = 0,05, ** p = 0,001, *** p = 0.0001.

5.2 HPSE inhibition influences EMT markers expression

To investigate the potential role of HPSE as a modulator of EMT, we planned to treat the cell with the specific inhibitor of HPSE: Roneparstat (SST0001) (Pala et al., 2016). In addition, FGF-2 and TGF-β1 are two well-known EMT activators (Masola et al., 2014; Suh et al., 2020). We also tested their effect alone or in combination with Roneparstat in MCF-7 and MDA-MB-231. To evaluate the activation of the EMT process we considered the expression of SNAI1 and SLUG, two EMT-associated transcription factors, but also Fibronectin (FN) and Vimentin (VIM) as mesenchymal markers, E-Cadherin (E-CAD) as epithelial marker. Gene expression was analysed at 6 and 12 hours, while protein expression was at 24 hours.

In MCF-7, gene expression analyses indicated that the treatment with Roneparstat alone did not affect the expression of EMT markers, except for SLUG expression at 12 hours (**Fig. 14A, 14B**). On the other hand, the treatment with FGF-2 and TGF-β significantly increased the expression of SNAI1 and SLUG at 6 and 12 hours, while the co-treatment with Roneparstat significantly reduced this augmentation (**Fig. 14A, 14B**). Also, FN expression was significantly increased after the treatment with both FGF-2 and TGF-β, with Roneparstat that significantly reduced this effect both at 6 and 12 hours (**Fig. 14C**). TGF-β, but not FGF-2, was able to induce a significant increase of VIM expression (**Fig. 14 D**) and the treatment with Roneparstat reported its expression at basal levels. The treatment with FGF-2 produced a slight reduction in E-CAD expression which was thwarted by Roneparstat (**Fig. 14 E**). Protein analysis confirms only partially the difference in gene expression. Roneparstat alone was able to interfere with the expression of VIM and E-CAD, but not with FN (**Fig. 14F, 14G, 14H**). FGF-2, but not TGF-β, was able to significantly increase the expression of FN, but the treatment with Roneparstat did not interfere with this increase (**Fig. 14F**). VIM expression was significantly increased both with FGF-2 and TGF-β, but the co-treatment with Roneparstat had a significant reduction only for TGF-β (**Fig. 14G**). The expression of E-CAD was significantly reduced only by TGF-β and Roneparstat modulated that reduction (**Fig.14H**).
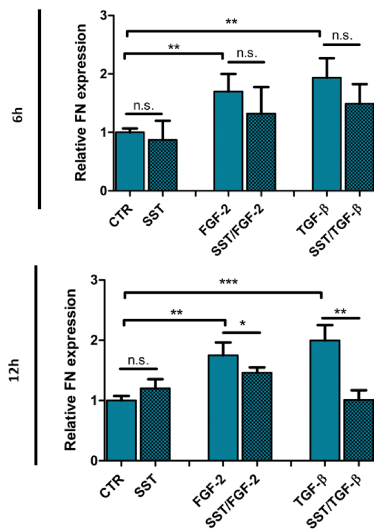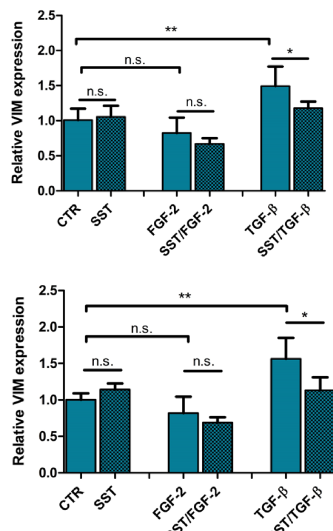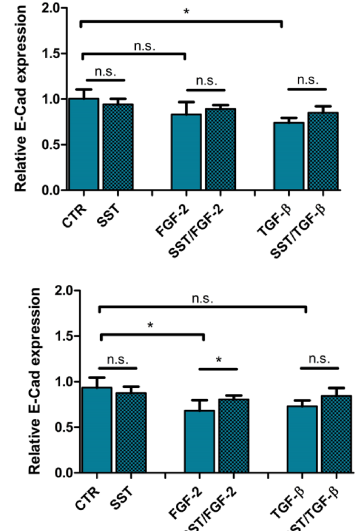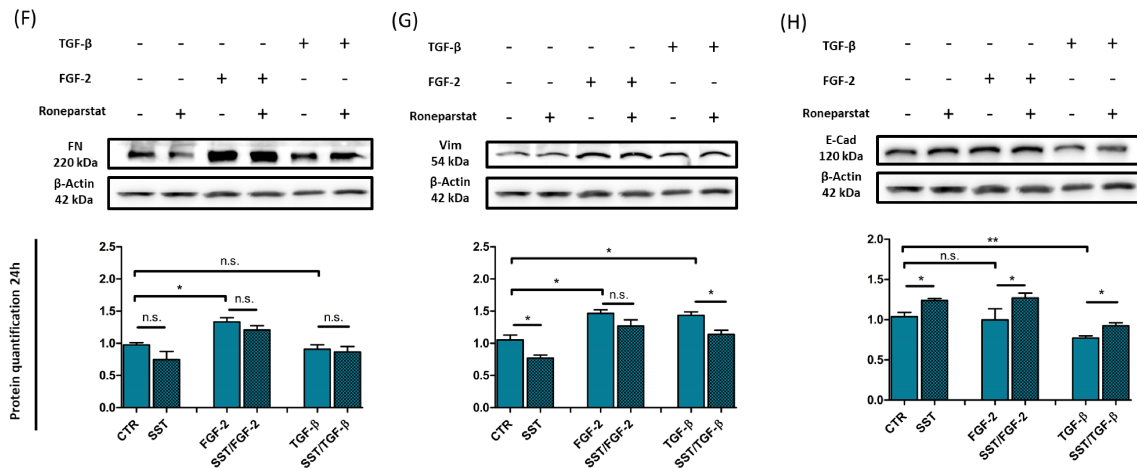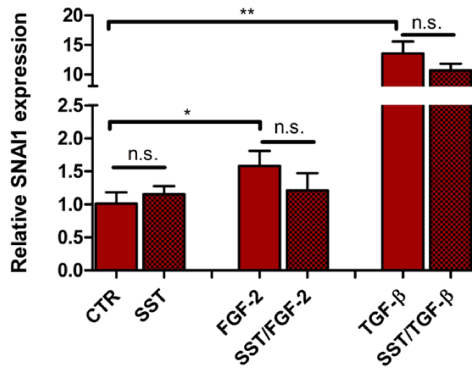
MCF-7
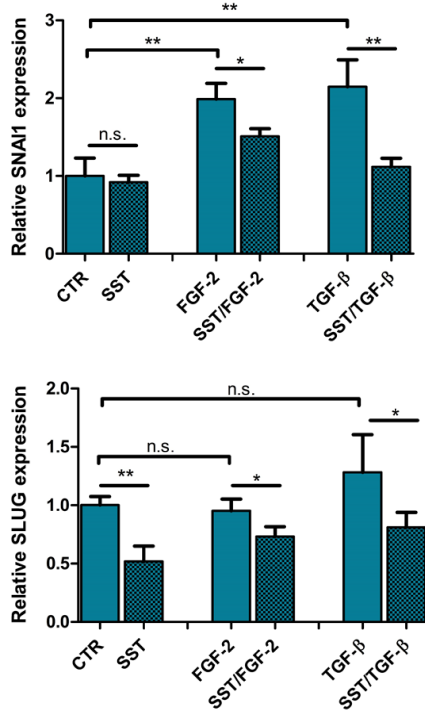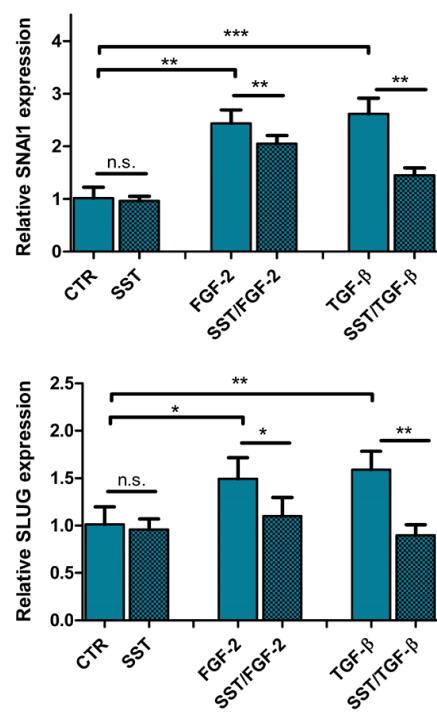
Figure 14 – Evaluation of EMT markers in MCF-7 cells by Real-Time qPCR and Western Blotting analysis. Gene and protein expressions at different time points of EMT-marker after treatment with or without Roneparstat (SST0001) (200 ug/mL), FGF-2 (20 ng/mL) and TGF-β (20 ng/mL). Snai1 (A), Slug (B), FN (C), Vim (D) and E-Cad (E) gene expression was evaluated by real-time PCR at 6 and 12 hours. Bars represent the mean ± standard deviation (SD); n = 6. Gene expression was normalized to GAPDH as the housekeeping gene. * $p \leq 0.05$; ** $p \leq 0.001$, *** $p \leq 0.0001$ vs. CTR. FN (F), Vim (G) and E-Cad (H) protein levels were evaluated by WB at 24 hours and normalized to β-Actin as housekeeping. Bars represent the mean ± standard deviation (SD); n = 3, * $p \leq 0.05$; ** $p \leq 0.001$

Moving to MDA-MB 231, the treatment with Roneparstat was able to reduce significantly SNAI1 and SLUG gene expression at the basal level at 12 hours. The treatment with FGF-2 and TGF-β both at 6 and 12 hours induced an increase of these two transcription factors. The contemporary treatment with Roneparstat inhibitor was able to reduce the increase of their expression in all the conditions analyzed (**Fig. 15A, 15B**). On the contrary, FN, VIM and E-CAD gene expression was not affected by Roneparstat treatment alone (**Fig. 15C, 15D, 15E**). FN gene expression was increased by TGF-β at 6 as well as at 12 hours, while FGF-2 action was detected only at 12 hours. HPSE-inhibition reduced all these increases (**Fig. 15C**). VIM gene expression was not affected at 6 hours, while was increased at 12 hours by TGF-β and modulated with the co-treatment with Roneparstat (**Fig. 15D**). Moreover, E-CAD gene expression was reduced with both FGF-2 and TGF-β at 12 hours, but Roneparstat did not affect this effect (**Fig. 15E**). Again, protein expression did not follow the gene expression pattern. Protein expression at 24 hours highlighted any effect related to Roneparstat or the co-treatment with FGF-2 and TGF-β (**Fig. 15F, 15G, 15H**).

MDA-MB-231

(A) Relative mRNA expression 6h

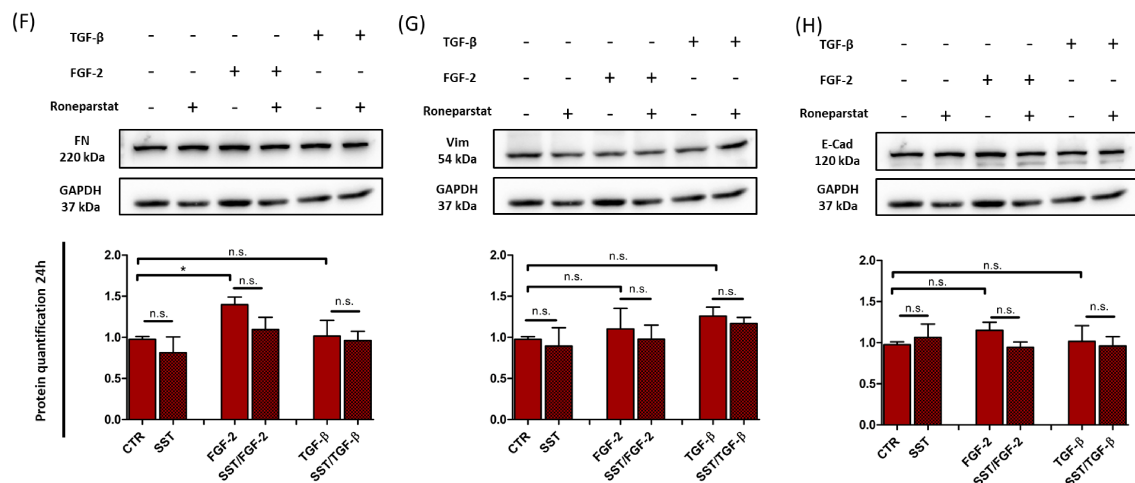(B) Relative mRNA expression 12h

(C)   (D)   (E)
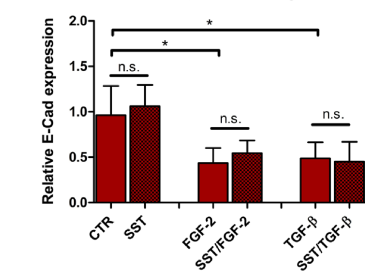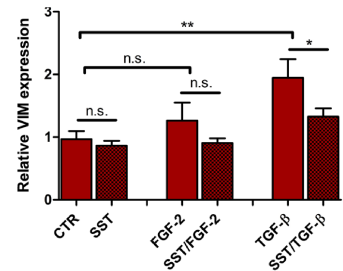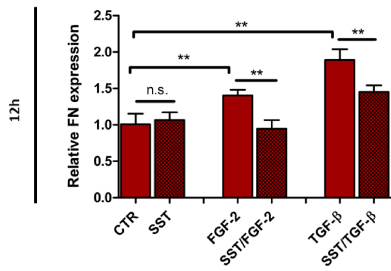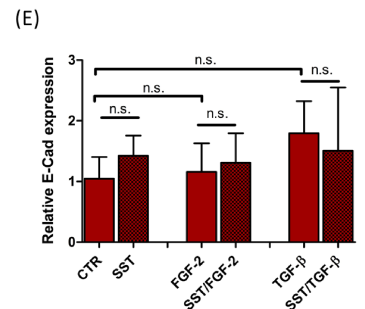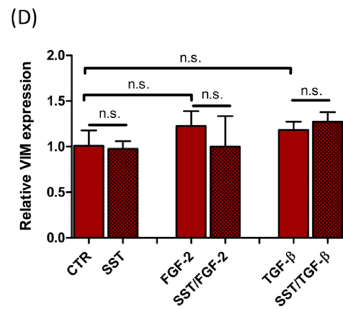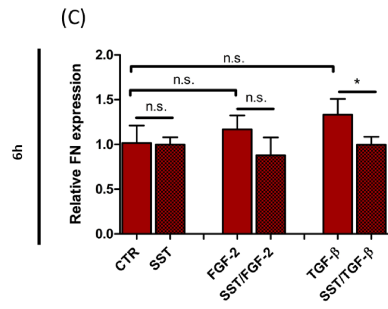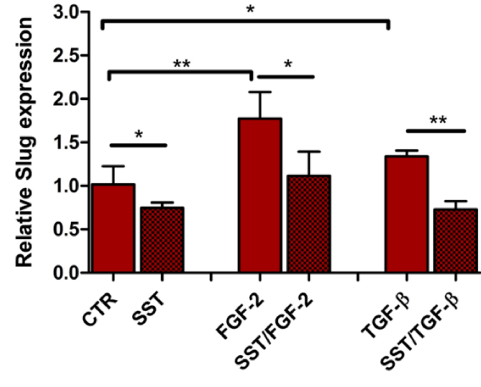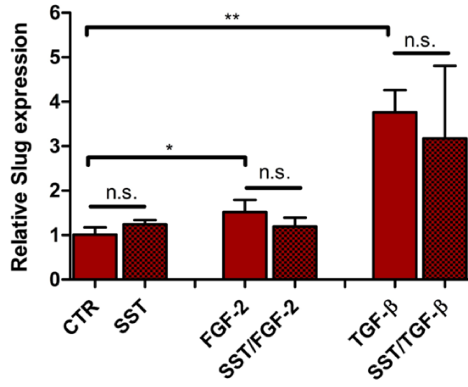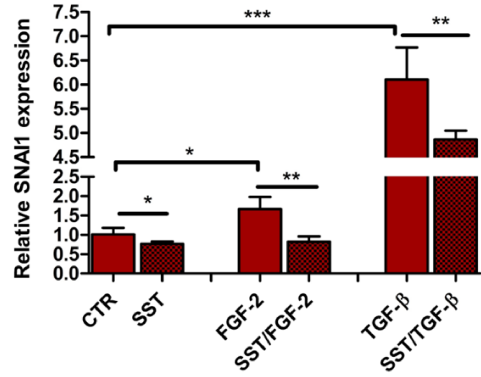
Figure 15 – Evaluation of EMT markers in MDA-MB 231 cells by real-time qPCR analysis and Western Blotting analysis. Gene and protein expressions of EMT-marker after treatment with or without Roneparstat (SST0001) (200 ug/mL), FGF-2 (20 ng/mL) and TGF-β (20 ng/mL). Snail (A), Slug (B), FN (C), Vim (D) and E-Cad (E) gene expression was evaluated by real-time PCR at 6 and 12 hours. Bars represent the mean ± standard deviation (SD); n = 6. The gene expression was normalized to GAPDH as the housekeeping gene. * $p \leq 0.05$; ** $p \leq 0.001$, *** $p \leq 0.0001$ vs. CTR. FN (C), Vim (D) and E-Cad (E) protein levels were evaluated by WB at 24 hours and normalized to β-Actin as housekeeping. Bars represent the mean ± standard deviation (SD); n = 3; * $p \leq 0.05$; ** $p \leq 0.001$

5.3 Inhibition of HPSE mitigate cell migration

A wound healing assay was used to evaluate the migration capacity of the cells. To do this, it is important to evaluate the best migration conditions for the cell type considered. For migration, it is important to set one condition that induces cell movement avoiding or reducing the proliferation rate acting on the medium formulation. In order to do this, we analyze the growth during the time of our cells at different time points with different serum concentrations to find the lower FBS concentration to reduce the proliferation in favor of migration (Tremel et al., 2009). The image shows that the MCF-7 cells, with reduced FBS concentrations, changed their morphology but seem to be alive at any time point considered. On the other hand, the MDA-MB-231 suffered in FBS-free and FBS-low a time-dependent condition. These results suggested that the best migration condition for MCF-7 and MDA-MB-231 was the complete medium supplemented with 1% FBS for 24 hours (**Fig. 16A, 16B**).
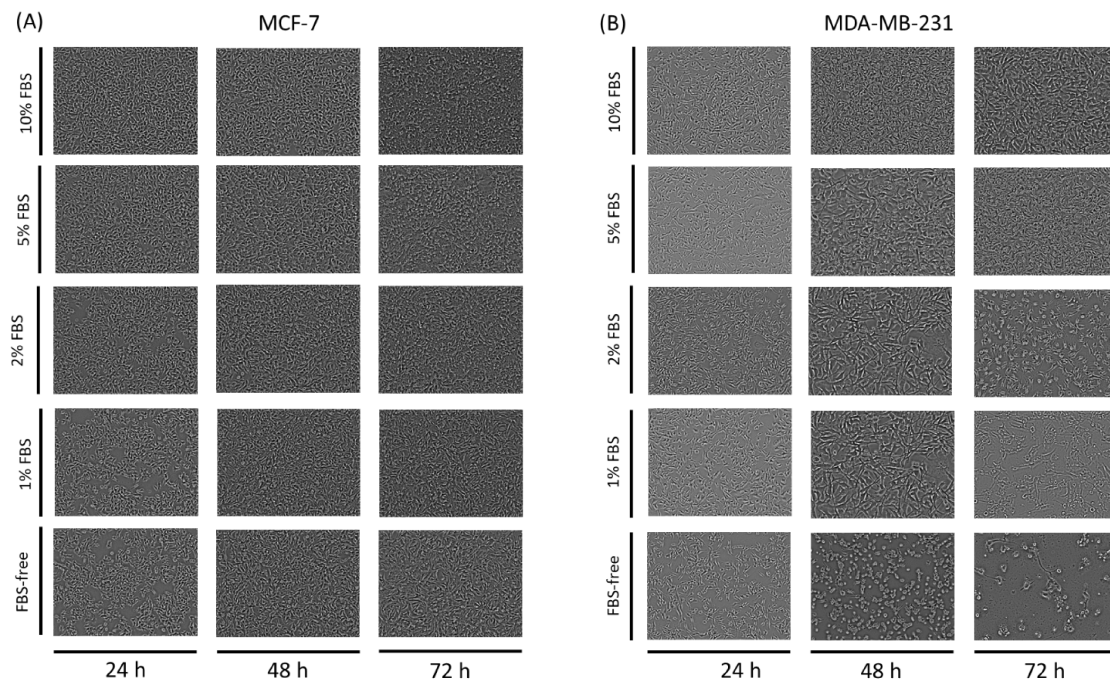
Figure 16 – Representation of growth condition for wound healing assay. MCF-7 (A) and MDA-MB-231 (B) picture at different time points and different serum concentrations. The images were taken at 10x and 20x magnification.

For the wound healing assay, the pictures were taken at time 0 immediately after the scratch was created in the monolayer cells, as well as after 24 hours. Migration was quantified as the difference in migration area between T24 and T0 (**Fig. 17A**).

In MCF-7 cells, Roneparstat did not affect the migration capacity of the cells. FGF-2 was able to promote a significant increase in migration and when Roneparstat was added, this effect was significantly reduced. TGF-β and Roneparstat did not modify the migration of MCF-7 cells (**Fig. 17B**).

In MDA-MB 231, HPSE inhibition significantly reduced the basal migration of the cells. Moreover, treatment with FGF-2 did not affect migration, whereas TGF-β reduced the migration of these cells (**Fig. 17C**).
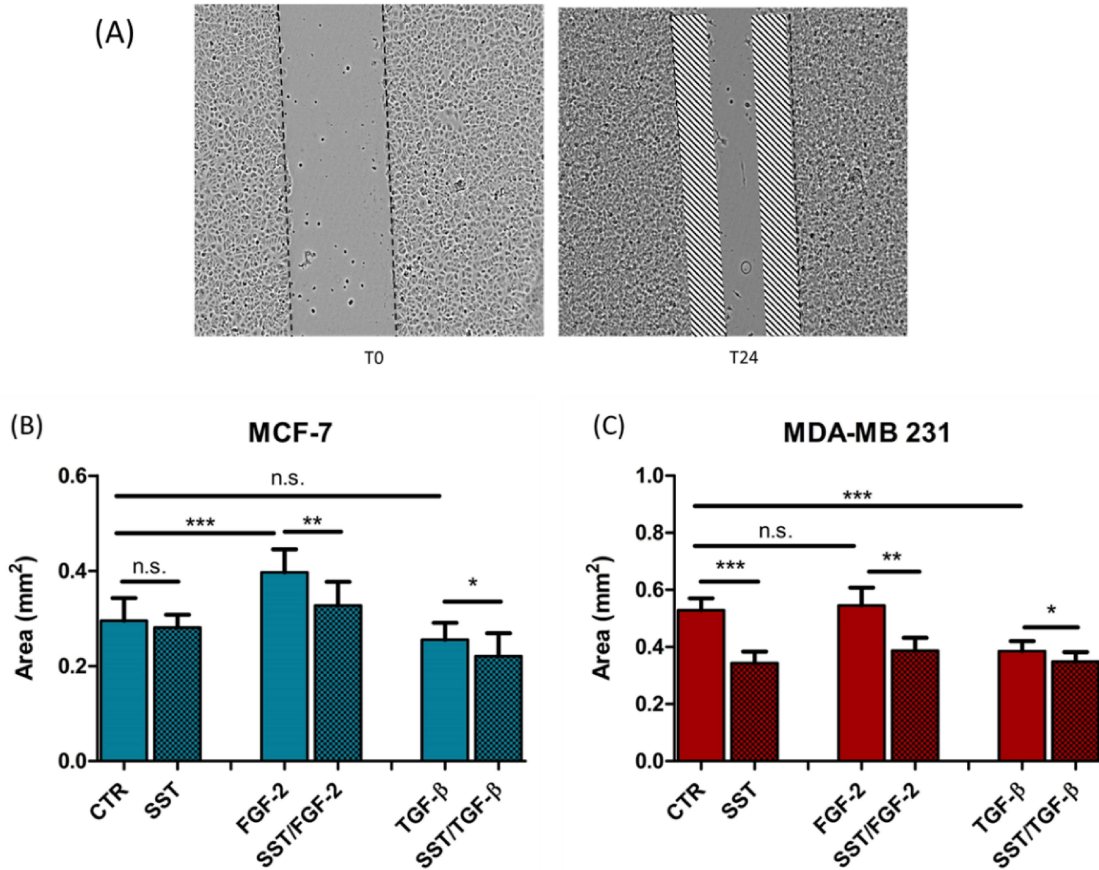
Figure 17 – Wound Healing Assay. (A), representation of wound healing assay and area quantification after the scar. MCF-7 (B) and MDA-MB-231 (C) quantification of migration capacity after the treatment with or without Roneparstat (SST0001) (200 ug/mL), FGF-2 (20 ng/mL) and TGF-β (20 ng/mL). Bars represent the mean ± standard deviation (SD); n = 8. * $p = 0.05$; ** $p = 0.001$, *** $p = 0.001$

In addition, to make sure that the experimental conditions used for the wound healing assay did not influence the viability of the cells, we performed also a viability test. In MCF-7 and MDA-MB-231 the treatment with Roneparstat, FGF-2, TGF-β or the combination of them, had no significant effect on cell viability (**Fig. 18A, 18B, 18C, 18D**).
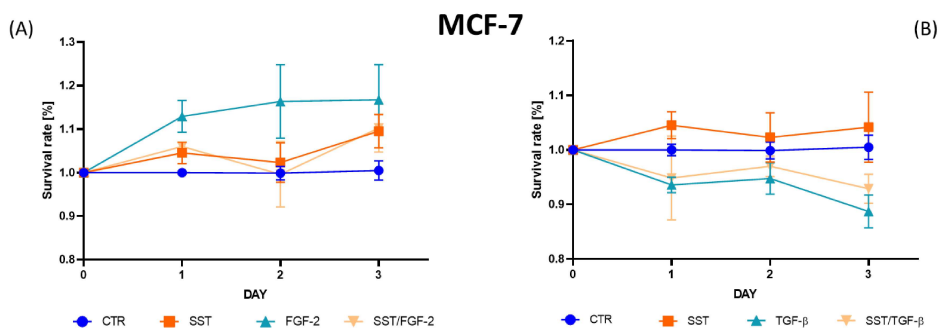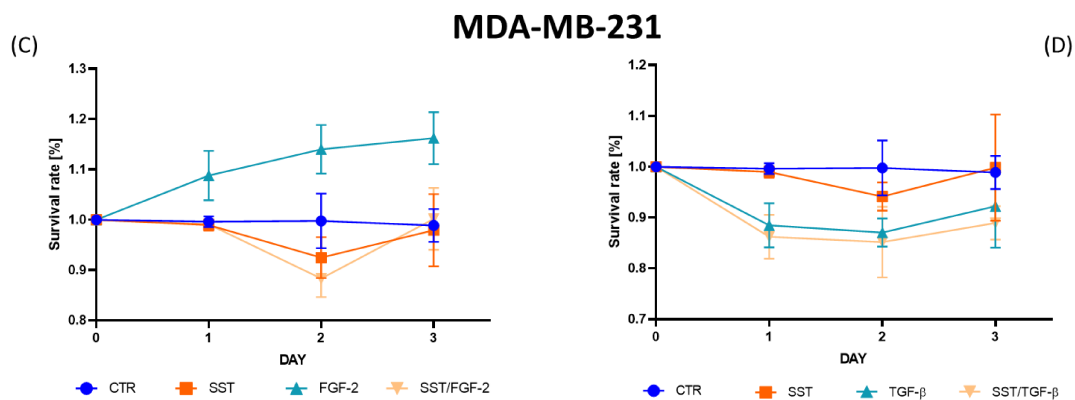
Figure 18 – Cell viability assay on MCF-7 and MDA-MB-231. Cell viability assay after the treatment with or without Roneparstat (SST0001) (200 ug/mL), FGF-2 (20 ng/mL) and TGF-β (20 ng/mL) in MCF-7 (A, B) and MDA-MB-231 (C, D).

5.4 HPSE Ko-cell lines by CRISPR/Cas9

HPSE enzymatic function is well-described, but this enzyme exhibits also a non-enzymatic activity that is poorly described. The non-enzymatic activity is based on the migration into the nucleus of the enzyme that can interact directly with DNA or influence the activity of many enzymes, especially HAT (Purushothaman et al., 2011). Roneparstat or other inhibitors produced can inhibit only the enzymatic activity of the enzyme. So, to study the influence of non-enzymatic HPSE in breast cancer, we planned to produce knock-out cell lines using the CRISPR/Cas9 technique. The ribonucleoproteins (RNPs), constituted by Cas9-GFP complexed with three different sgRNA target HPSE, were transfected in MCF-7 and MDA-MB-231 cells by liposome particle (**Fig. 19A**). The sgRNA sequence are showed in **Fig.19B**. After 24 h, the single cell solution of transfected cells expressing the GFP were isolated by FACS followed by an expansion period to establish a new clonal cell line (**Fig. 19C, 19D**).

(A)

After 24 h
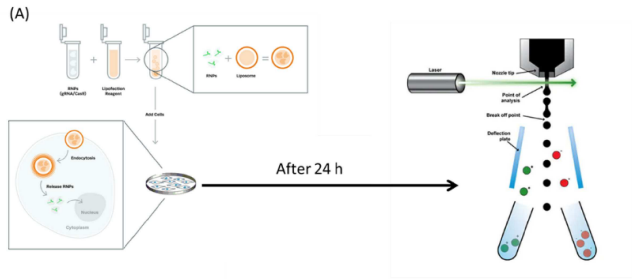
(B) **HPSE guide sequence**

Guide A: 5' – GUCCUGUGCUUGCGCAGGU
Corresponding DNA sequence: 5' – GTCCTGTGCTTGCGCAGGT
DNA localization: 5464 - 5482 (Exon 2)

Guide B: 5' – GUGGAGGAGAAGUUACGGU
Corresponding DNA sequence: 5' – GTGGAGGAGAAGTTACGGT
DNA localization: 20717 – 20735 (Exon 4)

Guide C: 5' – UCUUAGCCGUCUUUCUUCG
Corresponding DNA sequence: 5' – TCTTAGCCGTCTTTCTTCG
DNA localization: 29404 – 29422 (Exon 6)

(C)

Single cell Sorting MCF-7

(D)
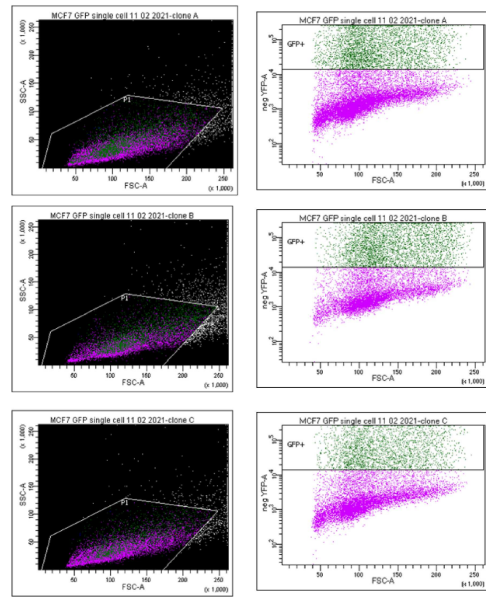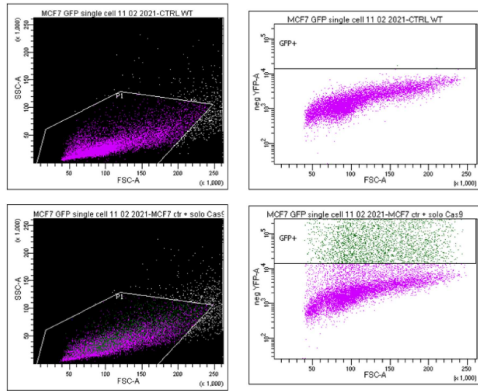
Single cell Sorting MDA-MB 231
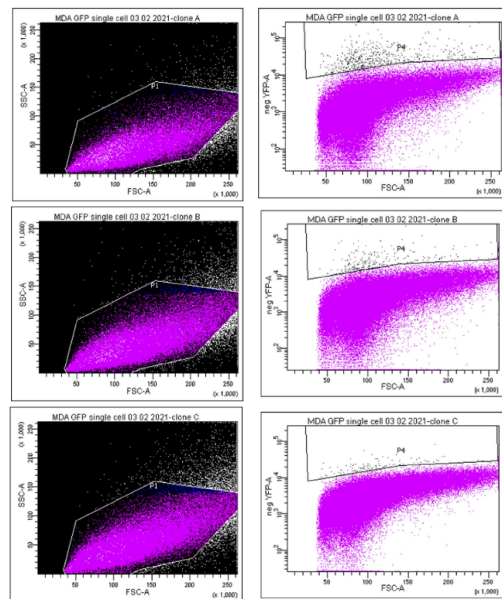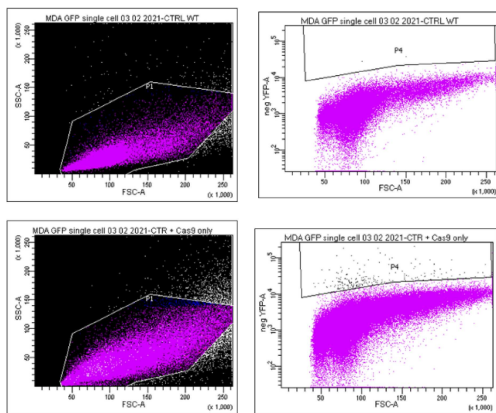
Figure 19 – CRISPR/Cas9 and single cell sorting. (A) show a schematic representation of the CRISPR/Cas9 approach used. In (B) were listed the three sgRNA sequences and the genome target. MCF-7 (C) and MDA-MB-231 (D) expressing GFP signal were isolated. The images showed the cytofluorimetric representation of the cells and the GFP-number of positive events (green dots) considering a negative control (no transfected cell lines) and positive control (cells transfected with the Cas9 alone for GFP signal).

It is noteworthy that different cell types can vary substantially in their responses to single-cell isolation, due to their phenotype and behaviour. To check the success of gene editing, gene expression analyses were performed using a specific primer for the amplification of the specific target region. **Fig. 20** shows the distribution of the HPSE gene expression analyses for all the clones obtained for each cell line. We obtained that only 33% of MCF-7 and 10% of MDA-MB-231 showed a consistent reduction of HPSE (**Fig. 20**). Among these clones, we selected some of them to analyse the protein expression to confirm the reduction also in protein levels. The clones that showed also a reduction in protein levels were selected for DNA sequencing. Unfortunately, the sequencing didn't confirm the genome modification.
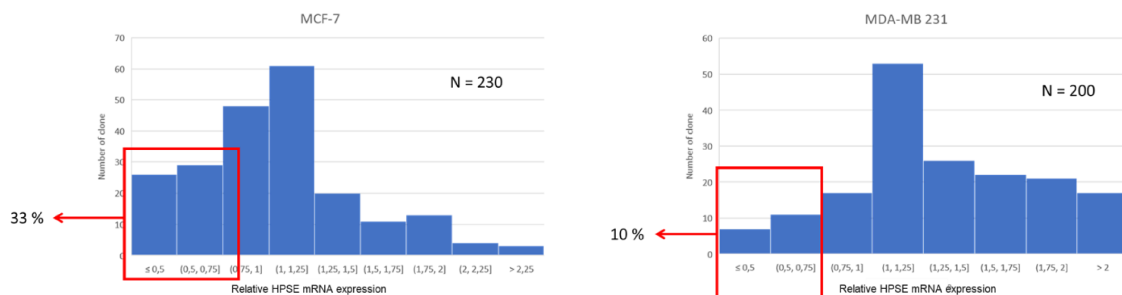
Figure 20 – Distribution of relative HPSE mRNA of MCF-7 and MDA-MB-231. The two graphs show the expression of HPSE in the clones analyzed by real-time qPCR. Only the clone with a significant reduction of HPSE expression compared with polyclonal cell lines (considered as CTR) was then analyzed by DNA sequencing.

5.5 Generation of stable HPSE overexpressing or silenced cell lines

To investigate how the HPSE levels influence the expression of EMT markers in breast cancer, the shRNA approach and the expression of the open reading frame (ORF) of HPSE were used as alternative strategies. Due to the differential expression of HPSE between the MCF-7 and MDA-MB-231, we decide to overexpress HPSE in MCF-7 and to silence and overexpress HPSE in MDA-MB-231. We use a liposome system carries the anti-HPSE shRNA lentiviral vector with puromycin resistance or the plasmid DNA true ORF- HPSE with G418 resistance. After the selection period with puromycin and G418 (according to the dose/death assay), gene and protein expression were analysed to confirm the results obtained. In MCF-7, results confirmed a significant upregulation at gene and protein levels confirmed by real-time PCR and WB compared to control cells (CTR) (**Fig. 21A, 21B**).
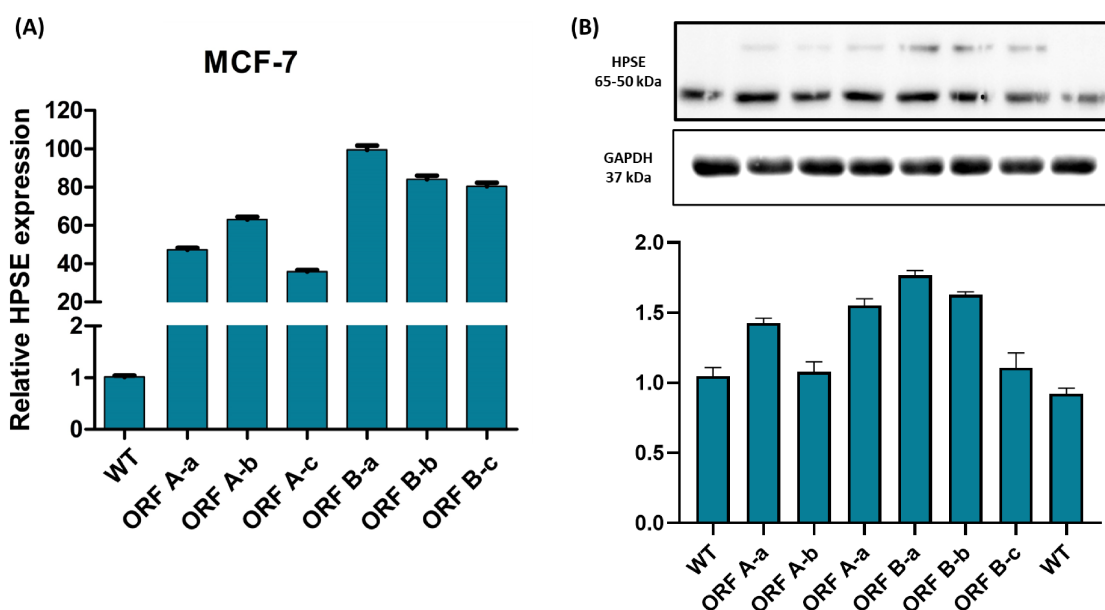
Figure 21 – Validation of HPSE overexpression in MCF-7 cell line. HPSE gene expression was evaluated by real-time PCR on different clones of MCF-7 cells **(A)**. Data were normalized to GAPDH expression. Means ± SD (error bars), n=4. WB analysis of HPSE in MCF-7 clones and relative quantification normalized to GAPDH **(B)**.

Moreover, gene and protein expression analyses of HPSE in MDA-MB-231 confirm that the cells transfected with the shHPSE system confirm a significant reduction, while a moderate upregulation in ORF transfected cells was found (**Fig. 22A, 22B**).
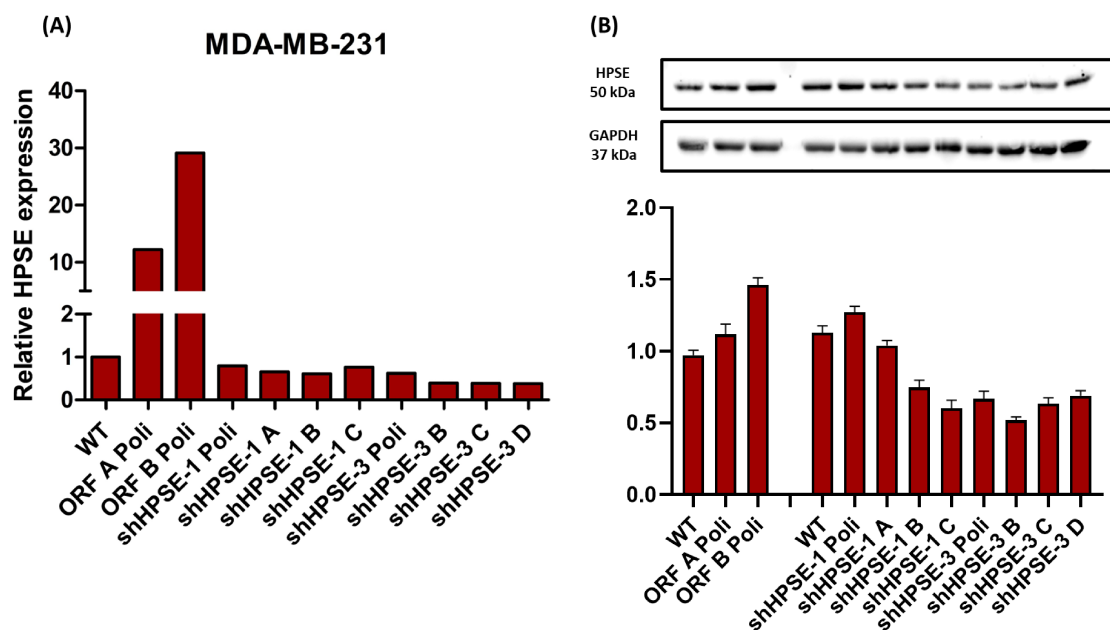
Figure 22 – Validation of HPSE overexpression and silencing in MDA-MB-231. HPSE gene expression was evaluated by real-time PCR on different clones of MCF-7 cells. Data were normalized to GAPDH expression **(A)**. WB analysis of HPSE in MCF-7 clones and relative quantification normalized to GAPDH **(B)**.

Due to the data obtained, we chose for the following experiments only the clones that expressed the higher or lower HPSE levels both in gene and protein expression. So, for MCF-7 we selected the clone ORF B-a, while for MDA-MB-231 the clone ORF-B Poli and sh-3D.

5.5.1 HPSE overexpression regulates EMT markers in MCF-7

In order to investigate whether HPSE was able to modulate EMT, the expression levels of the EMT markers were measured in MCF-7 WT compared to the overexpressing cells. In addition, we also evaluated the response after the inhibition of HPSE by Roneparstat (200 ug/mL) alone or in the presence of different concentrations of FGF-2 (10 ng/mL or 20 ng/mL). Due to the previous results, we planned to analyse EMT markers in gene expression at 24 hours and protein expression at 48 hours. Gene expression analysis after the treatment with Roneparstat, showed a reduction in the expression of HPSE in MCF-7 WT and MCF-7 overexpressing HPSE. In addition, FGF-2 was able to increase the expression of HPSE both in WT and overexpressing cells, but the co-treatment with Roneparstat was able to reduce this augmentation (**Fig.**

23A). HPSE influences many functions of SDC-1 and due its strict interplay, we also want to test the expression of SDC-1 in our cell model. Gene expression confirms that the SDC-1 was significantly upregulated in MCF-7 overexpressing HPSE. Furthermore, FGF-2 treatment increased the gene expression of SDC-1 which was reduced by the co-treatment with Roneparstat (**Fig. 23B**).

Figure 23 – HPSE and SDC-1 expression in MCF-7. HPSE (A) and SDC-1 (B) gene expression were evaluated by real-time PCR on different clones of MCF-7 WT and HPSE overexpressing cells at 24h after treatment with Roneparstat (SST0001) (200 ug/mL), FGF-2 (10 ng/mL) and FGF-2 (20 ng/mL) or in combination. Bars represent the mean $\pm$ standard deviation (SD); n = 6. The gene expression was normalized to GAPDH as the housekeeping gene. * $p \leq 0.05$; ** $p \leq 0.001$, *** $p \leq 0.0001$ vs. CTR.

We also evaluated the EMT-markers expression. The mesenchymal and epithelial markers considered, were respectively upregulated and downregulated in HPSE overexpressing cells (**Fig. 24**). The transcription factors SNAI1 and SLUG were upregulated after treatment with FGF-2. The inhibition of HPSE by Roneparstat treatment alone or in the presence of FGF-2 was able to reduce the increase, confirmed by real-time PCR (**Fig. 24A, 24B**). In addition, gene and protein expression of the epithelial marker E-Cad was downregulated in HPSE overexpressing compared with WT cells, confirmed by real-time and WB (**Fig. 24C, 24D**). By contrast, gene expression suggested Roneparstat and/or FGF-2 effects, that was marked in MCF-7 overexpressing cells, but WB analyses did not confirm any significant change (**Fig. 24D**). On the other hand, the mesenchymal marker FN was upregulated, confirmed at gene expression by real-time PCR and at protein level with WB (**Fig. 24E, 24F**).

FGF-2 induce an increase of FN in both the cell lines. Roneparstat alone did not influence the expression of FN, while the contemporary administration reduced the FGF-2 activation, analysed by real-time PCR and only partially confirmed in WB (**Fig. 24E, 24F**).

Figure 24 – Evaluation of EMT markers in MCF-7 cells by Real-Time qPCR and Western Blotting analysis. Gene and protein expressions show the different expression of EMT-marker in MCF-7 and HPSE overexpressing cells after treatment with or without Roneparstat (SST0001) (200 ug/mL), FGF-2 (10 ng/mL) and FGF-2 (20 ng/mL). Snail (A), Slug (B), E-Cad (C) and FN (E) gene expression were evaluated by real-time PCR at 24 hours. Bars represent the mean ± standard deviation (SD); n = 6. The gene expression was normalized to GAPDH as the housekeeping gene. $* p \leq 0.05$; $** p \leq 0.001$, $*** p \leq 0.0001$ vs. CTR. E-Cad (D) and FN (F) protein levels were evaluated by WB at 48 hours and normalized to GAPDH as housekeeping. Bars represent the mean ± standard deviation (SD); n = 3, $* p \leq 0.05$; $** p \leq 0.001$

5.5.2 HPSE expression in MDA-MB-231 and EMT markers

We investigated the role of HPSE and the relative expression of EMT markers in MDA-MB-231 WT compared to the overexpressing or silenced cells. Again, we evaluated the response to treatment with Roneparstat (200 ug/mL) alone or in combination with FGF-2 (10 ng/mL). Due to the previous results, we planned to analyse EMT markers in gene expression at 24 hours, and protein expression at 48 hours. Similar to the MCF-7, gene expression confirms that the expression of SDC-1 depends on the expression of HPSE. Indeed, the overexpression of HPSE in MDA-MB-231 promoted an upregulation in SDC-1 expression, while in cells silenced for HPSE, the expression of SDC-1 was downregulated (**Fig. 25A, 25B**). Furthermore, FGF-2 treatment increased the gene expression of SDC-1 in MDA-MB-231 WT and overexpressing HPSE, which was reduced by the co-treatment with Roneparstat (**Fig. 25A, 25B**).
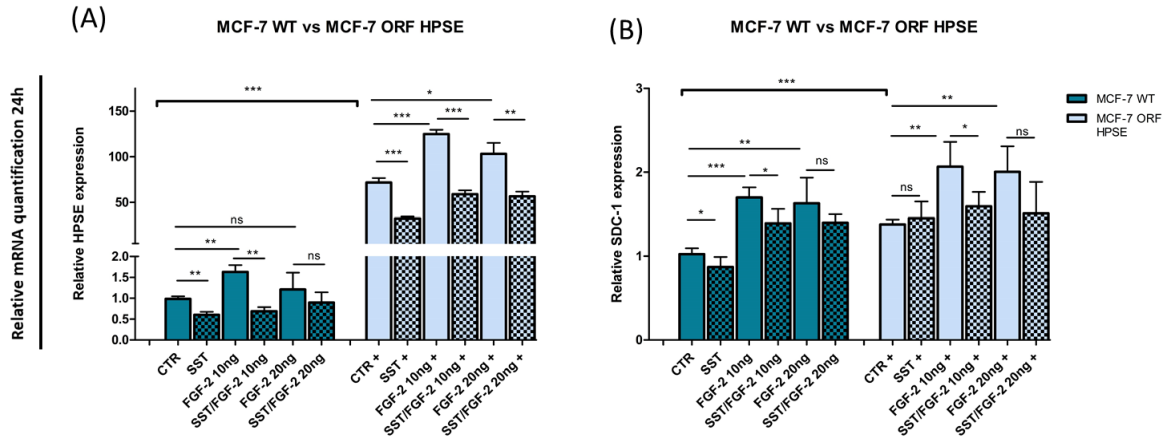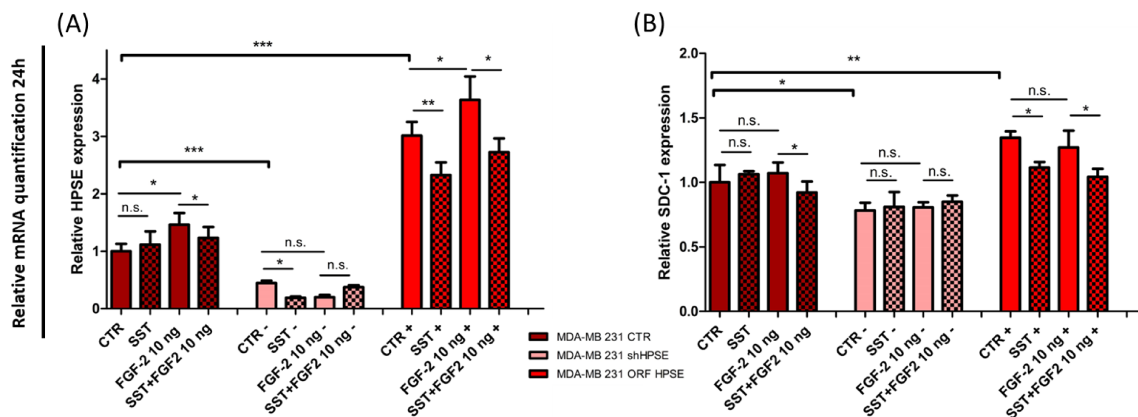

Figure 25 – HPSE and SDC-1 expression in MDA-MB-231. HPSE (A) and SDC-1 (B) gene expression were evaluated by real-time PCR on different clones of MDA-MB-231 WT, MDA-MB-231 silenced for HPSE and MDA-MB-231 overexpressing HPSE at 24h after treatment with Roneparstat (SST0001) (200 ug/mL), FGF-2 (10 ng/mL) and FGF-2 (20 ng/mL) or in combination. Bars represent the mean ± standard deviation (SD); n = 6. The gene expression was normalized to GAPDH as the housekeeping gene. $* p \leq 0.05$; $** p \leq 0.001$, $*** p \leq 0.0001$ vs. CTR.

On the other hand, all the EMT markers analysed seem to be not affected by HPSE overexpression or silencing. Gene expression of SNAI1 and SLUG was not significantly changed in all the conditions analysed, except for the overexpressing cells in which there was an increase in SNAI1, modulated by Roneparstat administration. In addition, SNAI1 expression mediated by FGF-2 was modulated by Roneparstat (**Fig. 26A, 26B**). E-Cad and Vim gene expression analyses by real-time PCR showed no significant change in all the conditions considered, confirmed also at protein levels by WB (**Fig. 26C, 26D, 26E, 26F**).
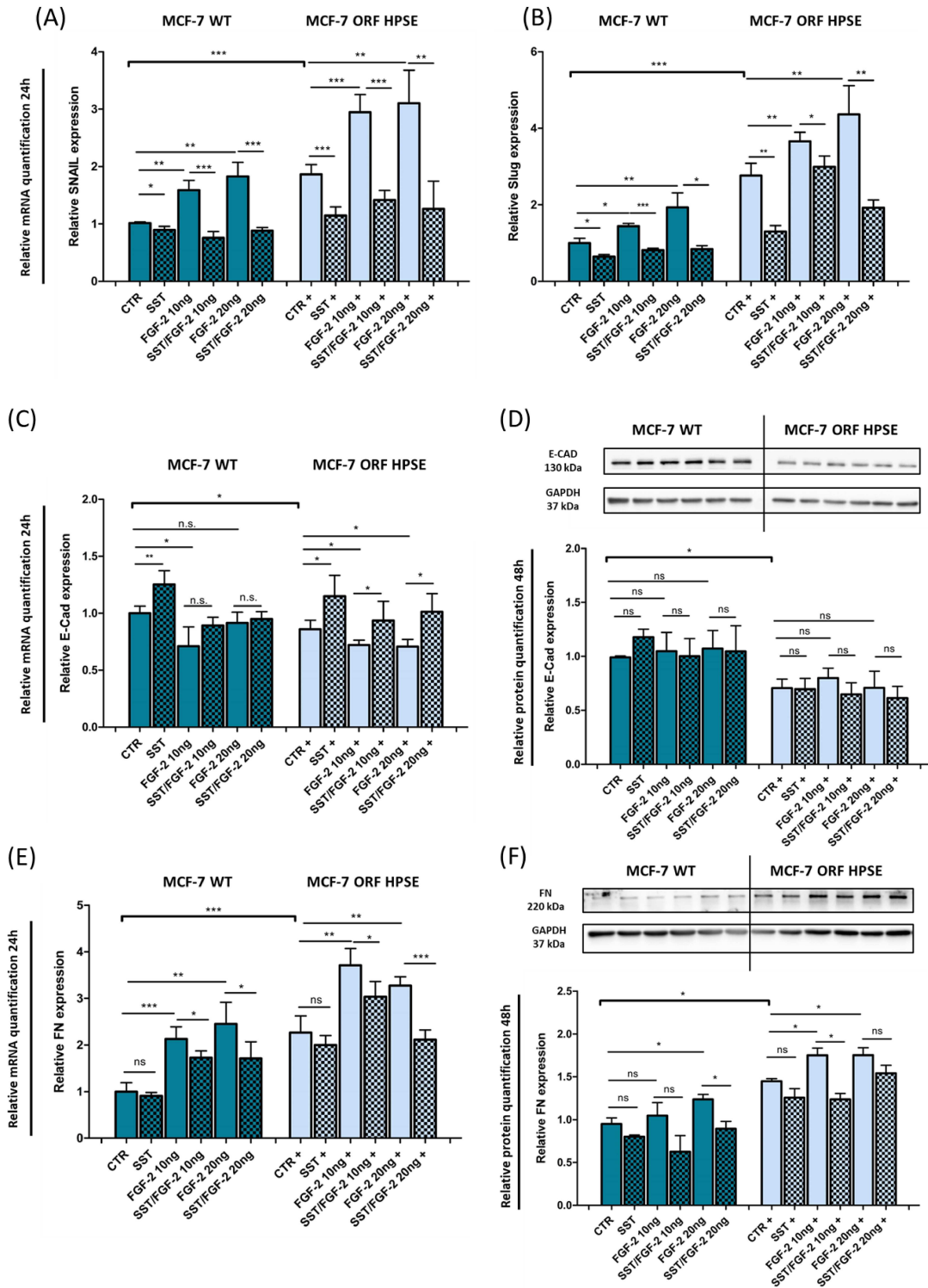
(A)

(B)

(C)

(D)
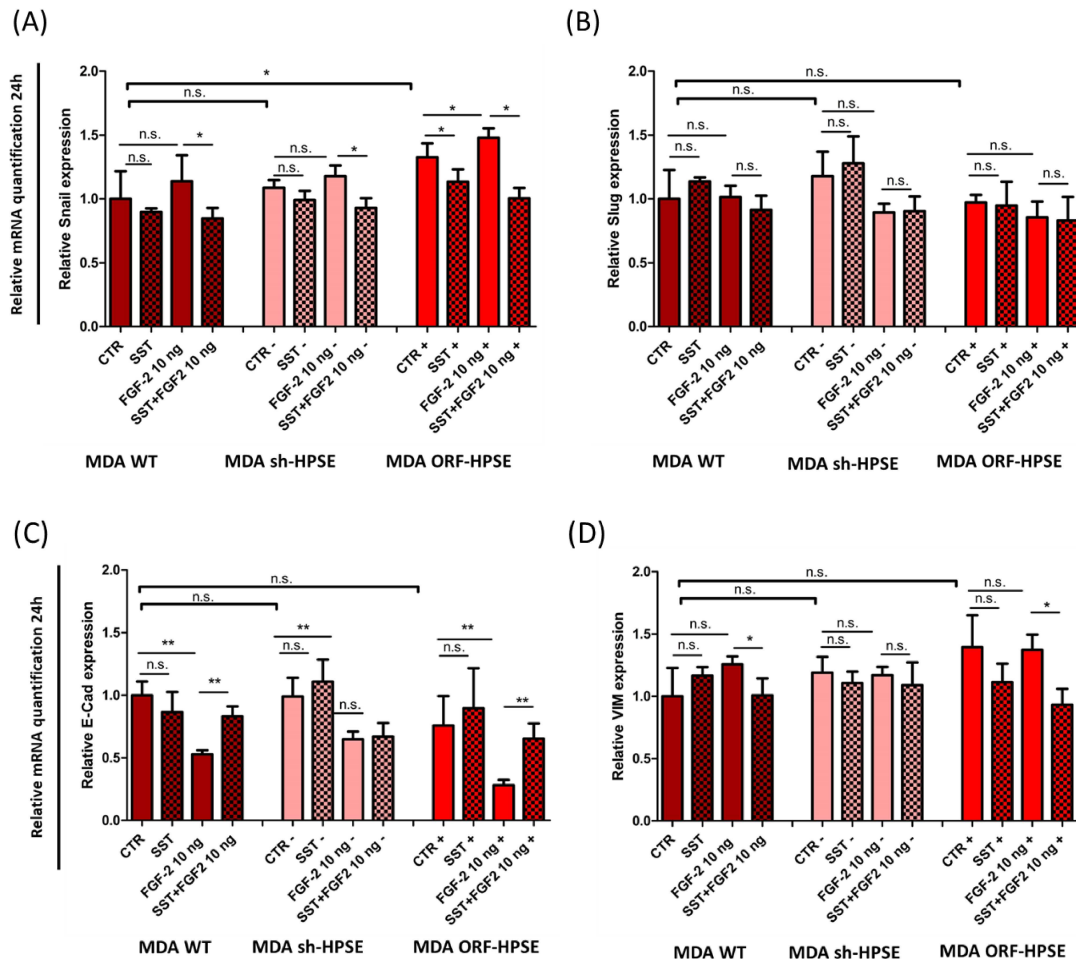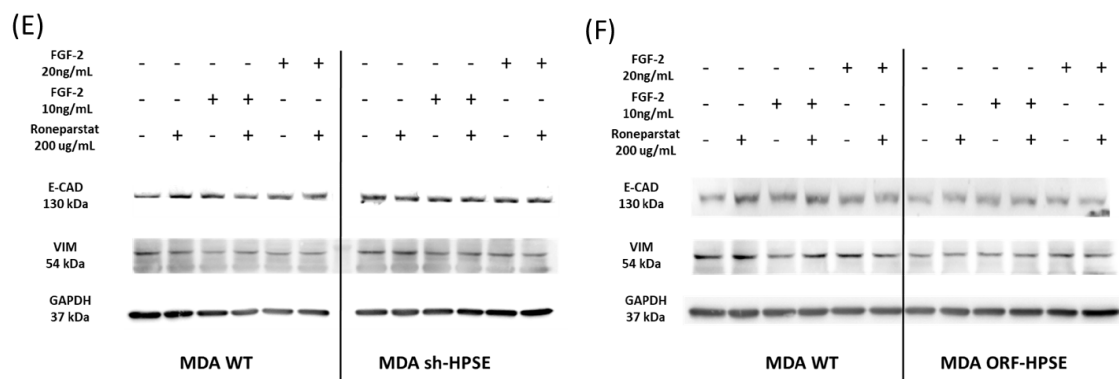
Figure 26 – Evaluation of EMT markers in MDA-MB-231 cells by Real-Time qPCR and Western Blotting analysis. Gene and protein expressions show the different expression of EMT-marker in MDA-MB-231 WT, HPSE silenced and HPSE overexpressing cells after treatment with or without Roneparstat (SST0001) (200 ug/mL), FGF-2 (10 ng/mL) and FGF-2 (20 ng/mL). Snail (A), Slug (B), E-Cad (C) and FN (D) gene expression was evaluated by real-time PCR at 24 hours. Bars represent the mean ± standard deviation (SD); n = 6. The gene expression was normalized to GAPDH as the housekeeping gene. * $p \leq$ 0.05; ** $p \leq$ 0.001, *** $p \leq$ 0.0001 vs. CTR. E-Cad and FN protein levels were evaluated by WB at 48 hours in MDA-MB-231 WT vs. HPSE silenced MDA-MB-231 (E) and MDA-MB-231 WT vs. HPSE overexpressing MDA-MB-231 (F). Data was normalized to GAPDH as housekeeping.

5.6 HPSE expression influences cell migration

The wound healing assay was performed to investigate the influence of HPSE on cell migration and was quantified as the difference in migration area between T24 and T0.

Starting from the MCF-7 WT, HPSE inhibition did not affect the migration capacity of the cells. FGF-2 was able to promote a significant increase in migration and when Roneparstat was added, this effect was significantly reduced. In HPSE overexpressing MCF-7, we find an increased migration capacity at the basal levels, but the inhibition with Roneparstat does not significantly regulate the migration. In addition, FGF-2 promoted a higher increase in the migration capacity compared to the control cells, but again the inhibition of HPSE was able to reduce its migration (**Fig. 27A**).

On the other hand, in MDA-MB 231, there were no significant differences between the WT, HPSE silenced or overexpressing cells, while the cells positively responded to FGF-2 treatment which in turn was able to increase the migration capacity in all the conditions. The inhibition of HPSE alone or after the treatment with FGF-2 was able to reduce the migration competence of the cells both in WT, HPSE silenced and overexpressing cells (**Fig. 27B**).
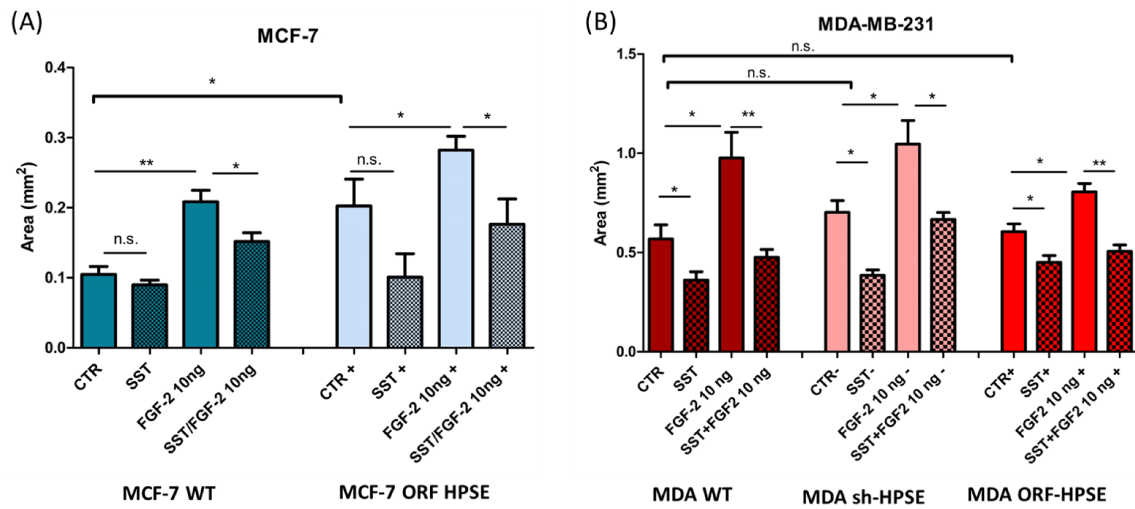
Figure 27 – Wound Healing Assay in MCF-7 and MDA-MB-231 cells. Cell migration was evaluated in MCF-7 WT vs. HPSE overexpressing MCF-7 (A) and in MDA-MB-231 WT vs. HPSE silenced MDA-MB-231 vs. HPSE overexpressing MDA-MB-231 (B). Quantification of migration capacity was quantified after the scar and the treatment with or without Roneparstat (SST0001) (200 ug/mL) and FGF-2 (10 ng/mL). Bars represent the mean ± standard deviation (SD); n = 8. * $p = 0.05$; ** $p = 0.001$

6. DISCUSSION AND CONCLUSIONS

Breast cancer is the most frequent malignant disease among women (Sung et al., 2021). The rates of metastasis and mortality in breast cancer patients have decreased in the last decades as a result of early diagnosis by mammographic screening and the implementation of systemic adjuvant therapy, but a unique therapy is difficult to develop (Torre et al., 2017). For non-metastatic breast cancer, different treatments based on chemotherapy, radiotherapy or endocrine therapies (now considered the gold standard therapy) have been developed (Waks & Winer, 2019). Unfortunately, for triple negative breast cancer (TNBC) the worst and most aggressive type, the treatment mainly relies on cytotoxic chemotherapy, which however resulted inadequate (Waks & Winer, 2019). Therefore finding of new target is crucial for the development of more efficient therapies

Metastases are a key point of malignant tumors, because set the basis for the colonization of other vital organs at distant sites (Fares et al., 2020). The process that sustains the metastatic dissemination is the EMT, an evolutionary mechanism that confers at the cancer cells the metastatic properties enhancing mobility, invasion, and progression (Mittal, 2017). Numerous determinants contribute to the EMT, with FGF-2 and TGF-β standing out as pivotal factors among them that deeply influence the process (Suh et al., 2020; Pang et al., 2016).

In recent investigations, researchers pointed out how the ECM composition and remodeling significantly influence directly or indirectly all the hallmarks of cancers, including tumorigenesis and metastasis. Several enzymes are involved in ECM turnover, with particular emphasis on HPSE, the sole endo-glucuronidase in mammals proficient in cleaving HS. It is now consolidated that HPSE is overexpressed in all human solid tumors, exerting multifaced functions in tumor progression (Jayatilleke & Hulett, 2020). In addition, several studies have shown that HPSE overexpression can induce EMT, a common event found in different disease settings such as multiple myeloma (Li et al., 2016) and renal fibrosis (Masola et al., 2015).

The EMT process is linked to the metastatic properties of cancer cells, supporting their dissociation from the primary tumor, and enhancing mobility and intravasation (Yeung & Yang, 2017). Previous work suggested that in breast cancer patients, HPSE overexpression correlated with sentinel node metastasis and that HPSE-positive tumors

(90%) showed a reduced level of HS deposition (Maxihimer et al 2002). Furthermore, HPSE expression was also significantly correlated to histology grade, lymph node status and lymphovascular invasion (Tang et al 2014). Compelling evidence establishes a strong correlation between the expression of this enzyme and EMT also in other solid tumors, as delineated in **Table 1**. In most cases, the inhibition of HPSE has demonstrated a decrease both in vitro and in vivo of EMT and metastasis (see chapter "HPSE inhibition").

Starting from this evidence, the goal of this project was to understand how the HPSE expression or its activity affects the EMT behaviour in different breast cancer cell lines. To inhibit HPSE activity we choose Roneparstat, a compound based on the glycol splitting of the non-sulfated hexuronic acids (GlcA and IdoA). The potent inhibitory capacity of Roneparstat, confirmed by dose-inhibition curves studies (IC$_{50}$ $\approx$ 3 nM), is owing to the strong HS homology which allows it an antagonist action but also a multiple protein-ligand interaction depending on its concentrations (Rivara e Pala, 2016). In addition, to better characterize the effect of Roneparstat in vitro, we also tested this drug in combination with FGF-2 and TGF-β as EMT activators.

It is noteworthy that in the breast cancer cells model a key regulator of EMT is FGF-2 and its receptor (Suh et al., 2020) and that HPSE enzymatic activity modulates the activation of the FGF-2 signalling pathway (Marchetti et al., 2006). Indeed, the activation of the FGF pathway depends on the binding to HS, a crucial step for dimerization and activation of FGF-receptor (FGFR) mediated by the formation of an FGF-FGFR-HS ternary complex (Goetz & Mohammadi, 2013). In addition, HS fragments derived from SDC-1 degradation by HPSE were shown to potentiate the FGF2 mitogenicity, which could further contribute to modulating the growth factor activity (Kato et al., 1998). TGF-β1 is known to modulate the EMT process in breast cancer (Pang et al., 2016). Interestingly, it has been proved that in a different pathological setting as well as renal fibrosis, HPSE is a regulator of EMT induced by FGF-2 and TGF-β in renal tubular cells (Masola et al., 2012, 2014). It is noteworthy that the epithelial cells (not only in cancers but also in other pathological conditions) respond to these two growth factors activating a group of transcription factors that activate the EMT, especially SNAI1 and SLUG (Strutz et al., 2002; Moustakas & Heldin, 2016).

In our in vitro studies we found that the expression of HPSE correlated with the different phenotypes of the two breast cancer cell lines analysed. In particular, the MDA-MB-231, defined as a triple negative phenotype with high metastatic potential showed higher HPSE expression levels compared to the poorly metastatic MCF-7. In addition, the MCF-7 showed a cobblestone morphology with a higher expression of the epithelial marker E-CAD, while the spindle-shaped MDA-MB-231 expressed more FN and VIM, two mesenchymal markers.

In line with this evidence, our preliminary data proved that the inhibition of HPSE with Roneparstat in MCF-7 was not able to influence the expression of the EMT markers SNAI1, SLUG, FN, VIM and E-CAD. On the other hand, in MDA-MB-231 which exhibited a higher level of HPSE, the inhibition of HPSE activity was able to reduce the gene expression of SNAI1 and SLUG, but not the other EMT markers.

So, the EMT process was evaluated by the treatments with FGF-2 and TGF-β, which were able to significantly increase the gene expression of SNAI1 and SLUG in both cell lines. In addition, the two cell lines showed a moderate increase in gene expression of FN and VIM and a reduction of E-CAD. Interestingly, the inhibition of HPSE obtained by the treatment with Roneparstat before the administration of FGF-2 or TGF-β reduced the increase of EMT gene expression, suggesting a role of HPSE in EMT activation.

Nevertheless, despite the gene expression differences, we confirmed it only partially at protein expression levels. In particular, MCF-7 showed an increase of EMT markers after the treatments with FGF-2 and TGF-β, an effect that was modulated after the inhibition of HPSE. On the contrary, no differences in protein levels were detected in MDA-MB-231.

Our pieces of evidence suggest that the difference between the two cell lines was owing not only to the different levels of HPSE but also the different levels of EMT markers correlated to the specific phenotype. The effect of HPSE inhibition promoted by Roneparstat administration, alone or in combination with EMT activators, suggested not only the involvement of HPSE in the EMT pathway but also its ability to interfere with the EMT-induction by different factors.

The acquisition of migration capacity is a common feature of the EMT process. To evaluate the effects of HPSE upon the migration trend of MCF-7 and MDA-MB-231, we performed a cell migration assay (also known as wound healing assay). This method involved the creation of a "wound" in a cell monolayer inducing cell migration to fill the gap, mimicking the same condition during wound healing in vivo. Considering our previous results, we observe a distinct cellular response after the inhibition of HPSE post-Roneparstat administration, which correlates with the different expression of HPSE between the two cell lines analysed.

Indeed, the HPSE inhibition was able to reduce cell migration on the higher-HPSE expressing MDA-MB-231, while no difference was found in the MCF-7. Then, to better characterize the migration capacity, the EMT process was stimulated by the treatment with FGF-2 in both cell lines. In MCF-7, the treatment with this growth factor induced an increase in cell migration, an effect that was significantly reduced by HPSE inhibition. In contrast, in MDA-MB-231 we did not find any change after FGF-2 treatment, probably related to the different phenotypes exhibited by this cell line.

Interestingly, unlike FGF-2, the TGF-β induced cytostatic effect in both the cell lines despite the proliferation was not affected in the condition analysed. It is known that TGF-β stimulation induced cytostatic effect in different non-neoplastic epithelial cells, as well as in endothelial cells, hematopoietic cells, neuronal cells and certain types of mesenchymal cells (Siegel & Massagué, 2003). In breast cancer the effects of TGF-β are context-dependent, reflecting how the intricate interplay between TGF-β pathways and the peculiar molecular subtypes influence the behaviour of MCF-7 and MDA-MB-231 (Zarzynska, 2014).

After this set of experiments, to better understand the potential role of HPSE in the EMT process, we chose to alter the expression of HPSE with a different approach. It is now consolidated that HPSE exhibit also a non-enzymatic activity through its capacity to translocate into the nucleus where it regulates gene expression (Nobuhisa et al., 2007; Stewart et al., 2015). The nuclear migration of HPSE is not surprising, considering that SDC-1 has also been discovered in the nucleus, which colocalized with HPSE (Zhong et al., 2009). Two different modes are proposed to explain the mechanism through which the nuclear HPSE can influence gene expression: the promotion of HAT activity by the cleavage of nuclear HS or by direct interaction with specific DNA sequence

(Purushothaman et al., 2011; Stewart et al., 2015). So, to discriminate the contribution of the enzymatic activity compared to the non-enzymatic on the EMT process, we tried to obtain knock-out cell lines for HPSE using the CRISPR/Cas9 technique. To suppress the expression of HPSE and minimize the off-target effects of the Cas9 protein, we used the ribonucleoprotein (RNP) system. In our approach, the Cas9 protein fused with the GFP was mixed with the sgRNA target for specific HPSE gene sequences. After the transfection, we analyzed HPSE gene expression by RT-qPCR only in GFP-positive cells. Some clones showed a reduction of HPSE gene expression but sequencing analysis failed to confirm the knockout both in MCF-7 and MDA-MB 231 cell lines. We suppose that a possible explanation of our failure could be due to the polyploid karyotype of these cells and the RNP system that is limited to the half-life of the complex (C. S. Wang et al., 2000; Y. Wu et al., 2020).

As alternative strategies to the knock-out, we planned to alter the expression of the enzyme inducing its overexpression and silencing. The general idea was to upregulate HPSE expression in MCF-7 which exhibits a lower level compared to the MDA-MB-231. On the other hand, we wanted to study the reduction of HPSE in MDA-MB-231 and evaluate a putative additional effect following HPSE overexpression. To do this, we induced a stable HPSE overexpression in cell lines by the transfection of a plasmid carried the open reading frame of the enzyme and the silencing exploiting the short hairpin RNA (shRNA) system.

Considering the overexpression of HPSE in MCF-7 cells, we found a significant reduction of E-CAD and an increase of SNAI1, SLUG and FN both at gene and protein expression. These data suggest that the overexpression of HPSE induces the increase of the EMT markers, reinforcing the idea that this enzyme influences this process. In addition, we find a higher migration capacity concerning the MCF-7 WT. To evaluate the enzymatic component of HPSE after the overexpression, we also inhibited the enzyme activity with Roneparstat. The inhibition of HPSE with Roneparstat alone in HPSE overexpressing MCF-7 cells was able to reduce the gene expression of the epithelial marker E-CAD. Furthermore, the inhibition of HPSE significantly reduced the increased migration capacity acquired by these cells. In addition, we also found that FGF-2 treatment induced a higher EMT activation in HPSE overexpressing cells, confirmed by the increase of SNAI1, SLUG and FN as well as the reduction of E-CAD.

In this context, the inhibition of HPSE by Roneparstat was able to interfere with FGF-2 activation, modulating the expression of the EMT markers. These shreds of evidence suggest that the upregulation of HPSE influences not only the phenotype and behaviour of MCF-7 but also contributes to phenotype switch by the upregulation of the EMT markers and the increase of migration capacity. The inhibition of HPSE by Roneparstat administration was able to modulate not only the expression of the EMT markers but also to influence the activation promoted by FGF-2 and reduce the migration capacity.

Moving to the MDA-MB-231, we found that both the silencing and overexpression of HPSE produced no dramatic changes in all the EMT-markers analysed. Additionally, the wound healing assay confirmed that there were no differences also on the migration capacity of the different clones compared to the WT. In addition, the FGF-2 treatment confirmed the previous results, inducing only a slight effect on the different cell lines. Furthermore, the inhibition of HPSE with Roneparstat alone or the co-treatment with FGF-2 reflected only a faint effect on EMT markers expression. On the whole, we can do two considerations on the results obtained for MDA-MB-231: the downregulation of HPSE induced was mild to appreciate a modulation on EMT process and that the overexpression of HPSE does not influence EMT in this cell type due to the high levels of the enzyme in WT cell. Furthermore, we can speculate on MDA-MB-231 that the different molecular subtypes could influence the expression of the EMT markers by the activation of different pathways which differ from those activated by HPSE.

An ample number of evidences suggest that HPSE can affect cell behavior and signaling by regulating the structure and function of HSPGs. Among the HSPGs, SDC-1 is known for its implication in tumor growth, angiogenesis and metastasis in breast cancer (Sheta & Götte, 2021). It is noteworthy that HPSE regulates both the level and location of SDC-1, enhancing SDC-1 synthesis and subsequent shedding (Yang et al., 2007). In addition, in a recent work, Huang et al pointed out that the upregulation of HPSE correlates with an increased expression and cleavage of SDC-1 in human breast tissue cultured ex-vivo (Huang et al., 2020). So, due to this dynamic interaction between HPSE and SDC-1 expression and how it could contribute to the EMT process, we also analysed the gene expression of SDC-1. We found that the overexpression of HPSE correlates with an upregulation of SDC-1 both in MCF-7 and MDA-MB-231. Consistently, the downregulation of HPSE in MDA-MB-231 induced a reduction of

SDC-1. Our results showed that the inhibition of HPSE by Roneparstat did not affect SDC-1 expression. In addition, the treatment with FGF-2 induced an increase in SDC-1 expression both in MCF-7 and MDA-MB-231. In contrast, the inhibition of HPSE was able to reduce the effect promoted by FGF-2 treatment.

Our results pointed out that the inhibition of HPSE enzymatic activity induced by Roneparstat affects the expression of EMT markers and the migration capacity of MCF-7 and MDA-MB-231. The effect and response of these breast cancer cell lines are strictly correlated to their specific phenotype, suggesting that HPSE expression plays a different role at different tumor stages. Indeed, the overexpression of HPSE in MCF-7 promoted the increase of all the EMT markers but also the migration capacity. In MDA-MB-231, which showed a higher HPSE expression, we did not find relevant differences, probably related to the different molecular subsets that could influence the EMT process differently. The treatment with HPSE inhibitors that are currently under clinical trial suggests that the inhibition of HPSE alone induced only a slight effect on tumor, while it was used as an adjuvant drug to ameliorate the response to chemotherapy as confirmed in many models. Interestingly, HPSE was overexpressed after treatment with radiation (Meirovitz et al., 2011) or chemotherapy drugs (L. Zhang et al., 2015; Lanzi et al., 2021). A lot of evidence collected by in vitro experiments reinforced this idea of combinatory administration to potentiate the therapy outcomes. For example, Zahavi et al. found that the treatment with Roneparstat alone had only a slight effect on MDA-MB-231, while in combination with other chemotherapy drug as adjuvant promoted a combinatory effect (Zahavi et al., 2021). Again, Lanzi et al found that Roneparstat showed a mild inhibition on CME-1 cell proliferation, a synovial sarcoma cell culture model, but exhibited a synergistic effect in combination with HDAC inhibitor (Lanzi et al., 2021). The combinatory activity suggests that HPSE inhibition is useful in reducing cancer resistance after chemotherapy treatment.

It is also important to understand that among all the inhibitors, no one can interfere with its non-enzymatic activity. Notably, in human renal cell carcinoma biopsies it was found a peculiar HPSE variant called HPSE-T5, a truncated and enzymatically inactive protein. Interestingly, this variant maintained pro-tumorigenic properties, enhancing cell proliferation, tumor growth and xenograft development (Barash et al., 2010). In addition, the expression of the C-domain (8c) of Hpse in a mouse mammary tumor virus

(MMTV) transgenic mice was able to promote tumor growth and metastasis but also to increase phosphorylation and signalling mediated by Akt, Stat5 and Src (Boyango et al., 2018). In addition, it was demonstrated by an immunofluorescent experiment, a clear nuclear localization of Roneparstat that colocalized with HPSE (Lanzi et al., 2021). Taking together these considerations, we can speculate that the enzymatic inhibition of HPSE could not affect its non-enzymatic functions and therefore further studies on HPSE expression are necessary.

We tried to obtain additional information by combining our results with the knock-out cell model. Unfortunately, we did not obtain any knock-out cell lines to discriminate the enzymatic and non-enzymatic role of HPSE in EMT and migration. In 2023, Jajatilleke and his group published a work 2023 which characterized tumor size, angiogenesis and metastasis in an MMTV-Hpse$^{-/-}$ mouse model. The researchers found that in MMTV-Hpse$^{-/-}$, the absence of Hpse reduced tumor growth, tumor size and vascularization (Jayatilleke et al., 2023).

In conclusion, our data confirm that different breast cancer cell lines show different levels of HPSE which correlated with their phenotype and metastatic potential. The inhibition of HPSE promoted by Roneparstat alone or after the treatment with FGF-2 and TGF-β, affects differentially the expression of EMT markers and migration, depending on epithelial or mesenchymal phenotype. In addition, HPSE expression influences the expression of EMT-associated genes and the migration capacity of the cells. Collectively, these results shed light on the complex role of HPSE, taking into consideration its enzymatic and non-enzymatic contribution to tumor growth, angiogenesis, progression and metastasis. The molecular mechanisms that regulate HPSE expression in most aggressive tumors are only partially known. A possible explanation has been identified for the genomic alteration of p53, which regulates the methylation and expression of many genes, comprising HPSE (Baraz et al 2006). Therefore, we cannot exclude the role of HPSE in aggressive tumor, considering the high heterogeneity which cannot be summarized with just the two cell lines analysed in this work.

Experimental and clinical research supports the involvement of HPSE in all stages of tumor formation, from initial stages to growth, metastasis to chemotherapy resistance. From this evidence and considering that once HPSE is inhibited no other molecule is able to perform a similar function, it follows that a pharmacological strategy aimed at its inactivation should be able to block the enzyme both inside the cell as well as in the extracellular environment. Future perspectives could focus on the development of a new class of highly specific inhibitors acting not only to its enzymatic action but also its non-enzymatic function. Nevertheless, some HPSE inhibitors have already entered clinical research and some significant results have been reported. Bearing in mind the role of HPSE in the release/activation of growth factors that provide favorable conditions for cancer cells growth, can be imagined a therapeutic approach of HPSE inhibitors to abrogate its support to the tumor microenvironment, instead of the treatment of the primary tumor itself. From what has emerged recently, in particular from the study of hematological tumors, it seems that HPSE is up-regulated after high-dose chemotherapy and that this facilitates the chemo-resistance of the tumor. This has led to the hypothesis that the use of HPSE inhibitors in combination with chemotherapeutic drugs may overcome initial chemo-resistance and increase the possibility of eradicating the tumor. Much remains to be done in this area but the combination of an anti-heparanase therapy with anti-cancer drugs may likely be the therapeutic route to be followed in the future.

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LIST OF PUBLICATIONS

1. Franchi M., Karamanos K. A., Cappadone C., Calonghi N., **Greco N**., Franchi L., Onisto M., Masola V. *(2023)*. Colorectal Cancer Cell Invasion and Functional Properties Depend on Peri-Tumoral Extracellular Matrix. *Biomedicines*, 11(7), 1788. https://doi.org/10.3390/biomedicines11071788

2. **Greco N.**, Onisto M., Alibardi L. *(2023)*. Protein extracts from regenerating lizard tail show an inhibitory effect on human cancer cells cultivated in-vitro. *Annals of anatomy = Anatomischer Anzeiger:official organ of the Anatomische Gesellschaft*, 250, 152115. https://doi.org/10.1016/j.aanat.2023.152115

3. Franchi M., Karamanos K. A., Cappadone C., Calonghi N., **Greco N.**, Franchi L., Onisto M., Masola V. *(2022)*. Substrate Type and Concentration Differently Affect Colon Cancer Cells Ultrastructural Morphology, EMT Markers and Matrix Degrading Enzymes. *Biomolecules*, 12(12), 1786. https://doi.org/10.3390/biom12121786

4. Masola V., **Greco N.**, Tozzo P., Caenazzo L., Onisto M. *(2022)*. The role of SPATA2 in TNF signaling, cancer and spermatogenesis. *Cell death & disease*, 13(11), 977. https://doi.org/10.1038/s41419-022-05432-1

5. Masola V., **Greco N.**, Gambaro G., Franchi M., Onisto M. *(2022)*. Heparanase: A Paramount Enzyme for Cancer Initiation, Progression, and Metastasis. The Extracellular Matrix and the Tumor Microenvironment. *Biology of Extracellular Matrix* 11:197-217. https://doi.org/10.1007/978-3-030-99708-3_8

6. Masola V., **Greco N.**, Gambaro G., Franchi M., Onisto M. *(2021)*. Heparanase as active player in endothelial glycocalyx remodeling. *Matrix biology plus,* 13:100097. https://doi.org/10.1016/j.mbplus.2021.100097