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TESI DI LAUREA

Exploring the role of extracellular Nicotinamide Phospho-Ribosyl-Transferase in breast cancer tumour microenvironment: insights from in vivo and in vitro investigations

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ABBREVIATIONS LIST

ATP adenosine triphosphate

CCR5 C-C chemokine receptor type 5

CTCs tumoral circulating cells

DNA deoxyribonucleic acid

EGF epidermal growth factor

EMT epithelial-to-mesenchymal transition

eNAMPT extracellular nicotinamide phospho-ribosyl-transferase

ER oestrogen receptor

ERK extracellular signal-regulated kinase

HER2 human epidermal growth factor receptor-2

iNAMPT intracellular nicotinamide phospho-ribosyl-transferase

IL interleukin

MAPK mitogen-activated protein kinase

MET mesenchymal-to-epithelial transition

MMP matrix metalloproteinases

NAD nicotinamide adenine dinucleotide

NAD+ nicotinamide adenine dinucleotide (oxidised)

NADH nicotinamide adenine dinucleotide (reduced)

NAM nicotinamide

NAMPT nicotinamide phospho-ribosyl-transferase

NAPRT nicotinic acid phospho-ribosyl-transferase

NF-κB nuclear factor κ-light-chain-enhancer of activated B cells

NMNAT nicotinamide mononucleotide adenyl-transferase

NMN nicotinamide mononucleotide

PARP poly ADP-ribose polymerase

PBEF pre-B cell colony enhancing factor

PECs peritoneal macrophages

PR progesterone receptor

PI3K phosphatidylinositol 3'-kinase

SCR scramble

SP-NAMPT signal peptide NAMPT

STAT3 signal transducer and activator of transcription 3

TCA tricarboxylic acid cycle

TGF-β transforming growth factor β

TNBC triple-negative breast cancer

TNF tumour necrosis factor

TLR4 toll-like receptor 4

VEGF vascular endothelial growth factor

1 INTRODUCTION

1.1 Breast cancer

Breast cancer is still the primary cause of cancer-related death in women worldwide, being a deeply heterogeneous, metastatic, and therapy-resistant disease (**Nolan et al., 2023**). With an estimated 2.26 million recorded cases in 2020, it is the most widespread malignant neoplasm in the world. Although it was considered a highly developed country-related disease in the past, in 2020, more than half of breast cancer diagnoses and two-thirds of breast cancer-related mortality took place in less developed regions of the world (**Wilkinson et al., 2021**).

Mammary carcinoma is a complex disease caused by the combination and accumulation of various genetic mutations, lifestyle, and environmental factors capable of modifying cell functions (**Taurin et al., 2020**). Some of the primary factors related to a major risk of developing breast cancer are sex, ageing, and prolonged oestrogen exposure.

Family history represents another risk factor; nearly 25% of incidences of all breast cancer are associated to familiarity. Women whose mothers or sisters has mammary cancer are more exposed to this malignancy.

Other risk factors are associated with habits and personal behaviours: smoking, excessive alcohol consumption, overweight, and obesity (**Sun et al., 2017**).

It has become clear that breast cancer is a family of illnesses with unique pathological, molecular, and clinical characteristics (**Asleh et al., 2022**).

1.1.1 Clinical and molecular aspects of breast cancer subtypes

"The 2013 St. Gallen International Breast Cancer Conference" published a classification of breast cancer molecular sub-types: luminal A, luminal B, HER2+ (human epidermal growth factor receptor-2 positive), HER2 overexpression, basal-like triple-negative breast cancer (TNBC) (**Li Yin et al., 2020**):

- Luminal A is the most prevalent type of breast cancer since it makes up 50–60% of mammary carcinoma cases. Despite the high prevalence, patients who are affected by this sub-type have a good prognosis. It is characterised by the presence of oestrogen receptors (ER) and progesterone receptors (PR) and by the lack of human epidermal growth factor receptor-2 (HER2) (**Eroles et al., 2012)**.
- Luminal B cancers (ER+ and/or PR+/HER2+) account for 20–30% of all mammary carcinomas. As compared to luminal A tumours, they show a more aggressive phenotype, a higher proliferative index, and worse outcomes in patients (**Eroles et al., 2012)**.
- The molecular sub-type of cancer characterised by the overexpression of HER2 accounts for 20% of all types of mammary carcinoma (**Kunte et al., 2020**).
- Triple-negative BC (TNBC) is defined by a lack of expression of ER, PR, and HER2. Using gene expression profiling analysis, TNBC has been classified as a basal-like breast cancer (BLBC) sub-type.

  As stated in epidemiological data, TNBC frequently appears in premenopausal women under 40 years old, who make up nearly 15-20% of all breast cancer patients. Unlike other breast cancer sub-types, life expectancy of patients affected by TNBC is shorter in time, with a mortality rate of 40%. TNBC is extremely aggressive, as roughly 46% of TNBC patients will develop distant metastases at the time of diagnosis (**Lyons et al., 2019**).

1.1.2 Therapy according to molecular subtypes

The advancement of target therapies for the treatment of breast cancer has progressed significantly over the past 25 years. The therapeutic choice depends on the sub-type of breast cancer and on the stage at the time of the diagnosis (**Jacobs et al., 2022**).

Since luminal breast cancers are typically oestrogen-dependent tumours, they can be successfully approached with therapies that differ from traditional chemotherapy and include aromatase inhibitors (AI), selective ER modulators (SERM), such as tamoxifen, and selective ER down-regulators, such as fulvestrant (**Eroles et al., 2012**).

Nowadays, cyclin-dependent kinase inhibitors such as CDK4/6I, are becoming increasingly relevant as treatment choices for HR+/HER2− metastatic advanced breast cancer. However, their effectiveness in luminal tumours is still under ongoing investigation (**Abdelmalak et al., 2022**).

The research for an effective HER2-positive breast cancer therapy has undergone significant development over the last two decades. Before the advent of monoclonal antibodies targeting HER2, breast cancer that was HER2-positive was associated with a poor prognosis (**Kunte et al., 2020**).

At present, the standard care for early therapy in patients affected by HER2-positive metastatic breast cancer (MBC) is represented by trastuzumab plus chemotherapy.

In the event of therapeutic resistance and disease relapse, dual HER2 antibody treatment, pertuzumab and trastuzumab, combined with chemotherapy is used (**Kunte et al., 2020**; **Swain et al., 2022**).

Despite effective and consolidated targeted therapies for luminal A, luminal B, and HER2+ tumours, research efforts are now focused on a better understanding of the biology of triple-negative breast cancer since the lack of molecular targets hampers the development of therapies specific for this type of tumour (**Asleh et al., 2022)**.

The specific molecular phenotype of TNBC makes it insensitive to endocrine therapy or molecular target therapy. For this reason, classic chemotherapy constitutes the first-line treatment for patients affected by this type of cancer.

“The National Comprehensive Cancer Network guidelines” suggest the combination of taxanes, anthracyclines, cyclophosphamide, cisplatin, and fluorouracil, although the effectiveness of this treatment is poor. Adding to this, in some countries, the integration of bevacizumab (anti-VEGF) has been proposed to implement the polychemotherapeutic approach to TNBC, but the survival time of patients hasn’t resulted in a notable increase.

At present, thanks to the advances in the classification of TNBC molecular types and genome sequencing, potential molecular targets have been identified among the PARP enzymes involved in the DNA repairing process and the immune checkpoint receptors responsible for tumour immune-mimesis, thus enabling the registration of PARP and checkpoint inhibitors (**Lyons et al., 2019**).

Immunotherapy, specifically, has prolonged the survival of patients with other solid tumours and represents a promising therapeutic strategy also for TNBC. The most efficacious immunotherapeutic approach involves the use of immune checkpoint inhibitors (ICIs) to enhance the proliferation and the cytotoxic potential of tumour-infiltrating lymphocytes (TILs) by blocking receptors such as the "Cytotoxic T lymphocyte antigen-4" (CTLA-4) or the "Programmed Death-1" (PD-1). Accordingly, the monoclonal antibodies developed against PD-1 (i.e., pembrolizumab, nivolumab), PD-L1 (i.e., atezolizumab, durvalumab, avelumab), and CTLA-4 (i.e., ipilimumab) have generated durable responses against various tumour types (**Keenan et al., 2020**).

Nonetheless, it is now clear that immunotherapy alone is not sufficient to treat TNBC. Therefore, ongoing research aims at the development of new regimens and therapeutic targets (**Yun Li et al., 2022**).

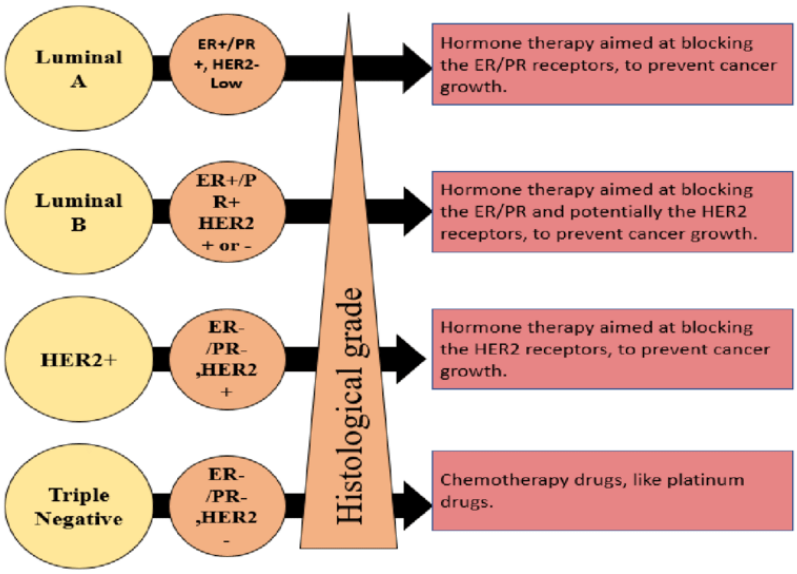


Figure 1: Receptors expressed, histological grade, and potential treatments of each breast cancer molecular subtype (Houseman et al., 2021)

1.2 Biosynthesis pathways and cellular metabolism of NAD

Nicotinamide adenine dinucleotide (NAD) is a metabolite and coenzyme involved as an electron donor in several metabolic pathways and cellular processes (**Covarrubias et al., 2021**), including cytosolic glycolysis, serine biosynthesis, the tricarboxylic acid cycle (TCA), oxidative phosphorylation, and cell redox state homeostasis maintenance.

Apart from its redox properties, NAD is also the substrate of numerous NAD-consuming enzymes with major roles in gene expression and cell signalling, such as poly ADP-ribose polymerases (PARPs) and the Sirtuin family (SIRT1–7).

In the last few years, an alteration in the intracellular levels of NAD has been related to numerous pathologic conditions, such as those connected to ageing and inflammation, including cancer. Therefore, maintaining NAD homeostasis appears crucial for normal cell function.

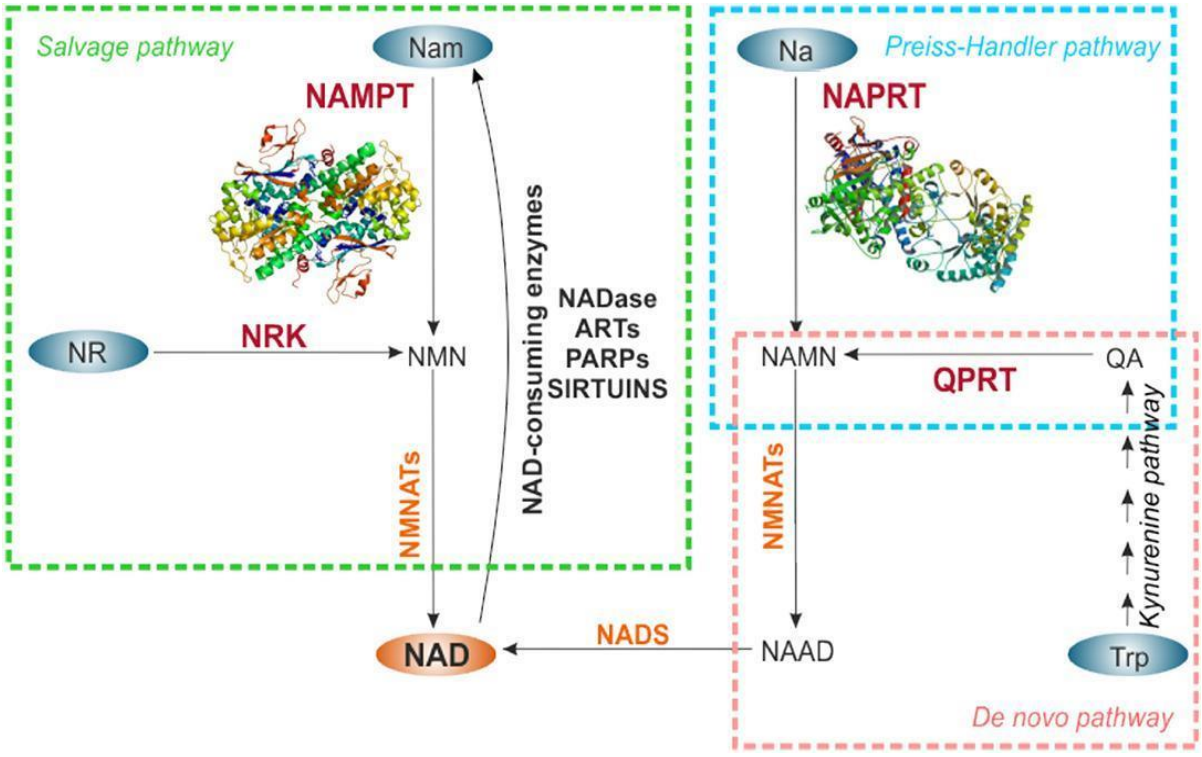


Figure 2: NAD biosynthetic pathways (Audrito et al., 2020)

The biosynthesis of NAD can take place primarily *via* three different pathways:

– The *de novo* synthesis, which starts with the catabolism of the amino acid tryptophan to quinolinic acid, that is further converted into Nicotinic Acid Mononucleotide (NAMN) by Quinolinate Phospho-Ribosyl-Transferase (QPRT) (**Audrito et al., 2020**).

- The Preiss-Handler pathway, which implies the conversion of dietary nicotinic acid (NA) into NAMN by the enzyme Nicotinic Acid Phospho-Ribosyl-Transferase (NAPRT); NAMN is then transformed into Nicotinic Acid Adenine Dinucleotide (NAAD) by the Nicotinamide Mononucleotide Adenyl-Transferase (NMNAT). This process is completed by converting NAAD into NAD^+ by Nicotinamide Adenine Dinucleotide Synthetase (NADS) (**Covarrubias et al., 2021**).

- The Salvage pathway, which starts with the condensation of nicotinamide (NAM) and Phospho-Ribosyl-Pyro-Phosphate (PRPP) into nicotinamide mononucleotide (NMN), as operated by the enzyme Nicotinamide Phospho-Ribosyl-Transferase (NAMPT); NMN is then converted into NAD.

Quantitatively, the salvage pathway is the most prominent in mammalian cells due to the higher availability of NAM in the bloodstream compared to nicotinic acid (**Audrito et al., 2020**); indeed, eukaryotic cells employ this pathway because of the constant requirement for NAD-consuming enzymes to facilitate various cellular and metabolic processes (**Dakroub et al., 2020**).

1.3 Nicotinamide Phospho-Ribosyl-Transferase (NAMPT)

NAMPT belongs to the class of dimeric type II Phospho-Ribosyl-Transferases, and it is an essential enzyme overexpressed in all mammalian tissues (**Gasparrini et al., 2022**), suggesting a crucial role in physiological processes (**Dakroub et al., 2020**).

NAMPT exists as an intracellular enzyme (iNAMPT), which is primly located in the cytoplasm, nucleus, and mitochondrion, and extracellular protein (eNAMPT), which circulates in serum and other extracellular fluids (**Tang et al., 2023**).

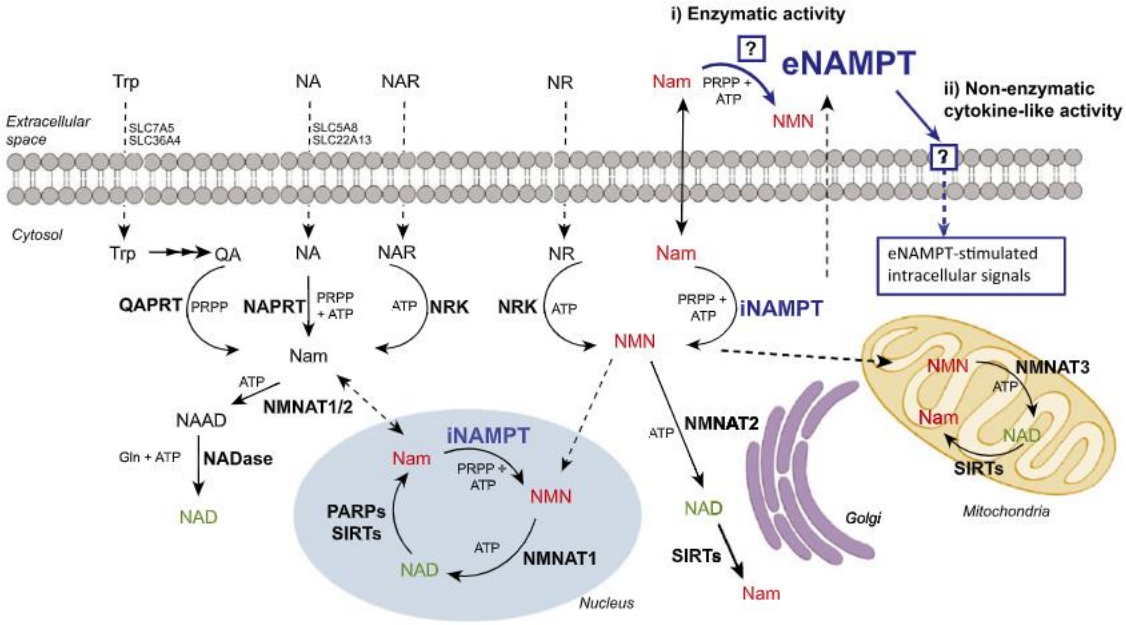


Figure 3: iNAMPT and eNAMPT differ in their cellular localization (Ambra et al., 2016)

1.3.1 iNAMPT

Intracellular NAMPT has a crucial role in the biosynthesis of NAD as an enzyme participating in the salvage pathway. Additionally, since it modulates the intracellular level of NAD, iNAMPT regulates the metabolic activities of NAD-consuming enzymes, such as PARPs and SIRTs, which play critical roles in apoptosis, DNA repair, and epigenetic regulation (**Audrito et al., 2020**).

The control of these metabolic activities, which involve NAD, and the continuous production of adenosine triphosphate (ATP) are essential for both normal tissues and malignant cells (**Wei et al., 2022**).

iNAMPT was discovered to be overexpressed in breast, ovarian, colorectal, prostate, and gastric cancers, as well as in melanoma and myeloma (**Pramono et al., 2020**). This overexpression parallels that of NAD-consuming enzymes such as PARPs and sirtuins, leading to the hypothesis that cancer cells require high levels of NAD for their growth and invasive activities (**Galli et al., 2020**). Consequently, perturbing NAD homeostasis by reducing NAD levels within tumour cells is believed to offer a fruitful strategy in cancer therapy (**Wei et al., 2022)**.

The potential of NAMPT inhibitors as anticancer agents was initially highlighted by Hasmann et al. in 2003 (**Galli et al., 2020**). Due to their notable anti-tumour effectiveness in preclinical models, first-generation NAMPT inhibitors, such as FK866 (also known as APO866) and CHS828 (also known as GMX1778), were tested in cancer patients. The first clinical trial started in 1999 but stopped in 2012 because of severe side effects and inefficacy.

The most severe NAMPT inhibitors' dose-limiting toxicity was thrombocytopenia, gastrointestinal, haematological, retinal, and cardiac toxicity. They also exhibited poor and variable pharmacokinetics (PKs). These limitations underscore the need to broaden the therapeutic potential and increase the efficacy of NAMPT inhibitors, leading to the development of second-generation compounds; the most exploited strategies for drug discovery were: the design of dual-target inhibitors employing a multitarget approach; improving tumour targeting by utilising antibody-conjugated drugs (ADCs); mitigating NAMPT's enzyme-independent tumour-promoting effects with NAMPT degraders; and harnessing proteolysis-targeting chimaera (PROTAC) technology (**Wei et al., 2022; Galli et al., 2020; Tang et al., 2023**).

1.3.2 eNAMPT

In addition to acting intracellularly as an enzyme, NAMPT can be secreted into the extracellular space, being referred to as eNAMPT, where it is supposed to act as a cytokine (**Wu et al., 2022**). The first report of its extracellular localization dates back to 1994, when Samal et al. described eNAMPT as a cytokine able to enhance pre-B-cell colony formation in conjunction with IL-7 and stem cell factor. According to this activity, the protein was named "pre-B-cell colony-enhancing factor" (PBEF) (**Garten et al., 2009**).

Subsequently, in 2005, a study identified eNAMPT as visfatin, a protein secreted by adipocytes that binds to the insulin receptor and mimics insulin activity in obese patients.

Nowadays, according to the HUGO Gene Nomenclature Committee, we can refer to visfatin and PBEF using the common name of eNAMPT (**Tanaka et al., 2007; Dakroub et al., 2020**). eNAMPT is secreted by multiple types of cells, including adipocytes, hepatocytes, cardiomyocytes, immune cells, and various cancer cells.

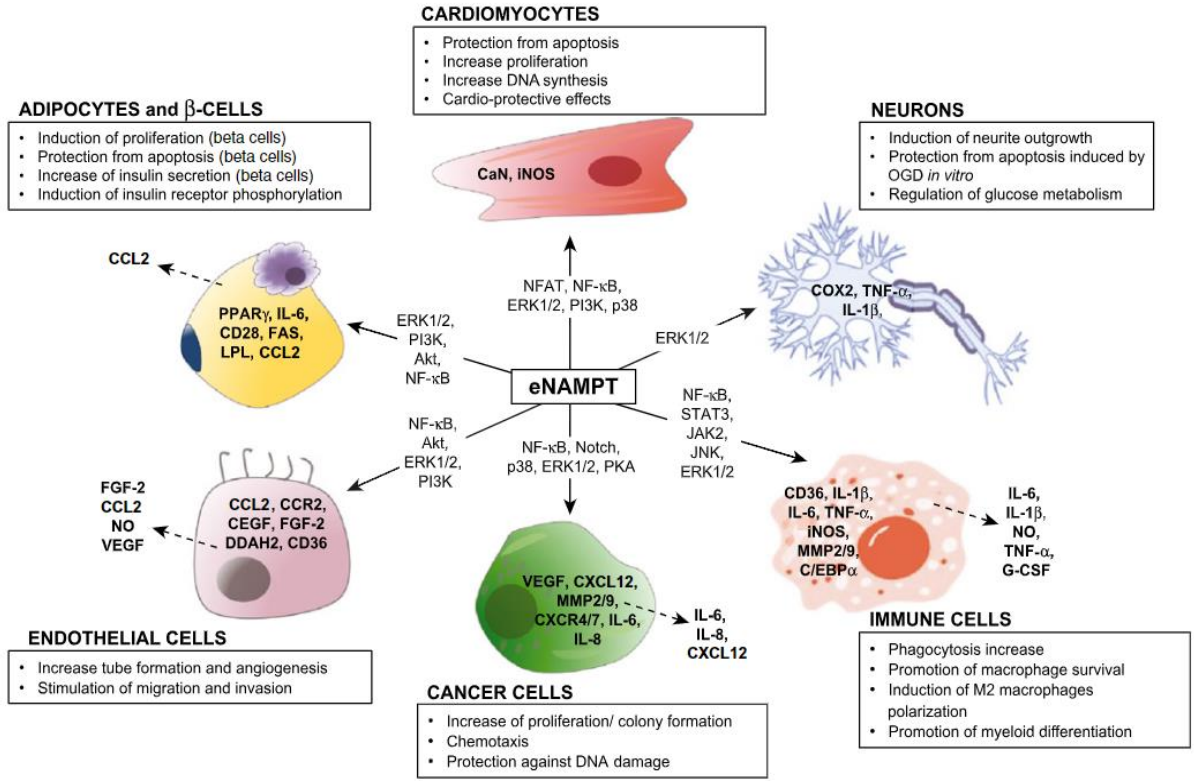


Figure 4: eNAMPT is produced by several cell types and works as a cytokine with autocrine and paracrine effects (Grolla et al., 2016)

The specific mechanism behind the release of eNAMPT is unclear due to its lack of a signal peptide sequence. Although Colombo et al. demonstrated that, in peritoneal macrophages (PECs), IFNγ-triggered eNAMPT release is impaired by brefeldin and monensin A (two

inhibitors of the classical ER-Golgi secretion pathway), thus suggesting it occurs *via* the canonical pathway (**Colombo et al., 2022**), currently the most accredited hypothesis identifies the "non-classical" secretory pathway as the main mechanism through which cells secrete NAMPT extracellularly (**Audrito et al., 2020**). Of note, eNAMPT has also been localised in secreted microvesicles (**Yoshida et al., 2019**).

As regards how the protein is directed towards extracellular release, the involvement of post-translational modifications, such as the deacetylation of lysine 53 operated by SIRT1, has been hypothesized by some authors (**Yoon et al., 2015**).

Serum NAMPT levels are elevated in pathological conditions such as obesity, non-alcoholic fatty liver disease, diabetes mellitus, and particularly in neoplasms. It is evident that metabolic stress conditions, such as hypoxia in melanoma cells and ischemia and oxygen-glucose deprivation (OGD) in neurons and glial cells, greatly stimulate eNAMPT release in these cells. eNAMPT is also released in response to inflammatory stimuli, such as pathogen-derived (LPS) and host-derived mediators (e.g., TNF-β, IL-1β, and IL-6) (**Grolla et al., 2016**).

Several studies support the hypothesis that eNAMPT engages an extracellular receptor; this seems to be confirmed by the significant and quick activation of several intracellular pathways, such as ERK and STAT3, and NMN's inability to duplicate the effect (**Travelli et al., 2018**).

The insulin receptor was the first hypothesised partner for eNAMPT (**Garten et al., 2008**); indeed, according to this study, eNAMPT would mimic the effects of insulin on adipogenesis, cellular glucose uptake, and blood glucose levels through binding to the insulin receptor. This raises concerns about its potential role in metabolic disorders, such as obesity and type 2 diabetes. However, there is currently no direct evidence of eNAMPT binding to the insulin receptor; in contrast, indirect molecular mechanisms are likely responsible for the insulin-like characteristics of eNAMPT. (**Garten et al., 2008**).

Another putative eNAMPT receptor is the C-C chemokine receptor type 5 (CCR5), as binding to this protein would block HIV entry into the cell (**Travelli et al., 2018**). However, more recently, it has been discovered that eNAMPT acts just as a natural antagonist of this receptor, suggesting that other receptors should be involved in transducing the activity of this cytokine (**Torretta et al., 2020**). In line with this speculation, Camp et al. showed that eNAMPT causes lung inflammation by directly interacting with Toll-like receptor 4 (TLR4) and triggering the activation of inflammatory pathways, including NF-κB (**Camp et al., 2015**). However, TLR4 isn't the only receptor that eNAMPT interacts with, as shown in a study conducted by Colombo et al., which investigated the impact of eNAMPT on myeloid cells. Indeed, it has been found

that eNAMPT-driven M1 macrophage activation occurs even in PECS derived from wild-type TLR4 knock-out (TLR4-KO) mice (**Colombo et al., 2022**). Further investigation is therefore required to determine if other receptors can mediate eNAMPT effects.

As already stated above, eNAMPT is elevated in a wide range of acute and chronic inflammatory conditions (**Audrito et al., 2020)**, therefore suggesting its use as a possible biomarker and therapeutic target in various diseases.

High levels of eNAMPT are found in serum and plasma of all cancer patients, solid or non-solid tumours; this condition appears to be linked to a more aggressive course of disease (**Grolla et al., 2016**). Thus, considering the association between eNAMPT and the worst prognosis, relying solely on iNAMPT inhibitors is no longer sufficient. As a result, strategies involving the direct neutralisation of eNAMPT using antibodies have become of paramount interest (**Audrito et al., 2020**).

This is the subject of the study conducted by Sun et al., in which treatments with a humanised monoclonal eNAMPT-neutralising antibody (ALT-100 mAb) were explored as a potential therapeutic approach to prevent the progression and reduce the lethality of prostate cancer (PCa). Also, Garcia et al. have developed a polyclonal neutralising antibody against eNAMPT that has proved to be useful in protecting against ventilator-induced lung damage (**Camp et al., 2015**).

In addition to the oncology field, the clinical value of an increase in eNAMPT levels has also been studied in the context of inflammatory diseases. Specifically, high serum levels of eNAMPT may help predict patients' unresponsiveness to anti-TNF therapies, as has been demonstrated for people affected by inflammatory bowel disease (IBD). In line with these findings, Colombo et al. developed a monoclonal eNAMPT-neutralising antibody named C269 that ameliorates symptoms in experimental murine colitis, either in acute or chronic conditions (**Colombo et al., 2020**).

According to these studies, the neutralisation of eNAMPT appears to be a potential treatment approach for a wide range of inflammatory and metabolic disorders (**Grolla et al., 2016**).

1.3.3 eNAMPT in breast cancer

The overexpression of both intracellular and extracellular NAMPT in cancer has revealed the dual role of this protein, both as a biomarker and promoter of tumour development.

As demonstrated for the intracellular form, eNAMPT seems to be involved in several types of cancer, including breast cancer. For instance, there is a correlation between eNAMPT serum

levels and TNM staging, histological grading, tumour volume, and lymph node metastasis in postmenopausal breast cancer women (**Assiri et al., 2015**).

eNAMPT is prominently present in the blood flow and tissues from mammary tumours and is associated with a worse clinical prognosis. It has been demonstrated that this protein plays a crucial role in the growth, invasion, and migration of breast cancer. In particular, invasive breast cancers overexpressing eNAMPT present with lymph node metastases (**Shackelford et al., 2013**).

It is common knowledge that angiogenesis plays a critical role in the progression of cancer and metastasis formation. eNAMPT regulates angiogenesis through mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3'-kinase (PI3K) pathways (**Adya et al., 2008**). It also upregulates those genes coding for matrix metalloproteinases (MMP-2, MMP-9) and vascular endothelial growth factor (VEGF), potentially promoting angiogenesis and metastasis in breast cancer (**Kim et al., 2010**).

Even inflammation participates in remodelling the tumour stroma, a process necessary for the effective migration and invasion of cancer cells. Confirming the crucial role of the tumour microenvironment in driving metastatic progression, eNAMPT promotes M2 differentiation in monocytic cells through the activation of the ERK/CXCL1 pathway and enhances EMT in breast cancer cells, followed by increased migration, tumour sphere formation, and stemness.

Given its role in cancer, eNAMPT is believed to contribute to autocrine, paracrine, and endocrine signalling (**Grolla et al., 2016**).

1.4 Epithelial-to-mesenchymal transition (EMT)

Epithelial-to-mesenchymal transition (EMT) is a complex biological process through which epithelial cells gradually assume a mesenchymal phenotype **(Lachat et al., 2021).**

EMT is part of physiological events such as embryonic development and wound repair process, in which inflammatory cells and fibroblasts are involved, but can also underlie pathological conditions such as fibrosis and cancer.

It is classified as type I EMT, related to embryologic development; type II EMT, which takes place during wound healing and fibrosis; and type III EMT, as it occurs in cancer cells **(Lachat et al., 2021).**

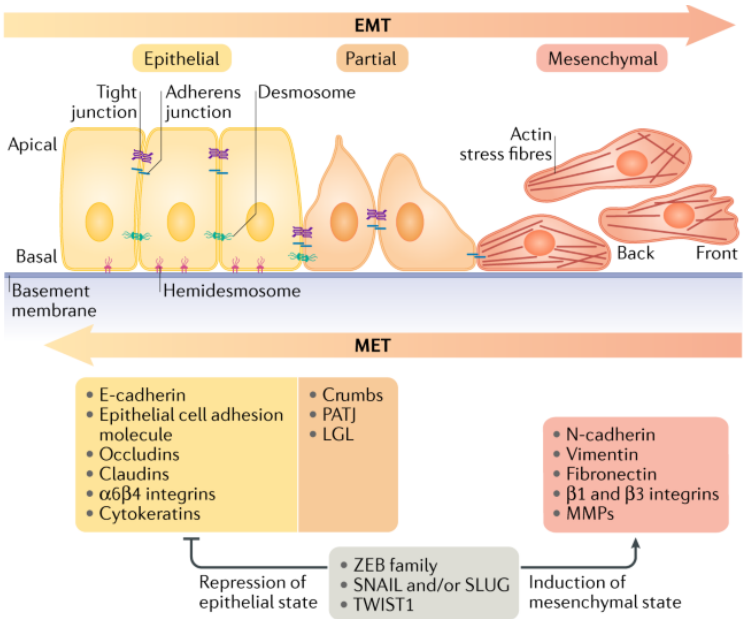


Figure 5: The epithelial-to-mesenchymal transition (EMT) consists of the activation of specific transcription regulators such as ZEB, SNAIL and TWIST, which inhibit the gene expression of molecules associated with the epithelial state (i.e. E-cadherin, Occludins, and Claudins) and induce the expression of genes associated with mesenchymal state (N-cadherin, Vimentin, and MMPs) (Dongre et al., 2019)

In the EMT process, tumour cells lose their epithelial properties (original polarity, adhesion molecule expression) and acquire mesenchymal properties (migratory capacity, invasiveness, resistance to apoptosis, secretion of matrix metalloproteases) **(Yan et al., 2018; Marconi et al., 2021).**

EMT and its opposite mechanism, named Mesenchymal-to-Epithelial Transition (MET), permit carcinoma cells to perform many of the stages of metastatic invasion of cancer cells, including the local invasion at the original tumour location, intravasation into blood vessels, migration *via* the bloodstream, extravasation into the parenchyma of distant tissues, and survival as micrometastatic deposits **(Zhang et al., 2018).**

EMT can be induced by multiple factors, such as cytokines and transforming growth factor β 1 (TGF-β1), a multifunctional cytokine that is responsible for cell differentiation, proliferation, and apoptosis and powerfully promotes EMT.

TGF-β1-induced EMT is mediated by three families of transcriptional repressors named Snail, ZEB, and Twist. These factors are activated through a Smad-dependent mechanism or an interaction with other pathways **(Yazaki et al., 2021).** They are responsible for the activation of mesenchymal markers including fibronectin, vimentin, and N-cadherin and the repression of epithelial markers like E-cadherin **(Elzamly et al., 2018)**. Indeed, a classic example of cancer-related EMT can be seen in the switch of cadherins from E- to N-type **(Gheldof et al., 2013).**

Of note, with regards to breast tumours, it has been observed that "basal-like" or "triple-negative" cancers, in particular, express high levels of vimentin, an intermediate filament protein typically found in mesenchymal cells and positively correlated with tumour invasiveness **(Elzamly et al., 2018)**. Moreover, among all types of breast cancer, TNBCs are the ones that most frequently express EMT markers, being also characterised by early recurrence, metastasis, and a poor prognosis.

Relevant to the purpose of this thesis project, it has been reported in the literature that eNAMPT induces EMT by increasing TGFβ1 production in the MCF10A cell line, which acquires a fibroblast-like morphology resulting from the down-regulation of E-cadherin and the up-regulation of N-cadherin, vimentin, and ZEB1.

It is therefore evident that developing new treatment approaches to target EMT and its consequences for cancer progression still requires a thorough understanding of these molecular interactions (**Soncini et al., 2014**).

2 PURPOSE OF THESIS

Breast cancer continues to be the most prevalent type of tumour worldwide and is responsible for the majority of cancer-related death among women (**Akram et al., 2017**).

It is a highly heterogeneous and resistant-to-treatment disease that results from the combination of several genetic changes and external circumstances that impact cellular processes (**Taurin et al., 2020**).

10–20% of breast cancer cases are represented by triple-negative breast cancer (TNBC), which presents the worst prognosis since its aggressiveness and the lack of specific receptors, such as receptors for oestrogen, progesterone, and HER2, make this tumour type particularly challenging to address with conventional therapies (**MacDonald et al., 2022**). In this context, it's important to find and develop new therapeutic targets to improve the available treatment options for TNBC patients.

Nicotinamide Phosphoribosyl Transferase (NAMPT) is an enzyme involved in one of the synthesis pathways of Nicotinamide Adenine Dinucleotide (NAD), but it is also secreted in the extracellular environment (named eNAMPT), where it exerts a cytokine-like action modulating the immune response (**Audrito et al., 2020**).

Elevated eNAMPT levels have been detected in several solid tumours, including breast cancer (**Gasparrini et al., 2022**). Hence our laboratory's interest in studying eNAMPT as a promising target for the treatment of TNBC.

My thesis specifically aims to investigate if eNAMPT is involved in metastasis formation by reprogramming triple-negative breast cancer cells.

3 MATERIALS AND METHODS

3.1 Cell culture

Murine mammary carcinoma cell lines 4T1 WT, 4T1 CL5 (a 4T1 subclone selected for its high potential for invasiveness and metastasis), EMT6, M158, and human breast cancer cell lines MDA-MB-231, MDA-MB-453, MDA-MB-468, MDA-MB-361, HCC38, and HCC70 were cultured, in adhesion on Petri dishes, in their respective growth mediums (Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS), 1% glutamine, and 1% penicillin/streptomycin. The media used for each cell line are detailed in Table 1.

For cell propagation as monolayers, culture media were removed, cells were washed with phosphate-buffered saline (PBS), detached with trypsin, and reseeded on Petri dishes, after removal of trypsin, and stored in an incubator supplied with 5% CO_2/95% air at 37°C.

Cell line	Medium
4T1	MEM
4T1 CL5	MEM
EMT6	DMEM
M158	DMEM
MDA-MB	DMEM
MDA-MB	RPMI
MDA-MB	DMEM
MDA-MB	DMEM
HCC38	RPMI
HCC70	RPMI

Table 1: cell lines and media. MEM, Minimum Essential Medium; DMEM, Dulbecco's Modified Eagle's Medium; RPMI, Roswell Park Memorial Institute Medium.

3.2 Western blot

Cell counting was performed using the Bürker Counting Chamber. $2x10^5$ cells were plated into a 12-well plate and treated for different time lengths according to each experiment protocol. After the treatment, cells were gently washed with PBS and added with 1 mL of RIPA lysis buffer (Tris-HCl 50nM pH=7.2, NaCl 150mM, EDTA 0.5mM, NP40 0.1%), supplemented with a cocktail of protease (PIC 1M, PMSF 100nM) and phosphatase (NaF 1M, Na_3VO_4 1M) inhibitors. The lysis was performed using a plastic cell scraper. Lysates were collected into microfuge tubes and centrifuged at 13,000 rpm for 15 minutes at 4°C. Bradford protein assay (Sigma-Aldrich) was performed to obtain the protein quantification.

Samples were resolved through 10% SDS-PAGE and transferred to a nitrocellulose membrane (Trans-Blot Turbo Transfer System, Bio-Rad).

Membranes were then saturated with a milk-blocking buffer (5% milk in TBS/0.1% Tween composed of TRIZMA BASE 121g and NaCl 90g), for 1 hour at room temperature, to saturate unspecific binding sites and incubated, overnight at 4°C, with the primary antibody. Anti-Vimentin (Invitrogen), anti-E-cadherin, anti-N-cadherin, anti-Zeb1, anti-Snail (Cell Signalling), and anti-β-Actin (Sigma-Aldrich) primary antibodies were used.

After 1 hour of incubation with the secondary antibodies (anti-mouse or anti-rabbit), enhanced chemiluminescence (ECL) solution (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific) was added to the membrane, and the corresponding signal was detected using the ChemiDoc Imaging System (Bio-Rad).

Densitometry was analysed using the Image Lab BioRad programme.

3.3 Analysis of eNAMPT release in cell medium

$6.0x10^5$ cells were plated into a 6-well plate for 24 hours. The day after, the fully-complemented medium was removed, and cells were incubated for 4 hours with a serum-free medium. Next, the medium was collected and centrifuged at 1200 rpm for 5 minutes at 4°C. Supernatants were concentrated using 30 kDa Cut-Off filters (VIVASPIN500 Sartorius, Germany). Fifteen-microliters aliquots of supernatant were collected to perform Western Blot analysis. The anti-NAMPT primary antibody (AdipoGen) was used to quantify eNAMPT levels.

3.4 Generation of *in vivo* mammary carcinoma model

In vivo animal experiments were authorised by the animal ethical committee of Università del Piemonte Orientale.

$7.0x10^3$ 4T1 CL5 cells were injected into the mammary gland fat pat of female Balb/C mice previously anaesthetized with 4% isoflurane (Sevoflurane). Tumour growth was monitored starting from day 15.

Mice were sacrificed on days 7, 14, and 28. Mice were weighed on the day of sacrifice, and the tumour mass, lungs, and blood were collected.

Solid tissues were subjected to mechanical and enzymatic dissociation, involving incubation with collagenase at 4°C for 30 minutes and filtration through cell strainers. Blood was centrifuged, plasma was stored for further analysis, and cells were collected.

Tumour, lung, and blood cells were seeded into a 6-thioguanine-rich medium to perform the assay for “clonogenicity”.

4 RESULTS AND DISCUSSION

4.1 Preliminary section

The 4T1 murine breast cancer cell line is well-known for its severe degree of invasiveness and predisposition to spontaneous metastasis, especially in the lungs, closely matching the behaviour of human triple-negative breast carcinoma.

My thesis laboratory engineered a specific subclone of this cell line (i.e., 4T1 CL5) capable of releasing a massive quantity of extracellular NAMPT without altering its intracellular counterpart. To generate this cell line, cells have been infected with a lentiviral vector carrying the green fluorescent protein (GFP) gene, used to monitor the infection, and the NAMPT gene fused, at the 5'-end, with the sequence coding for the signal peptide (SP) of the immunoglobulin, in order to allow the constitutive release of the chimeric protein. Accordingly, this cell line was re-named 4T1 SP-NAMPT.

Notably, a FLAG®-tag was added to the 5'-end of the SP sequence to allow exogenous eNAMPT discrimination from the native protein.

A "control" cell line was also generated, named Scramble (SCR), which was transduced with a lentiviral vector carrying only the GFP gene.

4.2 eNAMPT increases the metastatic potential of 4T1 cells *in vivo*

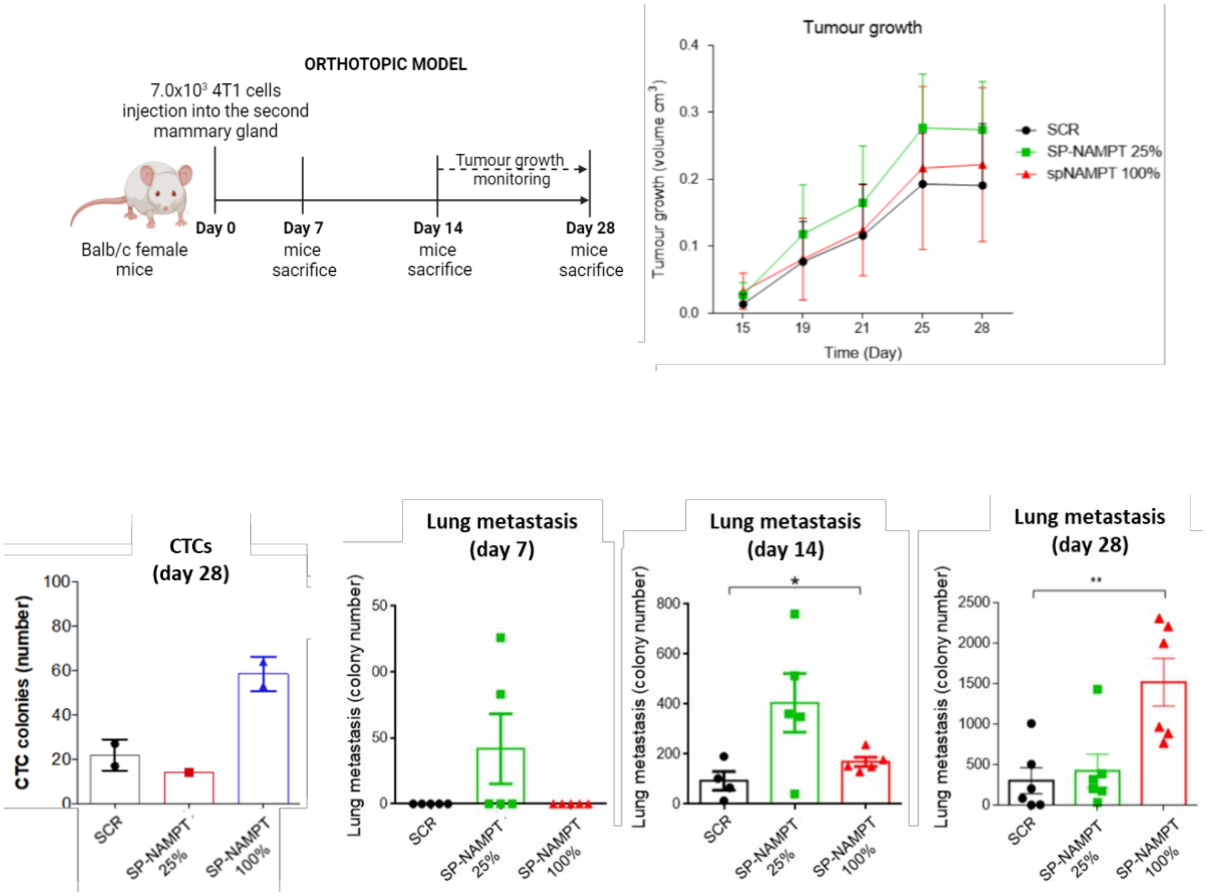


Figure 1. Generation of the murine 4T1 SP-NAMPT model of breast cancer; tumour growth was monitored between days 14 and 28 (A); blood cells and dissected lung tissue were plated into 6-thioguanine-enriched medium (taking advantage of 4T1 resistance to 6-thioguanine); and the number of colonies was calculated performing the clonogenic assay (B).

The 4T1 SCR and SP-NAMPT cells were used to create an orthotopic *in vivo* model of breast cancer in mice to evaluate whether eNAMPT is able to affect tumour growth and the metastatic potential of the 4T1 cell line.

By injecting different percentages of SCR and SP-NAMPT cells into the mammary gland pads of female Balb/C mice, we generated three experimental groups, named:

- SCR, composed of mice injected only with SCR cells;
- SP-NAMPT 25%, where grafts were obtained using 25% of SP-NAMPT cells and 75% of SCR cells
- SP-NAMPT 100%, injected exclusively with SP-NAMPT cells.

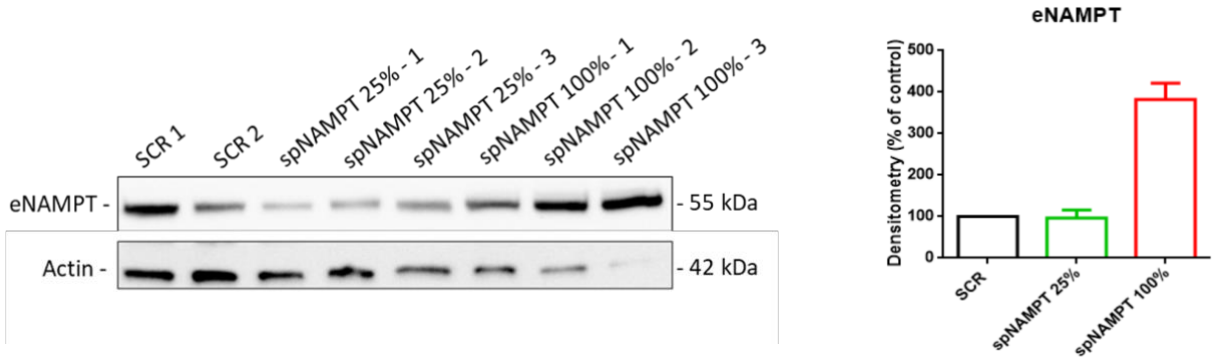
To evaluate the tumorigenicity of these cells, tumour mass outgrowth was monitored between days 14 and 28, and sacrifices were carried out on days 7, 14, and 28. Moreover, on the day of sacrifice, tumours, lungs, and blood cells were collected and seeded into a 6-thioguanine-rich medium (taking advantage of 4T1 resistance to 6-thioguanine) to perform the "clonogenic assay".

As shown in Fig. 1, although any variation in tumour volume was documented between the three experimental groups, a higher number of lung metastases was generated in the SP-NAMPT 25% group on days 7 and 14, and in the SP-NAMPT 100% group at day 28.

Regarding the circulating tumour cells (CTCs), i.e., that subset of neoplastic cells with metastatic potential detectable in the bloodstream, they were increased at day 28 in SP-NAMPT 100% mice, suggesting that the more elevated dose of eNAMPT may allow tumoral cells to spread from the primary mass.

In other words, data obtained from *in vivo* experiments suggested that tumours exposed to a NAMPT-enriched microenvironment may increase their migratory capacity, making it easier to reach other districts of the organism.

A



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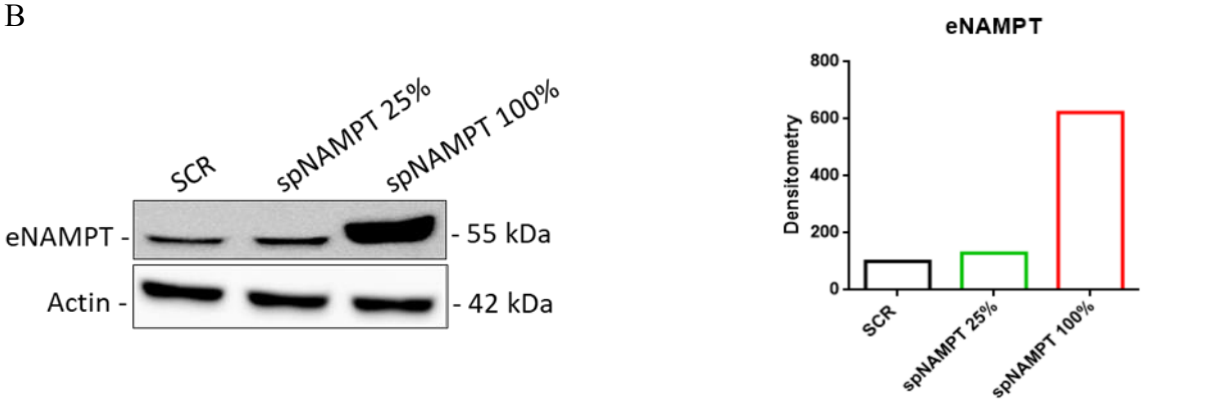


Figure 2. Western Blot analysis of eNAMPT release on tumours (A) (n = 1) and circulating tumour cells (B) (n = 4) after 28 days of in vivo experiment. The densitometric analysis of the bands is related to the Actin of each sample. Actin is a structural protein used in gene housekeeping to normalise gene expression of the protein.

Of note, *ex vivo* assessment of eNAMPT levels using the Western blot technique allowed us to demonstrate that tumour-derived cells and the CTCs preserved their ability to produce and release consistent amounts of this cytokine even after 28 days of *in vivo* growth (Fig. 2A and B).

4.3 Acute treatment of 4T1 CL5 cells with recombinant NAMPT (rNAMPT) does not induce changes in EMT markers

Inspired by the results of in vivo experiments, we were interested in discerning whether the increase in lung metastasis in SP-NAMPT 100% mice could be due to a direct influence of eNAMPT on tumour cells. In this regard, we treated 4T1 CL5 wild-type (WT) cells with rNAMPT to investigate the mechanism behind the increase in metastasis formation induced by eNAMPT.

As already discussed in the introduction, EMT is a pivotal mechanism employed by tumour cells to undergo phenotypic alterations, transitioning from an epithelial state to a mesenchymal one. This transition enables them to have enhanced mobility and invasive capabilities, particularly towards new tissues and organs (***Park et al., 2022***).

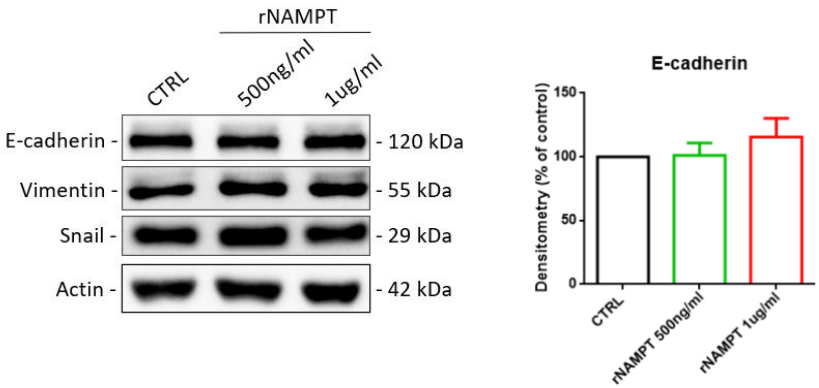
Based on these assumptions, my specific involvement in the project was to study the expression of key markers associated with the EMT process, such as E-cadherin among the epithelial markers and Vimentin and Snail1 for the mesenchymal signature.

First of all, to explore the timing and dose of eNAMPT that may induce phenotypic changes in tumour cells, I adopted two different strategies:

- 24-hour treatments (fig. 3 A) of cells with two different concentrations of rNAMPT (500 ng/mL and 1 µg/mL),
- time course of treatments (12, 24, and 48 hours) with 1 µg/mL rNAMPT.

However, in both experimental settings, no significant modulation was documented upon treatment with rNAMPT, suggesting that, at least, a single, short exposure to this cytokine is not able to induce EMT in our cell model.

A



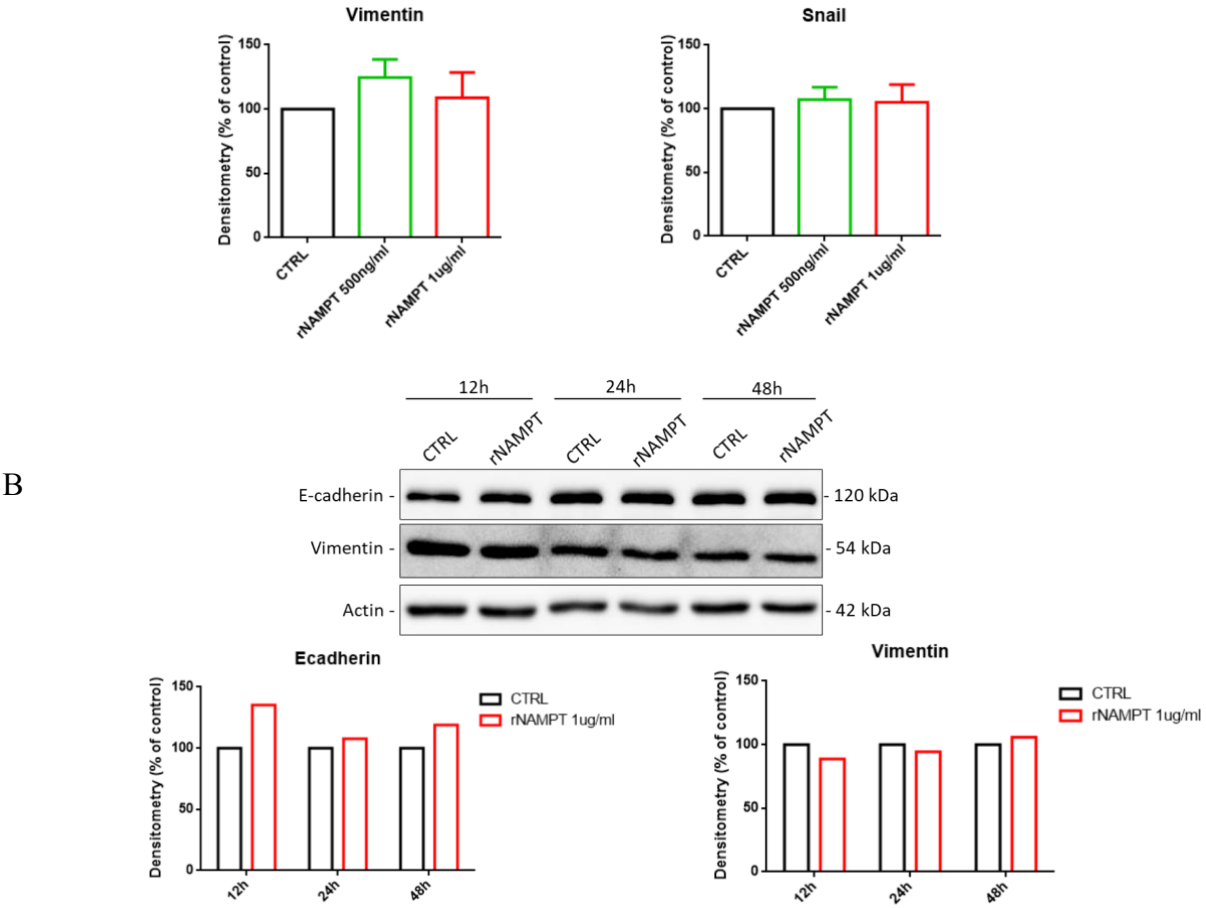


Figure 3. Western blot analysis of EMT-markers expression of 4T1 CL5 cells treated with rNAMPT for 24 hours (A) (n = 4), and for 12, 24, and 48 hours (B) (n = 1).

The densitometric analysis of the bands is compared to the Actin in each sample and then analysed by setting the CTRL to 100%. Actin is a structural protein used in gene housekeeping to normalise gene expression of the protein.

4.4 Distinct breast cancer cell lines release different amounts of eNAMPT

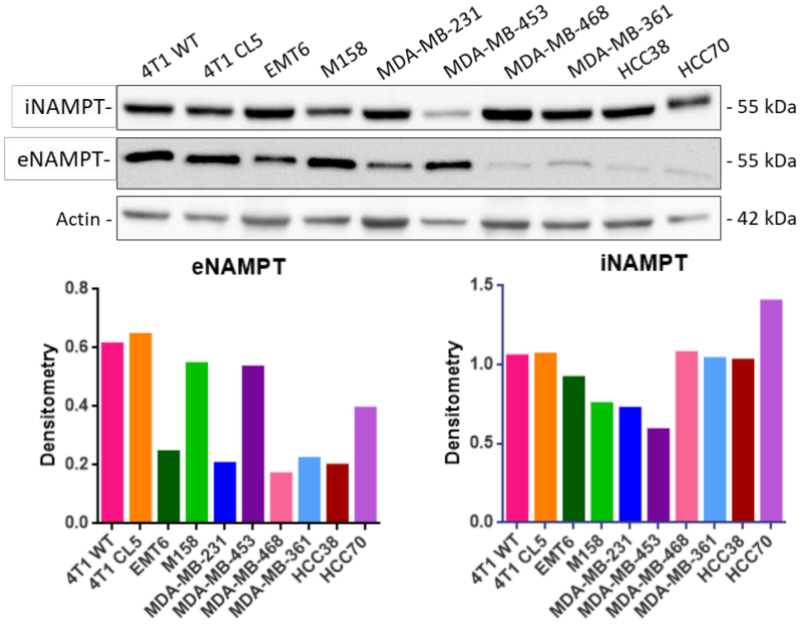
Given the insufficient results obtained with 4T1 cells in the acute setting, I measured eNAMPT levels in a panel of breast cancer cell lines in an attempt to identify a correlation between the epithelial/mesenchymal phenotype and the levels of eNAMPT. In this regard, Western blot analysis was performed to evaluate, contemporaneously, the protein levels of eNAMPT and of E-cadherin, Vimentin, and Snail1, which were used as phenotype signatures (fig. 4 A-B)

All the cell lines tested were cultured under 2D growth conditions in the absence of rNAMPT, aiming to delineate their behaviour under basal conditions.

The data obtained are summarised in the table in Figure 4C, in which the phenotype and the origin of each cell line were annotated as reported in the literature.

As it is evident from the histograms in Fig. 4A, the release of eNAMPT is characterised by high variability between the cell lines and no apparent correlation with the expression of epithelial *vs* mesenchymal markers.

A



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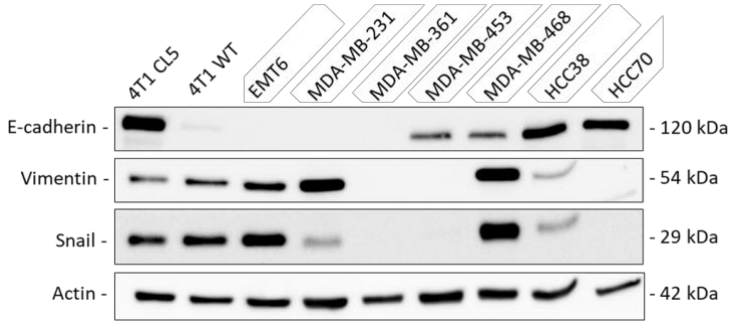
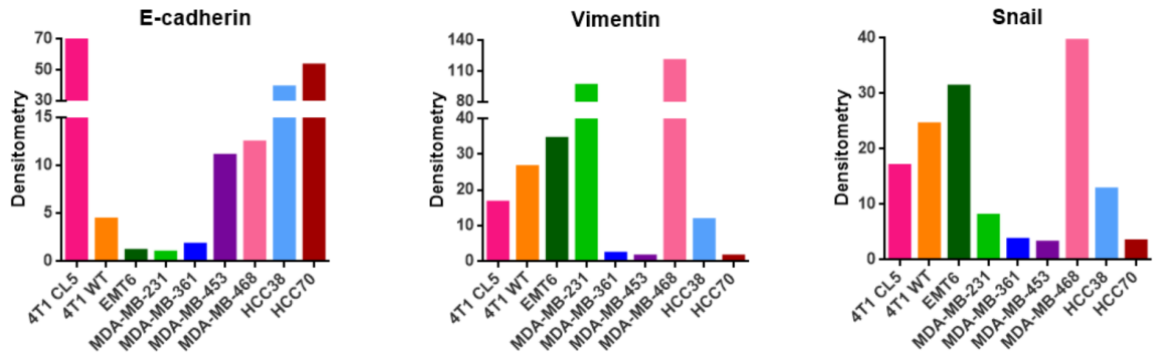




C

	E-cadherin	Vimentin	eNAMPT levels	Characteristics from literature	Derivation
4T1 WT			HIGH	Mesenchymal	Mammary tumour of Balb/c mouse
4T1 CL5			HIGH	Mesenchymal	A more aggressive clone of 4T1 cell line
EMT6			LOW	Epithelial	Alveolar nodule of Balb/c mouse
MDA-MB-231			LOW	Mesenchymal	Human pleural effusion
MDA-MB-361			HIGH	Mesenchymal	Human brain
MDA-MB-453			LOW	Mesenchymal	Human mammary gland
MDA-MB-468			HIGH	Mesenchymal	Human pleural effusion
HCC38			LOW	Epithelial	Human mammary gland
HCC70			HIGH	Epithelial	Human ductal carcinoma

20 40 60 80 100 120

Figure 4: Western Blot analysis of eNAMPT release (A) and EMT marker expressions (B) in different breast cancer cell lines (n = 1); table summarising data obtained on E-cadherin and Vimentin expression, release of eNAMPT, and characteristics from the literature of all the various cell lines considered (C).

The densitometric analysis of the bands is compared to the Actin in each sample. Actin is a structural protein used in gene housekeeping to normalise gene expression of the protein.

Nevertheless, it should be noted that all these cell lines are reported in the literature as metastatic breast cancer models, while an elevated expression of E-cadherin has been also documented in highly invasive tumour types (**Jinesh et al., 2017**), thus questioning the hypothesis according

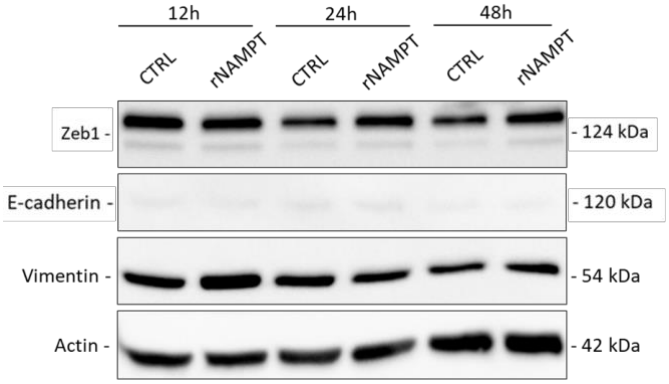
to which only the mesenchymal phenotype is associated with the metastatic potential of these tumours. Indeed, this comes to light when analysing the data summarised in Fig. 4C.

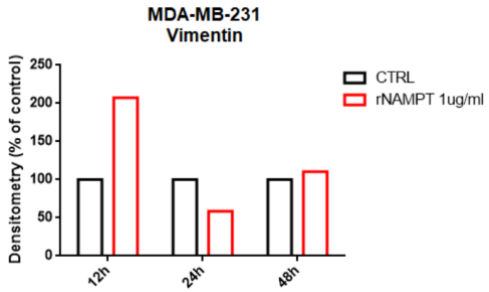
4.5 Short-timing treatments with rNAMPT do not induce EMT marker modulations in breast cancer cell lines.

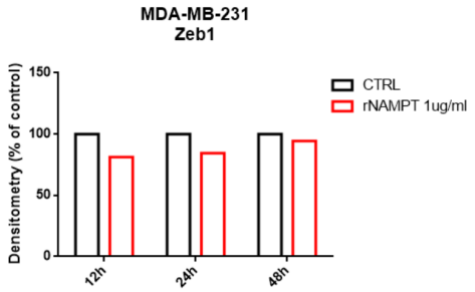
The high variability in the expression of E-cadherin and Vimentin, as well as in the release of eNAMPT, highlighted by previous experiments, suggests that the expression of a specific EMT marker compared to another depends, above all, on the type of cell line tested. Therefore, I decided to perform further experiments using only three cell lines: the EMT6 and M158 cell lines, as mouse cell models that release low and high levels of eNAMPT,respectively; and MDA-MB-231, a human breast cancer cell line chosen to highlight possible differences between the murine and human contexts.

Cell lines were treated with rNAMPT (1 µg/mL) at different time points (12, 24, and 48 hours) in order to evaluate, *via* Western blot, the expression of EMT markers, as already done for the 4T1 CL5 cell line.

A







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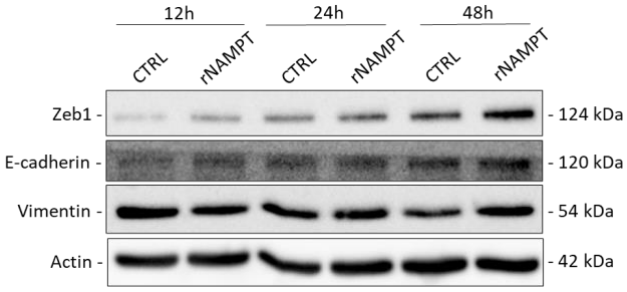
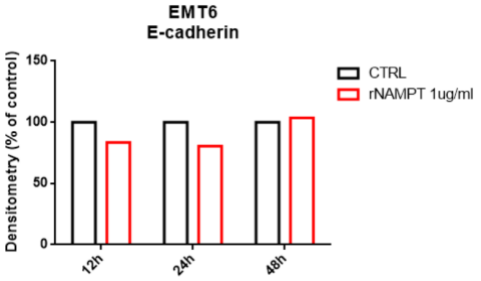

12h
24h
48h
CTRL
rNAMPT
CTRL
rNAMPT
CTRL
rNAMPT
Zeb1 -
E-cadherin -
Vimentin -
Actin -
- 124 kDa
- 120 kDa
- 54 kDa
- 42 kDa


EMT6
E-cadherin
Densitometry (% of control)
150
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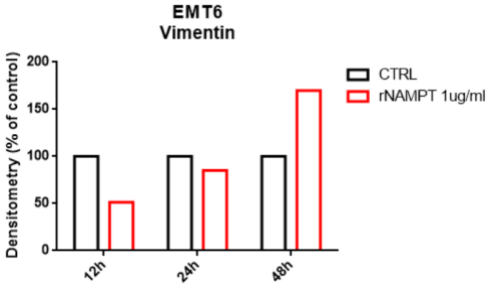
50
0
12h
24h
48h
CTRL
rNAMPT 1ug/ml


EMT6
Vimentin
Densitometry (% of control)
200
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12h
24h
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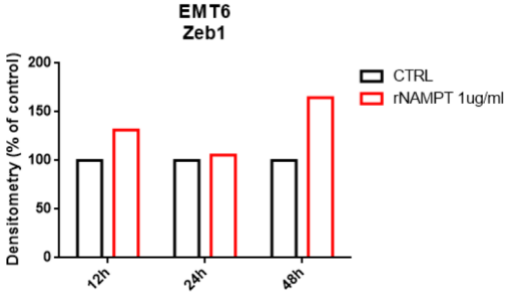
rNAMPT 1ug/ml


EMT6
Zeb1
Densitometry (% of control)
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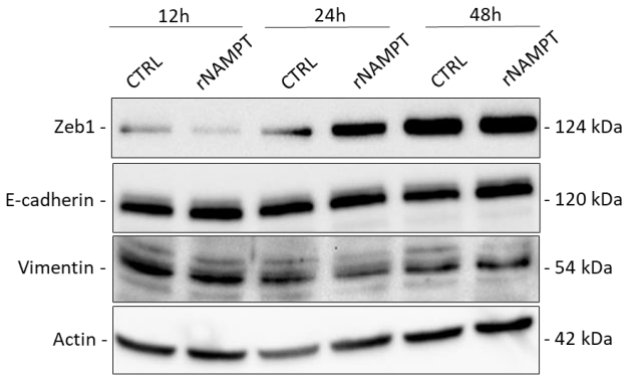
12h
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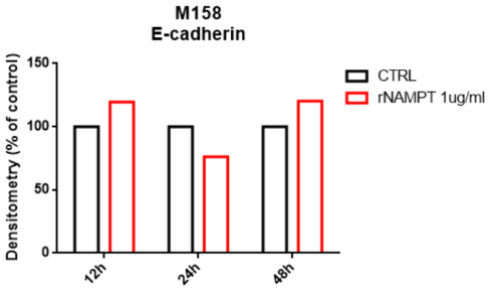
Zeb1 -
E-cadherin -
Vimentin -
Actin -
- 124 kDa
- 120 kDa
- 54 kDa
- 42 kDa


M158
E-cadherin
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rNAMPT 1ug/ml

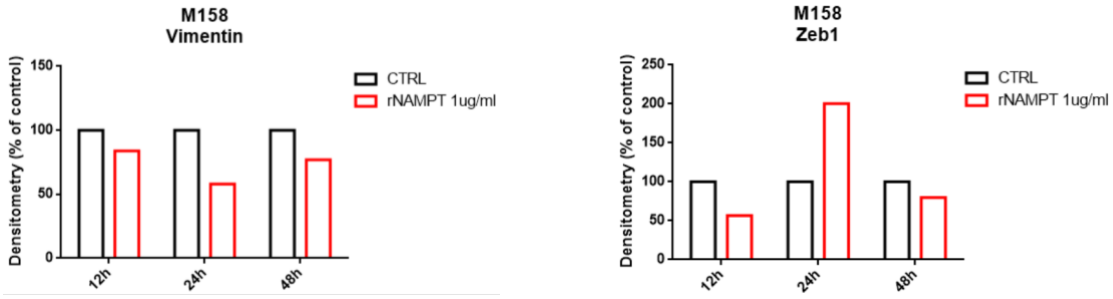

Figure 5. Western blot analysis of EMT marker expressions in MDA-MB 231 (A), EMT6 (B), and M158 cells (C) treated with 1μ rNAMPT at 12, 24, and 48 hours; cells treated only with the "NAMPT buffer" were used as controls (n = 1). The densitometric analysis of the bands is compared to the Actin in each sample and then analysed by setting the CTRL to 100%. Actin is a structural protein used in gene housekeeping to normalise gene expression of the protein.

In the literature, evidence suggests that eNAMPT induces EMT by increasing TGFβ1 production. TGF-β1-induced EMT is mediated by transcriptional repressors such as Snail and ZEB **(Yazaki et al., 2021)**, thus making it even more relevant to examine the role of Zeb1 in this context**.**

In addition to evaluating the expression of E-cadherin Vimentin and Snail, we also considered the expression of another EMT marker, the Zinc finger E-box binding homebox 1 (Zeb1) protein, as this is responsible for transcriptional repression of E-cadherin (**Wu et al., 2020**), providing further insight into the characterization of the epithelial-to-mesenchymal transition process.

The data represented in Figure 5 show that short exposure to rNAMPT does not induce a significant modulation of EMT markers in any of the cell lines analysed, confirming the results obtained with 4T1 CL5 cells.

Of note, for MDA-MB-231 cells, it was not possible to detect the expression of E-cadherin, most likely due to their strong mesenchymal commitment.

4.6 Chronic exposure of 4T1 CL5 cells to eNAMPT causes a significant modulation of EMT markers

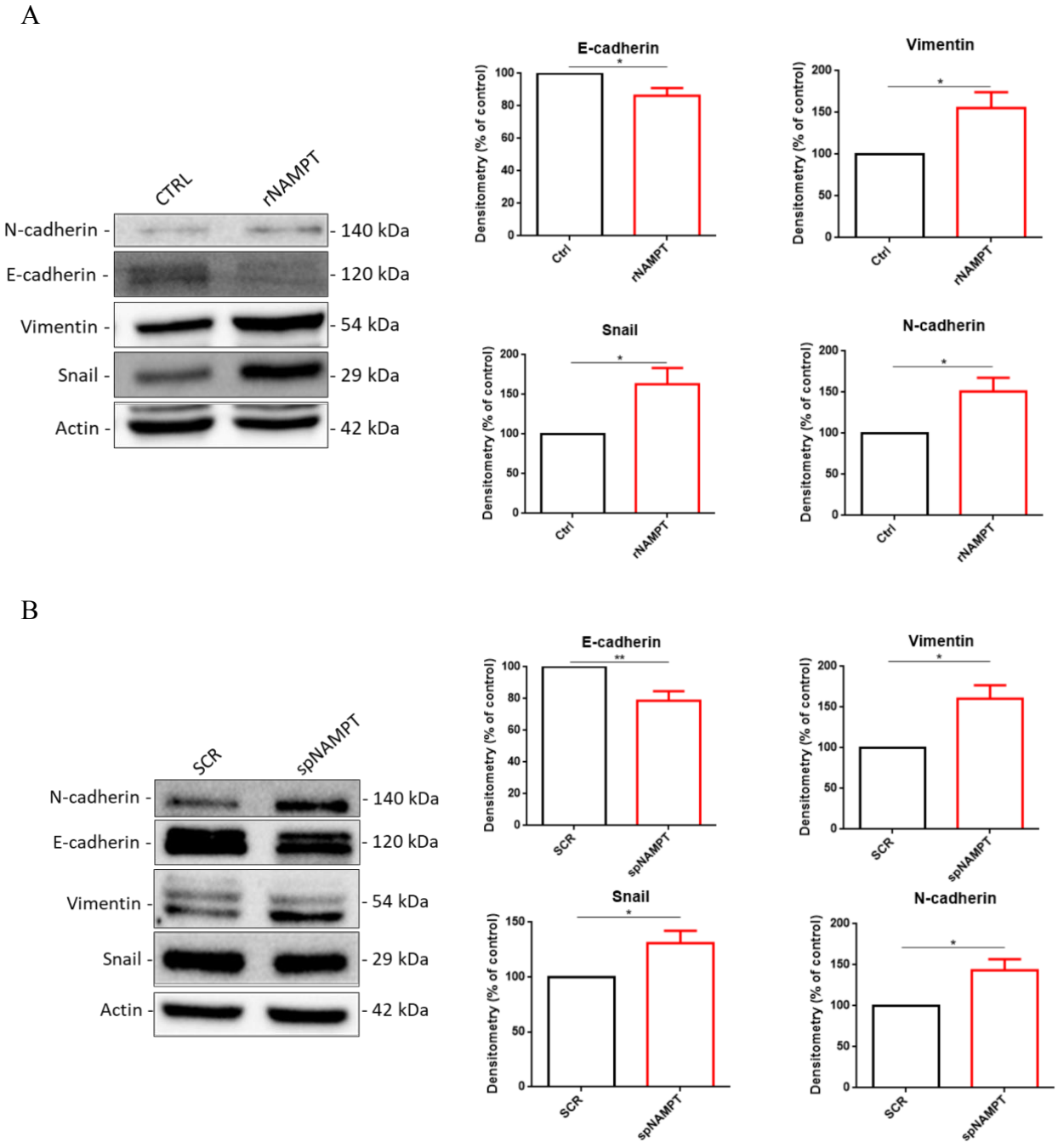

Figure 6. Western blot analysis of EMT marker expressions in 4T1 CL5 cells treated with rNAMPT for 7 days or NAMPT buffer as control (A); Western Blot analysis of EMT marker expressions in 4T1 SCR and SP-NAMPT cells (B).

Given that, in all the cell lines tested, a single acute treatment with rNAMPT was not able to modulate the expression of EMT markers (up to 48 hours post-administration), I decided to evaluate the effect of chronic treatments. This was also inspired by Soncini et al.'s article in

which MCF10A cells were exposed to eNAMPT for 14 days, suggesting that this cytokine needs a longer time to exert its effects in tumour reprogramming.

In particular, two different strategies were adopted: i) the administration of exogenous rNAMPT1 (μg/mL) to 4T1 CL5 WT cells every two days, for seven days in total; ii) the use of the 4T1 SP-NAMPT cell line, which can secrete eNAMPT continuously for the entire length of the experiment (3 weeks).

In this set of experiments, the evaluation of EMT markers was extended to N-cadherin and Snail1. One of the hallmarks of EMT is indeed the switch between E-cadherin and N-cadherin expression (the latter being a typical mesenchymal marker), as well as the activation of the transcription factor Snail1 (**Yazaky et al., 2021**). Of note, Zeb1 is poorly expressed in 4T1 cells and therefore was not evaluated.

Western blot analysis of EMT marker expressions (Fig. 6A) revealed that seven-day treatments with rNAMPT induce an increase in E-cadherin levels and a decrease in Vimentin, N-cadherin, and Snail1 expression, according to the conventional EMT process. Importantly, the same results were obtained also by culturing the 4T1 SP-NAMPT cell line for three weeks (Fig. 6B), a timing that reproduces the duration of the *in vivo* experiments; in this case, a comparison was made with the expression of the same EMT markers in the SCR cell line, kept in culture for the same amount of time.

Taken together, all these data suggest that tumour cells submitted to an eNAMPT-enriched microenvironment are more likely to escape from the primary mass, enter the circulation system, and subsequently reach secondary metastatic sites, such as the lungs. Moreover, we suggest that EMT reprogramming may be the mechanism acting behind the acquisition of the metastatic potential of breast cancer cells, well documented *in vivo* since chronic exposure of 4T1 CL5 WT cells to eNAMPT modifies the expression of EMT markers towards a mesenchymal phenotype.

Nonetheless, EMT is an extraordinarily complex process in which tumour cells can exhibit different characteristics, resulting in a varied landscape linked to their phenotype and their capacity for invasion and metastasis (***Park et al., 2022***). Thus, it is evident that the effect of eNAMPT on the expression of distinct EMT markers, such as E-cadherin and Vimentin, may change depending on the cellular model investigated.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The specific mechanism through which eNAMPT influences breast cancer development must be further explored but existing evidence suggests its potential role in promoting cell survival, proliferation, or other pathways contributing to the oncogenic phenotype **(Audrito et al., 2020)**. The results obtained offer insights into the impact of eNAMPT on tumour reprogramming towards a more metastatic phenotype, both *in vivo* and *in vitro*.

Although there appears to be no significant change in the size of the primary tumour, *in vivo* data reveal a substantial increase in lung metastasis formation in mice transplanted with SP-NAMPT 4T1 cells. Additionally, the elevated presence of circulating tumour cells in this cohort of mice after 28 days from transplantation suggests that high levels of eNAMPT may facilitate the migration of tumour cells from the primary mass to the metastatic site.

As stated in the introduction, EMT is an extraordinarily complex process through which tumour cells lose their epithelial characteristics and acquire mesenchymal features, resulting in a wide range of phenotypes and propensity to invasion and metastasis (***Park et al., 2022***).

However, in this regard, rNAMPT did not induce significant changes in EMT markers, at least *in vitro,* when 4T1 WT cells were treated acutely. This suggests either that short-term treatments are not sufficient for phenotypic modifications in tumour cells, or that EMT requires other signals originating from the tumour microenvironment.

On the other hand, assessment of eNAMPT release by various breast cancer cell lines reveals substantial variability in the levels of this cytokine with any clear association with a specific EMT phenotype. It is therefore evident that the differential expression of EMT markers such as E-cadherin and Vimentin is cell-type specific.

Conversely, it appears that chronic exposure (seven days) of 4T1 WT cells to rNAMPT causes a significant modulation of EMT markers (e.g. increased levels of E-cadherin and decreased levels of Vimentin, N-cadherin, and Snail 1), suggesting that only reiteration of treatments with this cytokine is needed to reprogram breast cancer cells.

Taken together, our results suggest that tumour cells exposed to an eNAMPT-enriched microenvironment exhibit an enhanced ability to escape from the primary tumour mass, thus entering the bloodstream to reach distant secondary sites, while EMT may be required for acquisition of this metastatic potential.

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