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Blood-brain barrier: role in brain metastasization of breast cancer

Ana Rita da Costa Garcia

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Dissertação orientada por:

Prof.ª Dra. Maria Alexandra Brito

Prof. Dr. Cláudio Moreira Gomes

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Abstract

Insufficient understanding of the mechanisms behind blood-brain barrier (BBB) extravasation by breast cancer (BC) cells, during the metastatic cascade precludes the development of therapeutics to prevent brain metastasis formation in BC patients. Among all BC subtypes, the triple negative BC has the fewest therapeutic options due to the lack of well-defined molecular targets. Allied to this fact, BBB restricted permeability renders current treatment of patients with brain metastasis unsuccessful and account to the poor prognosis of the affected 2 million people worldwide. Therefore, it is emergent to act even before the BC cells colonize the brain parenchyma. For that, additional studies are required in order to detail the events and molecular players involved in the interplay between BC cells and brain endothelium. In particular, it is important to establish a temporal scale that comprises each step of extravasation, to detail the precise phenotype of both cell types, to determine the alterations occurring in brain endothelium, to examine the mechanism of BBB transposition and to elucidate the players involved in the malignant-endothelial interaction. To fill these gaps in the current knowledge, we outlined the following aims for this thesis: to establish a time and spatial-course profile of BC cells and BBB endothelial cells along extravasation, to identify the players involved in extravasation, to evaluate endothelial-mesenchymal transition occurrence in brain endothelium, to characterize the transmigration pathways used by BC cells for the BBB transposition and to evaluate the integrity of the barrier along this process. For our studies, we established a new and human BC brain metastasization *in vitro* model, composed by human brain microvascular endothelial cells (HBMEC), that mimics BBB, and human adenocarcinoma cells (MDA-MB-231 Br4), a triple negative BC cell line prone to metastasize in brain. To evaluate the interplay between the two cell types at temporal and spatial levels, live-cell imaging confocal microscopy and automated microscopy were performed in order to replicate and monitor the intercellular dynamics along 24 h, with images acquisition every hour. Our results showed that after 1 h, the malignant cells undergo morphological changes, revealed by an increase of their perimeter and a decrease in circularity and roundness, acquiring an invasive and migratory phenotype. These alterations were accompanied by the cytoplasmatic extensions development in BCCs. In addition, during this process, the BC cells showed the ability to quickly and efficiently intercalate the endothelial monolayer. Moreover, our data indicate that carcinoembryonic antigen (CD66adecb) and integrin-β1 (CD29) are not involved in extravasation, but the cluster of differentiation 44 (CD44) and intercellular adhesion molecule 1 (ICAM-1) appears as extravasation players, particularly relevant in BMECs. On the other hand, this process promotes alterations in cells metabolism and an increase in the percentage of BC cells that express glucose transporter 1 was observed. Simultaneously, after 3 h of interaction, was observed two endothelial populations with different levels of glucose transporter 1 expression. In addition, during the extravasation process, the endothelial cells undergo the endothelial-mesenchymal transition, revealed by an increase of neuronal-cadherin and myosin light chain kinase expression, as well as the elongation of these cells, acquiring an invasive phenotype. Concomitantly, a progressive disruption of the endothelial monolayer integrity was evident and correlated with alterations in adherens junctions protein, β-catenin, allowing paracellular transmigration though the barrier. Interestingly, during the malignant-endothelial cells interaction, the BC cells seems to engulf endothelial cells components. Additional experiments using cell supernatants (both from CellTracker™ Red-labelled endothelial cells and from mixed cultures) showed that BC cells exhibit a red labelling, further suggesting the engulfment of endothelial-secreted components. Allied to this fact, using a Nanosight tracking analysis, a decrease of microparticles concentration was more noticeable in mixed cultures comparatively to endothelial cultures was observed, as well as after the incubation of BC cells with endothelial culture medium. Together, these studies contribute for a better understanding of BC cells trafficking across brain microvascular endothelium, an essential step for the development of novel strategies to avoid extravasation of malignant cells into the brain and thus to prevent brain metastases formation.

Keywords: Blood-brain barrier; brain metastasis; breast cancer; endothelial-mesenchymal transition; extravasation.

Resumo

A falta de conhecimento profundo acerca dos mecanismos subjacentes ao extravasamento da barreira hematoencefálica (BHE) pelas células de cancro da mama, durante a cascata metastática, impede o desenvolvimento de terapias que previnam a formação de metástases cerebrais nos pacientes com cancro da mama. Entre todos os subtipos de cancro da mama, o cancro da mama triplo negativo é o que apresenta o menor número de opções terapêuticas, devido à falta de alvos moleculares definidos. Aliada a este facto, a restrita permeabilidade da BHE torna, atualmente, o tratamento de pacientes com metástases cerebrais num grande desafio, o que explica as pequenas taxas de sucesso e o mau prognóstico de cerca de 2 milhões de pessoas afetadas em todo o mundo. Neste sentido, torna-se urgente atuar previamente à colonização das células de cancro da mama no cérebro. Para tal, é necessário a realização de estudos adicionais que detalhem os mecanismos e os principais intervenientes moleculares na interação entre as células de cancro da mama e o endotélio cerebral, tendo como principais focos de estudo o estabelecimento de uma escala temporal que inclua cada etapa do processo de extravasamento (rolamento, adesão e transmigração/transposição), investigação detalhada do fenótipo de ambos os tipos celulares ao longo do tempo, determinação de alterações ao nível do endotélio e das células de cancro da mama, compreensão dos mecanismos de transposição da BHE e por fim, a elucidação dos marcadores moleculares envolvidos no processo de interação entre as células endoteliais da microvasculatura e as células de cancro da mama. Com base nestas necessidades, com este trabalho objetivamos estabelecer um perfil temporal e espacial das células de cancro da mama e células endoteliais ao longo do extravasamento, identificar os ligandos moleculares que intervém neste processo, pretende-se também avaliar a ocorrência da transição endotelial-mesenquimal ao nível do endotélio, caracterizar as vias de transposição da BHE usadas pelas células de cancro da mama e em simultâneo, avaliar a integridade da barreira ao longo do tempo. Para os nossos estudos, começámos por estabelecer um novo modelo humano de metastização de células de cancro da mama no cérebro *in vitro*, para o qual usámos um modelo simplificado da BHE *in vitro*, composto por células humanas endoteliais da microvasculatura cerebral (HBMEC) e ao qual adicionámos as células de adenocarcinoma humano (MDA-MB-231 Br4), uma linha celular de cancro da mama triplo negativo com propensão para a metastização no cérebro. Para avaliar temporal e espacialmente a interação entre as células endoteliais e tumorais, realizámos microscopia confocal e automatizada das culturas puras e mistas, que permitiu monitorizar e replicar a dinâmica celular ao longo de 24 h com aquisição de imagens a cada hora. Os nossos resultados mostraram que, após 1 h de interação, as células tumorais sofrem alterações morfológicas, evidenciadas por um aumento do seu perímetro e uma simultânea diminuição da circularidade e arredondamento, bem como a formação de extensões destas células, conseguindo estas uma maior área de contacto no endotélio. Todas estas características revelaram a aquisição de um fenótipo invasivo pelas células de cancro da mama nos tempos iniciais de interação entre os dois tipos celulares. Para além disso, recorrendo a softwares específicos, foi projetada a distância migrada pelas células, o que sugere que as células tumorais, durante o extravasamento, migraram maiores distâncias até às 3 h de interação entre os dois tipos celulares. Os nossos resultados demonstraram também que as células de cancro da mama, apresentam sobretudo um tipo de migração coletivo. Através da análise espacial das células de cancro da mama em relação à monocamadas endotelial, foram observadas duas posições distintas, designadas por "fora" quando as células tumorais se encontram completamente em cima da monocamada endotelial e "intercaladas" quando estas células se encontram parcial ou totalmente dentro da monocamada, definidos como estadio 1 e 2, respetivamente. Os resultados obtidos demonstraram que as células de

cancro da mama rápida e eficientemente intercalam-se entre as células endoteliais, logo após 1 h de interação com as células endoteliais. Após 3 h, foi possível observar que algumas células de cancro da mama, migraram por baixo das células endoteliais. De seguida, com o intuito de caracterizar e identificar as proteínas envolvidas no processo de extravasamento, recorreu-se à técnica de citometria de fluxo. Este estudo permitiu inferir que o antigénio carcinoembrionário (CD66adecb) e a β1 integrina (CD29) não estão envolvidos neste processo, enquanto que o cluster de diferenciação 44 (CD44) e a molécula de adesão intercelular 1 (ICAM-1) revelaram ter um papel importante no extravasamento, ao nível das células endoteliais, contrariamente ao que acontece nas células malignas. De seguida, realizámos o mesmo tipo de análise para o transportador de glucose 1, de modo a perceber se a interação entre as células tumorais e endoteliais influência o metabolismo destas células, visto que é uma das principais fontes de energia de ambos os tipos celulares. Curiosamente, este estudo evidenciou que o extravasamento promove o aumento do número de células de cancro da mama que expressam este transportador. Relativamente às células endoteliais, a percentagem de células que expressam este transportador de glucose é semelhante nas culturas endoteliais e mistas ao longo do tempo, mas às 3 h de interação observou-se a formação de duas populações endoteliais distintas relativamente aos níveis de expressão deste transportador. De seguida, e para determinar se as células endoteliais sofrem um fenómeno recentemente descrito, designado por transição endotelial-mesenquimal, resultante da interação com as células malignas, foram feitos estudos de imunofluorescência, a partir dos quais avaliámos a expressão de reconhecido marcador mesenquimal, como a caderina neuronal e um marcador de migração celular, como a cinase de miosina de cadeia leve. De acordo com os resultados obtidos, após 3 h de interação entre as células endoteliais e tumorais observou-se um aumento da intensidade de fluorescência de ambos os marcadores estudados. Em simultâneo, estas células adquiriram uma morfologia alongada, evidenciando um fenótipo invasivo, que consequentemente facilita a ocorrência do extravasamento. Sendo este um processo altamente dinâmico, de seguida avaliámos a integridade da BHE através da monitorização das culturas endoteliais e mistas ao longo do tempo, o que revelou que a área coberta por células endoteliais diminui ao longo do tempo, também evidenciado pelo aparecimento de zonas com ausência de células endoteliais na monocamada. Através da marcação da β-catenina, uma das principais proteínas das junções aderentes, nas monocamadas endoteliais e culturas mistas, foi possível concluir que a disrupção da BHE está relacionada com a disrupção deste tipo de junções evidenciada primeiro pela formação de pequenos buracos na marcação, seguida da deslocalização desta proteína juncional da membrana para o citoplasma e núcleo e culmina na observação de grandes espaços entre as células endoteliais. As pontes de contacto entre células endoteliais que se mantiveram encontram-se altamente pressionadas. Curiosamente, ao longo deste estudo, de forma inesperada, percebemos que durante a interação entre os dois tipos celulares, as células de cancro da mama parecem incorporar componentes provenientes das células endoteliais. A partir de estudos adicionais, nos quais transferimos meios de cultura (provenientes de culturas mistas e endoteliais, cujas células endoteliais foram previamente marcadas com CellTracker™ Red CMTPX), observámos que as células de cancro da mama exibiram uma marcação vermelha, principalmente na região perinuclear. Curiosamente, apresentaram um maior número de vesiculas no seu interior, quando utilizado o meio de cultura endotelial. Paralelamente, a concentração de partículas em suspensão em cada um dos meios de cultura antes e após incubação com as culturas tumorais foi avaliada, utilizando o Nanosight. Estes estudos revelaram que após 3 h de incubação, as culturas mistas apresentam uma menor concentração de partículas quando comparadas com as culturas endoteliais. Para além disso, após a incubação das células tumorais com o meio de cultura endotelial, verificámos uma diminuição da concentração de micropartículas em suspensão, comparativamente à sua concentração antes da incubação. Estes resultados evidenciam assim a possível capacidade das células tumorais para incorporaram componentes das células endoteliais durante o extravasamento. No seu conjunto, estes estudos contribuíram para uma melhor compreensão do processo de extravasamento das células de cancro da mama triplo negativo

através da BHE, a qual é essencial para o desenvolvimento de novas estratégias terapêuticas que previnam a transposição da BHE pelas células malignas e assim impedir a formação de metástases cerebrais.

Palavras-chave: Barreira hematoencefálica; metástase cerebral; cancro da mama; transição endotelial-mesenquimal; extravasamento.

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Abbreviations

α-SMA	Alpha-smooth muscle actin
AJ	Adherens junction
Ang-2	Angiopoietin-2
BBB	Blood-brain barrier
BC	Breast cancer
BCBM	Breast cancer brain metastases
BCC	Breast cancer cell
BM	Basement membrane
BMEC	Brain microvascular endothelial cell
BMP	Bone morphogenetic protein
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
BSA	Bovine serum albumin
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CEC	Cerebral endothelial cell
CHEK2	Checkpoint kinase 2
CNS	Central nervous system
DMEM	Dulbecco's modified Eagle's medium
EC	Endothelial cell
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
EndMT	Endothelial-mesenchymal transition
ER	Oestrogen receptor
E-selectin	Endothelial selectin
FBS	Fetal bovine serum
FSP-1	Fibroblast-specific protein-1
GFP	Green-fluorescent protein
GLUT	Glucose transporter
HBMEC	Human brain microvascular endothelial cell
HBSS	Hank's Balanced Salt Solution
HER2	Human epidermal growth factor receptor 2
HUVEC	Human umbical vascular endothelial cell
ICAM	Intercellular adhesion molecule
IgCAM	Immunoglobulin superfamily of cell adhesion molecules
IL-6	Interleukin 6
IL-1β	Interleukin 1 beta
JAM	Junctional adhesion molecule
LAM	Leukocyte adhesion molecule
L1-CAM	Neural cell adhesion molecule L1
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
MUC1	Mucin 1
N-cadherin	Neuronal cadherin
NTA	Nanoparticle tracking analysis
NK	Natural killer

NVU	Neurovascular unit
RPMI	Roswell Park Memorial Institute
PBS	Phosphate-buffered saline
PE	Phytoerythrin
PFA	Paraformaldehyde
PSGL	Platelet selectin glycoprotein ligand
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
SEM	Standard error of mean
sLe^x	Syalyl Lewis x
SP	Neuropeptide substance P
SRS	Stereotactic radiosurgery
STK11	Serine/threonine kinase 11
TEM	Transendothelial migration
TGF-β	Transforming growth factor beta
TJ	Tight junction
TN	Triple negative
TNBC	Triple negative breast cancer
TNF-α	Tumor necrosis factor alpha
TP53	Tumor protein p53
VCAM	Vascular cell adhesion molecule
VE-cadherin	Vascular endothelial cadherin
VLA	Very late antigen
vWF	von Willebrand factor
WBRT	Whole brain radiation therapy
ZO	Zonula occludens

I. INTRODUCTION

The introduction of this dissertation is divided in three parts. The first part entitled "Breast Cancer" overviews breast cancer biology with emphasis on brain metastases development. The second part designated as "Interplay between tumour cells and endothelial cells: a key step for extravasation", which details the extravasation process, focusing on each step that comprises this process, as well as its molecular players. Finally, the last section named "Endothelial-mesenchymal transition: a novel and key role in blood-brain barrier remodelling during tumour cells extravasation" gives prominence to changes at the blood-brain barrier that facilitate brain colonization.

1.1 Breast Cancer

Every year, several statistical and epidemiological studies are published evidencing cancer as a nefarious disease, a leading cause of death worldwide and one of the most significant barriers to increasing life expectancy. Breast cancer (BC) is a malignant tumour that usually starts with the uncontrolled proliferation of epithelial cells in the mammary ducts (as reviewed in [1]). BC represents the most commonly diagnosed and the leading cause of neoplastic disease in women, with an incidence of more than 2 million new cases and 626,679 deaths estimated in 2018 [2]. In contrast, in males, this type of cancer is considered a rare disease, accounting for less than 1% of all BC cases diagnosed [3].

Usually, the process of neoplastic transformation that leads to BC development is influenced by two distinct groups of risk factors. About 5 to 10% of all BC cases have origin in gene mutations and are called "hereditary BC cases". These mutations mainly affect the genes known as breast cancer gene 1 and 2 (*BRCA1* and *BRCA2*). In general, women with *BRCA1* mutation have a 55-65% lifetime risk of developing cancer, whereas with *BRCA2* mutated, the lifetime risk is reduced to 45% [4]. Importantly, these mutations have played a relevant role in cancer development, once the main function of these genes comprise cell damage repair mechanisms and adequate cells growth. Although with lower BC risk than the *BRCA* mutations, inherited mutations in many other genes (e.g. *TP53, CHEK2, PTEN* and *STK1*1) can also lead to BC development [5]. The second group, called the "non-genetic" cases, has an unknown disease causality but risk factors include age, family history, race, ethnicity, proliferative breast lesions, lifestyle and personal behaviour [4].

Regardless of what factor leads to BC development, this type of cancer is recognized as a complex, multifaceted disease encompassing a great variety of entities that differ among patients (intertumor heterogeneity) and even within each individual tumour (intratumor heterogeneity). According to this heterogeneity, there is a considerable variation in clinical, morphological and molecular attributes of tumours, which serve the basis for disease classification, tumorigenicity, treatment resistance and metastatic potential evaluation [6]. Firstly, one of the most important and distinctive BC categorizations correlated with the tumour develops, and its designation is determinant by the specific cells that are affected. In this context, the BC is designated by carcinoma when BC arises from the epithelial components of the breast (for example, the cells that line the terminal ducts), whereas if it arises from the stromal components of the breast (including myofibroblasts and blood vessel cells) is named as sarcoma. Among these two types, breast carcinomas develop more frequently [7, 8]. Within the large group of breast carcinomas, there are various denoted types based on their invasiveness, which can be classified as non-invasive (or *in situ*), invasive, or metastatic [9]. However, the most widely used classification criterion to distinguish the various types of BC considers non-anatomical factors and distributes BC types according to the expression of receptors in three major subtypes: luminal, human epidermal growth factor receptor 2 (HER2) positive, and basal like. Luminal tumours are characterized by being positive for estrogen and progesterone receptors (ER and PR, respectively), and the majority respond positively to hormonal interventions. The $HER2^{+}$ tumours make up 10-15% of BCs and are characterized by the absence of ER and PR expression and the high expression of the HER2 and

proliferation gene clusters. This subtype of BC can be effectively controlled with a diverse array of anti-HER2 therapies. The third and more aggressive subtype is designated as basal-like tumours, also known as triple-negative breast cancer (TNBC). This subtype represents about 15-20% of BC diagnosed cases and a complex molecular landscape once is characterized by the lack of both hormone receptors and HER2 expression, which results in a few therapeutic options. Moreover, your aggressive/highly proliferative nature culminates in a poor prognosis and heterogeneous behaviour in BC patients [10]. All these reasons make it urgent to explore and characterize this BC subtype, in order to develop new therapeutic strategies and decrease the mortality associated with their development.

1.1.1 Metastatic Breast Cancer: Focus on Brain Metastasis

Although there are already many treatment options for BC in early stage, including surgery, radiotherapy and chemotherapy [11], the major concern associated with this cancer is the lack of timely diagnosis that usually culminates with the appearance of metastases in distant organs. In this context, the central nervous system (CNS) metastases from BC occur in 15-50% of patients, representing the second most frequent cause of parenchyma metastasization [12, 13]. Previous studies have identified subgroups of patients with triple negative (TN) and $HER2^{+}$ BC as having an increased risk for brain metastases formation [1, 14]. Hence, the HER2-positivity and triple-negativity, as well as age, tumour stage, number of metastatic sites (n>2) and large tumour size are considered determinant risk factors for brain metastases development [14]. In patients with these BC subtypes, the prevalence of brain metastases is 30-40% [15]. After the brain metastases establishment, the most common symptoms are headache, limb weakness, altered neurocognitive function, seizures and ataxia, which are directly correlated with poor prognosis of disease and high mortality of the patients [16].

1.1.1.1 Going to the Brain: Metastatic Cascade

It is well known that BCCs, as well other tumour cells, have preferential secondary organs for metastasis. Some evidences have confirmed that metastatic localization does not occur randomly but rather in preferred sites under the control of a multitude of microenvironmental, cellular and molecular factors, known as metastatic organotropism [17]. According to the theory of Stephen Paget, called “seed and soil”, both the intrinsic properties of BCCs (the “seed”) and the host organ microenvironment (the “soil”) are important to determine the efficiency of organ-specific metastasis [18]. Based on this, the BCCs seems to have a preference for brain, as well for bone, lung and liver metastasization.

Under physiological conditions, the mammary epithelium architecture is a well-defined structure and is ensured by the presence myoepithelial cells layer, followed by the basement membrane (BM) and finally the extracellular matrix (ECM) [1]. However, this scenario changes dramatically, when the normal breast epithelial cells proliferate massive and uncontrollably within the mammary ducts, forming large carcinogenic masses that culminates in a primary breast cancer development. When non-invasive malignant cells acquire migratory and invasive properties, they grow beyond the ductal structure in order to spread into other tissues. For that, BCCs need to undergo a series of steps, commonly named as the metastatic cascade. This multi-step process comprises major steps: (1) invasion of the BM and ECM and cell migration; (2) intravasation that comprises the exit of malignant cells of the primary tissue and enter the circulation through transendothelial migration (TEM); (3) survival in the circulation; (4) extravasation from the vasculature to secondary tissue; and finally (5) colonization at secondary tumour sites [19], as depicted in **Figure 1.1**. Importantly, each stage of metastasis imposes different, often harsh conditions and energetically taxing challenges for the tumour cells to complete. As the cascade progresses, the number of viable tumour cells which survive and successfully complete each stage decreases precipitously, making this a very inefficient process. In this context, it is estimated that more

than 99.98% of disseminated cancer cells die before a metastasis could form [19]. The first step required for the formation of BC metastases involves BCCs escape from the primary tumour site by the alteration of cell plasticity and increased motility that allow the passage of malignant cells through myoepithelial cells, BM and ECM that constitute tumour growth barriers [1]. In this context, some studies suggested that when the tumour progresses into an invasive form, the tumour cells undergo a phenotypical change from typical cuboidal to an elongated spindle shape, with loss of cell-cell and cell-BM adhesion and gain a migratory ability [20, 21], which are features of the epithelial-mesenchymal transition (EMT) process occurrence. This mechanism also positively influences tumour cell resistance to anoikis (i.e. induction of apoptosis related to loss of adhesion) [22]. The subsequent migration of tumour cells depends on the local proteolytic degradation of these structures [1] by matrix metalloproteinases (MMPs) allowing the extension of cellular projections known as invadopodia. Posteriorly, dynamic interaction between cancer cells and surrounding microenvironment guide the tumour cells.

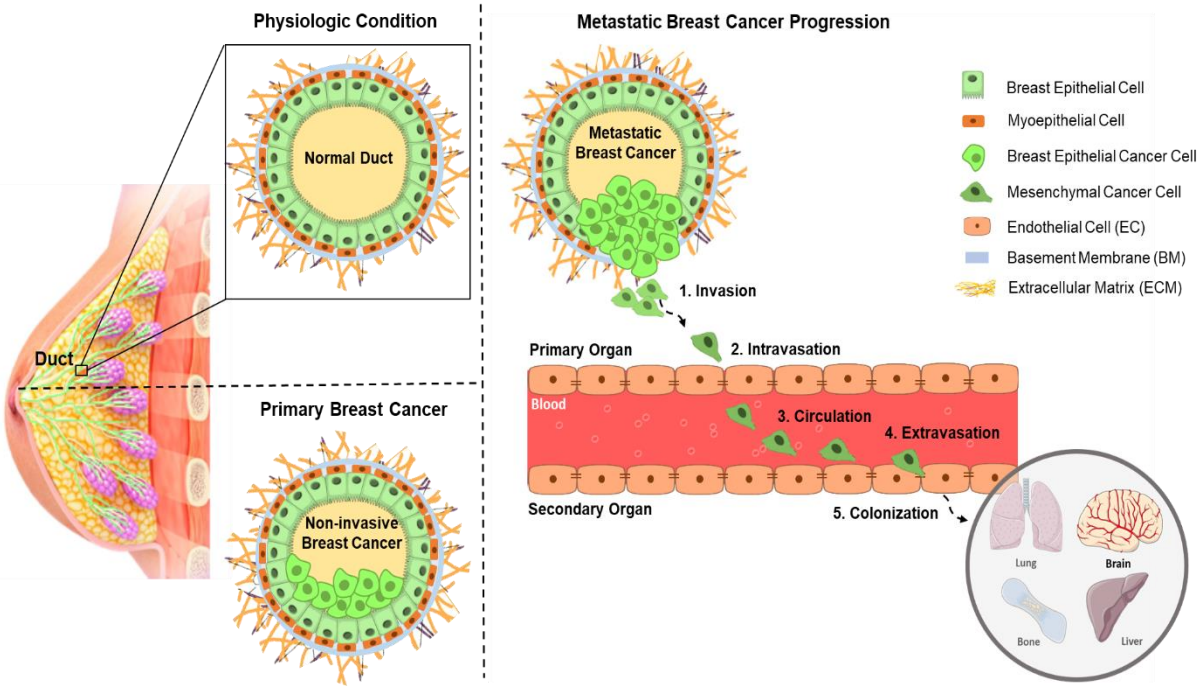

Figure 1.1. Schematic representation of breast cancer and metastasis development from mammary ducts. In normal breast, the mammary duct is lined by polarized epithelial cells that are surrounded by a layer of myoepithelial cells, followed by two structures, the basement membrane (BM) and extracellular matrix (ECM). The primary breast cancer is characterized by the massive breast cancer cells (BCCs) proliferation inside the mammary ducts, called non-invasive BCCs. The metastatic BC is developed when non-invasive malignant cells acquire invasive properties and grow beyond the ductal structure, starting the metastatic cascade. The metastatic cascade is comprised by several and sequential steps: initially, tumour cells migrate into adjacent tissues, referred to as the local invasion, a step that involves the breakdown of the BM and invasion into the surrounding ECM (1); then cells enter the circulation (2), a process known as intravasation (2); once inside the blood vessels, tumour cells need to survive the shear stress resulting from the blood flow and evade clearance by the immune system to successfully reach distant organs (3); tumour cells then attach to endothelial cells (EC), which facilitates their extravasation into the target organ (4); finally, cells colonize to the secondary organs (5), preferentially bone, liver, lung and brain.

Once malignant cells undergo EMT and are able to detach from the primary tumour and invade surrounding tissue, to reach distant organs, the cells need to enter in bloodstream in a process named intravasation. Importantly, in this step of the metastatic cascade, it is crucial the interaction between BCCs and endothelial cells (ECs), the cells that constitute the vessel walls, but also their interaction with endothelium-associated cells (e.g. perivascular cells), reinforcing the vascular structure. Once within the circulatory system, the fluid shear stress and the presence of immune cells such as natural killer (NK) cells can result in death of a great number of tumour cells [1]. These obstacles make the tumour cells circulation in blood the limiting step of the metastatic cascade. For BCCs to reach and colonize the parenchyma, extravasation must occur, which involves the arrest of cancer cells in specific

capillary beds, followed by the transposition of the BBB microvasculature that culminates in breast cancer brain metastases (BCBM) development. Among all steps of the metastatic cascade, this one is still poorly studied and characterized, especially when it occurs at the BBB level, a barrier with unique properties.

After colonization of the brain by BCCs, without any treatment, the expected survival time of patients with brain metastasis is about one month after diagnosis [23]. Regarding the classical methodologies, surgical resection of metastasis revealed to be in prone of the survival benefit in patients, especially those with a single and accessible brain lesion, with good functional status and with absent or controlled systemic disease [23]. Patients that present multiple intracranial lesions and poor performance status, the surgical option does not exist and the first-line option for patients with BCBM is whole-brain radiation therapy (WBRT). This hypothesis is considered once BCCs are radiosensitive. However, although this method offers symptomatic relief, the development of neurocognitive dysfunction over time is a significant problem for the survivors patients with brain metastasis [24]. On the other hand, the stereotactic radiosurgery (SRS) is an alternative for single metastasis in inaccessible areas or in some patients who are for some reason not fit for surgery. This procedure involves the localized delivery of high doses of radiation and, in fact, SRS is less invasive and a more efficient method than WBRT [25], but it can be associated with complications such as the development of radiation necrosis and the delivery of high doses of focal radiation can limit further therapeutic options upon disease progression [26]. Thus, the treatment of metastases remains controversial, because even with the appropriate therapy, neurologic symptoms are currently observed and not fully reversible.

Recently, more selective and targeted treatment methods have been developed as chemotherapy-based anticancer agents. However, in contrast with peripheric organs, the brain presents the BBB, which separates the brain from the peripheral circulation and constitutes a big challenge for efficient drugs delivery [27]. Due to the difficulty of treating metastases when they are already formed inside the brain, the development of strategies that prevent the arrival of metastatic cells into the brain parenchyma becomes urgent, and the future relies in personalized therapies targeted to specific tumour molecular pathways, such as those involved in BBB transposition, cell-cell adhesion and/or angiogenesis [28].

1.2 Interplay between tumour cells and endothelial cells: a key step for extravasation

As mentioned earlier, the BC progression towards brain metastasis occurs by a metastatic cascade that includes the malignant cells passage across the BBB, by the process known as extravasation [1]. The extravasation is considered a multi-step process that was first described and associated with leukocytes, i.e. T lymphocytes, NK cells, neutrophil and monocytes, during inflammatory response [29, 30]. However, regardless the type of cells, the extravasation process can be divided into three sequential steps. In the first step, the cells slackly attach to the vascular endothelial cells, due to this loose interaction, the extravasating cells are still pulled along with blood stream, which results in a rolling motion of the cells on the vascular surface. In the second step, the cells tightly attach to the ECs and after that the tumour cells can actively transmigrate through the endothelial barrier, by transendothelial migration or diapedesis process. Although these processes are well characterized for leukocytes, these cells and tumour cells present differences at the molecular, physiological and mechanical levels [30]. Allied to this fact, the complexity and specificity of the endothelial structure involved in brain metastasization, makes this process unique and still poorly characterized.

1.2.1 Blood-brain barrier: a big challenge for brain metastasization

The BBB is described as a dynamic and complex vascular structure that separates the CNS from the peripheral blood circulation, ensuring a constant internal environment inside the brain of vertebrates

to safeguard normal cerebral functions and brain homeostasis. Brain capillaries that compose the BBB are primarily formed by a continuous and non-fenestrated single layer of brain microvascular endothelial cells (BMECs), considered the anatomic basis of BBB [27], that present unique features when compared with ECs of the rest of body. BMEC lining the vascular wall have a more extensive and strong intercellular adhesion network that seals the paracellular spaces between the blood and brain [27]. This fact combined with the absence of fenestrations, the reduced number of vesicles and the negative surface charge of ECs, prevent the traffic of solutes into the brain parenchymal space [31]. However, ECs also present specific transporters on their surface, relevant for brain supply and they have a higher mitochondrial content (8-11% of the cytoplasmic volume) when compared with tissues from non-BBB regions (2-5% of the cytoplasmic volume) [32], being a critical feature to generate energy in order to carry out physiological functions in BBB. To limit the entry of immune cells into the CNS, these cells express low level of leukocyte adhesion molecules (LAMs). Nevertheless, BMECs have a differential vascular metabolism, generating a barrier by altering the physical properties of molecules, which can change their reactivity, solubility, and transport properties [33].

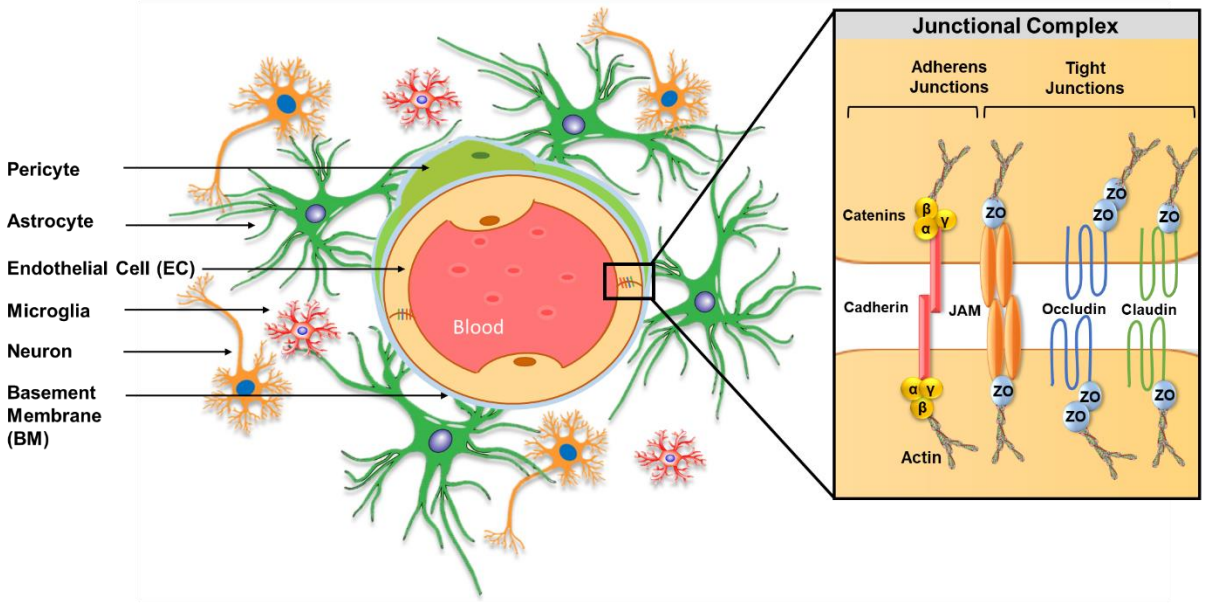

Figure 1.2. Schematic representation of the blood-brain barrier and of the neurovacular unit. The blood-brain barrier (BBB) is formed by endothelial cells (ECs) that line brain capillaries and control the passage of solutes between the brain and blood circulation. Astrocytes, pericytes, microglial cells, neurons and the basement membrane (BM) are the cellular and non-cellular components of the neurovascular unit (NVU) that interact with endothelial cells and provide functional and structural support. One of the most relevant features of the BBB is its extremely strong intercellular adhesion network, which is established mainly by tight junctions (TJs) and adherens junctions (AJs) between brain microvascular endothelial cells (BMECs). This strong adhesion seals the paracellular space between the blood and brain and prevents the passage of a variety of substances between these two compartments, accounting to the restricted permeability. The tight junctions (TJs) comprises occludin, claudins and junctional adhesion molecules (JAMs), which bind to accessory cytoplasmatic proteins such as zonula occludens (ZO), that subsequently interact with cell cytoskeleton by actin intermediate. The adherens junctions (AJs) are formed by cadherin-catenin complexes that provide structural integrity of the BBB and are necessary for tight junction formation.

Other cell types located adjacent to BMECs (including astrocytes, pericytes, microglia and neurons) are also known to contribute to BBB function, being part of the neurovascular unit [34] (**Figure 1.2**). As mentioned before, one of the most relevant features of the BBB is its extremely strong intercellular adhesion network, which is established mainly by tight junctions (TJs) and adherens junctions (AJs) between BMECs [27]. TJs contain at least 40 different proteins. Some of these proteins, such as the claudins, occludin and junctional adhesion molecules (JAMs) are transmembrane proteins that form intercellular homophilic and heterophilic adhesions, whereas others such as zonula occludens (ZO) family, AF6, cingulin, 7H6 and others are intracellular plaque proteins that form a scaffold between

the transmembrane proteins and the actin cytoskeleton [35, 36]. On the other hand, the AJs are multi-protein complexes such as cadherin-catenin complexes that play an important role in endothelial permeability regulation. Particularly, in BMECs, the AJs are distributed along the cleft frequently intermingled with TJs [37]. Altogether, these properties make these cells responsible to regulate CNS homeostasis.

1.2.2 Adhesion of malignant cells to the blood-brain barrier endothelium

Similarly to leukocytes, the tumour cells extravasation is mainly dependent on the occurrence of sequential interactions and crosstalk between adhesion molecules expressed on the ECs, tumour cells and other circulating cells [38]. For the immune cells it is known that the extravasation process is initiated by a transient contact of the circulating tumour cells with the BBB endothelium followed by rolling with the reduced velocity along the vascular wall leading to its arrest, polarization, and subsequent robust interaction is required for this attachment to resist swift blood flow and crawling to sites permissive for diapedesis [39]. About tumour cells, what is known is that these cells mimic mechanisms used by leukocytes, but the adhesion players involved in tumour cell extravasation are somehow different, suggesting additionally or alternative non-leukocyte-like mechanism [30]. In this context, several studies have shown that a wide range of ligands and receptors including selectins, integrins and members of the immunoglobulin superfamily of cell adhesion molecules (CAM), like intercellular CAM (ICAM) and vascular CAM (VCAM), contribute to the adhesion process [38, 40].

1.2.2.1 Selectins and its ligands promote the rolling and initial attachment to endothelium

Like leukocytes, the rolling of cancer cells during the first steps of extravasation is mediated by selectins. Selectins are vascular cell adhesion molecules and are a family of three C-type lectins: P-, L- and E – selectin. Although all the selectins have been shown to contribute for brain metastases, only P- and E-selectin are expressed by ECs [41] when stimulated by inflammatory cytokines secreted by cancer cells or cancer cell-associated leukocytes [42]. In contrast, tumour cells do not express selectins, but their respective ligands.

P-selectin, formally designated by CD62P, and its ligands are responsible for the first interaction among cells in the bloodstream, since they are also expressed on platelets surface. One of the most known P-selectin ligands is platelet selectin glycoprotein ligand-1 (PSGL-1 or CD162) expressed by both leukocytes and cancer cells. This connection is responsible by platelet-cancer cell aggregate formation, which leads to the adhesion of cancer cells to stimulated ECs [43] and simultaneously promote the survival of tumour cells in circulation by the modulation of NK cells function [44]. The pro-metastatic action of platelets can be organ-specific. For instance, platelets clearly enhance the metastasis of BC to the lung [45] but there is no current evidence of the same effect in brain metastasis. L-selectin (also known as CD62L) has a distinct role in the tumour cells extravasation. This selectin is responsible for the establishment of a metastatic niche due to the recruitment of bone marrow derived cells such as leukocytes to metastatic site. L-selectin deficiency attenuates metastasis formation [46].

Despite the importance of the other selectins, the initial attachment is mainly mediated by endothelial (E) – selectin (also named CD62E), a heavily glycosylated transmembrane protein, expressed by endothelial cells. E-selectin is expressed within the first hours after exposure to inflammatory mediators. Several ligands of this selectin have been identified, but few in the context of BCCs adhesion to the endothelium and even less when it comes to brain endothelium. It is known that BCCs express some ligands of E-selectin family such as cluster of differentiation (CD)44 and CD24, but their interaction and key role in BCCs extravasation across the BBB remains unclear [40, 47, 48]. On the other hand, comparing with well-established adhesion processes of other tumour cells, the cooperation between CD44v (CD44 variant) and carcinoembryonic antigen (CEA) has been reported in

human colon cancer cells adhesion process [49]. Moreover, heparin, which inhibits selectin-mediated interactions was shown to delay a melanoma brain metastasis formation [50]. Additionally, prostate cancer cells express glycoprotein and glycosphingolipid structures containing sialyl Lewis X (sLe^x) epitopes to adhere to E-selectin on bone marrow ECs, promoting metastatic dissemination into the bone. However, the participation and contribution of these molecules in BCCs extravasation across BBB is still unknown.

Importantly, other studies revealed that cadherins, calcium-dependent homophilic cell-cell adhesion molecules, such as neuronal (N)-cadherin, have been observed to play a minor role in the regulation of rolling and adhesion of MDA-MB-468 human breast carcinoma cells by means of N-cadherin/N-cadherin interaction in the pulmonary endothelium [30]. Thus, the study of the molecules involved in first stages of adhesion process is very important to trace the mechanisms adjacent to the extravasation process and uncover possible targets for preventive therapeutic approaches.

1.2.2.2 Complexes Integrins-IgCAMs promote firm attachment to endothelium

After establishing the first interaction, a firm adhesion to the endothelium must occur. In general, integrins are heterodimeric proteins consisting of an α and a β subunit that mediate cell-cell and cell-ECM interactions. However, in tumour cells extravasation context, the integrin ligands are normally designated as non-extracellular matrix ligands, such as immunoglobulin-like cell adhesion molecules (IgCAMs) that can be expressed by both tumour cells and ECs (**Figure 1.3**). The expression of these adhesion molecules is also involved in their attachment to the platelets [51].

Among several known endothelial adhesion molecules, the VCAM-1 (also known as CD106) has been found to be involved in this process. It has been shown that VCAM-1 is aberrantly expressed in BCCs and that it can bind to its natural ligand very late antigen (VLA-4 or α4β1 integrin). Moreover, this binding appears to be responsible for the metastasis of BCCs to lung, bone and brain. Thus, the α4β1 integrin – VCAM-1 interaction represents a potential therapeutic target for metastatic BCCs [30, 52]. *In vitro* assays have also shown the involvement of α4ß1 integrin in melanoma, osteosarcoma and kidney cancer cells adhesion to the vascular endothelium [53]. Other studies with human MDA-MB-231 BCCs injected into the vasculature of zebrafish embryos were found to adhere to the intraluminal vessel wall via β1 integrins [54]. As well VCAM-1, the ICAM-1 (also known CD54), a transmembrane glycoprotein expressed by several cell types, including ECs, has been demonstrated as a potential player in metastatic ability of BCCs [55, 56]. One of your recognized ligands is Mucin 1(MUC1), a glycoprotein found in BCCs [57]. Also, αVβ3 integrin (also known as vitronectin receptor) expressed by BCCs is involved in the adhesion process. Its ligand is neural cell adhesion molecule L1 (L1-CAM) and its expression can be observed in ECs or platelets [58], but the interaction between these molecules in the context of BCCs extravasation and brain metastasis establishment remains unclear and is one of the major gaps that prevents detailed characterization of tumour cells extravasation.

1.2.3 Transendothelial Migration

While tumour cells use various strategies to survive in the hostile intravascular environment, their metastatic potential eventually depends on their ability to rapidly extravasate into surrounding tissue [59]. The TEM is considered the last step of extravasation process and in the BCBM context is characterized by the release of the tumour cells from the microvasculature into the brain microenvironment [1] (**Figure 1.3**). Despite the scarce information about the extravasation process, it is known that the duration of TEM by cancer cells towards the brain is significantly shorter *in vitro* (6 to 18 hours) than *in vivo* (3 to 5 days), probably due to different conditions in the vasculature once they are much more complex than what can be mimicked *in vitro* [60].

In general, the transmigration can be achieved by two possible pathways: the cells can either move between the ECs, which is termed the paracellular or junctional route, or they can migrate through ECs, which is termed the transcellular route. Leukocytes can use both routes in the brain endothelium as well, but much less is known about the transmigration routes used by tumour cells, especially when the secondary organ is the brain parenchyma [30]. However, the predominant mode of tumour cells extravasation appears to be the paracellular migration, during which cancer cells migrate between two ECs, a process requiring cellular rearrangements and disruption of inter- endothelial cell-cell junctions [59]. In this regard, *in vitro* studies demonstrated that melanoma cells coming in contact with cerebral endothelial cells (CECs) disrupted the TJ and AJs of these cells and use the paracellular transmigration pathway [61]. Moreover, another study indicated that the metastatic melanoma cells initiate disruption of human pulmonary endothelial cell-cell junctions and promote the formation of intercellular gaps in endothelial monolayers in the first minutes of direct co-culture. This disruption was associated with vascular endothelial (VE)-cadherin phosphorylation, which can lead to an increase in vascular permeability [62]. Simultaneously, it has been demonstrated that N-cadherin plays a key role in this process by the formation of an adhesion complex that lacks β-catenin [63].

The cadherins are Ca^{2+}-dependent cell adhesion molecules and can exist under two forms at the BBB: the vascular endothelial (VE)-cadherin (also known as CDH5 or CD144) and/or N-cadherin (or CDH2). While VE-cadherin is endothelium-specific [64], N-cadherin is typically expressed in mesenchymal cells [65]. In cancer pathogenesis, cadherins were proved to control the balance between suppression and promotion of invasion, being VE-cadherin reported to function as an invasion suppressor and being downregulated in most carcinomas, while neural cadherin can act like a suppressor or promoter depending on the cellular environment [66]. In microvasculature, VE-cadherin may signal through β-catenin, which binds cadherin cytoplasmic tail and α- catenin, which in turn links the complex to actin cytoskeleton. Particularly relevant for BBB integrity is the association between β-catenin and Wnt proteins [67], promoting the β-catenin/Wnt signalling, which in turn leads to an increased TJ proteins expression [68].

Regarding the BCCs transmigration it is not completely clear whether these malignant cells migrate preferentially paracellularly or transcellularly. In fact, transcellular migration of tumour cells has only been described in case of BCCs during intravasation in an *in vitro* vascular network [69] and migration through umbilical cord ECs [70].

In the extravasation context, few studies have been performed, but these point that human BCCs lines use preferentially the paracellular route of TEM into the extravascular stroma since cancer cells are typically much larger than ECs and leukocytes [59]. More recently, associated with paracellular transmigration, an additional step was identified in BCCs extravasation process that occurs even before the complete endothelial transmigration. In this, the tumour cells are incorporated into the endothelial monolayers, thus leading to their disruption [71]. This phenomenon had already been described as tumour-endothelial cells mosaic formation by Tremblay et al., when investigated the colon cancer cells transmigration mechanism through the vascular endothelium [72]. Even with this evidence, further studies are still needed to better characterize this process for BCCs.

Other studies indicate that tumour cells, including BCCs, produce and secrete some molecules that interfere with junctional complexes organization and expression of the junctions' proteins. One of these molecules is the neuropeptide substance P (SP), which contributes to BMECs activation and consequent secretion of tumour necrosis factor alpha (TNF-α) and angiopoietin-2 (Ang-2). These, in turn, lead to alterations in localization and distribution of BBB junctional proteins, such as ZO-1 and claudin-5, resulting in an increase of barrier permeability [73]. Also, cathepsin S has been shown to specifically mediate BBB transmigration of BCCs via proteolytic processing of the JAM [74].

Another important aspect associated with tumour cells transmigration is correlated with their morphology during interaction with ECs. Morphologically, the BCCs have the ability to form

specialized structures called podosomes to facilitate their transit across the endothelial monolayer, contributing for the paracellular transmigration, due to the EC junctions opening. These podosomes have been described as "invadosome-like portrusions" that are commonly referred to as invadopodia in cancer cells. This phenomenon was firstly described in intravasation process, but more recently *in vivo* studies demonstrated that the disruption of these structures results in decreased extravasation efficiency and an abrogation of metastasis [59].

Regardless of the pathway used by tumour cells in TEM, in contrast to leukocytes tumour cells are supposed not to leave the endothelium intact after their migration. Tumour cells are much larger than leukocytes and it would be difficult to squeeze between ECs without any damage. Therefore, it was postulated that during transmigration, the endothelium is irreversibly damaged, which is a consequence of the induction of apoptosis, mainly by the loss of cell-cell contacts [75]. Yet, others authors demonstrated that the absence of endothelial apoptosis in TEM regions indicates that this process does not cause the complete destruction of the barrier [60]. Therefore, further studies are needed in order to examine the BBB integrity after their transposition by tumour cells.

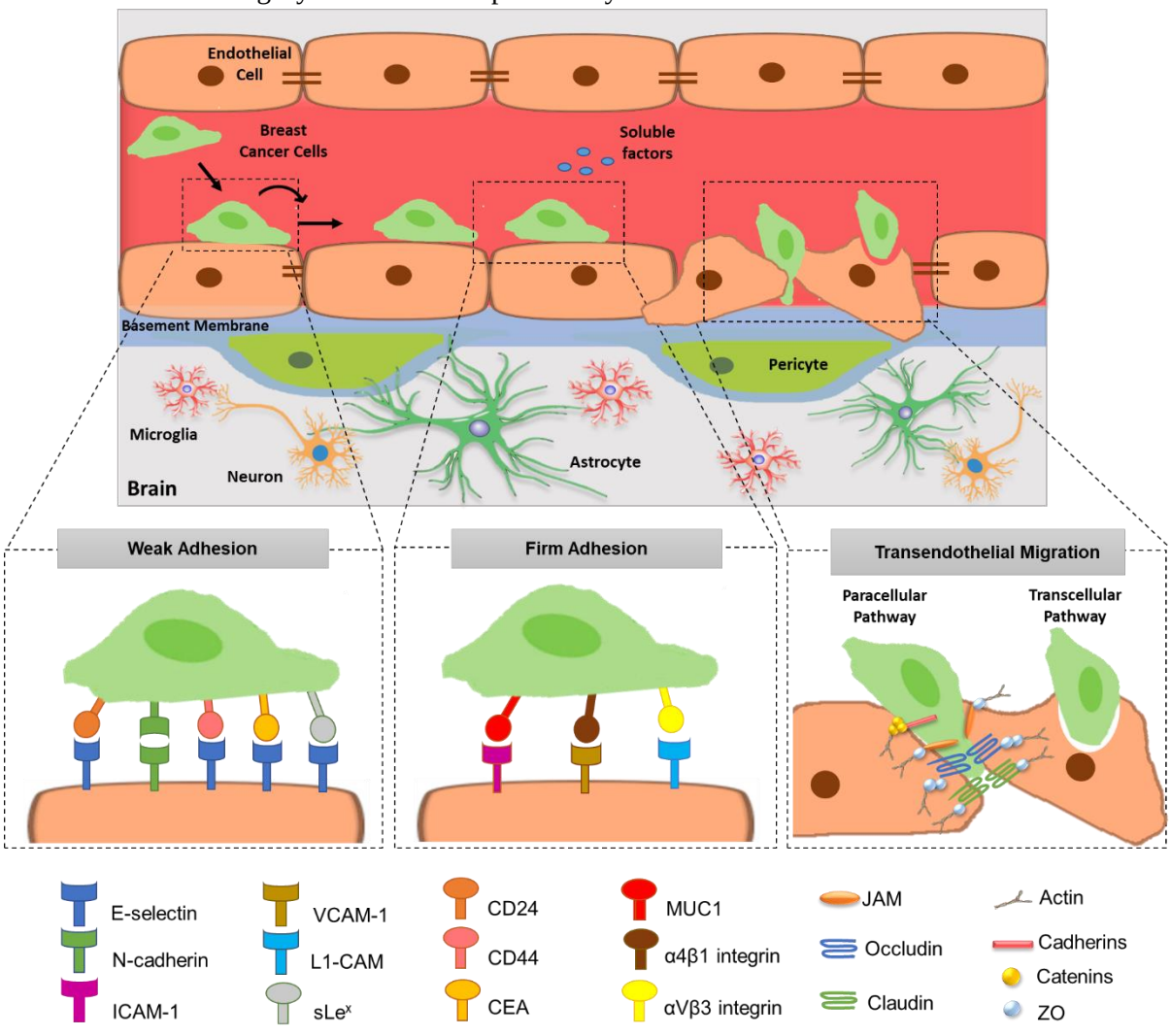

Figure 1.3. Schematic representation of metastatic cancer cells extravasation. Breast cancer (BC) progression towards brain metastasis occurs by a metastatic cascade that includes malignant cells passage across de blood-brain barrier (BBB), known as extravasation. The migration of BC cells (BCCs) across the BBB includes the occurrence of mechanisms of rolling (weak adhesion), adhesion and transendothelial migration (TEM)/diapedesis. Sequential steps in tumour cells migration are controlled by interactions between specific molecules on tumour cells and their counter-receptors on endothelial cells (ECs). In bloodstream, the first interaction between cells facilitates the rolling. Endothelial (E)-selectin expressed by activated ECs has many possible partners expressed by tumour cells, such as cluster of differentiation (CD)44, CD24, carcinoembryonic antigen (CEA) and syalyl Lewis x (sLe^x). Also, neuronal (N)-cadherin/N-cadherin interactions participate in the weak adhesion step. These molecules contribute to the early adhesion stages of tumour cells to the endothelium. The interaction of selectins

with their ligands present on malignant cells results in a catch bond that switches from rolling and adhesion of the cells to firm adhesion by further interactions with other adhesion molecules such as integrins. The integrin ligands, in adhesion processes, are normally designated as non-ECM ligands, such as immunoglobulin-like cell adhesion molecules (IgCAMs). The tumour cells express some ligands such as α4β1 (or very late antigen 4; VLA-4) and MUC1 that interact with vascular CAM (VCAM)-1 and intercellular CAM (ICAM)-1, respectively, expressed by ECs. Similarly, also αVβ3 integrin (vitronectin receptor) is expressed by malignant cells, including breast cancer cells, and is involved in the adhesion process. One of its ligands is neural cell adhesion molecule L1 (L1-CAM) and its expression can be observed in ECs and platelets. The TEM is considered the last step of extravasation process and in the BCBM context is characterized by the release of the tumour cells from the microvasculature into the brain microenvironment. The transmigration can be achieved by two possible pathways: the cells can either move between the ECs, which is termed the paracellular or junctional route, or they can migrate through an EC, which is termed the transcellular route. The predominant mode of tumour cells extravasation appears to be the paracellular migration, during which cancer cells migrate between two ECs, a process requiring cellular rearrangements and disruption of inter-endothelial cell-cell junctions.

1.3 Endothelial-mesenchymal transition: a novel and key role in blood-brain barrier remodelling during tumour cells extravasation

Recently, has been described that ECs undergo endothelial/mesenchymal transition (EndMT), a process with certain similarities with the better understood epithelial-to-mesenchymal transition. Just as occurs during normal development, during postnatal EndMT, ECs acquire mesenchymal characteristics, such as an elongated, fibroblastoid morphology, increased motility, cytoskeletal modifications and cell-to-cell junction rearrangement. EndMT may lead to ECs acquiring a variety of different mesenchymal fates. At the cellular level, EndMT consequences include altered ECs junction organization, loss of cell polarity, and increased cell proliferation and migratory capacities. This results in a number of pathological consequences of considerable clinical significance in several diseases, such as cancer [76, 77] (**Figure 1.4**).

These features, mainly the ECs junction organization and the loss of endothelial markers, has been reported as the major contribute for the disruption of adherens and tight junctions between neighboring ECs, allows the metastatic cells to squeeze between and pass through the endothelial cell layer and contribute for the tumour cells dissemination to secondary organs.

The EndMT might play a more relevant role during tumour cell extravasation in organs with a more compact organization of the ECs, such as the brain. In this regard, this transition has already been reported in some ECs lines, such as human umbilical vascular ECs (HUVECs) in co-culture with melanoma cells [76].

1.3.1 Loss of endothelial traits and gain of mesenchymal features

In physiological conditions, BMECs express a distinct set of biomarkers that allow to distinguish them from other cells types, including the expression of VE-cadherin, claudin-5, von Willebrand Factor (vWF) and cytokeratins. However, during EndMT, the expression of endothelial markers is dramatically reduced. Furthermore, this reduction is accompanied by the gain of mesenchymal markers such as fibroblast-specific protein-1 (FSP-1), alpha-smooth muscle actin (α-SMA), vimentin, and N-cadherin. These changes in protein expression cause the loss of ECs adhesion and stimulate alterations in cytoskeletal composition and organization, inducing a striking change in cell morphology giving rise to elongated and spindle shaped cells. These newly formed mesenchymal cells are highly invasive and migratory [78]. It is known that EndMT is required for metastatic BC TEM to occur [76]. However, the mechanism by which this phenotypic and morphological transition in BBB is induced is still poorly understood, but is well described for EMT, a similar process that occurs in BCCs early stages of metastatic cascade, allowing invasion and subsequent intravasation.

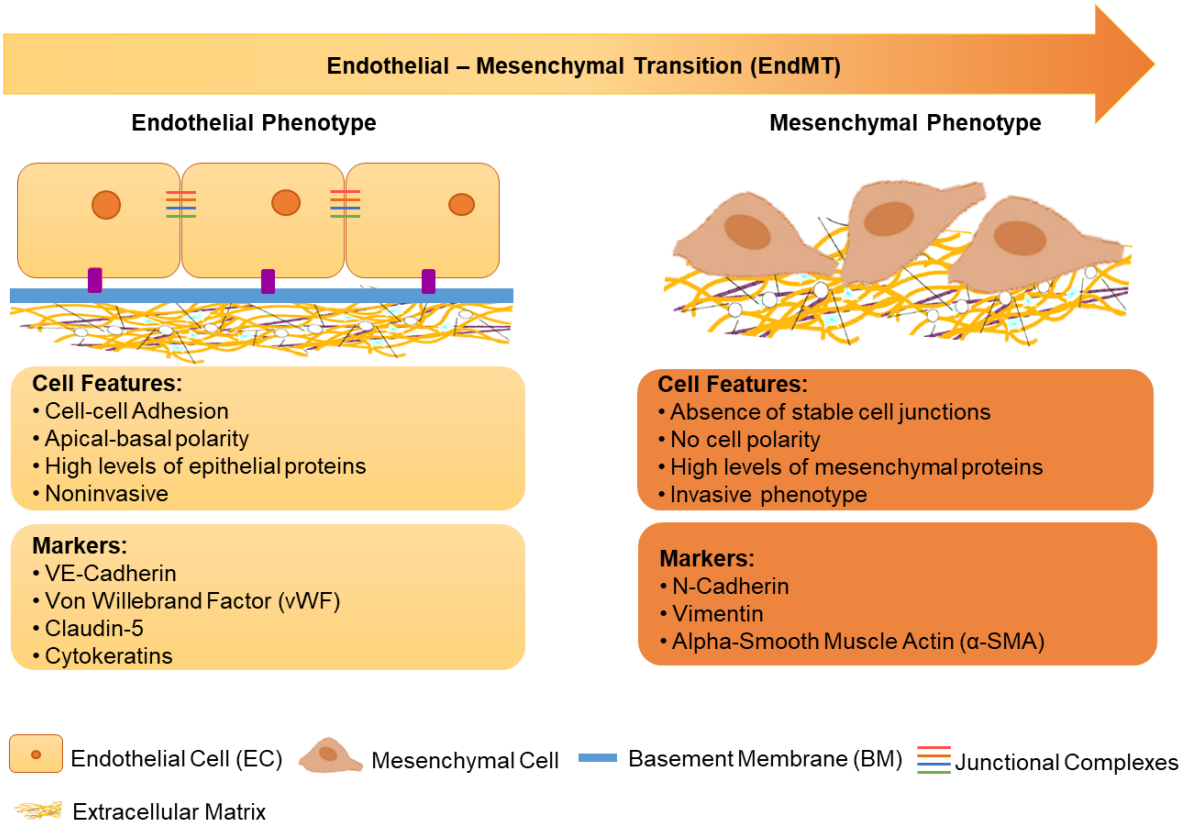

Figure. 1.4. Schematic representation of endothelial-mesenchymal transition (EndMT), a process by which endothelial cells lose their endothelial features and acquire mesenchymal properties. Under physiologic conditions, endothelial cells of the blood-brain barrier (BBB) interact with each other due to the presence of numerous junctional complexes. However, the proximity of tumour cells to the endothelium during the extravasation process promotes the release of inflammatory mediators, leading to the activation of transcription factors and, subsequently, endothelial cells undergo EndMT. During this process, the expression of endothelial markers is reduced and accompanied by the gain of a mesenchymal profile. Consequently, these cells lose their adhesion, polarity and non-invasive nature and acquire invasive and migratory properties. These changes lead to BBB disruption, and favour tumour cells extravasation.

II. AIMS

The metastatic progress of BCCs and their interaction with BMECs is a process poorly characterized. As such, this work will test the hypothesis that, during extravasation, the interaction established between BCCs and BMECs leads to morphologic and phenotypic alterations in both cell types, resulting in the expression of distinct pairs of ligands/receptors, which can be possible targets for new therapeutic strategies in order to prevent BCBM formation. Therefore, with this study, we aim to disclose the extravasation step in the metastatic cascade by profiling the ligands involved and establishing the phenotypic changes in BMEC and BCCs that have a direct influence on the mechanism of BCBM formation. To achieve this global aim, specific objectives were outlined, directed to:

1) Establish a time-course profile of BCCs and BMECs along extravasation;
2) Characterize BCCs adhesion mechanisms to BBB;
3) Evaluate endothelial-mesenchymal transition in BMECs during extravasation;
4) Characterize BCCs transmigration pathways through BBB;
5) Evaluate the BBB integrity along extravasation;

This investigation work will contribute to an important goal in Neurooncology of reducing brain cancer by launching the basis for the development of novel therapeutic approaches focused in the

targeting of BCCs and/or ECs during extravasation. Overall, this project will contribute to improve life expectancy ad quality of BC patients.

III. MATERIAL AND METHODS

3.1 Cell Lines and Culture Conditions

3.1.1 Endothelial Cell Line

In the present study, the cell line HBMEC (Human Brain Microvascular Endothelial Cells), kindly provided by Prof. Kwang S. Kim, Division of Infectious Diseases, Baltimore, was used as a simplified *in vitro* model of the BBB endothelium. This cell line proved to be the most suitable one for *in vitro* studies, being considered the best *in vitro* BBB model, concerning barrier tightness [79]. This human cell line was immortalized and routinely cultured in our lab as previously described [80-84]. Briefly, HBMEC were plated in coated coverslips with collagen type I (Corning, 3900 µg/mL) at a concentration of 50 µg/mL, in the 12 and 24-well plates for flow cytometry and immunocytochemistry and coated collagen I gel (1.5 mg/mL) in µ-Slide 8 well glass bottom (Ibidi, Martinsried, Germany), for live-cell imaging analysis, respectively at a density of 5×10^4 cells/mL and incubated at 37°C and 5% CO_2, for 72 h. Cells were cultured in RPMI 1640 (Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS - Biochrom AG), 10% NuSerum IV (Corning), 1% non-essential amino acids (NEAA, Biochrom AG), 1% minimal essential medium (MEM) vitamins (Sigma-Aldrich), 1mM sodium pyruvate (Biochrom AG), 2mM L-glutamine (Biochrom AG) and 1% antibiotic-antimycotic solution (Sigma-Aldrich). Endothelial cell cultures were maintained at 37°C in a humid atmosphere enriched with 5% CO_2. In our studies, HBMEC were used between passage 19 and 29.

3.1.2 Adenocarcinoma Cell Line

The MDA-MB-231 Br4 cell line (in short MDA231 Br4), kindly donated by Dr. João Barata, Instituto de Medicina Molecular, IMM, Lisbon, was used. The MDA231 Br4 is an epithelial, breast adenocarcinoma cell line that was derived from a metastatic pleural effusion. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS - Biochrom AG), 2mM L-glutamine (Biochrom AG) and 1% antibiotic-antimycotic solution (Sigma-Aldrich). These cultures were maintained at 37°C in a humid atmosphere enriched with 5% CO_2. In our experiments, these cells were used between passage 13 and 42.

3.2 Breast Cancer Brain Metastasization *in vitro* Model Establishment

As an *in vitro* model that mimics the BC brain metastasization, mixed cultures of HBMEC and MDA231 Br4 cells were implemented. In preliminary experiments different BCCs concentrations were tested: 2×10^3, 1×10^4, 5×10^4, 1×10^5, 5×10^5, 1×10^6 and 2×10^6 cells/mL. The optimal density of MDA231 Br4 cells was defined as $1x10^5$cells/mL in plate and 5×10^4 cells/mL in Ibidi support. For mixed cultures experiments, MDA231 Br4 cells were plated on top of HBMEC (cultured as described above). In parallel, assays were run with each cell type cultured alone, as controls. The time of incubation was assay-dependent.

3.3 Live-Cell Imaging

In order to comprehensively disclose the interplay between BCCs and BMECs along time, live-cell imaging confocal and automated microscopy were performed, in collaboration with Dr. Hugo

Botelho and Professor Rui Malhó, FCUL Microscopy Facility. For that, the established BC brain metastasization *in vitro* model (described above) was used. The HBMEC were seeded on collagen I gel (1.5 mg/mL) coated in μ-Slide 8 well glass bottom. The collagen gels were performed by the sequential adding and mixing of PBS (10x), 1M NaOH, dH_2O and the rat tail type-I collagen. All gel components except collagen must be pre-cooled. The gel polymerizes in the incubator at 37°C, during 1 h. After 72 h of endothelial seeding, the endothelial monolayers were marked with CellTracker™ Red CMTPX Dye (2.5 μM) in RPMI for 30 min, at 37°C and 5% CO_2, in order to distinguish the two types of cells. After this time, the MDA231 Br4 were added to endothelial cultures in live-cell imaging medium (30 mM HEPES in Hank's Balanced Salt Solution, HBSS, without phenol red, pH 7.0-7.4). As mentioned above, single cell type cultures were run in parallel (controls). The time-lapse image acquisition of single and mixed cultures was performed using Leica TCS SP8 confocal microscope LAS X 3.5.2.18963 (Leica Biosystems, Germany) at 37°C and 5% CO_2. Images from two different positions per well were acquired every hour until 24 h, using a 20x dry objective (1024x1024 pixel image, 32 z-stacks with 1.3 mm interval). Images were acquired at the Faculty of Sciences of University of Lisbon's Microscopy Facility, a node of the Portuguese Platform for BioImaging (reference PPBI-POCI-01-0145-FEDER-022122).

The data analysis was performed using ImageJ (NIH, USA) and Icy (Institut Pasteur and France BioImaging, France) softwares, in order to evaluate the tumour cells morphological parameters, the tumour cells position relatively to the endothelium by the construction of 3D and orthogonal representations, the distance migrated by tumour cells, and to examine the endothelial monolayer integrity along extravasation. Morphological parameters, such as cells perimeter, roundness and circularity in single and mixed cultures were evaluated over time, based on analysis of four cells per position, at four different positions, as well as the migrated distance by these cells on endothelium, using the equation:

$$d = \left| \sqrt{\left(x_f - x_i\right) + \left(y_f - y_i\right)} \right| \quad \text{(i)}$$

where the d is the tumour cell migrated distance of the same cell at different timepoints, x_i and y_i are the coordinates of cell initial position and x_f and y_f are the coordinates of the final position. The tumour cells displacement was 3D represented and quantified on Imaris 9.3.1, using volume and surface modules.

The tumour cells position relatively to endothelium along time was monitored based on analysis of fifty tumour cells per position, considering cells that were above the endothelium level (out cells), or partially or fully intercalated within the endothelial monolayer (stage 1 or stage 2, respectively). The data shown represents analysis of data collected from two independent experiments. Finally, the endothelial monolayer integrity along time was quantified based on the comparation of the HBMEC covered area in single and mixed culture.

3.4 Flow Cytometry

To evaluate the ligands involved in MDA231 Br4 cells extravasation across the BBB, GLUT1, CD44, CD54, CD66adecb and CD29 positive cells were analysed in single and mixed cultures incubated for 0.5, 1, 3 and 6 h. The cultures were washed with versene, followed by incubation with trypsin-EDTA (1x, Sigma-Aldrich) for about 3-4 min. The trypsin activity was inhibited by the addition of FBS and the cells were pelleted by centrifugation at 500 g for 5 min at room temperature, resuspended in flow buffer (2% FBS in phosphate-buffered saline, PBS). Afterwards, each cell suspension was incubated with the fluorescent labelled antibodies GLUT1, CD44, CD54, CD66adecb and CD29 conjugated with phytoerythrin (PE) (1:50, Immunotools) for 20 min at 4°C. Flow cytometry was performed in Guava easyCyte 5HT Base System cytometer (Merck Millipore).

Data analyses were performed, using FlowJo software (FlowJo LLC). In the representative dot plots, the HBMEC and MDA231 Br4 cells were distinguish by the signal of GFP, which was considered negative before the 10^2 and positive after this value on the logarithmic scale on the x axis. Thus, for all the markers and conditions studied, the total BMECs population was defined by the GFP negative (-) gate, whereas the total tumour population was defined by the GFP positive (+) gate. The positivity for each marker in both populations was evidenced by a second gate defined above 10^2 on the logarithmic scale on the y axis.

3.5 Immunocytochemistry

Immunostaining studies were performed to evaluate the endothelial-mesenchymal transition and BBB disruption occurrence in mixed cultures at 0.5, 1, 3 and 6 h after the beginning of two cell lines interaction, as well in single cultures. Thus, the endothelial cell marker, β-catenin, the mesenchymal marker, neuronal (N)-cadherin and a cell mobility marker, myosin light chain kinase (MLCK) expression was analysed. Firstly, the cell cultures were fixed with 4% of paraformaldehyde (PFA, Sigma-Aldrich) for 20 min at room temperature. Following fixation, cells were permeabilized with 0.3% Triton X-100 (VWR International), except the cell cultures that will be labelled with anti-N-cadherin, and blocked with 3% bovine serum albumin (BSA, Sigma-Aldrich) in PBS, during 60 min at room temperature. Cells were incubated overnight at 4°C with the primary antibodies and thereafter with the corresponding secondary antibodies for 60 min at room temperature (for complete antibodies information, see Table 8.1 in supplementary material). Both primary and secondary antibodies were diluted in blocking solution. Nuclei were counterstained with Hoechst dye 33342 in PBS (1:1000, Thermo Fisher Scientific), for 10 min at room temperature. Between incubations, cells were washed three times with PBS. Cells were examined using an Olympus BX60 microscope and Hamamatsu's image acquisition and analysis software. Images were acquired at the Faculty of Sciences of University of Lisbon's Microscopy Facility, as mentioned above.

Post-acquiring treatment was performed using ImageJ and Icy softwares, for the measuring of proteins intensity endothelial cells elongation. For the fluorescence intensity and cells elongation analysis, five HBMEC per image were outlined with polygon tool.

3.6 Endothelial Cells Components Engulfment Analysis

In order to verify the incorporation of endothelial components by MDA231 Br4 cells during extravasation process, it was performed a similar test to live cell imaging in 12-well plates. 24 h before the start of the mixed cultures, the MDA231 Br4 cells were plated at a density of $5x10^4$cells/mL in 12-well plates. Endothelial and mixed cultures were incubated for 3 h at 37°C in a humid atmosphere enriched with 5% CO_2. After this time, both culture medium was transferred to MDA231 Br4 cells culture and incubated for 3 h. After reaching the incubations times, the culture medium was collected and conserved at -80°C for Nanosight assay and the cell cultures were fixed with 4% PFA in PBS.

3.7 Nanoparticle Tracking Analysis

The particle concentration (particles/mL) of collected culture medium was analysed using Nanoparticle Tracking Analysis (NTA) by NanoSight NS300 (Malvern Instruments, Shanghai, China) under a collaboration with Dr. Bruno Costa-Silva from the Champalimaud Foundation, which utilizes the properties of both light scattering and Brownian motion in order to measure the amount of particles in liquid suspension. Ideal measurement concentrations were found by pre-testing the ideal particle concentration value (15-30 particles/mL). All samples described in previous section were diluted in sterile PBS (1:4) to a final volume of 1 mL, immediately prior to analysis. Quantification was performed

using NanoSight with parameter setup of five captures per sample, 30 s per capture with 16 camera level, temperature 25°C, syringe pump speed 15, and detect threshold 5.

3.8 Statistical Analysis

Results were analysed using GraphPad Prism® 7.0 (GraphPad Software, San Diego, CA, USA) and are expressed as means ± SEM. The live-cell imaging results represents the average of two independent experiments and a two-tailed Student's *t* test was used to compare the BCCs morphological parameters in pure and mixed culture. The same test was applied to the evaluation of endothelial components engulfment by BCCs (n=1). In addition, two-way ANOVA, was used to evaluate the expression of adhesion markers in BMECs and BCCs in single and mixed cultures by flow cytometry (n=3) and mesenchymal markers in BMECs by the immunofluorescence analysis (n=3). P values less than 0.05 were considered statistically significant.

IV. RESULTS

The characterization of the extravasation process of BC is still poorly understood, mainly at the level of interaction between BC cells and BMECs, due to the complexity of this mechanism coupled with the fact that each tumour behaves differently. Based on this, we began our work by the establishment of a human BC brain metastasization *in vitro* model, which is composed by human brain microvascular endothelial cells (HBMECs) monolayers, comprising the anatomical basis of the BBB, and the triple negative breast cancer cells - MDA231 Br4 – labelled with GFP, that present brain tropism, a feature that makes our model unique and specific.

4.1 Triple negative breast cancer cells acquire an invasive and migratory phenotype in early extravasation events

In order to evaluate the interplay between MDA231 Br4 cells and HBMEC at temporal level, live-cell imaging confocal microscopy and automated microscopy was performed in order to replicate and monitor the intercellular dynamics along time (**Figure 4.1**). In this context, because some studies have demonstrated that tumour cells intravasation and extravasation occurs predominantly within the first 24 h of tumour cell introduction in culture systems [85, 86], our time window was within 24 h, imaging at every hour. Our results showed that after 1 h of interaction between the two cell types, the BC cells undergo morphological changes, from rounded to elongated morphology (**Figure 4.1A**), which is less noticeable in BCCs single cultures (**Figure 4.1B**).

Morphological parameters such as BC cells perimeter, roundness and circularity were analysed along time (**Figure 4.1C-E)** and in fact, we observed that tumour cells in mixed culture revealed an increase of their perimeter as early as after 1 h of interaction relatively the beginning of culture (0 h; $p<0.001$), as well comparatively to control at the same timepoint (2 to 6 h, $p<0.05$ and at 24 h, $p<0.001$) (**Figure 4.1C**). Simultaneously, it was observed a decrease of tumour cells roundness and circularity (**Figure 4.1D and E**). Regarding the cell roundness, the BC cells revealed a decrease of their round-shape after 1 h of interaction relatively the beginning of culture (0 h; $p<0.001$), as well comparatively to control at the same timepoint (at 2, 4, 5 and 6 h, $p<0.05$; 3 and 24 h, $p<0.01$) (**Figure 4.1D**). A similar profile was notorious in circularity and after 1 h, the BCCs presented a decrease of this parameter relatively the beginning of mixed culture (0 h; $p<0.01$), as well comparatively to control at the same timepoint (at 2, 3, 5, 6 and 24 h, $p<0.05$; 4 h, $p<0.001$) (**Figure 4.1E**). These changes were accompanied by the formation of highly dynamic intravascular cytoplasmatic extensions, mainly after 2 h of interaction, that allow them to contact a larger area of the endothelium (**Figure 4.1A**, white arrows).

These findings suggest that interaction between these two cell types promotes morphological changes in BCCs, which lead to the acquisition of invasive properties in malignant cells.

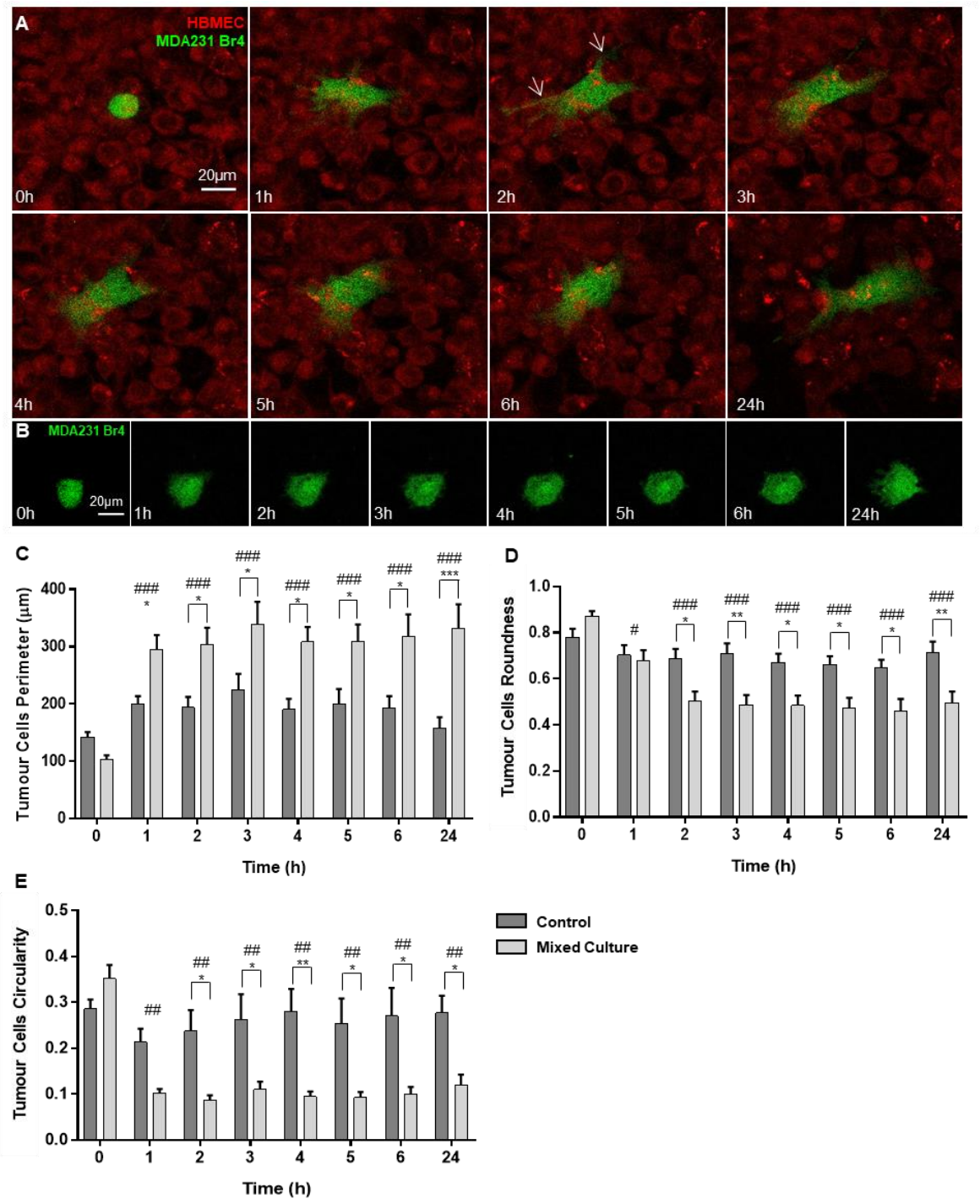

Figure 4.1. MDA231 Br4 cells acquire an invasive phenotype during extravasation. High resolution time-lapse confocal microscopy (20x) of mixed cultures composed by MDA231 Br4 cells (tagged with GFP; green) and HBMEC confluent monolayer (labelled with CellTracker™ Red CMTPX Dye; red) **(A)** and MDA231 Br4 cells single culture (control) **(B)** at different timepoints (0, 1, 2, 3, 4, 5, 6 and 24 h). Morphological parameters such as tumour cells perimeter **(C)**, roundness **(D)** and circularity **(E)** were analysed at the same timepoints. Data are given as means ± SEM (n = 2). Statistical significances are denoted as *P<0.05, **P<0.01 and ***P<0.001 for mixed culture vs control at the same timepoint and $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 for mixed culture of indicated timepoints vs. mixed culture at 0 h. Arrows indicates the cytoplasmatic cell extensions.

Once tumour cells have acquired an invasive phenotype, usually associated with migratory properties, next we assess the distance migrated by BCCs over time. The total distance travelled by BCCs is shown in Figure 4.2A through a colour gradient, in which each colour represents the distance travelled each hour over the 24 h.

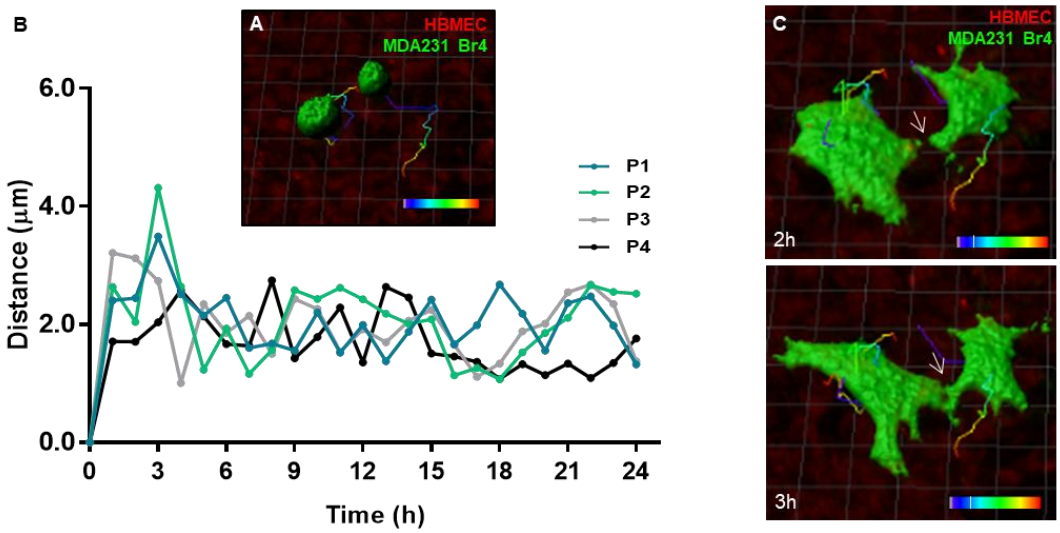

Figure 4.2. MDA231 Br4 cells acquire a migratory phenotype during extravasation. 3D representation of the total distance migrated by MDA231 Br4 cells (tagged with GFP; green) along 24 h by the colour gradient (**A**). Semi-quantitative analysis of MDA231 Br4 cells migrated distances along 24 h (**B**). Each line in B represents the distances mean of four MDA231 Br4 cells in four different positions on ibidi wells. Representation of tumour cells collective migration (**C**). Arrows indicates the contact points between tumour cells.

We quantified the distance migrated by the BCCs every hour (**Figure 4.2B**) and our results showed that tumour cells migrated more longer distances up to 3 h of interaction with brain endothelium, comparing with the migrated distance after this time (**Figure 4.2B)**. Moreover, our results showed that the BCCs migration is mainly collective, establishing direct contact with other malignant cells (**Figure 4.2C**, white arrows). These findings suggest that the interaction between BCCs and BMECs also leads to the acquisition of a migratory properties in BCCs. All of these alterations can potentiate the extravasation occurrence at early timepoints.

4.2 Triple negative breast cancer cells localize at different endothelium levels during the extravasation process

Extravasation is considered a complex process and is defined by the sequential occurrence of three main steps: the rolling (also defined as weak adhesion), adhesion and transmigration through the endothelium [87]. Since the previously obtained results showed a highly dynamic interaction between MDA231 Br4 cells and HBMEC, the next step of our study consisted in the evaluation of spatial BCCs distribution on the endothelium. To this end, MDA231 Br4 cells were plated over HBMEC monolayers and monitored by live-cell imaging confocal microscopy (**Figure 4.3**), focusing now in early timepoints.

Our results revealed that along time BC cells change their positioning relatively to endothelium (**Figure 4.3A and B**). Spatially, it were defined two distinct positions, denominated as out, when the BC cells are positioned above the endothelial monolayer or intercalated, when the tumour cells are positioned partial or fully within endothelial monolayer. Moreover, we defined that the intercalated position can be divided in two different stages accordingly with the degree of BC cells interleaving: stage 1, which corresponds to a rounded morphology and the tumour cells was not completely intercalated with endothelial cells and stage 2, when BC cells present an elongated form and are fully at the same level as the endothelial cells.

At temporal level, we observed that at 0 h of interaction between the two cell types, 100% of BC cells were completely out of the endothelium. However, after 1 h of interaction, it was observed that the percentage of out BC cells reduced dramatically to 10%, concomitantly with the increase of the percentage of BC cells intercalated in stage 1 and 2 (42 and 48%, respectively). Importantly, after 2 h of interaction, 88% of the BC cells were intercalated in stage 2, that increase at 3 h to 90% (**Figure 4.3C**), by passing the out BC cells to intercalated stage 1 and BC cells stage 1 to stage 2. Importantly, orthogonal projections showed that BCCs were in fact spreading between BMECs until 2 h, but after 3 h, some BCCs migrating underneath them (**Figure 4.3A and B**).

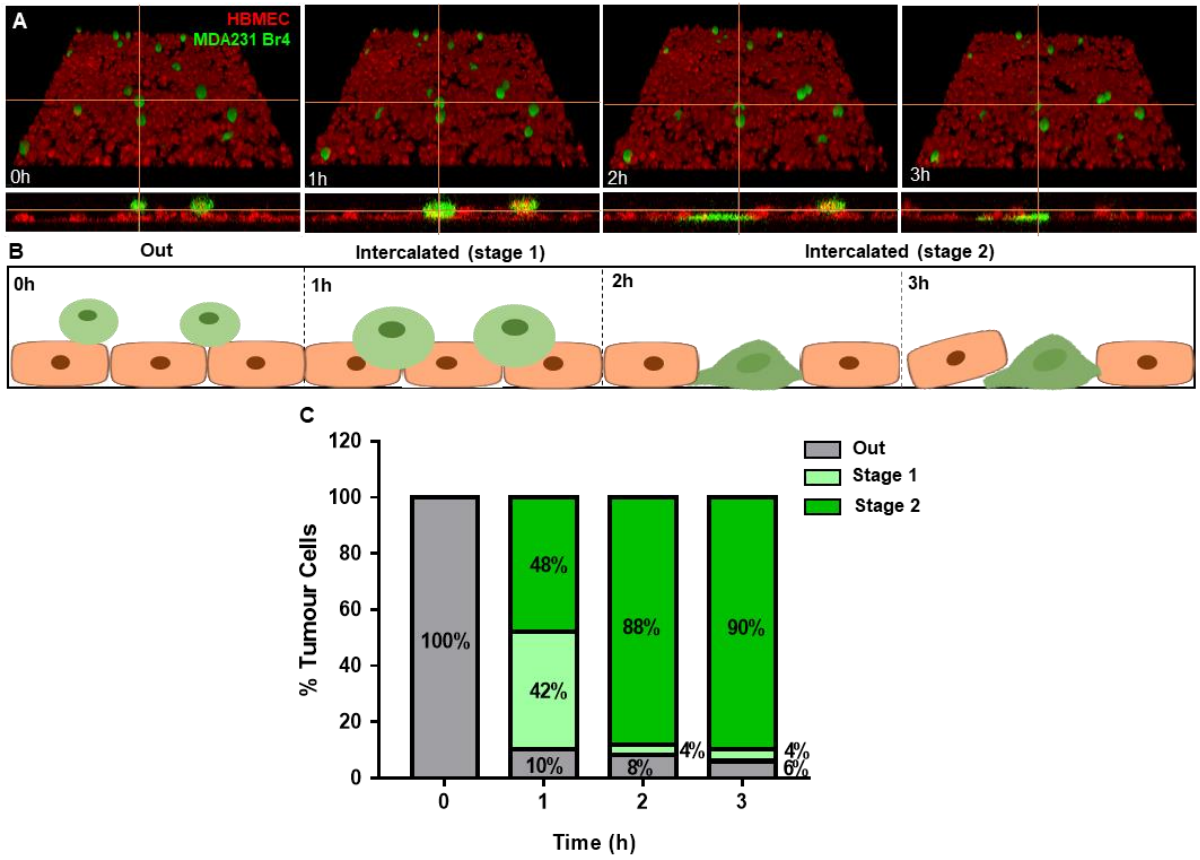

Figure 4.3. MDA231 Br4 cells localize at different endothelium levels during extravasation. 3D and orthogonal visualization of MDA231 Br4 cells (tagged with GFP; green) interaction with HBMEC confluent monolayer (labelled with CellTracker™ Red CMTPX Dye; red) at 0, 1, 2 and 3 h **(A).** Schematic representation of tumour cells position relatively to confluent HBMEC cultures (out, intercalated stage 1 and intercalated stage 2, corresponding the tumour cells over, partially or completely inserted in the endothelial monolayer) **(B)**. Semi-quantitative analysis of tumour cells positions relatively to endothelium (out and intercalated stage 1 and stage 2) **(C)** at the same timepoints.

Our study demonstrated that after the beginning of interaction between MDA231 Br4 cells and HBMEC, the malignant cells have the ability to quickly and efficiently intercalate within the endothelial monolayer, which indicates that the complete extravasation can occur at early timepoints of interaction. Importantly, since the previous results have shown that after 2 h, most of the tumour cells were elongated and already intercalated in the endothelium, we defined four important timepoints for the next studies: an early (0.5 h), that aims to include the adhesion process, two timepoints close to the time of complete extravasation (1 and 3 h) and a late (6 h) timepoint.

4.3 CD54 and CD44 were identified as players in extravasation process

Since previous results revealed that most of the BCCs are already completely intercalated in the endothelial monolayer after 3 h of interaction with BMECs, we expected that the cell adhesion process

occurs before this time and the BCCs migrate for the possible extravasate site. On the other hand, it is known that the extravasation process of tumour cells is mainly dependent on the sequential crosstalk between adhesion molecules expressed by endothelial cells, tumour cells and other circulating cells. So, the next step of this work was to identify the adhesion ligands in both endothelial and tumour cells, involved in the extravasation process. For that, we analysed the expression of some cell surface proteins, such as carcinoembryonic antigen (also known CEA or CD66adecb; in short CD66), ICAM-1 (CD54), CD44 and integrin β1 chain (CD29) by flow cytometry in single and mixed culture to ascertain the cells expressing the ligands at the timepoints previously defined (**Figure 4.4**). The carcinoembryonic antigen is a recognized E-selectin ligand involved in tumour cells rolling [88], which is the first step of tumour cells adhesion to the endothelial monolayer, as already mentioned. Our results clearly demonstrated that BCCs express this ligand in both single and mixed cultures, but no differences were noted during the interaction with endothelium. In contrast, the expression of this ligand was not observed in BMECs in any studied condition (**Figure 4.4A**), which indicates that this surface protein may be tumour cell specific and do not have a relevant role in BCCs extravasation process.

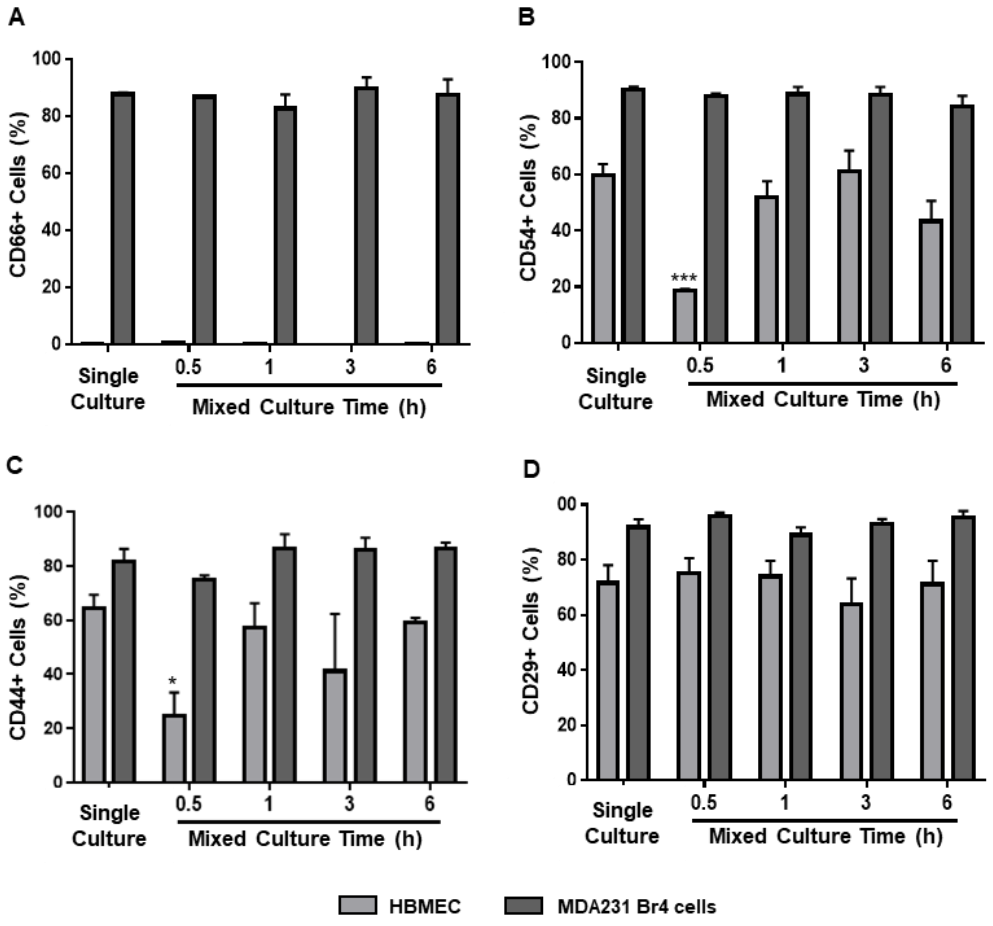

Figure 4.4. CD54 and CD44 were identified as players in MDA231 Br4 cells extravasation process. The adhesion players involved in MDA231 Br4 cells extravasation process was evaluated by flow cytometry. Semi-quantitative analysis of the HBMEC and MDA231 Br4 CD66 positive (+) (A), CD54+ (B), CD44+ (C) and CD29+ (D) cells. Data from flow cytometry are given as means ± SEM (n=3). Statistical significances are denoted as *P<0.05, **P<0.01 and ***P<0.001 for HBMEC (in single culture) vs HBMEC (in mixed culture).

Additionally, the CD54, also known as ICAM-1, is a cell surface glycoprotein of the immunoglobulin superfamily that is expressed at a low basal level in endothelial cells. Additionally, this glycoprotein has been positively correlated with a more aggressive tumour phenotype and metastatic

potential in BCCs [89]. The expression of CD44, a non-kinase transmembrane glycoprotein, has been recognized in several cell types including BCCs. In this context, some studies demonstrated that the loss of CD44 attenuated tumour cell adhesion to ECs *in vitro* and promotes an increase of efficiency of post-intravasation events and distant metastasis *in vivo* [90]. However, the CD44 expression has also been identified on the surface of ECs, which is correlated with vessels integrity and stability [91]. In this regard, our data for these two adhesion molecules demonstrated that the percentage of BCCs CD54+ or CD44+ in single and mixed culture is similar. In contrast, we observed that in early timepoint (0.5h of mixed culture), the percentage of HBMEC CD54+ and HBMEC CD44+ populations decrease significantly in mixed culture in comparison to the same population in single endothelial culture (**Figure 4.4B and C**). Analysing the mixed cultures, our results demonstrated that HBMEC CD54+ population increases until 3 h and a decrease was observed at 6 h (**Figure 4.4B**). Regarding HBMEC CD44+ population in mixed cultures, it increases to 1 h and a decrease is observed at 3 h of interaction between two cell types (**Figure 4.4C**).

In addition, it is known that the integrins, like the CD29, is a surface adhesion molecule that plays an important role in adhesion and migration of BCCs [92]. Analysing the expression of this molecule, our quantitative data indicated that no differences were observed in their expression in both BMECs and BCCs populations in pure and mixed culture (**Figure 4.4D**).

These findings indicates that CD66 and CD29 are not involved in this process, but CD44 and CD54 appears as players in BCCs extravasation process.

4.4 Triple negative breast cancer cells extravasation requires the increased of glucose input

During cancer progression, tumour cells adapt their metabolism to meet the energetic and biosynthetic demands that accompany the increased proliferation and colonization of distinct metastatic sites. The metabolic strategy used by triple negative breast cancer cells is the glycolysis, as brain microvascular endothelial cells [93]. It is known that in BMECs, the glucose transporter (GLUT) 1 is responsible for glucose supplying for the brain, which is considered the main energy source [27]. Also, expression of GLUT1 was identified in BCCs and at high level in TN BCCs [94]. However, it is unclear how interaction between BMECs and BCCs during extravasation influence both cells types metabolism. Thus, the next step in this work was to examine the glucose transporter (GLUT) 1 expression profile in endothelial and breast populations, during extravasation process, by flow cytometry (**Figure 4.5**).

As shown in Figure 4.5, in single cultures both endothelial and BCCs populations, express high levels of GLUT1. Given that the HBMEC population is gate-defined in GFP- and MDA231 Br4 cells is gate-defined in GFP+, our results demonstrated about 98% and 73% of the HBMEC and MDA231 Br4 cells population, respectively, is GLUT1+. Importantly, the BCCs population does not appear to be fully confined to the GFP+ population, a few percentages was observed within GFP- population (**Figure 4.5A**). In mixed cultures, although both endothelial and BCCs population continue to express GLUT1, BCCs expression levels of GLUT1 increased significantly compared to those observed in single cultures ($p<0.001$) (**Figure 4.5A-C**). Regarding endothelial population, an interesting observation in this study was that our quantitative results indicated that the percentage of cells that express GLUT1 is similar in all timepoints of mixed culture, as well in pure culture (**Figure 4.5A-C**). However, differences within endothelial population were observed, mainly at 3 h, where two populations with different levels of GLUT1 expression are formed (**Figure 4.5C and D**).

These observations suggest that the interaction between the two cell types affects BCCs metabolism. Moreover, this interaction leads to the formation of two endothelial populations according to GLUT1 expression levels, mainly at 3 h.

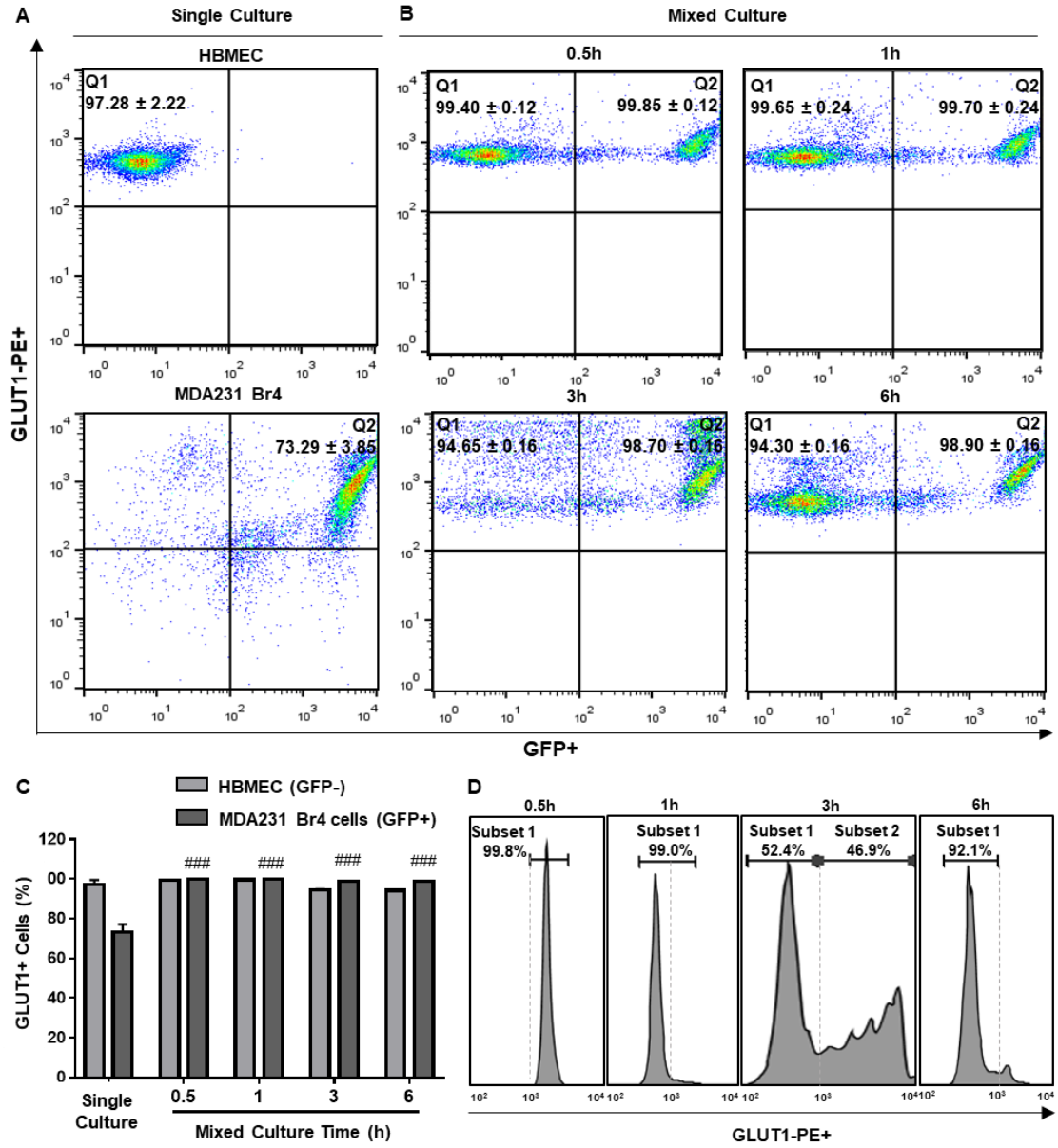

Figure 4.5. Increased glucose input to MDA231 Br4 cells during extravasation. Flow cytometric analysis of the percentage of HBMEC and MDA231 Br4 GLUT1 negative (-) and positive (+) cells in single and mixed cultures. Representative dot plots of GLUT1 expression profile of HBMEC and MDA231 Br4 cells population in pure culture at the basal levels (**A**) and mixed cultures (**B**) at the indicated timepoints. Semi-quantitative analysis of GLUT1-positive cells was performed in each condition (**C**). HBMEC GLUT1+ population heterogeneity analysis in mixed culture (**D**).Data are given as means ± SEM (n=3). For these experiments, we considered that the total HBMEC population is green fluorescent protein (GFP)- and the MDA231 Br4 cells population is GFP+. The percentages presented are result of the gate in each population for GLUT1+ and are presented in dot plots as mean of the percentage of GLUT1-positive cells of one representative experiment. Statistical significances are denoted as $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ for MDA231 Br4 cells (in single culture) vs MDA231 Br4 cells (in mixed culture).

4.5 Brain microvascular endothelial cells undergo Endothelial-Mesenchymal Transition

It is well known that in pathological conditions, such as cancer, the endothelium can be affected in several ways. Recently, some authors demonstrated that the last step of extravasation – transendothelial migration – may be related to one of these ways through the occurrence of endothelial-to-mesenchymal transition (EndMT) in ECs. Allied to this fact, some evidences suggest that EndMT is a necessary process for the BC cells transmigration to occur [76]. However, this process is still little explored in this particular context. In order to evaluate if EndMT occur in HBMECs by interaction with MDA231 Br4 cells, we performed the immunofluorescence analysis of a well-known mesenchymal

marker, N-cadherin, in single endothelial and mixed cultures (**Figure 4.6**). The results showed that the N-cadherin was expressed by BMECs in single and endothelial cultures (**Figure 4.6A and B**). However, in early timepoints of interaction, such as 0.5 and 1 h, in BMECs the N-cadherin fluorescence intensity decrease in mixed cultures in comparison to the single cultures ($p<0.01$) (**Figure 4.6C**). In contrast, after 3 h of interaction between two cell types, a significantly increase of the N-cadherin fluorescence intensity relatively to control ($p<0.001$) was observed (**Figure 4.6C**). Curiously, at 6 h no differences in N-cadherin fluorescence intensity were observed relatively to their control (**Figure 4.6C**). Looking only at the mixed culture, we observed that there exist significant differences between all the studied timepoints relatively to 3 h of mixed culture ($p<0.001$) (**Figure 4.6C**). These findings suggest that along the interaction with BC cells, the endothelial cells acquire mesenchymal markers, which expression peaks at 3 h of interaction.

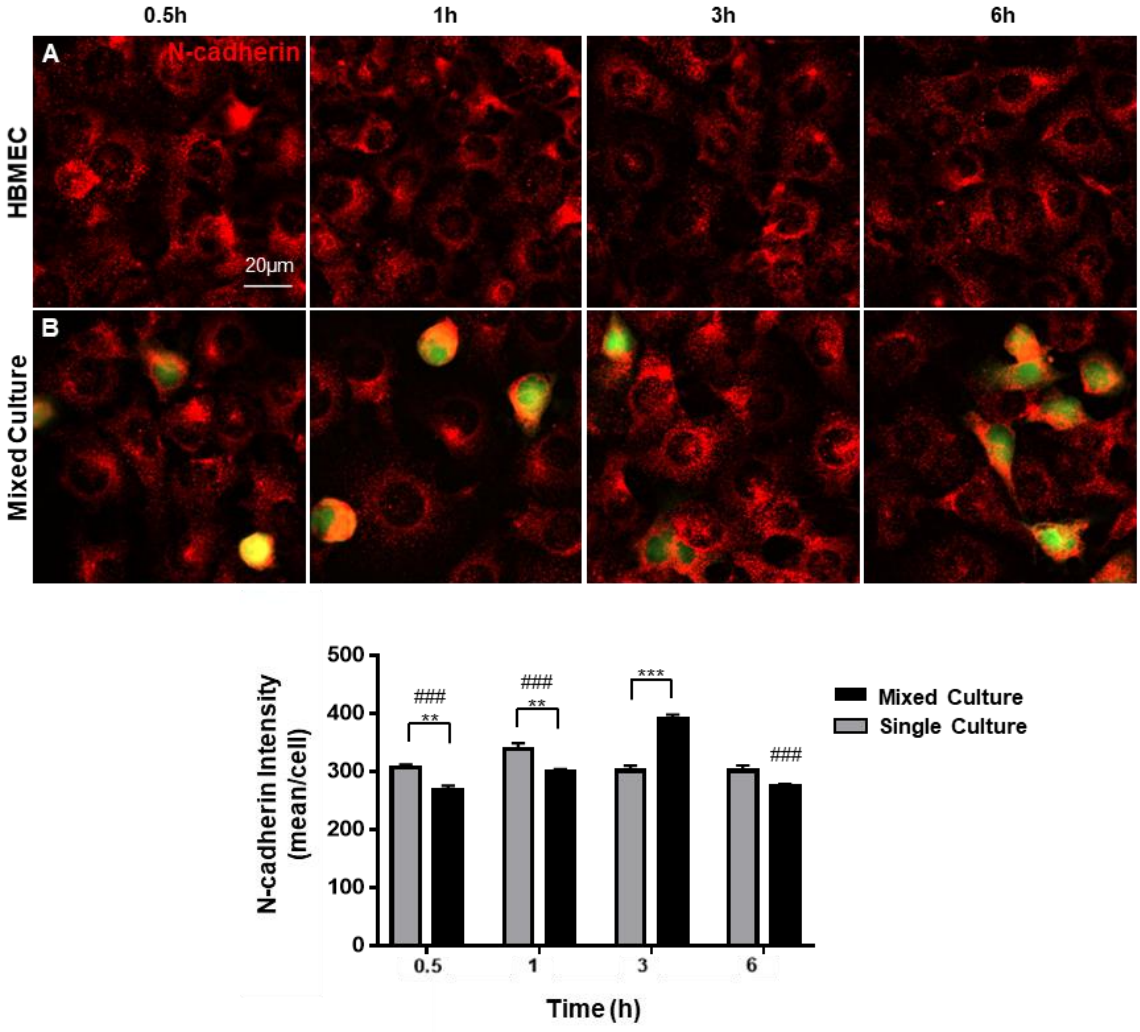

Figure 4.6. HBMEC acquire mesenchymal markers by the interaction with MDA231 Br4 cells. Immunocytochemistry analysis of neuronal-cadherin (N-cadherin, red) was performed in HBMEC single **(A)** and mixed culture with MDA231 Br4 cells (tagged with GFP; green) **(B)** at 0.5, 1, 3 and 6 hours (h). N-cadherin expression profile in endothelial and mixed cultures was quantified by the mean intensity/HBMEC (five cells/field) **(C)** at the same timepoints. Data are given as means ± SEM (n=3, 10 field/condition). Statistical significances are denoted as *P<0.05, **P<0.01 and ***P<0.001 for mixed culture vs single culture at the same timepoint and $^{\#}$ P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 for differences in mixed culture relative to 3 h of mixed culture.

In addition to the expression of mesenchymal markers, like N-cadherin, one of the consequences of EndMT is the perturbation of adhesive structures and consequently, the cell stability [76]. The effect of the presence of BC cells in endothelial monolayers was investigated for the same timepoints by

immunocytochemical analysis of the cytoskeleton-associated protein, MLCK (**Figure 4.7**). Protein expression and cell morphology were evaluated. Our data indicated that after 1 h of interaction with BCCs, the MLCK fluorescence intensity in HBMEC increased, relatively to control culture ($p<0.05$), but a more significantly increase was noted at 3 h of mixed culture, comparatively to control ($p<0.001$) at the same timepoint (**Figure 4.7A-C**). These differences were not observed in the earliest and late timepoints (0.5 and 6 h, respectively). Analysing mixed culture, we observed an increase of the MLCK fluorescence intensity along time, which expression peaks at 3 h of interaction. Significant differences between all the studied timepoints relative to 3 h of mixed culture ($p<0.001$) (**Figure 4.7C)** were observed. In terms of cell morphology, at 3 h, HBMEC in mixed cultures revealed an increased elongation, comparatively to control ($p<0.01$) (**Figure 4.7D**).

Altogether, these findings reveal that the interaction with BCCs, endothelial cells gain mesenchymal markers and an elongated morphology, from which they acquire invasive properties, resulting from the crosstalk with triple negative BC cells.

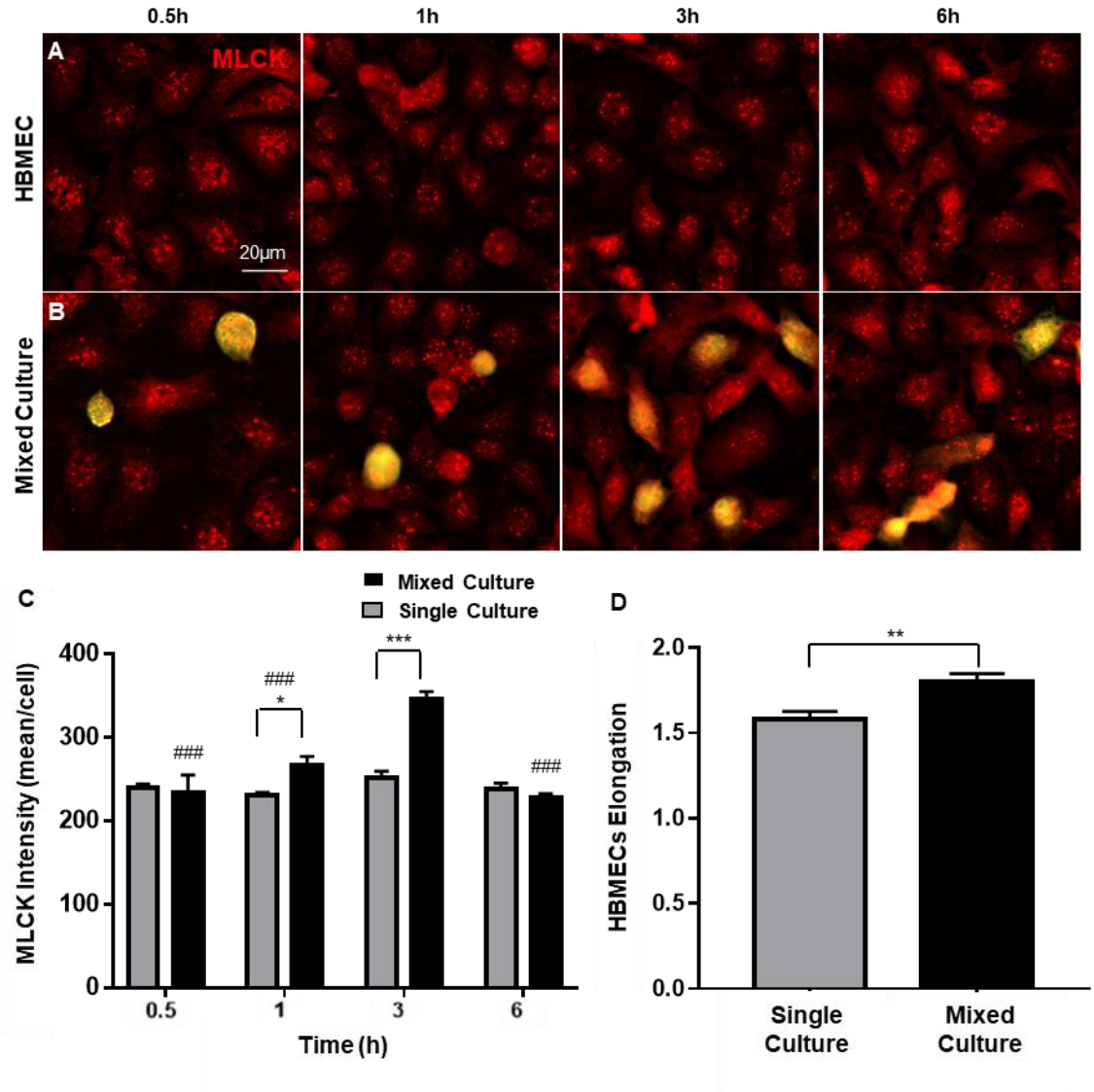

Figure 4.7. HBMEC acquire invasive properties by the interaction with MDA231 Br4 cells. Immunocytochemistry analysis of myosin light chain kinase (MLCK, red) was performed in HBMEC monocultures **(A)** and mixed culture with MDA231 Br4 cells (tagged with GFP; green) **(B)** at 0.5, 1, 3 and 6 hours (h). MLCK expression profile in endothelial and mixed cultures was quantified by the mean intensity/HBMEC (five cells/field) **(C)** at the same timepoints. Differences in HBMEC elongation at 3 h were evaluated **(D)**. Data are given as means ± SEM (n=3, 10 fields/condition). Statistical significances are denoted as denoted as *P<0.05, **P<0.01 and ***P<0.001 for mixed culture vs single culture at the same timepoint and $^{\#}$ P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 for differences in mixed culture relative to 3 h of mixed culture.

4.6 Breast cancer brain metastasization involves BBB disruption at paracellular level

Since our previous results have shown that the interaction between the triple negative breast cancer cells and endothelial cells is a highly dynamic process, we then examined the integrity of endothelial monolayers, that mimic BBB, during this process (**Figure 4.8**). First, in our live-cell imaging confocal microscopy experiments, we observed that the area covered by endothelial cells decreases over time in mixed cultures (AUC = 2026±29.4) compared to endothelial monocultures (control; AUC = 2175±28.4). In fact, we detected that after 24 h of interaction, about 27% of monolayer is disrupted (**Figure 4.8A**).

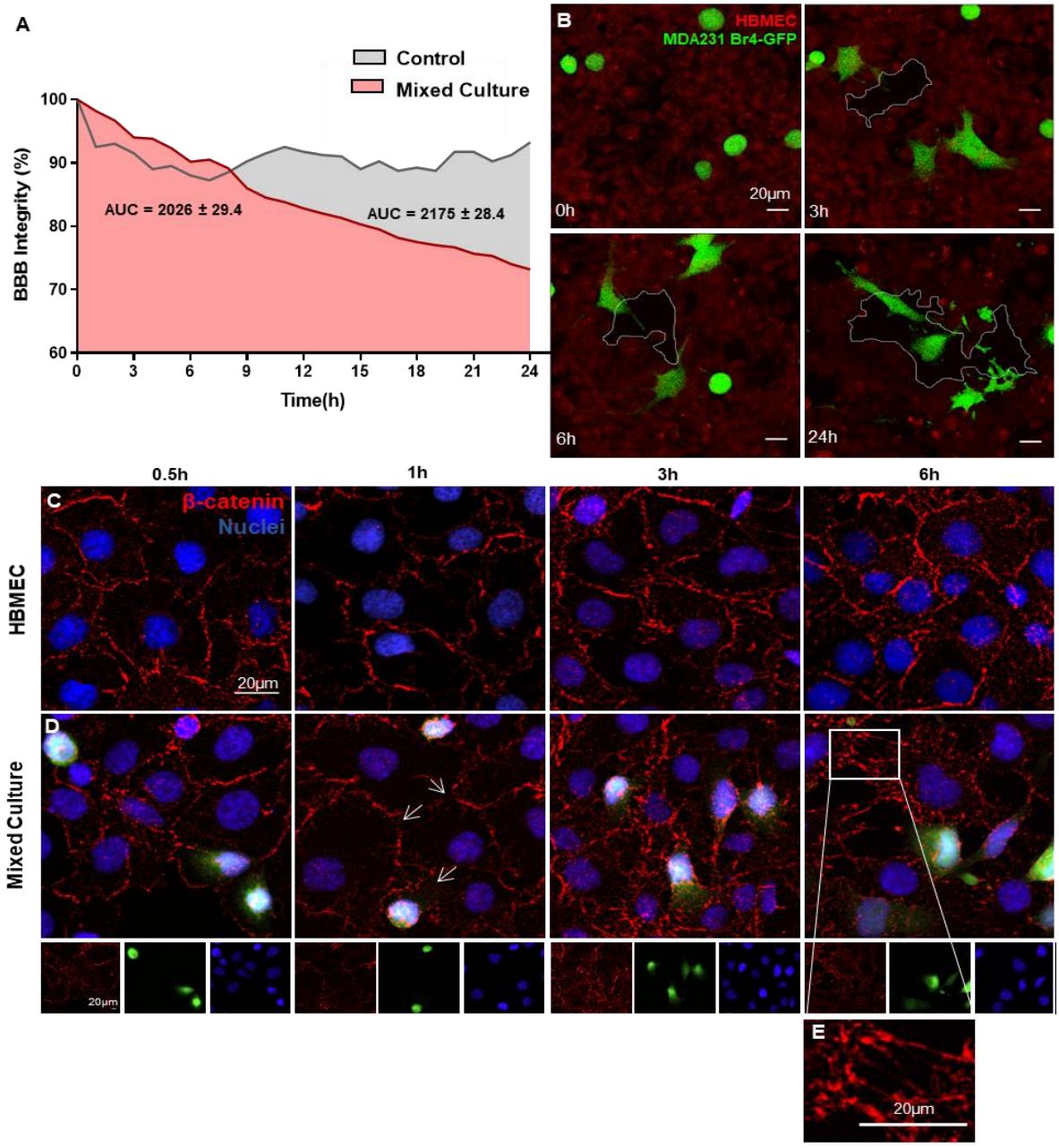

Figure 4.8. HBMEC and MDA231 Br4 cells interaction decrease the blood-brain barrier (BBB) integrity. The effect of malignant-endothelial cells interaction on BBB integrity was evaluated in mixed and endothelial cultures (control) by live-cell imaging microscopy and area under curve was determined **(A)** along 24h. Representation of endothelial gaps (circled with white line) **(B)**. Immunocytochemistry analysis of β-catenin was performed in HBMEC monolayers **(C)** and mixed cultures

(D) at 0.5, 1, 3 and 6 hours (h). Visualization of β-catenin junctions between adjacent endothelial cells at 6h **(E).** Arrows indicates gaps localization.

Simultaneously, we investigated the presence of gaps in the endothelial monolayer and analysing the same endothelial region, we observed that at the beginning of mixed cultures the monolayer was intact (without gaps), whereas after 3 h, the endothelial monolayer presents some gaps (circled with white line), whose size increases over time (**Figure 4.8B**). Interestingly, we observed the presence of tumour cells or part of them (some protrusions) within endothelial gaps (**Figure 4.8B**).

The AJ protein β-catenin is known to contribute to endothelial barrier sealing and maintenance. To assess the changes in human brain endothelium AJ induced by the interaction with tumour cells, the expression of β-catenin was examined by immunocytochemistry in mixed and endothelial cultures (**Figure 4.8C-E**). Our results demonstrated that after 0.5 h of interaction between malignant and endothelial cells, the β-catenin labelling is mainly found in the endothelial cell membrane and differences relatively to control were not noticeable (**Figure 4.8C and D**). After 1 h, the β-catenin was still observed at the cell membrane level, but some gaps have been observed in the labelling (indicated by white arrows). In contrast, after 3 h of interaction it was no possible to quantify the presence of intercellular gaps in β-catenin labelling, since the AJ protein was distributed throughout the cell and was not predominantly in cell membrane, as noted in the early timepoints (0.5 and 1 h). Finally, at 6 h of interaction, we not only observed that the β-catenin labelling was distributed all over the cell, but also detected the endothelial cells detachment from the monolayer, forming holes, where the tumour cells are predominantly located. Although some adjacent endothelial cells were able to maintain junction regions, they are highly stretched, forming fibrils that joins two cells (**Figure 4.8E**).

These findings suggest that malignant-endothelial cells interaction compromises BBB integrity over time, mainly affecting the proteins of adherent junctions in endothelial cells.

4.7 Endothelial components engulfment as a possible disruptive extravasation mechanism

Curiously, in our live-cell imaging experiments we observed that the BC cells exhibit a red labelling, mainly in perinuclear region, after the interaction with endothelial cells (**Figure 4.1A)**, that had been previously marked with CellTracker™ Red CMTPX Dye. With these observations we hypothesized that the malignant-endothelial cells interaction could promote endothelial cells components' engulfment by malignant cells.

In order to confirm this hypothesis, we performed additional experiments using culture medium, both from CellTracker™ Red-labelled endothelial and mixed cultures (**Figure 4.9**). Thus, after 3 h of endothelial and mixed culture incubation, we transferred the culture media to tumour cells cultures (**Figure 4.9A and D**). These cultures were incubated during 3 h and then were observed by immunofluorescence. Our results showed that tumour cells acquired red labelling in their perinuclear region as small vesicles with both culture media (**Figure 4.9C and F**), which was not observed in the respective controls in which culture media were added and removed immediately (**Figure 4.9B and E**). The number of red vesicles inside the BC cells was quantified, using an Icy software tool (spot detector) and differences were observed between incubations with different culture media. Surprisingly, our quantification revealed that the number of vesicles in tumour cells was higher when incubated with endothelial culture medium compared to the incubation with mixed culture medium (endothelial culture medium: 33.38 ± 3.2 vesicles; mixed culture medium: 17.7 ± 1.7 vesicles, $p<0.01$) (**Figure 4.9G)**.

In order to understand if the presence of BC cells influences the concentration of suspended particles, the next step in our study was to check for differences in particle concentrations in both culture media, before and after incubation with the tumour cells, using NanoSight equipment (**Figure 4.9H and I**). Interestingly, our results demonstrated that after 3 h of endothelial and mixed culture incubation (that correspond to the particle concentration that was added later to the tumour cultures), the particles concentration in endothelial cultures was about 3 times higher than mixed cultures particles

concentration ($4.4\times10^9 \pm 6.2\times10^7$ and $1.5\times10^9 \pm 1.1\times10^7$particles/mL, respectively, p<0.001) (**Figure 4.9H**).

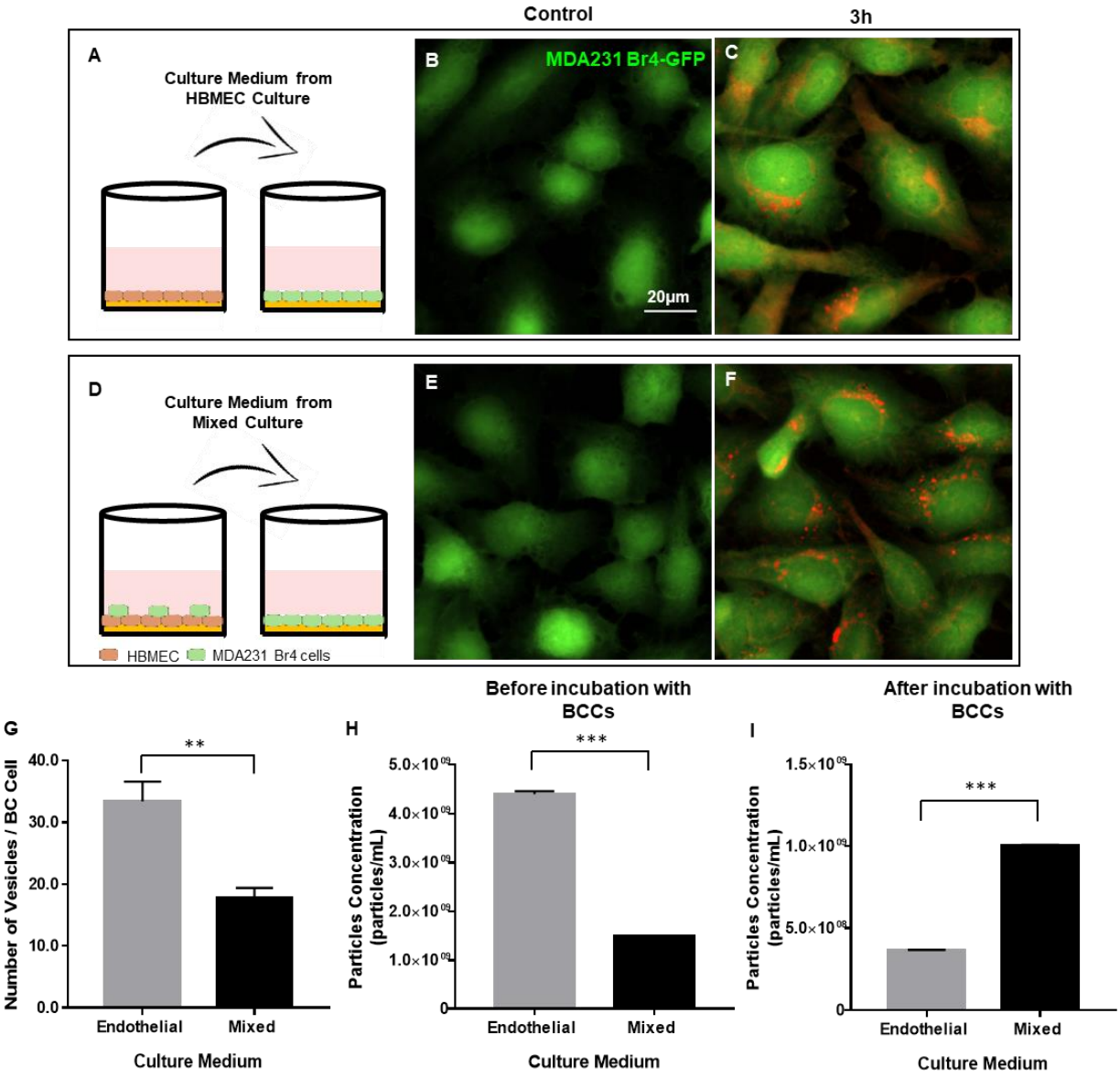

Figure 4.9. Malignant-endothelial cells interaction may promote endothelial cells components' engulfment. Immunofluorescence analysis of endothelial vesicles (red) incorporation by MDA231 Br4 cells (tagged with GFP), after the incubation with culture medium from endothelial (**A-C**) and mixed culture (both incubated during 3h) (**D-F**), during 0 h (control) (**B**, **E**) and 3 h (**C**, **F**). The number of vesicles per BC cell (**G**) after 3 h of incubation with endothelial and mixed culture medium was analysed. The concentration of particles population present in culture medium was quantified using NanoSight (**H**, **I**). Semi-quantitative analysis of particles concentration (particles/mL) in endothelial and culture medium after 3 h of incubation (**H**) and MDA231-Br4 cells culture medium after 3 h of incubation with endothelial and culture medium (**I**). Data are given as means ± SEM (n=1, 10 replicates/condition). Statistical significances are denoted as denoted as *P<0.05, **P<0.01 and ***P<0.001 for endothelial culture vs mixed culture.

Additionally, when we incubated tumour cells with endothelial and mixed culture medium during 3 h, we observed differences in particles concentration. While the particle concentration with the mixed culture medium remains similar (before incubation: $1.5\times10^9 \pm 1.1\times10^7$; after incubation: $1.0\times10^9 \pm 4.0\times10^6$ particles/mL), with the endothelial culture medium a significant decrease in its concentration (before incubation with BCCs: $4.4\times10^9 \pm 6.2\times10^7$; after incubation with BCCs: $3.6\times10^8 \pm 3.5\times10^6$ was observed (**Figure 4.9I,** p<0.001). Thus, our findings indicated that the triple negative BC cells interaction with BBB endothelial, cells may promote endothelial cells components' engulfment.

V. DISCUSSION

The CNS is a common site for BC metastasis. Development of brain metastases has a very poor prognosis despite the extensive therapeutic efforts. Therefore, it is very important to understand the mechanisms and molecular players involved in BCCs extravasation through the BBB, which could eventually help to develop preventive strategies against the formation of BCBM.Thus, in the present study we established the time- and spatial-course profile of BC cells and BBB endothelial cells along extravasation, identified the players involved, evaluated the endothelial-mesenchymal transition occurrence in brain endothelium, characterized the transmigration pathways used by BC cells for the BBB transposition and evaluated the integrity of the barrier along this process. For these studies, we established a new human *in vitro* model of BC brain metastasization, which relies in the use of mixed cultures composed by HBMEC, to mimics the BBB, and the triple negative human adenocarcinoma cell line - MDA-MB-231 Br4 - labelled with GFP, as BCCs with brain tropism, a feature that makes our model unique and specific.

We analysed the time-course of tumour cells extravasation into the brain parenchyma in pure and mixed cultures and our results demonstrated that after 1 h of interaction between the two cell types, the tumour cells undergo morphological alterations, revealed by an increase of their perimeter and a decrease of their roundness and circularity. These changes were accompanied by the formation of highly dynamic intravascular cytoplasmatic extensions that allow them to contact a larger area of the endothelium. Our understanding of tumour cells extravasation has been informed to a significant extent by well-characterized mechanisms of immune cell diapedesis. In this context, some studies have reported that these cells form specialized structures, called podosomes, which facilitate their transit across the endothelial layer [95]. Tumour cells also have the ability to form podosomes that are commonly referred to as invadopodia [96]. Initially, the visualization and characterization of invadopodia formation in models of BC have suggested that invadopodia are key mediators of the intravasation process [97], but it has now been reported, the formation of these structures also in extravasation process for some BC cell lines [59], which corroborates our results. Interestingly, invadopodia is considered a tight network formed by structural proteins and their disruption results in decreased extravasation efficiency and abrogation of metastasis [59, 98]. Thus, the morphological alterations like these have been pointed as possible therapeutic targets in order to prevent the formation of metastasis. Taken together, these results suggest that interaction with BMECs, promotes the tumour cells invasiveness, which is critical for successful cancer cell dissemination. Allied to this, the tumour cells acquire a migratory phenotype, more noticeable until 3 h of interaction with BMECs, which potentiates the migration of these cells to possible extravasation sites. Nowadays, collective cell migration has been reported to connect with cancer migration, and even metastases [99]. Our results are consistent with this data and we observed that BCCs move more when they make contact through invadopodia with other BCCs.

It is known that regardless of the cell type, the extravasation process comprises three main steps: the rolling, which is characterized by the establishment of the first interactions between ligands and the corresponding receptors expressed in each of the cell types that promote the slowdown of cells; the adhesion step, in which cells firmly adhere to the endothelium and transendothelial migration, defined by the passage of cells through the endothelium. Moreover, in other studies, an additional step was identified in BCCs extravasation process that occurs even before the complete endothelial transmigration. In this context, the tumour cells are incorporated into the HUVEC monolayers, leading to their disruption [71]. This phenomenon had already been described as tumour-endothelial cells mosaic formation by Tremblay et al., when investigated the colon cancer cells transmigration mechanism through the HUVEC endothelium [72]. In this sense, we further observed that MDA231 Br4

cells present two distinct positions relatively to endothelium, denominated as out, when the BCCs are localized above the endothelial monolayer and intercalated, when the tumour cells are positioned partial or fully within endothelial monolayer, defined as stage 1 and 2, respectively. After 1 h, the majority of BCCs are intercalated within endothelial monolayer. These results indicate that MDA231 Br4 cells rapidly and efficiently intercalate within endothelial monolayer. Contrary to what has been shown by Tremblay et al., for the colon cancer cells intercalation [72], BCCs primarily form a mosaic within endothelial monolayer, but after 3 h, we observed that the part of BCCs were underneath to BMECs, which promoted their detachment. Thus, the quick intercalation can be considered an additional step of BCCs extravasation through the BBB, which potentiate the complete extravasation in early timepoints.

Based on these results, the BCCs appear to complete the extravasation process faster than other tumour cells. For instances, the melanoma cells change their morphology after about 60-120 min, becoming flattened and elongated, and completed the transmigration 15-30 min after these alterations development [61]. Importantly, other studies demonstrated that 6 h of interaction between BCCs, such as MDA231 and MCF-7, and brain ECs (D3), not enough for the most of these cells to transmigrate [100]. These differences with the BCCs mentioned earlier, may be associated with the fact that BCCs used in the present study have brain tropism, which makes this model unique, able to mimic better the metastatic organotropism.

Similarly to leukocytes, the tumour cells extravasation is mainly dependent on the occurrence of sequential interactions and crosstalk between adhesion molecules expressed on the ECs, tumour cells and other circulating cells [38]. Curiously, our findings indicate that carcinoembryonic antigen and integrin-β1 are not involved in extravasation, but the CD44 and ICAM-1 appear to play a relevant role in BCCs extravasation. Regarding ICAM-1, shortly after the beginning of mixed culture, the BMECs practically do not express this surface protein. However, along the time of interaction between the two cell types, its expression increases in BMECs, culminating in the peak at 3 h. In addition, 0.5 h after the beginning of mixed culture, the CD44 practically do not expressed by BMECs, but an increase in their expression was observed at 1 h and a further decrease was observed at 3 h. These results show that these surface molecules were differentially expressed along the time of interaction between BCCs and BMECs, which indicates that these molecules appear to participate in different stages of extravasation. In fact, the CD54, also known as ICAM-1, is a cell surface glycoprotein in the immunoglobulin superfamily that is expressed in ECs and has been positively correlated with a more aggressive tumour phenotype and metastatic potential in BCCs [89]. Also, the CD44 expression has also been identified on the surface of ECs, which is usually correlated with vessels integrity and stability [91]. On the other hand, in our study, the ICAM-1 and CD44 levels in BCCs remains elevated throughout the interaction with BMECs, indicating that both molecules do not play a relevant role in this interaction. These results can be explained by the fact that among the all BC subtypes, the ICAM-1 and CD44 expression has been observed mainly in TNBC [101, 102].

It is known that during cancer progression, tumour cells adapt their metabolism to meet the energetic and biosynthetic demands that accompany the increased proliferation and colonization of distinct metastatic sites. It is also known that in BMECs, the glucose transporter (GLUT) 1 is responsible for glucose supplying for the brain, which is considered the main energy source [27]. Also, expression of GLUT1 was identified in BCCs and at high level in TNBC [94]. Additionally, GLUT1 expression is associated with poor prognostic in BC cases [103], acting as a regulator of signalling cascades in the tumorigenesis of BC [94], making it a possible new therapeutic target. Our findings are consistent with previous data and provide an evidence that extravasation process require an increase of the percentage of BCCs that express this glucose transporter, possibly increasing the tumour cells metabolism in order to complete extravasation through brain endothelium. The results obtained were as expected, as we previously realized that BCCs acquire rapidly a highly invasive and migratory phenotype, resulting in the loss of a higher amount of energy. On the other hand, no significant differences were observed in

the percentage of BMECs GLUT1+ throughout the extravasation, but especially at 3 h, two endothelial subpopulations were observed with different GLUT1 expression levels. Thus, the GLUT1 expression in BMECs seems to be affected especially when at the times corresponding to the intercalation of BCCs in the endothelial monolayer and the detachment of these cells. Consequently, it may affect the barrier integrity and the number of BMECs expressing this metabolic marker.

Recently, some studies have shown that in organs with more compact endothelial organization, the EndMT that culminates in endothelial remodelling is considered a prerequisite for cancer dissemination [76]. One of the main features described for EndMT is the reduction of endothelial markers, which is accompanied by the gain of mesenchymal markers such as fibroblast-specific protein-1 (FSP-1), alpha-smooth muscle actin (α-SMA), vimentin, and N-cadherin [78]. In this context, our findings showed that after 3 h of interaction between HBMECs and MDA231 Br4 cells, an increase of N-cadherin was observed in BMECs in mixed cultures, evidencing the EndMT occurrence. In addition to the expression of mesenchymal markers, like N-cadherin, one of the consequences of EndMT is the perturbation of adhesive structures and consequently, the cell stability [76]. Thus, we evaluated the expression of MLCK, a cytoskeleton-associated protein correlated with invasive and migratory cells properties [104], which are two important features of mesenchymal cells. An increase of MLCK expression and an elongated morphology were observed in BMECs, evidencing the gain of invasive phenotype.

Simultaneously, the co-culture with MDA231 Br4 cells induced visible HBMEC monolayer damage, which increases with long incubation times. After 24 h, about 27% of BBB is disrupted in mixed culture. In this work we evaluated the expression of one of the AJ proteins, β-catenin, for two main reasons. The first is that EndMT is also characterized by loss of endothelial markers in BMECs and on the other hand, the BBB disruption is also usually associated with the loss or disruption of the junctional complexes. Our immunocytochemical analysis demonstrated that β-catenin labelling changes during the extravasation process. In early timepoints of extravasation the β-catenin is mainly found in cell membrane, as well as in controls. However, some labelling gaps appear after 1 h and after 3 h, timepoints at which this AJ protein is distributed throughout the cell and is not predominantly in cell membrane. Finally, at 6 h of interaction, we detected the endothelial cells detachment in the monolayer, forming holes, where the tumour cells are predominantly located. Based on these observations, the present study demonstrated that MDA231 Br4 cells extravasation is facilitated by the EndMT occurrence in HBMEC, revealed by an increase of mesenchymal markers and a decrease of endothelial markers expression, such as β-catenin. Simultaneously, this process promoted the relocation of β-catenin, compromising the barrier integrity. Although some adjacent endothelial cells were able to maintain junction regions, they are highly stretched, forming fibrils that joins two cells. On the other hand, these observations are in accordance with previous results showing that – in contrast to leukocytes – the BCCs do not leave the endothelium intact during extravasation.

Regarding the transendothelial pathways used by BCCs, the predominant mode of tumour cells extravasation appears to be the paracellular migration, during which cancer cells migrate between two ECs, a process requiring cellular rearrangements and disruption of inter- endothelial cell-cell junctions [59]. Given this evidence, our results point to the use of the paracellular pathway in the MDA231 Br4 cells extravasation through BBB. However, the transcellular pathway cannot be completely ruled out, since there is evidence that MLCK activation is related to the transcellular passage of tumour cells in the intravasation process [69].

The microparticles and exosomes are regarded both biomarkers and mediators of many forms of pathology, including neurovascular inflammation. In the vasculature, many cell types can generate microparticles, including ECs [105]. On the other hand, some studies have demonstrated that BCCs, such as MDA231 and MCF-7, also release microparticles, which participate to a functional crosstalk with ECs that culminates in an increased secretion of ECs microparticles that sustain tumour cells [106].

In addition, in patients with BC, decreased levels of ECs-derived microparticles have been shown to be associated with better overall survival [107]. In this regard, in the present study, a new and potential crosstalk malignant-endothelial cells mechanism was identified.

Our preliminary studied have shown that after interaction of BCCs with BMECs previously marked with CellTracker™ Red CMTPX Dye, malignant cells exhibited a red labelling, mainly in the perinuclear region. Interestingly, our quantitative results revealed that the number of vesicles in BCCs was higher when incubated with endothelial culture medium compared to the incubation with mixed culture medium. Additionally, the particles concentration in endothelial cultures it is about 3 times higher than in mixed cultures, which potentially indicates that the BCCs in mixed culture can incorporate some BMECs released-particles. Moreover, after the incubation of tumour cells cultures with both culture media, we observed that the particles concentration in mixed culture remains similar, whereas with the endothelial culture medium a significant decrease in its concentration was observed. These observations revealed for the first time that BCCs may be engulf some components released by BMECs. In fact, the engulfment of endothelial particles mechanism was only described for macrophages, when interact with mouse pupillary membrane [108]. Further studies need to be done to understand if this mechanism is not only directed to suspended particles but also to intact cells and eventually BCCs, could use this as strategy to more easily transpose to BBB.

VI. CONCLUDING REMARKS

The present work shows that extravasation of BCCs across the BBB endothelium, is a very complex and dynamic process involving the interaction between both cell types that elicit morphological and phenotypic alterations. Together these studies contribute for a better understanding of BC cells trafficking across brain microvascular endothelium, an essential step for the development of novel strategies to avoid extravasation of malignant cells into the brain and thus to prevent brain metastases formation. However, further studies are still needed to understand how extravasation-associated mechanisms like EndMT that occur in BMECs during BCCs extravasation can be modulated in order to abrogate the BCBM development. In this context, it would be to establish the mediators or inhibitors of the EndMT, using a variety of inflammatory mediators, including the TGF-β, bone morphogenic proteins (BMPs), the tumour necrosis factor-α (TNF-α) and interleukin (IL)-1β and IL-6, known to be involved in this process [109-111]. Moreover, there is only evidence that tumour cells use the paracellular route to extravasate into the brain. However, the MLCK activation in ECs have been described to be involved in transcellular pathway during intravasation process. Thus, the study of other proteins related with transcellular migration, such as caveolin-1 could be a starting point.

Further studies are also needed to understand the exact role of ECs microparticles engulfment by BCCs during extravasation. In order to complete this study, we will determine if the particles within the BCCs are endothelial, by marking a recognized endothelial marker, vWF and the same studies will be repeated to corroborate our preliminary studies. Hopefully, clarification of these issues will allow to timely establish preventive approaches to avoid the occurrence of BCBM and delay the cancer dissemination.

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VIII. SUPPLEMENTARY MATERIAL

Table 8.1. Summary of the antibodies and experimental conditions used for immunofluorescence analysis.

Marker	Target Protein	Primary Antibody	Dilution	Secondary Antibody	Dilution
Endothelial	**β-catenin**	Thermo Fisher Scientific, #71-2700, Rabbit	1:100	Alexa Fluor® 555 Thermo Fisher Scientific, #A21428, Goat Anti-Rabbit	1:500
Mesenchymal	**N-cadherin**	Thermo Fisher Scientific, #PA519486, Rabbit	1:200		1:500
Cell mobility	**MLCK**	Thermo Fisher Scientific, #PA515177, Rabbit	1:100		1:500

N-cadherin: neuronal-cadherin; MLCK: myosin light chain kinase.