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TESI DI DOTTORATO

EVALUATION OF DRIVERS OF METASTATIC DISSEMINATION

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ABSTRACT

Neutrophil extracellular traps (NETs) are complex structures released by activated neutrophils that have been reported to be involved in metastatic dissemination. The aim of our study was to evaluate cancer cell adhesion to NETs and to determine whether cancer cell exposure to NETs can trigger the epithelial-to-mesenchymal transition (EMT), thereby promoting increased migration and invasion of tumor cells.

Various cancer cell lines were subjected to a solid-phase adhesion assay using plates coated with NETs, with or without the inclusion of antibodies against the α5β1 Integrin or CCDC25 receptor. After incubation for 1 or 2 hours, the proportion of adherent cells was calculated in relation to the total cell number. To evaluate the induction of EMT, cancer cells were treated with NETs for up to 48 hours and cell lysates were then subjected to western blot for the evaluation of E-cadherin, Vimentin, Snail, Slug, ZEB 1, and TWIST 1 protein levels, as well as Notch 1 and cleaved Notch 1. Moreover, untreated and NET-treated cancer cells underwent migration assays using transwell inserts in 24-well plates, with fetal bovine serum (FBS) serving as a chemoattractant.

Adhesion of cancer cells to NET-coated plates ranged between 54% and 93% that was significantly higher than that observed in uncoated plates. The addition of blocking antibodies targeting α5β1 or CCDC25 receptors markedly inhibited cell adhesion to NETs. Prolonged exposure of EGFR-driven lung cancer cell line to NETs led to EMT activation that was shown to be mediated by the upregulation and activation of Notch 1 and confirmed by elevated levels of EMT markers. This transition resulted in a notable loss of epithelial characteristics and a corresponding reduction in the expression of the oncogenic driver. Furthermore, cell migration was significantly enhanced in NET-treated cells compared to untreated controls.

Our study showed the dynamic role of NETs that may provide a microenvironment rich in DNA and fibronectin, facilitating the adhesion of many cancer cells at distant locations where prolonged exposure to NETs initiates EMT by activating the Notch 1 signalling pathway, thereby increasing the migratory and invasive potential of cancer cells. Moreover, these findings provided insight into how the immune and inflammatory microenvironment can directly alter the sensitivity of cancer cells to oncogene-targeted agents.

Key words: **NETs; EMT; Adhesion of cancer cells; Metastasis; Migration; TME.**

ABBREVIATIONS

8-OHdG	8-hydroxy-2'-deoxyguanosine
CAFs	cancer-associated fibroblasts
CCL3	chemokine (C-C motif) ligand 3
$CD4^+$ T	T helper cells
$CD8^+$ T	cytotoxic T cells
cfDNA	cell-free DNA
citH3	citrullinated histone H3
CTCs	circulated tumor cells
CTSC	cathepsin C
CXCL-1	chemokine (C-X-C motif) ligand
CXCR2	CXC chemokine receptor 2
E-Cadherin	epithelial cadherin
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
EVPs	extracellular vesicles and particles
FBS	fetal bovine serum
G-CSF	granulocyte colony-stimulating factor
HCC	hepatocellular carcinoma
HMGB1	high mobility group box 1
IL	interleukin

M1	anti-tumor macrophages
M2	pro-tumor macrophages
MET	mesenchymal-epithelial transition
MPO	myeloperoxidase
N1	anti-tumor neutrophils
N2	pro-tumor neutrophils
N-Cadherin	neural cadherin
NE	neutrophil elastase
NETs	neutrophil extracellular traps
NSCLC	non-small-cell lung cancer
PMN	pre-metastatic niche
ROS	reactive oxygen species
TAMs	tumor-associated macrophages
TANs	tumor-associated neutrophils
TGFβ	transforming growth factor beta
TLR9	Toll-like receptor 9
TME	tumor microenvironment
TNFα	tumor necrosis factor
TWIST	twist basic helix-loop-helix transcription factor
ZEB	Zinc-finger E-box-binding homeobox

1. INTRODUCTION

Cancer is one of the leading causes of morbidity and mortality worldwide, with metastasis being responsible for approximately 90% of cancer deaths (1, 2). The formation of metastases occurs through a multi-step process that allows the spread of cancer cells from the location of the primary tumor to distant sites in the body. Primary tumors before their spreading can often be successfully cured. On the contrary, cancer that extends beyond its original site is often resistant to treatment and almost inevitably evolves into a terminal illness. Despite the significant progress in understanding the mechanisms of metastatic dissemination, treatment of metastatic disease remains an unmet need (2, 3).

During the metastatic cascade, cancer cells are reported to undergo a sequence of events including detachment from the primary tumor, invasion of surrounding stroma, intravasation, dissemination through blood vessels, extravasation and colonisation of distant organs (4, 5). These steps could be interconnected and influenced by a variety of intrinsic biochemical, biological and molecular factors as well as microenvironmental conditions (6).

The first step of metastatic spread is the detachment of the cancer cells from the primary tumor (2). In anchorage-dependent cells, detachment from the extracellular matrix (ECM) or disruption of cell-cell interactions, induces a process called anoikis, a type of programmed cell death. In this way, it is ensured that detached cells do not spread far from their primary place to other incorrect location (7-9). In order to survive cell detachment from ECM and avoid anoikis, metastatic cells need to acquire new properties. In the context of malignant epithelial tumors, the activation of the epithelial-mesenchymal transition program is the most frequent mechanism used by cancer cells to change their features. In fact, EMT allows the acquisition of mesenchymal traits and loss of epithelial phenotype that ultimately promotes anchorage-independent survival, migration, invasion and ability to degrade ECM (2, 10).

1.1. Epithelial-mesenchymal transition

The epithelial-mesenchymal transition (EMT) is a fundamental process for embryogenesis, development, wound healing and tissue regeneration. Apart from physiological conditions, EMT is also reported to be the major driver of malignant progression of many types of carcinomas. Cancer cells with an activated EMT program have increased tumor-initiating properties, enhanced motility, ability to disseminate, and elevated resistance to therapy (11).

Cells in the epithelial state maintain apical-basal polarity. They are linked together by stable cell-to-cell tight and adherens junctions and desmosomes, while stable binding to basement membrane is guaranteed by hemidesmosomes. Intercellular interactions involve various adhesion proteins including epithelial cadherin (E-Cadherin) (12). The activation of EMT program implies the disruption of cell-cell and cell-matrix adhesion, the loss of cellular polarity and cytoskeletal remodelling that ultimately induce the progressive loss of epithelial phenotype and the acquisition of a mesenchymal-like phenotype with marked changes in cell morphology **(Figure 1.)**. Moreover, the molecular reprogramming of epithelial cells results in a reduction of the epithelial marker E-Cadherin and an increase of mesenchymal markers such as neural Cadherin (N-Cadherin), Vimentin and Fibronectin (11). The expression of EMT-related genes shows a high variability since it is affected by the cell or tissue type and the level of advancement towards the mesenchymal state.

Several transcription factors (TF) are involved in the execution of the EMT program and expression of EMT-related genes. They include Zinc finger protein Snail 1, and Zinc finger protein Snail 2 (known also as Slug), Zinc-finger E-box-binding homeobox 1 and 2 (ZEB 1 and ZEB 2) and Twist basic helix-loop-helix transcription factor 1 and 2 (TWIST 1 and TWIST 2) (12, 13). Snail 1 and Slug downregulate the expression of E-Cadherin and upregulate the expression of N-Cadherin, and high levels of both transcription factors are associated with an increased metastatic spread (14-16). In patients with hepatocellular carcinoma (HCC), blood levels of Snail 1 mRNA were much higher in patients with extra-hepatic metastases compared to those of nonmetastatic HCC patients (17). ZEB also downregulates E-Cadherin and upregulates N Cadherin (13, 18). ZEB 1 was reported to have a prominent role in the invasion and metastatic spread of pancreatic cancer (19). An increased expression of ZEB 2 was found at the invasive front of colorectal cancer

and was associated with metastatic disease indicating its potential role as a prognostic marker (20). TWIST 1 reduces E-cadherin expression and boosts N-cadherin levels, which results in decreased cell adhesion and enhanced cell motility (11). Elevated TWIST 1 expression was reported to facilitate the induction of EMT, whereas inhibition of TWIST 1 reduced tumor growth and metastatic progression (21). In breast cancer models, the full depletion of TWIST 1 effectively blocked EMT, partially restored sensitivity to multiple drugs in vitro, and enhanced the effectiveness of Adriamycin in vivo breast cancer models (22).

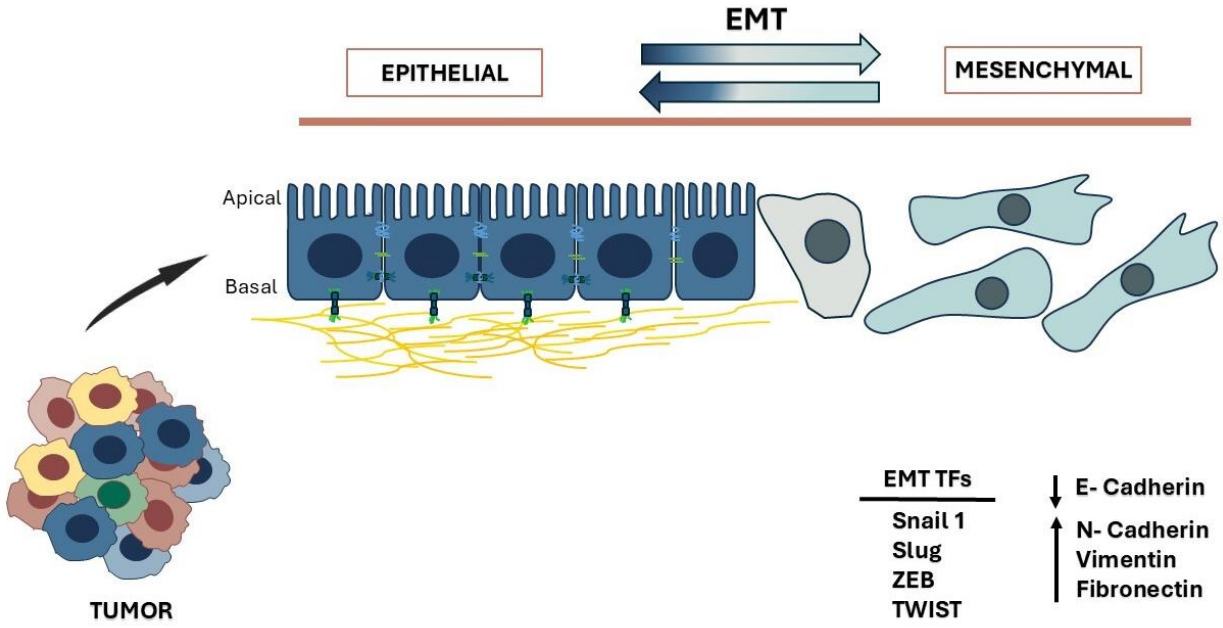

Figure 1. The epithelial-mesenchymal transition (EMT) process in tumor. Epithelial tumor cells with apical-basal polarity are connected by various adhesion proteins and structures called tight and adherens junctions and desmosomes while their connection with the extracellular matrix is ensured by hemidesmosomes. When EMT is activated in epithelial tumor cells, transcription factors like Snail 1, Slug, ZEB, and TWIST are upregulated and will orchestrate the phenotypic changes characterized by the reduction of the epithelial markers such as E- Cadherin and increase of mesenchymal markers like N- Cadherin, Vimentin and Fibronectin.

EMT is not a two-step process in which cancer cells can switch between epithelial and mesenchymal states but rather a process that drives cancer cells through a continuous spectrum of states characterized by the partial acquisition of mesenchymal traits and retention of some epithelial features. Different signalling pathways or cross-talk between them can induce the

activation of EMT program and this can lead to the expression of different EMT markers and different degree of mesenchymal transition. Epithelial cells very rarely become fully mesenchymal. In many cases, they express both, epithelial and mesenchymal markers or lose epithelial markers without expressing mesenchymal markers. Tumor cell plasticity during EMT is context-dependent (11, 23). It was noticed that single tumor cells could undergo EMT acquiring different intermediate states, thus causing phenotypic heterogeneity inside tumors (24). Breast and lung cancer-derived CTCs, along with metastatic cancer cells, frequently present an incomplete EMT (25, 26). Different EMT states could potentially provide tumor cells with better survival rates since they should adapt to different environments during metastatic dissemination (24). In the PyMT-driven breast cancer models, Snai 1 and TWIST 1 were needed to confer metastatic competence to cancer cells (27, 28) while in the pancreatic cancer model, depletion of ZEB 1 suppressed stemness and colonization capacity of cancer cells (19).

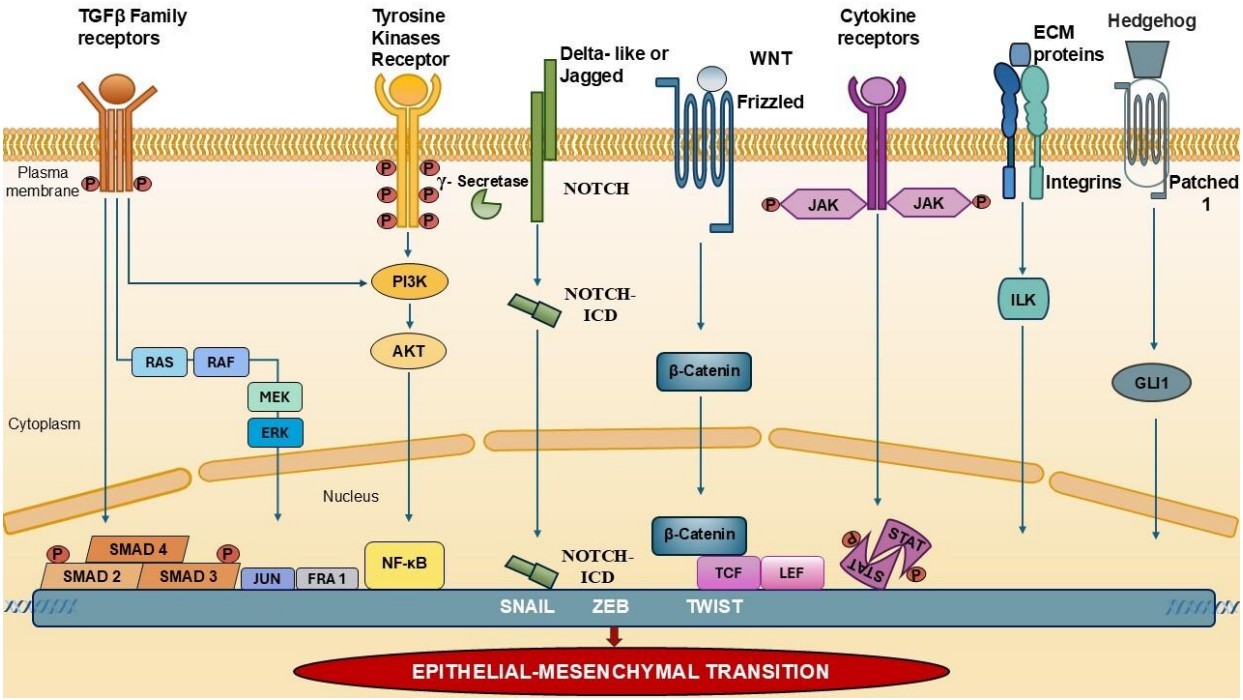

Figure 2. Signalling pathways involved in inducing EMT program. TGF-β, WNT, Notch, Integrin, PI3K-AKT, and Hedgehog (HH) signalling pathways may involve various mediators to activate EMT TF leading to the acquisition of partial or full mesenchymal cell properties. Factors involved in inflammation also could activate cytokine receptors thus-promoting EMT process (Modified from reference (12, 13))

EMT is not a definitive but a reversible process in which tumor cells can re-acquire the epithelial phenotype through the mesenchymal-epithelial transition (MET). Some evidence indicates that EMT is regulated epigenetically (29). Signals from the stroma surrounding the tumor, which is similar to the stroma found in inflammation and would healing, may trigger the activation of transcription factors involved in EMT that in turn will orchestrate various aspects of the EMT programme (12). On the other hand, carcinoma cells that express EMT-related markers can alter the behaviour of different stromal components, collectively shaping the tumor microenvironment (TME) (30). Several signalling pathways such as TGF-β, Wnt, Notch, Integrin, PI3K-AKT and Hedgehog (HH) cand induce EMT and are also reported to be involved in the tumor-microenvironment interactions **(Figure 2)** (13). Interestingly, EMT can enable the tumor to evade the immune system and cause resistance to radiation, chemotherapy and immunotherapy. Thus, EMT is not only a way for cancer cells to acquire new properties but also a way of TME to affect the behaviour of cancer cells (11, 12, 31).

1.2. Role of tumor microenvironment

Cancers are intricate structures composed of tumor cells and various non-cancerous cells, all situated within a vascularised modified extracellular matrix. The non-cancerous components of TME comprise a large variety of cell types including cancer-associated fibroblasts (CAFs), immune cells, tumor endothelial cells, pericytes, and other tissue-resident cells. These non-cancerous cells, that had been previously considered not influencing cancer cells, were shown to interact with each other and to affect cancer cell behaviour. In fact, the dynamic interplay between all cell types within TME can significantly affect tumor development, metastatic dissemination, and efficacy of therapies. This interplay can vary between patients and is influenced by several factors including organ type, histology, tumor stage, and properties of cancer cells. The ability of cancer cells to attract and reprogram non-cancer cells in TME along with the modulation of vasculature and ECM promotes the development of an environment that favours tumor growth. The interactions in TME can be mechanical, through cell-cell or cell-ECM contacts or paracrine, which involve several cytokines, chemokines, growth factors, and proteases (6, 32). More recently

extracellular vesicles and particles (EVPs) have been reported to be involved in this cell-cell communication (33).

Cancer-associated fibroblasts (CAFs) are primarily involved in the deposition and remodeling of ECM. They are activated by exposure to inflammatory mediators including Transforming growth factor beta (TGFβ), Interleukin 1 (IL-1), IL-6, and Tumor necrosis factor TNFα (6). CAFs activity determines the mechanical properties of ECM that can directly affect signalling and behaviour of cancer cells. Furthermore, fibrosis can result from enhanced activation of CAFs and this may impair immune cell recruitment thus promoting immune evasion and intratumoral diffusion of drugs (34-36). CAFs were also shown to induce EMT of tumor cells trough cytokine and growth factors secretion (37, 38). A large panel of immune cells is present in the TME that evolves along with tumor cells and is responsible for the anti-tumor immune response in the different phases of tumor progression. Furthermore, several immune cells present in the tumor environment can affect cancer cell behaviour by inducing EMT. T helper ($CD4^+$ T) cells were reported to activate the EMT program in pancreatic cancer (39) while cytotoxic T (**(**$CD8^+$ T) cells were shown to induce the formation of mesenchymal breast cancer cells with stem cell-like characteristics (40). Macrophages present in the TME can have either anti-tumor (M1) and pro-tumor functions (M2) (41). In particular, M2, through activation of different signalling pathways, can induce EMT of cancer cells leading to cancer progression (42, 43). Neutrophils which infiltrate the TME were shown to influence significantly cancer cell behaviour. Tumor-associated neutrophils (TANs) were reported to promote tumor progression by inhibiting the adaptive immune response within TME. However, similarly to tumor-associated macrophages (TAMs), TANs can be divided into anti-tumor N1 and pro-tumour N2 subclass and depending on the characteristics of TME, neutrophils can acquire N1 or N2 phenotype (44).

The majority of non-cancerous cell types in TME are also present in inflammation. However, in the inflammatory response, the various cell types and mediators exerts their functions in a highly organised manner and the whole process is finalized to restore tissue integrity. Cancer-associated inflammation is a less finalized process and usually becomes chronic and damaging. In many cases, chronic inflammation causes drug resistance and determines the outcome of cancer therapy including chemotherapy, radiotherapy and immunotherapy (45-47).

1.3. Neutrophil extracellular traps in cancer

Although the primary role of neutrophils is to combat infectious agents of different origins and sizes through phagocytosis and the activity of intracellular proteins they can also release neutrophil extracellular traps (NETs) with a process known as NETosis. Since the first discovery of NETs and the determination of their role in limiting pathogen dissemination and killing microbes (48, 49), it has been found that NETs can be released in different pathological conditions. This opened a completely new field of investigation for understanding NETs' role in various diseases, including cancer (50).

NETs are weblike structures, composed of DNA and nuclear, cytosolic and granular proteins (51). The diversity of NETs proteins which decorate NETs-DNA structure is huge and depends on the stimulus that induces NETosis. Patients that suffer from the same illness could differ in NETs proteins. Thus several markers are considered for NETs characterisation including Myeloperoxidase (MPO), Neutrophil elastase (NE) and Histones, which are usually present in NETs' structure (48, 52). Considering the complex composition of NETs, their role in cancer progression and metastatic spread may be highly relevant.

The process of NET extrusion can be regulated in TME by many factors including hypoxia, tumor-derived chemokines and inflammatory cytokines (53). Neutrophils can extrude NETs in the extracellular space not only during inflammation and infection but also in response to different molecules derived from tumor cells and CAFs (54). TAN-derived NETs are found in TME. Samples of human and animal pancreatic, breast, liver and gastric cancer as well as metastatic tumors were shown to be positive for NETs presence (55). Granulocyte colony-stimulating factor (G-CSF) (56, 57) and IL-8 from cancer origin were reported to induce NETs formation in TME. IL-8 and receptor CXC chemokine receptor 2 (CXCR2) trigger neutrophils to extrude NETs. Furthermore, release of NETs was associated with the upregulation of the Toll-like receptor 9 (TLR9) signalling pathway and cancer promotion (58, 59). Chemokine (C-X-C motif) ligand 1 (CXCL-1), CXCL-2, CXCL-5 and CXCL-6, released from tumors bind to their specific cell surface receptors on neutrophils thus inducing additional NETs formation in TME (60). In the hypoxic tumor environment, gastric cancer attracts neutrophils and though the involvement High mobility group box 1 (HMGB1), NETs can promote tumor growth (61). Recently, it has been

reported that cathepsin C (CTSC) secreted by tumors regulates IL-6 and chemokine (C-C motif) ligand 3 (CCL3) secretion leading to chemoattraction of neutrophils in the premetastatic niche of breast cancer cells into lung thus promoting colonization of distant organs. The same study showed that CTSC through activation of other signals also induces reactive oxygen species (ROS) production and NETs formation contributing to metastatic dissemination (62).

In colorectal and breast cancer patients, circulating cell-free DNA (cfDNA) has been measured in serum samples through a straightforward nucleic acid staining technique, offering an additional marker for cancer evaluation. In some cancer patients, high levels of citrullinated histone (citH3) were associated to poor survival while NETs themselves were linked to the progression of colorectal cancer (63, 64). Measuring levels of multiple NETs markers such CitH3, MPO and NE along with cfDNA in the blood may allow to obtain prognostic information in cancer patients. However, normal and pathological levels of these markers in different cancer types and stages were not determined yet. Since measuring cfDNA is less specific, evaluation of circulating cfDNA/MPO and cfDNA/citH3 has been proposed to obtain more specific results (65).

Based on the growing number of evidences that NETs can promote tumor growth and progression, an increased interest to use NETs as targets for cancer therapy emerged in the last years (66). Several drugs inhibiting NETosis or targeting NETs components are currently under investigation. For instance, DNase I has been used for digestion of NETs DNA (67). Anti-tumor effect and safety of recombinant DNAse I has been reported for patients with head and neck cancer (NCT00536952) and myeloid leukaemia (NCT02462265) enrolled in clinical trials. However, several compounds can have side effects on the immune system thus the interaction between NETs and tumor cells, the receptors involved in NETs recognition and downstream signalling pathways should be carefully considered in the evaluation of new therapeutic approaches (66, 67).

1.4. Neutrophil extracellular traps in metastatic dissemination

Although many efforts have been focused for years on the understanding the mechanisms of metastatic dissemination, new knowledge is still arising and constitutes the basis for the development of innovative therapeutic approaches in cancer patients.

To pass through different steps of the metastatic cascade, tumor cells adopt various phenotypic states and recruit nearby immune and stromal cells in the TME promoting their growth and escape from the immune system (4). It is now widely recognized that tumor spread and growth at secondary sites are not random events, but instead are governed by the relationship between the "seeds" (cancer cells) and the "congenial soil" (the target organ for metastasis)(2).

Emerging evidences suggest that NETs can influence tumor progression and several steps of metastatic dissemination (68). They can act as a substrate for adhesion of cancer cells potentially promoting their entrapment at distant organs (69). In recent years, some authors reported that NETs can induce EMT in both normal and malignant cells (70-72)**.** Tumor cells that become mesenchymal can acquire migratory and invasive abilities (55). However, little is known about the signaling pathway responsible for NET-induced EMT and some clues indicate the involvement of HMGB1, present in the structure of NETs (73) and activation of IL-1β/EGFR/ERK pathway in pancreatic cancer cells (74).

Once tumor cells enter the bloodstream, they are attacked by immune cells (75). To increase their chances of survival, circulating tumor cells (CTCs) may form clusters or associate with immune cells (76). Indeed, CTCs associated with neutrophils have been shown to take advantage of neutrophil growth factors and cytokines. In late stages of breast cancer, CTC clusters linked with neutrophils have been associated with disease progression (77). Recent studies showed that after surgical removal of hepatocellular carcinoma, CTCs in the bloodstream increase (66, 78, 79). If NETs are present in the circulation they can mechanically entrap circulating tumor cells and increase vascular permeability via elastase-mediated degradation of the intercellular junction protein VE-cadherin. The subsequent activation of β-catenin signalling may lead to tumor cell extravasation and growth at distant sites (79-81). Extravasation of tumor cells usually occurs in the pre-metastatic niche (PMN) where a favourable environment may promote colonisation of distant sites. The formation of PMN implies the modulation of ECM and infiltration of different

cell types including immune cells. Tumor cells that disseminate to distant sites can establish a secondary tumor or enter the dormancy (66, 82). NETs are reported to be present in the pre-metastatic niche where may favour the recruitment of tumor cells (83). Furthermore, some studies showed that dormant cancer cells can be awakened by NETs (84). When cancer surgery is complicated by postoperative infection, the accumulation of NETs at inflammatory sites is associated to a worse prognosis (72).

Interestingly, NETs could have different functions depending on the type of cancer (68). A deeper understanding of the interactions between cancer cells and NETs may lead to the development of new therapies aimed at blocking immune evasion mechanisms and preventing metastatic dissemination (55).

1.5. Neutrophil extracellular traps in adhesion of cancer cells

Cell adhesion is a physiological process required in healthy tissues for homeostasis maintenance. It involves cell-to-cell and cell-to-ECM interactions that occur through the expression of adhesion molecules, a large family of molecules including Cadherins, Integrins, Selectins, and Immunoglobulins. Adhesion molecules, apart from their role in physically sticking the cells together and maintaining contact with ECM, can also act as receptors, activating different signalling pathways. Cancer progression depends on the adhesion molecules and their modulation. In fact, expression and activity of adhesion molecules, especially Integrins and Cadherins, are needed for cell migration and invasion thus promoting metastatic dissemination (85).

The host group previously found that fibronectin is present inside the web-like structure of NETs and acts as a substrate for binding of cancer cells to NETs through Integrins α5β1 and αvβ3 expressed on the surface of cancer cells (69). In another study, the host group demonstrated that elevated levels of Integrins α5β1, αvβ3 and αvβ5 in different cancer cell lines significantly increased cell adhesion to NETs whereas lower levels of α5β1 prevented cell attachment to NETs (86). Other authors reported that Integrin β1 is present also in the structure of NETs indicating that both Fibronectin and Integrin β1 are important for cancer cell adhesion to NETs (79). β1-Integrin-dependent CTC adhesion to NETs in postoperative inflammation in various cancer models was reported to promote colonisation (66, 78, 79). Entrapment of cancer cells was showed to enhance invasive properties of cancer and metastatic spread of hepatocellular carcinoma (HCC) cells by

internalisation of NETs via toll-like receptor TLR4/9, and further activation of TLR4/9-COX2 signalling (87). It was recently reported that also NETs-DNA is involved in interaction with tumor cells. The transmembrane receptor CCDC25 was shown to recognize NETs-DNA rich in 8-hydroxy-2'-deoxyguanosine (8-OHdG). The binding caused activation of the ILK-ß-Parvin-RAC1–CDC42 signalling pathway with the consequent enhancement of motility and invasion ability of breast cancer cells (88). The same study also showed that NETs-DNA accumulated in the liver metastasis of patients with breast or colon cancer where it serves as a chemoattractant for the CTCs via the CCDC25 receptor (88). Although previous studies showed that blocking the β1 Integrins abolishes the motility of invading tumor cells (89), treatment of cancer patients with Integrin inhibitors alone did not significantly improve their clinical outcome thus indicating the need for designing appropriate combination therapy (90).

2. AIMS OF THE STUDY

NETs have been shown to mechanically entrap cancer cells at distant sites thus promoting colonisation of distant organs. Moreover, sporadic reports in the literature indicate that NETs can induce EMT in certain cancer cells even though the mechanisms involved are largely unknown. Therefore, there is an urge to investigate how NETs drive metastatic dissemination and the mechanisms underlying this process. In this study, we started from the evaluation of cancer cell adhesion to NETs focusing on the role of NETs-DNA sensing cell surface receptor CCDC25 and Integrin β1 chain that were reported to bind different components of NETs. Then we hypothesized that NETs can activate the EMT program of cancer cells thus enhancing the migration and invasion of cancer cells.

Based on these considerations, the aims of the present study are:

1. evaluate whether NETs can modulate the adhesion properties of a panel of cancer cell lines including EGFR-driven lung cancer cells;
2. determine the role of cell surface CCDC25 receptor and Integrin β1 chain in cancer cell adhesion to NETs ;
3. investigate whether NETs can induce EMT in a panel of cancer cell lines and identify the signalling pathways involved in this process;
4. evaluate whether NETs can enhance migratory properties of cancer cells

3. MATERIALS AND METHODS

3.1. Cell culture

Differentiated human acute promyelocytic leukemia HL-60 cell line (ATCC Cat# CCL-240, RRID:CVCL0002) was used as a stable source of NETs. HL-60 cell line was cultured in Iscove's DMEM medium- IMDM (Corning, Mediatech, Inc, Manassas, VA, USA). EGFR-driven H1975 (ATCC Cat# CRL-5908, RRID:CVCL_1511), KRAS-mutated A549 (ATCC Cat# CCL-185, RRID:CVCL_0023) non-small cell lung cancer cell lines, MCF-7 (ATCC Cat# HTB-22, RRID:CVCL_0031) breast cancer cells and human fibrosarcoma HT-1080 (ATCC Cat# CCL-121, RRID:CVCL_0317) cancer cells were used to evaluate NETs role in adhesion, EMT and migration of cancer cells. H1975 cell line was cultured in the RPMI 1640 culture medium (Corning, Mediatech, Inc, Manassas, VA, USA), while MCF7, A549 and HT1080 cell lines were cultured using Dulbecco's Modified Eagle Medium- DMEM (Corning, Mediatech, Inc, Manassas, VA, USA) medium, all supplemented with 10% FBS. All cell lines were maintained in a humidified incubator at 37°C and 5% CO_2

H1975 cells are characterized by an activating point mutation in exon 21 (L858R) and also possess T790M mutation within the EGFR kinase domain causing resistance to EGFR-tyrosine kinase inhibitors (91, 92). The A549 cell line carries a homozygous c.34G>A/p.G12S KRAS mutation (93, 94), while MCF-7 cancer cells express both estrogen and progesterone receptors, lacking HER2 amplification. HT-1080 fibrosarcoma cells are known for their high invasiveness and metastatic potential.

3.2. Production of NETs

HL-60 cells were differentiated into neutrophil-like cells by treatment with 1.3% dimethylsulfoxide (DMSO) added in IMDM, for 7 days as described previously (69). To verify differentiation, the expression of neutrophil markers CD11b (Agilent Cat# R084101, RRID:AB_579547) and CD177 (Miltenyi Biotec Cat# 130-126-423, RRID:AB_2889477) was evaluated using FACS analysis. Then, differentiated HL-60 cells (dHL-60) were treated with 25μM of Calcium ionophore (A23187, Sigma-Aldrich, St. Louis, MO, USA) for 4h in a humidified incubator at 37°C and 5% CO2 to induce NETs release. Conditioned medium was recovered and centrifuged at 310xg for 10 min at 4°C. Cell-free NETs-enriched supernatant was collected and centrifuged at 18000xg for 10 min at 4°C. After centrifugation, the pellet which contains NETs was resuspended in 100 μl of cold PBS. Double-stranded DNA (NETs-DNA) concentration was determined using a NanoDrop ND-1000 spectrophotometer with V3.5.2 software (NanoDrop Technology, Cambridge, UK). NET suspensions were stored at 80°C until used. To perform experiments in triplicate multiple NET preparations were required.

3.3. Cell characterization

H1975, A549, MCF-7 and HT1080 cell lines were characterized for the expression β1 chain of Integrin family and CCDC25 receptor by Western blot in basal conditions and after treatment with NETs at 0,5 μg/ml for up to 48h. Then, cells were subjected to solid phase adhesion assay.

3.4. Cell adhesion assay

To determine cancer cell adhesion to NETs, 24-well flat-bottomed plates were coated with 5μg of NETs resuspended in 200μl of PBS, using previously prepared cell-free NET suspensions, then incubated overnight at 4°C. As negative controls, PBS and conditioned medium from dHL-60 cells were used. An additional negative control was obtained by pre-treatment of NET-coated wells with 10 μl of DNase I (10000 UI/ml, Roche, Mannheim, Germany) for 15 minutes at room

temperature. After incubation, all plates were washed with cold PBS then each well was treated with serum-free medium containing 1% BSA for 1 hour at room temperature to block non-specific binding. Subsequently, $3x10^5$ cells were seeded in each well and allowed to adhere for 1 or 2 h in a humidified incubator at 37°C and 5% CO_2. After incubation, non-adherent cells were removed whereas adherent cells were counted and the results were expressed as a percentage of the total cell count. At least three independent experiments were conducted in duplicate for each cell line, using different NET preparations. Furthermore, to investigate the roles of α5β1 Integrin and CCDC25 receptor in cell adhesion to NETs, $3x10^5$ cells were pre-incubated with 5 μg/ml of blocking antibodies against α5β1 Integrin (Millipore Cat# MAB2514, RRID:AB_94626) or CCDC25 (Thermo Fisher Scientific Cat# PA5-54735, RRID:AB_2639443) in 300 μl of serum-free medium with 1% BSA for 1 hour at 37°C and 5% CO2. The adhesion assay was then carried out as described above.

3.5. Cell treatment with NETs

We preliminarly tested whether cancer cells can remain adherent to uncoated plates when treated with NETs in suspension for 48 h. Briefly, $5x10^5$ H1975 and HT1080 cells were seeded in each well using uncoated 6-well plates and allowed to adhere overnight at 37°C and 5% CO2. Then adherent cells were incubated with NET suspension (0,5 μg/ml of NETs) for 48 h in a humidified incubator at 37°C and 5% CO_2. At the end of incubation, adherent and non-adherent viable cells were counted.

Then we evaluated whether treatment with NETs could cause the activation of EMT in adherent cancer cells. Therefore, cancer cells were allowed to adhere to uncoated plates as previously described and then were incubated with 0.5 μg/ml of NET suspension in serum-free medium for 4h, 24h and 48h in a humidified incubator. After treatment, whole cell lysates were prepared and Western blot analysis was performed to detect EMT markers.

3.6. Immunoblot analysis

Whole cell lysates were obtained by adding RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Thermo Scientific Inc.) to cells on ice. Cell suspension was then homogenized by passages through a 26-gauge needle and then centrifuged at 16,000xg for 30 minutes at 4°C. After centrifugation, supernatants were collected and protein concentration was determined by the Bradford method (Bio-Rad). For Western blot analysis, proteins were separated via gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes. Additional incubation of membranes with blocking buffer (5% non-fat dry milk /TBS-T) was performed to prevent non-specific binding. Membranes were then incubated overnight at 4^{o}C with specific primary antibodies recognizing E-Cadherin (mouse monoclonal 1:1000; Santa Cruz Biotechnology Cat# sc-8426, RRID:AB_626780), Vimentin (mouse monoclonal 1:1000; Abcam Cat# ab8069, RRID:AB_306239), Fibronectin (rabbit polyclonal 1:1000; Thermo Fisher Scientific Cat# PA5-29578, RRID:AB_2547054), N-Cadherin (mouse monoclonal 1:1000; Santa Cruz Biotechnology Cat# sc-59987, RRID:AB_781744), Integrin β1 (mouse monoclonal 1:1000; Santa Cruz Biotechnology Cat# sc-13590, RRID:AB_627008), CCDC25 (mouse monoclonal 1:1000 Santa Cruz Biotechnology, Inc. #515201), GAPDH (rabbit monoclonal 1:1000; Cell Signaling Technology Cat# 2118, RRID:AB_561053), Vinculin (mouse monoclonal, 1:1000; Santa Cruz Biotechnology Cat# sc-73614, RRID:AB_1131294), α Tubulin (mouse monoclonal, 1:1000; Santa Cruz Biotechnology Cat# sc-5286, RRID:AB_628411), p-EGFR (rabbit monoclonal, 1:1000; Cell Signaling Technology Cat# 3777, RRID:AB_2096270), EGFR (mouse monoclonal, 1:1000; Santa Cruz Biotechnology Cat# sc-373746, RRID:AB_10920395), p-AKT (mouse monoclonal, 1:1000; Cell Signaling Technology Cat# 4051, RRID:AB_331158), AKT (rabbit, polyclonal; 1:1000; Cell Signaling Technology Cat# 9272, RRID:AB_329827), phospho-p44/42 MAPK (ERK 1/2) (rabbit polyclonal; 1:1000; Cell Signaling Technology Cat# 9101, RRID:AB_331646), p44/42 MAPK (ERK 1/2) (rabbit polyclonal; Cell Signaling Technology Cat# 9102, RRID:AB_330744), Cyclin D1 (rabbit polyclonal; 1:500; Cell Signaling Technology Cat# 2922, RRID:AB_2228523), p-ILK (rabbit polyclonal, 1:1000; Millipore Cat# AB1076, RRID:AB_10807157), ILK (mouse monoclonal; 1:500; Santa Cruz Biotechnology Cat# sc-20019, RRID:AB_627807), CDC42 (rabbit polyclonal, 1:1000; Millipore Cat# 07-1466, RRID:AB_1977123) SNAI1 (rabbit polyclonal;

1:1000; Millipore Cat# ABD38, RRID:AB_11213147), ZEB1 (rabbit polyclonal; 1:1000; Sigma-Aldrich Cat# SAB3500514, RRID:AB_10896384), SLUG (SNAI2) (rabbit polyclonal; 1:1000; Merck; #ABE993), TWIST1 (rabbit polyclonal, 1:1000; Sigma-Aldrich Cat# T6451, RRID:AB_609890), Notch 1 (rabbit monoclonal, 1:1000; Cell Signaling Technology Cat# 3608, RRID:AB_2153354) and Cleaved Notch 1 (rabbit monoclonal, 1:1000; Cell Signaling Technology Cat# 4147, RRID:AB_2153348). Secondary antibody Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Labs Cat# 111-035-144, RRID:AB_2307391) and Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Labs Cat# 115-035-003, RRID:AB_10015289). A commercially available ECL kit (GE Healthcare) was used to reveal the reaction and images were obtained using the ChemiDoc imaging system (Image Lab, Bio-Rad). The protein signals from the western blot were quantified using morphodensitometric analysis via ImageJ software (NIH, Bethesda, MD, USA). The area and mean intensity of each band were calculated and normalized against values of the corresponding loading control. The data are presented as relative protein levels for each NET-treated sample, compared to its corresponding internal untreated control.

3.7. Cell migration assay

The migration assay was performed to test if treatment with NETs could enhance migration of EGFR-driven H1975 lung cancer cells. Therefore, cells were pre-treated for 24h with 0.5 µg/ml of NETs or NETs digested with DNase I. Then the migration assay was performed by using 24-well plates and the transwell inserts (Corning) with 8.0 µm pore size. Untreated and treated H1975 cells ($5x10^4$) were resuspended in 150 µl of serum-free RPMI-1640 and seeded in the upper compartment while in the lower compartment we added 600µl of RPMI-1640 without FBS or with 3% and 10% of FBS as chemoattractant. Cells were allowed to migrate for 16 h in the humified incubator at 37°C and 5% CO2. At the end of incubation, cells that did not migrate in the upper compartment were removed with a cotton swab while migrated cells that crossed the membrane were fixed with 70% ethanol, stained with crystal violet and counted. Five randomly chosen microscopic fields were selected for counting and the results were expressed as a mean number of cells per field.

In parallel experiments, we tested the chemotactic properties of the NETs by adding 5µg/ml and 15µg/ml of NETs in serum-free RPMI-1640 medium in the lower compartment and untreated H1975 cells were seeded in the upper compartment. Pre-treatment of NETs with DNase I was used as a negative control. After migration for 20 h at 37°C and 5% CO_2, migrated cells were counted as previously described.

3.8. Statistics

The software MedCalc for Windows, version 10.3.2.0 (MedCalc Software, Mariakerke, Belgium) was used for statistical analysis. Means from unpaired data were compared by Student's t-test. Differences among multiple groups were evaluated by analysis of variance (ANOVA) followed by pairwise comparisons. Chi-squared test was used for the analysis of categorical data. A probability value of <0.05 was considered statistically significant.

4. RESULTS

4.1. Characterization of tumor cell lines

Different cancer cell lines were characterized for the expression of β1 chain and CCDC25 by Western blotting in basal condition and after 4h, 24h and 48h of cell exposure to NET suspension. **Figure 3** shows the levels of β1 chain and CCDC25 in basal conditions and in response to NET exposure.

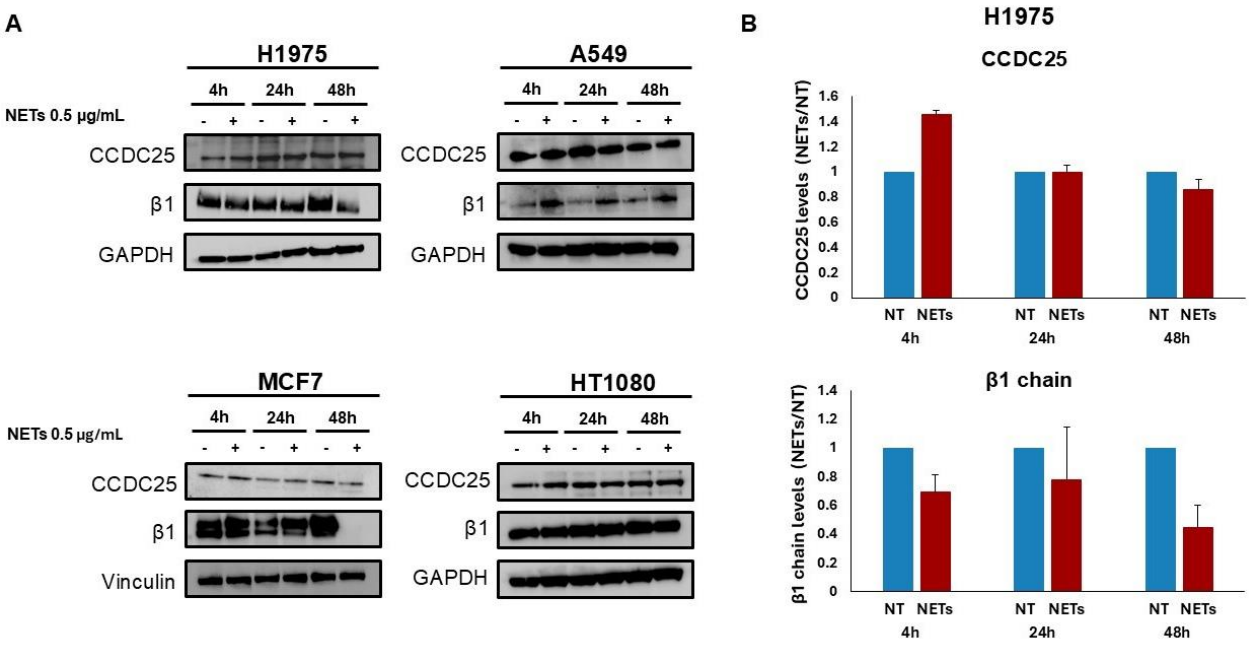

Figure 3. Levels of CCDC25 and β1 chain expression in different cancer cell lines. A. Cells were treated or not with 0.5 µg/mL of NETs for 4h, 24h and 48h and then subjected to Western blot analysis. GAPDH or Vinculin were used as equal loading**. B.** Results of quantitative analysis of β1 chain and CCDC25 in H1975 cell line. The data are expressed as the relative levels of protein in each NET-treated sample as compared to the corresponding untreated internal control. NET-treated samples showed up to a 60 % reduction of β1 chain levels whereas CCDC25 levels showed fluctuations without a specific pattern.

CCDC25 was expressed in all the cell lines tested and its levels remained substantially unchanged in response to NET exposure. Also β1 chain was expressed in all the cell lines tested but its levels were strongly reduced in response to NET exposure in H1975 and MCF7 cells. In A549 cells a slight increase of β1 chain levels were observed after treatment with the NETs while in HT1080 cell line NETs treatment did not cause any changes of β1 chain **(Figure 3A)**. Quantitative analysis of the expression of CCDC25 and β1 chain was performed in H1975 cell line and results are shown in **Figure 3B.**

4.2. NET-dependent adhesion of cancer cell lines

Cell adhesion to NETs was tested by incubating untreated cancer cell lines with NET-coated plates for 1 or 2 h in a humidified incubator at 37°C and 5% CO2. Adherent cells were expressed as a percentage of total cell number. PBS and conditioned medium of dHL-60 were used as negative controls along with pre-treatment of NET-coated wells with DNase I. **Figure 4A** shows the results of the solid-phase adhesion assay performed in all conditions for each cell line.

Cell adhesion to NET-coated plates varied between 54% and 93% depending on the cell line and it was significantly higher than that obtained in uncoated plates pre-incubated with PBS and CM. Cell adhesion to NETs decreased significantly when NET-coated wells were subjected to pre-treatment with DNase I. P values for each condition and cell line are reported in **Figure 4A**. The different ability of cell lines to specifically adhere to NETs may depend on many factors including their origin, phenotypic features and especially molecular profile.

Since the CCDC25 receptor was reported to bind the DNA component of NETs (88) and α5β1 Integrin was reported to bind Fibronectin included in the NET structure (69), we determined whether an excess of antibodies against CCDC25 and α5β1 could prevent cell adhesion to NETs. **Figure 4B** shows that in H1975 cells both antibodies caused a statistically significant reduction of cell adhesion to NETs similar to that obtained with disruption of NET structures with DNase I. These findings indicate that both CCDC25 and α5β1 can be involved in cell binding to NETs.

Then we tested whether the prolonged incubation of adherent cells with NET suspensions may cause a reduction of cell adhesion. After 48h of incubation with 0.5 μg/ml of NET suspension, we found that 87.7% of H1975 viable cells lost their adhesion to uncoated plates whereas only 7.8

% of untreated H1975 cells ($\chi 2 = 125$, $p < 0.0001$) were detached at the same time point **(Figure 4C)**. When we tested HT1080 cells that did not show any changes in the expression levels of β1 chain and CCDC25 after 48h exposure to NETs, we found that 9.9% of NET-treated viable cells lost their adhesion to uncoated plates as compared to 3.7% of untreated cells ($\chi 2 = 1.92$, $p = 0.1658$). Therefore, when NETs are used as an adhesion substrate in a solid-phase adhesion assay, they promote cell attachment. However, prolonged exposure to NETs may promote cell detachment.

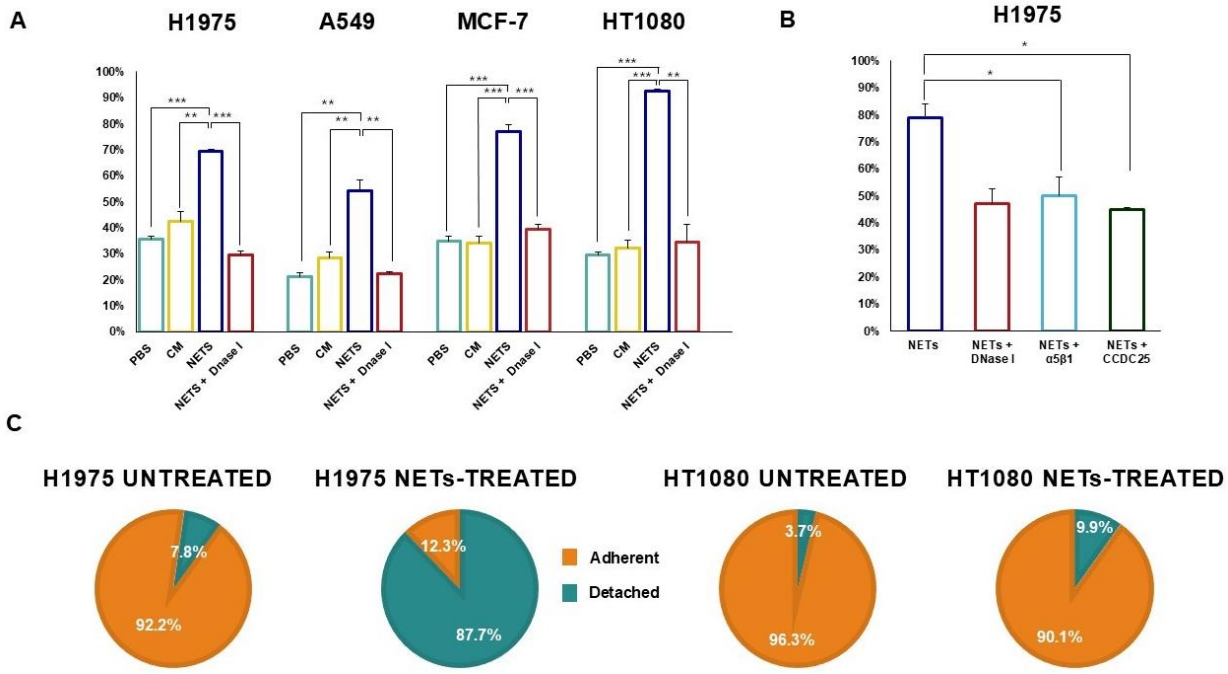

Figure 4. Cell adhesion of different cancer cell lines in the presence or absence of NETs. Cells were seeded and allowed to adhere to NET-coated multi-well plates for 1 or 2 h depending on the cell line. As a negative control, it was used plates coated with PBS or a conditioned medium of dHL-60 and NET-coated plates pre-incubated with DNase I. Results are expressed as a percentage of the total cell number in each well. In parallel experiments, adherent cells were exposed to 0.5 µg/mL NETs for 48 h followed by counting of adherent and detached viable cells. **A**. Cell adhesion (mean ± SE) was obtained in H1975 (2h) and A549 (1h) lung cancer cell lines as well as breast cancer cells MCF7 (2h) and HT1080 fibrosarcoma (1h) cell lines. **B**. Cell adhesion (mean ± SE) to NET-coated plates obtained in H1975 cells pre-incubated with blocking antibodies against α5β1 Integrin and CCDC25 receptor. **C**. Percentage of adherent and detached viable cells exposed or not to NETs in the medium for 48 h. Statistical significance: * $p<0.05$; ** $p< 0.01$; ***$p< 0.001$

4.3. NET-induced EMT in selected cancer cell lines

Based on these observations, adherent cancer cells were incubated with 0.5 µg/ml of NET suspension in serum-free medium for 4h, 24h and 48h and then tested for the expression of EMT markers by Western blot analysis. **Figure 5** shows the levels of E-cadherin, Vimentin and Fibronectin in H1975 and A549 lung cancer cell lines, MCF7 breast cancer cell line and H1080 fibrosarcoma cells.

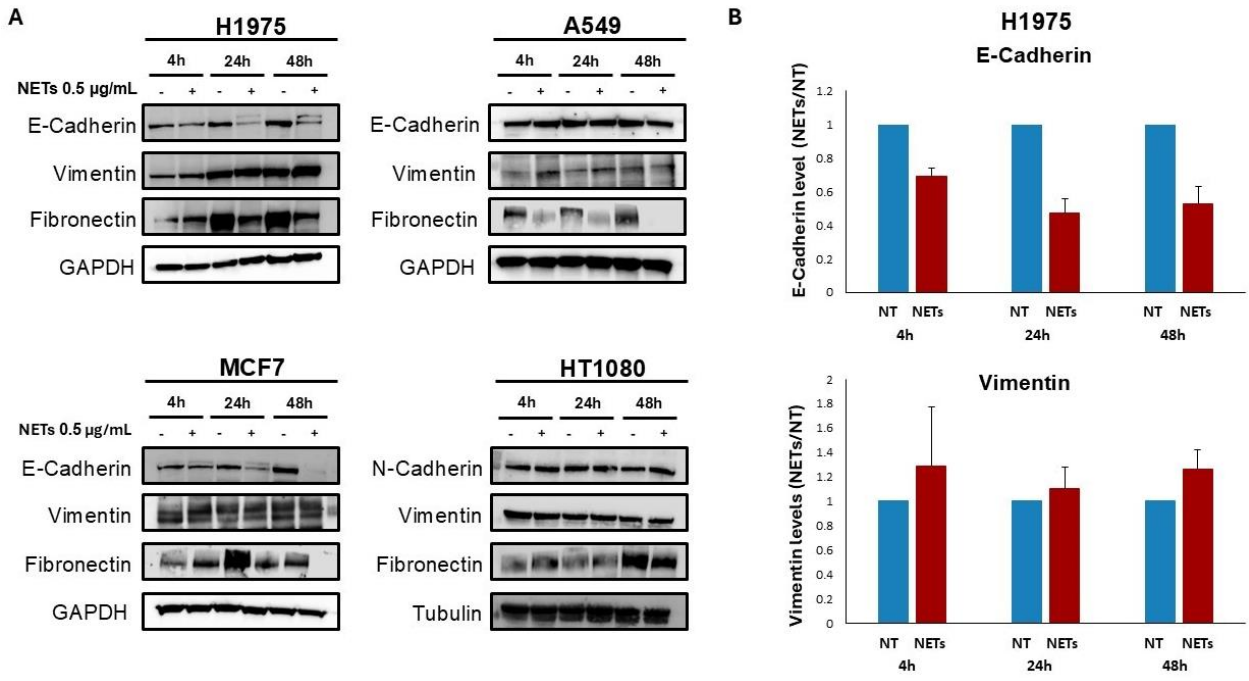

Figure 5. **Expression of EMT markers in response to NET treatment. A.** Levels of E-cadherin, Vimentin, N-cadherin and Fibronectin in different cancer cell lines exposed or not to 0.5 µg/mL NETs for 4h, 24h and 48h. GADPH and Tubulin were used as equal loading. **B**. Results of quantitative analysis of E-cadherin and Vimentin in H1975 cells. The data are expressed as the relative levels of protein in each NET-treated sample as compared to the corresponding untreated internal control. NET-treated samples showed up to a 53% reduction of E-cadherin levels whereas Vimentin levels were increased.

Exposure to NETs caused a decrease of E-cadherin in H1975 and MCF-7 cells and an increase of Vimentin in H1975 cells indicating the activation of the EMT program in those cells. Similar findings, although less prominent, were found in A549 lung cancer cells. The same EMT markers were unchanged in untreated and NET-treated HT1080 cancer cells. Fibronectin levels decreased in NET-treated H1975, MCF-7 and A549 cells as compared to untreated control while in HT1080 cells there was a slight reduction in levels of expression after 48h of NETs treatment **(Figure 5A)**. The results of the quantitative analysis of E-cadherin and Vimentin levels in H1975 cells are shown in **Figure 5B**.

The loss of the epithelial phenotype in H1975 cells was confirmed by the strong reduction of total and phosphorylated EGFR levels and by the downregulation of the EGFR downstream signalling pathway 48 h after NETs exposure **(Figure 6.).**

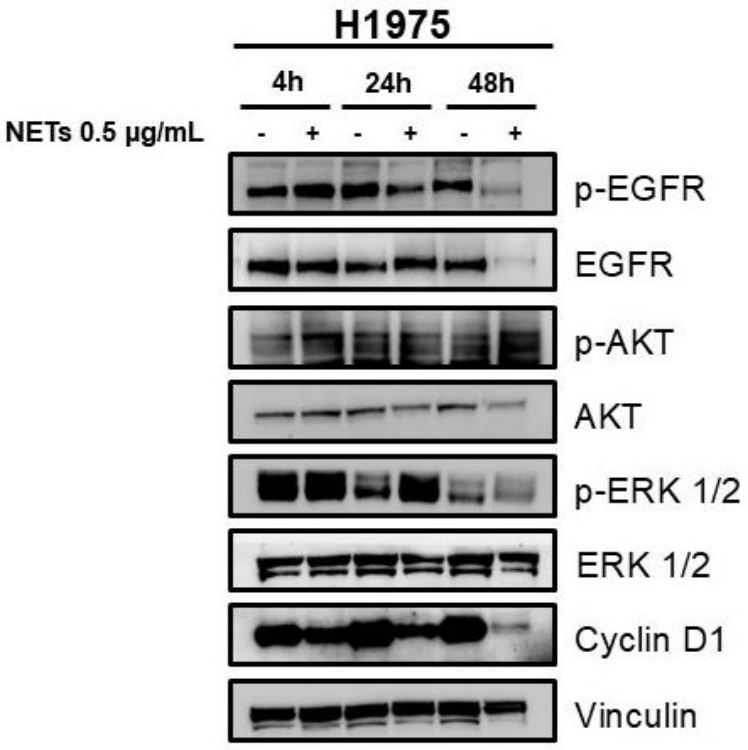

Figure 6. Loss of epithelial phenotype in EGFR-driven lung cancer H1975 cells exposed to NETs. H1975 cells were incubated with 0.5 µg/mL NETs for 4h, 24h and 48h. Levels of p-EGFR, EGFR, p-AKT, AKT, p-ERK 1/2, ERK 1/2 and Cyclin D1 were determined. Vinculin were used as equal loading.

Interestingly levels of Cyclin D1 indicating cell proliferation rate was decreased already at 4 h after exposure to NETs. Similarly, NET-treated MCF-7 showed a decrease of phosphorylated EGFR as compared to untreated control at 48h (data not shown). These findings taken together indicate that exposure of EGFR-driven lung cancer cell lines to NETs induces the activation of the EMT program and loss of the epithelial phenotype.

4.4. NET- dependent enhancement of cell migration

Cell migration experiments were performed using 24-multiwell plates with transwell and FBS as chemoattractant **(Figure 7.)**. Untreated and NET-treated H1975 cells were seeded in the upper compartment and allowed to migrate. An additional negative control was represented by H1975 exposed to DNase-treated NETs. When no chemoattractant was added in the lower compartment, migration of NET-treated cells was enhanced as compared to that of untreated cells although differences did not reach statistical significance **(Figure 7A)**. When 3% FBS was added to the medium and used as a chemoattractant, cell migration was significantly different in the 3 groups (F-ratio= 28.036, p=0.01) with NET-treated cells reaching the highest values **(Figure 7B)**. When the chemoattractant was 10% FBS added to the medium, an even higher difference in the cell migration was observed between NET-treated cells and the other 2 groups (F-ratio=76.185, p=0.003) **(Figure 7C)**. In fact, cell migration was 1.7, 2.1, and 2.7 folds higher in NET-treated cells as compared to untreated cells when no chemoattractant, 3% FBS and 10% FBS was used, respectively. Similar results were obtained when NET suspensions were used as a chemoattractant. When using 15ug/ml of NETs, a 3-fold increase of cell migration was observed as compared to negative controls.

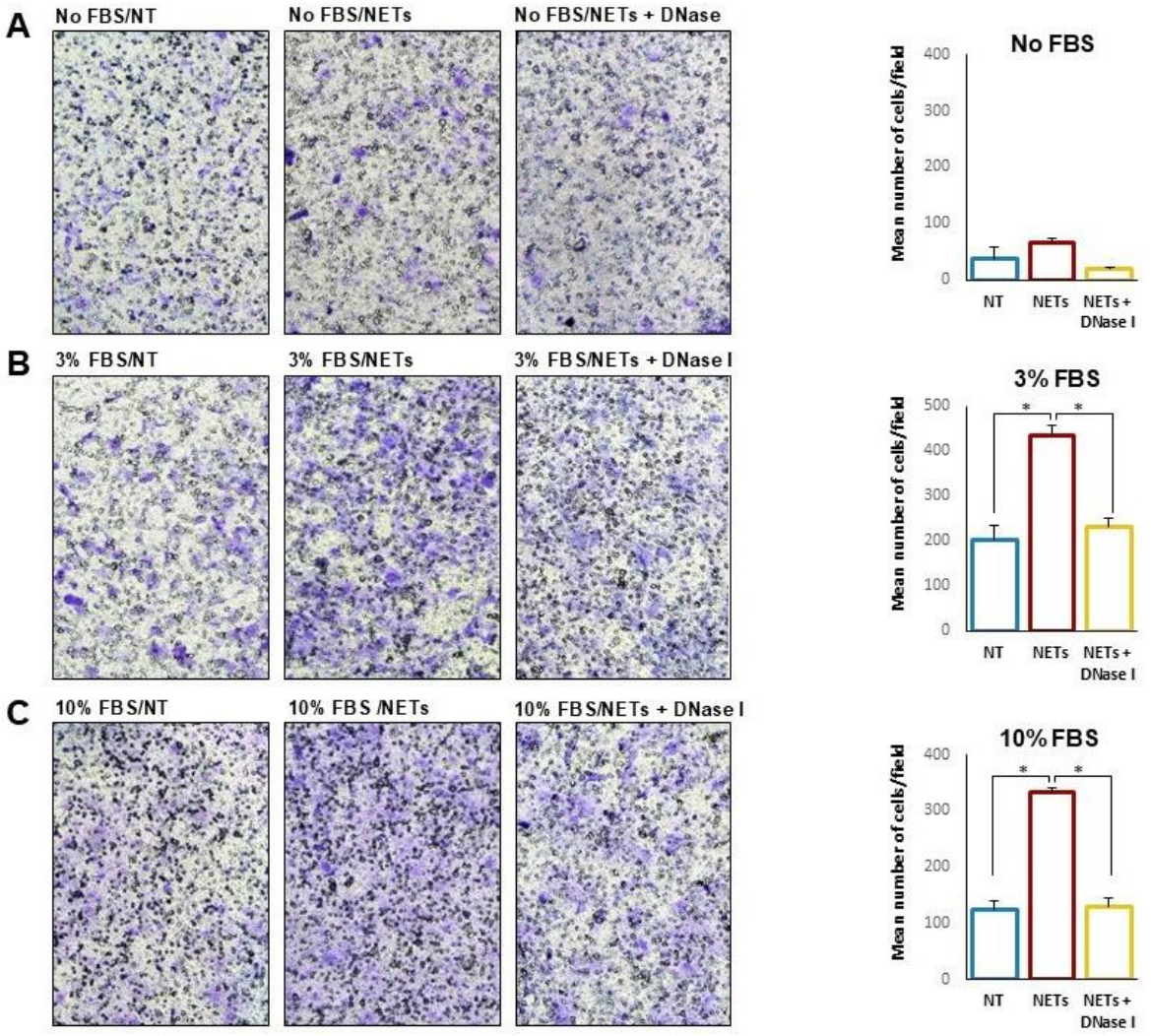

Figure 7. **NET-dependent enhancement of cell migration.** Untreated or NET-treated H1975 cells were seeded in the upper compartment of transwell and allowed to migrate for 16 h. No chemoattractant, 3% FBS or 10% FBS was added to the medium in the lower compartment. On the left, representative images showing migrated cells (100X Magnification) obtained with no FBS **(A)**, 3% FBS **(B)** and 10% FBS **(C)** in the lower compartment. On the right, the corresponding graph showing the mean number/field (± SE) of migrated cells obtained in each condition. Cell migration was 1.7 **(A)**, 2.1 **(B)**, and 2.7 **(C)** folds higher in NET-treated cells as compared to untreated cells when medium without serum, or supplemented with 3% FBS and 10% FBS, respectively, were used as chemoattractant. Statistical significance: * $p<0.05$.

4.5. Signalling pathways involved in NET-driven EMT

Since CCDC25 and α5β1 Integrin are the main receptors mediating cell adhesion to NETs, we first tested the activation of their downstream pathways by determining the levels of total Integrin-linked kinase (ILK), phosphorylated ILK and CDC42 in H1975 cells. No significant changes of phosphorylated ILK were observed in NET-treated H1975 cells whereas total ILK levels were decreased after 48h of NETs treatment. CDC42 levels were unchanged in response to NET treatment **(Figure 8A)**.

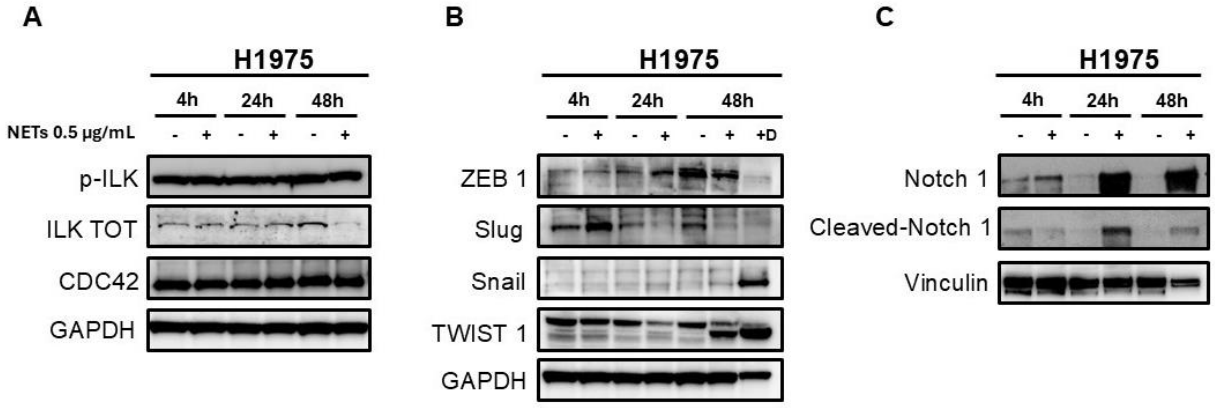

Figure 8. **Levels of signaling mediators in EGFR-driven H1975 cells exposed to NETs. A**. Levels of p-ILK, ILK and CDC42 in cells exposed or not to 0.5 µg/mL NETs for 4h, 24h and 48h. **B**. Levels of transcription factors in adherent cells exposed or not to treatment with NETs for 4h, 24h and 48 h (lanes 2,4,6) and in viable cells that were detached after 48h treatment with NETs (lane 7, +D). **C**. Levels of total Notch 1 and cleaved Notch 1 in H1975 cells exposed or not to NETs for 4h, 24h and 48h. GAPDH and Vinculin were used as equal loading.

Then, the transcription factors ZEB 1, Slug, Snail and TWIST 1 were tested in adherent cells exposed to treatment with NETs for 4, 24 and 48 h **(Figure 8B, lanes 2,4,6)** and in cells that were detached after 48 h of treatment with NETs **(Figure 8B, lane 7 +D)**. ZEB 1 expression was enhanced at 24 h. Slug levels were increased at 4 h. Levels of Snail were slightly increased at 24 h and 48 h in adherent cells and strongly upregulated in detached cells after 48h of treatment. TWIST 1 was strongly enhanced in adherent and detached H1975 cells exposed to NETs.

To clarify how the signalling of EMT activation reaches transcription factors, we tested levels of total Notch 1 and cleaved Notch 1 in H1975 cells. We selected Notch 1 because it was reported to be one of the main drivers of the EMT program in several cancer cells and was up-regulated in H1975 cells when they grow as tumor spheres (95). **Figure 8C** shows an increase of total Notch 1 in H1975 cells at all time points and an increase of cleaved Notch 1 at 24 and 48 h post-treatment.

5. DISCUSSION

The initial description of NETs as actor of host immunity was expanded by the evidences that NETs have a role in different human diseases including cancer and this raised additional questions. Among the points raised, it is central to understand how the complex interplay between NETs released by activated neutrophils and cancer cells can affect cancer progression. A major mechanism by which NETs may drive cancer progression involves the enhanced adhesion of tumor cells, mediated by β1 Integrins, to NETs (79, 86). In the present study, we investigated other potential mechanisms by which NET can affect the destiny of cancer cells and we tested whether they can induce a sequence of events leading to dissemination of cancer cells at distant organs.

Our study demonstrated that NETs act as a binding substrate for all the cancer cell lines examined. Moreover, the prolonged exposure of cancer cells to NETs triggered the activation of EMT in H1975 and MCF7 cells, leading to increased migratory capacity in H1975 cells. The dual function of NETs in facilitating cell adhesion and boosting the migratory behaviour of cancer cells, may reflect the complex dynamics of NET interactions within the peripheral vasculature of cancer patients. Circulating NETs may accumulate in small blood vessels, creating a microenvironment rich in DNA and fibronectin that serves as a scaffold for the attachment of various cancer cells expressing the CCDC25 receptor and the β1 Integrin chain. The prolonged adhesion to NETs can activate the EMT process, increasing the migratory capabilities of cancer cells and contributing to the formation of pre-metastatic niches. Likewise, circulating tumor cells can interact with NETs present in the blood and may acquire a mesenchymal phenotype, facilitating their spread to distant organs.

Our study investigated EMT activation in a panel of cancer cell lines and showed that while all tested cell lines can adhere to NETs, only a few of them were capable of activating the EMT program after treatment with NETs, at least at the dosage used in our study. These findings indicate that some types of cancer cells more easily activate the EMT program probably because of the permissive status of their signalling network.

To the best of our knowledge, our study was the first to reveal that Notch 1 was promptly upregulated in response to NETs and subsequently activated through cleavage, leading to upregulation of cleaved Notch 1 **(Figure 9.)**. This suggests that Notch 1 may play a pivotal role in NET-mediated induction of epithelial-to-mesenchymal transition (EMT), particularly in oncogene-driven non-small-cell lung cancer (NSCLC). Currently, the signaling pathway causing the upregulation and activation of Notch 1 is uncertain but we can suppose that the cross-talk with the Integrin signalling pathway, the close contact between cells that simultaneously bind to NETs, or modulation by other signalling pathways such as TGF-β and NF-kB/NLRP3 (72, 96) can have a role. Furthermore, there is the possibility that cancer cells have redundant mechanisms for EMT activation in response to NETs. In any case, the transition is complete after 48 hours of treatment, as evidenced by the expression levels of specific transcription factors.

The activation of the EMT pathway through Notch 1 implies the downregulation of the EGFR signalling cascade, resulting in a significant reduction in the expression of the oncogene driver and the development of resistance of cancer cells to EGFR inhibitors. Our results provide an example of how the immune and inflammatory microenvironment can directly influence cancer cell sensitivity to oncogene-targeted therapies. A recent study using animal models of breast cancer demonstrated that chemotherapy could lead to the recruitment of neutrophils in lung metastases. The subsequent release of NETs was shown to confer resistance to chemotherapy through the activation of TGF-β (97). Investigating whether TGF-β activation in metastatic breast cancer cells leads to the upregulation of Notch 1 would be of great interest. The interaction between cancer cells and neutrophils has been extensively studied, revealing that neutrophils can either support or inhibit cancer progression depending on the context. In this study as well as in our findings, the key observation is that neutrophils, along with other immune or inflammatory cells in the tumor microenvironment, can modulate tumor response to both targeted therapies and conventional chemotherapy.

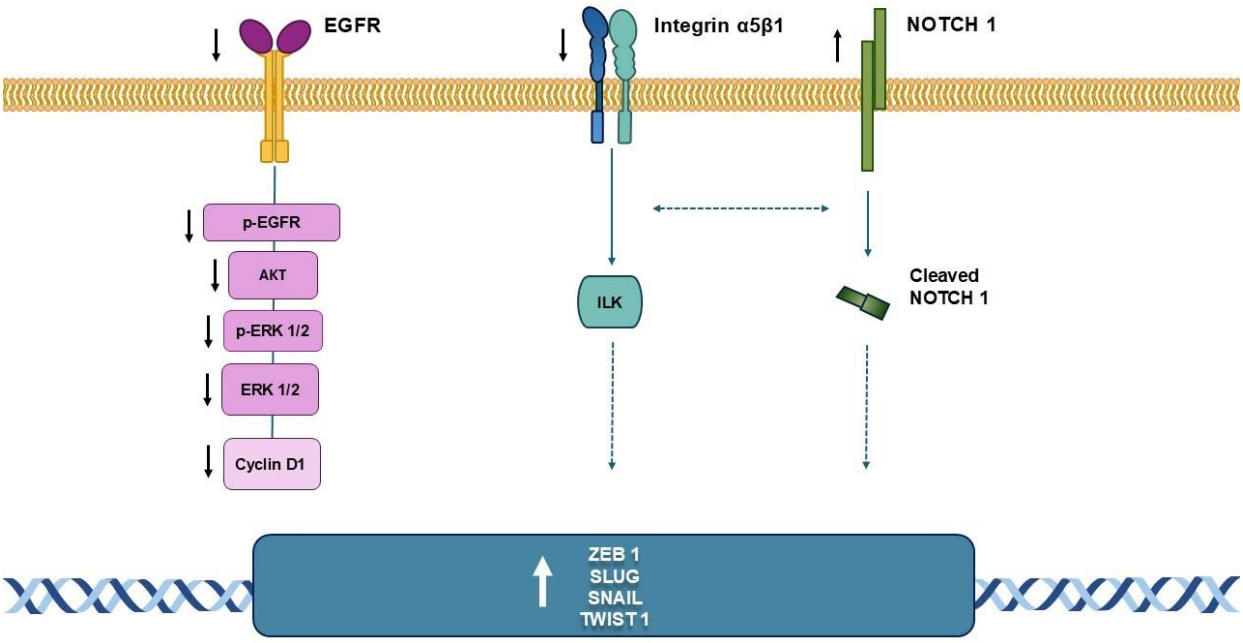

Figure 9. **Signalling pathway in EGFR-driven lung cancer H1975 cells upon treatment with NETs.** Exposure of cancer cells to NETs for a prolonged time leads to downregulation of Integrin β1 and upregulation of NOTCH 1 and Cleaved NOTCH 1. Levels of expression of EGFR, p-EGFR, AKT, p- ERK 1/ 2 and ERK 1/ 2 as well as Cyclin D1 are decreased indicating loss of epithelial properties. Expression levels of TF ZEB1, Slug, Snail and TWIST 1 are increased.

Our study was the first to simultaneously investigate the role of β1 chain and CCDC25 receptor in cancer cell adhesion to NETs, demonstrating that both molecules are equally effective in promoting cell attachment. These findings indicate that cancer cells utilize multiple mechanisms for their adhesion to NETs. However, it is still unclear whether either of these receptors plays a role in the activation or upregulation of Notch 1 receptor. Previous studies have highlighted the interaction between Integrins and Notch1 pathways (98), especially β1 Integrin and the Notch 1 pathway (99) during tissue development. Moreover, non-canonical Notch 1 activation has been linked to other signalling pathways in various types of cancer (100), and ILK has been identified as an upstream regulator of Notch 1 in leukemic cells (101). Further studies such as silencing the β1 chain or CCDC25 receptor, could help clarify how each contributes to Notch 1 activation.

6. CONCLUSIONS

In conclusion, our study showed that cancer cell adhesion to NETs is associated with the upregulation and activation of Notch 1, which subsequently triggers the epithelial-to-mesenchymal transition program. The acquisition of a mesenchymal phenotype increases the migratory ability and invasiveness of cancer cells at metastatic sites, thus contributing to tumor progression.

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I wish to honour the memory of those who are no longer with us but whose love, wisdom, and values continue to guide me every day. Though they are not here to witness this milestone, their influence remains a cornerstone of who I am and the work I have accomplished. This achievement is a tribute to their enduring presence in my heart.

Finally, I dedicate this work to those who are yet to come, including the children who will one day bring new light and joy into our family. May this achievement serve as a testament to the power of dreams, hard work, and love. To you, the future, I hope this work inspires you to pursue your own passions and embrace life with curiosity and courage.

SPECIAL THANKS TO ME:

This accomplishment is as much yours as it is mine.