Unraveling the Molecular Pathogenesis of Brain Metastatic Disease

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Unraveling the Molecular Pathogenesis of Brain Metastatic Disease

Het ontrafelen van de moleculaire pathogenese
van hersenmetastatische ziekte

Thesis

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Pai, mãe e mana, a minha tese de doutoramento é para vocês!
(Pai… espero que agora já mereça um frasquito de mel…☺)

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Chapter 1

General Introduction - Brain Metastasis

BRAIN METASTASIS

Brain metastasis is the most serious complication of cancer. The development of brain metastases is estimated to occur in 20% of patients with cancer [1, 2] whereas autopsy studies suggest an incidence as high as 40% [3-5]. This discrepancy seems to be the sequel of the fact that only part of cancer patients undergo brain MRI screenings, because they developed neurological symptoms, or brain metastases need to be ruled out prior to application of particular treatment protocols. In addition, brain scanning to exclude the existence of cerebral metastasis is not standard in most treatment guidelines for solid tumors.

Although any type of cancer can metastasize to the brain, lung - and breast cancers and melanomas most frequently give rise to cerebral metastases [1, 5]. The median time to the diagnosis of brain metastasis varies and is greatly influenced by the molecular subtype and stage of the disease. One study has reported a median time for brain metastasis to develop of 44 months for breast cancers, and 11 months for lung cancers [6]. In some instances, brain metastases cause the first symptoms in patients with otherwise undiagnosed advanced-stage primary tumors. Brain metastases are treated with multimodal therapies, such as the combination of surgery, radiotherapy, chemotherapy, immunotherapy and targeted therapies. The therapeutic approach for patients with brain metastases depends on the number and location of the metastases, as well as the lineage and stage of the primary tumor [7]. The prognosis after the development of brain metastases is usually dismal, with an overall survival of two years from diagnosis [8]. Regardless of multimodal therapies, brain metastases eventually progress. Neurological disease is the cause of death in up to 52% of patients with brain metastases [9, 10].

The pathogenesis of metastasis

Tumor cells spread from their primary site, through the blood stream (hematogenous route), to the brain microvasculature, where extravasation of tumor cells allows further complex cross-talk between tumor cells and the new microenvironment. Ultimately, neovascularization will concede the successful establishment of a new metastatic niche.

The “metastatic cascade” is a highly selective mechanism which consists of a series of sequential and interrelated steps, each of which preceding the formation of distant metastases. Primary tumor cells that are to become metastatic, must undergo an epithelial-mesenchymal transition (EMT) [11], endowing tumor epithelial cells with enhanced migratory capacity, increased resistance to apoptosis, as well as elevated expression of various enzymes. These features will elicit tumor cell detachment from the parental tumor mass, invasion through host stroma and migration into the circulation or the lymphatic channels. In order to survive the turbulence of the bloodstream, single cells often engage with different blood cells to form cell clumps [12-15]. Once in the bloodstream, these aggregations will eventually get arrested in the capillary

beds of receptive organs (vessel branches of cerebral capillaries in the case of brain metastasis), settle and grow within the vessels or, most frequently, penetrate the vessels and migrate into the organ parenchyma, where proliferation and neovascularization will enable the establishment of micrometastasis. Finally, vascular supply and evasion of host immune cells [16], in parallel with mesenchymal-epithelial transition (MET) within the new "niche", will elicit the formation of clinically detectable metastases. Occasionally, this metastatic cascade may be initiated from previously established metastases [17].

Organotropism – Historical perspectives

The distribution of metastases is determined by the interaction between tumor cells and the microenvironment of specific organs. The pattern of targeted organs is remarkably variable and depends on, aside from intrinsic features of the primary tumor, the organ-specific circulation pattern and characteristics of the local endothelial cells. Cancer types may have predilection for particular tissues (e.g. prostate cancer to bone) or organs (e.g. pancreatic cancer to liver), or display a particular sequential colonization of organs (e.g. colorectal cancer metastasizes first to the liver, to be followed by lungs and brain, respectively). Cancers that metastasize to brain (i.e. lung and breast cancers and melanomas) will usually also colonize various organ sites, sequentially or synchronously [18, 19]. Nevertheless, organotropism is not a rigid phenomenon – although specific patterns of metastases are clear – and different hypothesis, regarding the theory behind organotropism, have emerged over the years.

In 1889, Stephen Paget was the first to hypothesize that the predisposition of cancer cells to spread to specific organ sites was not just random but determined by two factors: the cancer cell ('seed') and the new host site ('soil') [20], suggesting a cancer cell-specific affinity for the microenvironment of certain organs. The theory of 'seed' and 'soil' implicates compatibility of tumor cells with the microenvironment of the metastatic site. Another hypothesis, by pathologist James Ewing [21], suggested that metastatic dissemination is mechanically driven, i.e., metastatic spread relies exclusively on the circulatory patterns between primary tumor site and metastatic site. A third hypothesis, by Duda D.G. et al., proposes that cancer cells bring their own microenvironment to the metastatic site [22]. In a lung metastasis model, it was shown that host-derived stromal cells cooperate with carcinoma cells in successful seeding, when carried over from the primary site within tumor clumps. The suggested presence of tumor-associated fibroblasts from elsewhere was confirmed in brain metastases of various carcinomas. Demonstration of the direct involvement of the primary tumor 'soil' in metastasis, has conceptual and clinical implications for the colonization steps in tumor metastasis establishment. The current widely accepted hypothesis is the 'seed-and-soil' theory, and its definition resides on three main principles. Firstly, neoplasms are, regarding their proliferation, angiogenesis, invasion and metastatic potential, biologically heterogeneous [23, 24] and different metastases of the same primary tumor may have different clonal origins within the same primary tumor

mass [25, 26]. Secondly, the establishment of metastasis is reserved for cancer cells which successfully completed all the metastatic steps [23]. Thirdly, metastatic tumor cells are dependent on the 'crosstalk' with keepers of homeostasis [27]. To this point, researchers have sought to understand how molecular and genetic traits, inherent to the primary cancer cell and secondary microenvironment, influence spread and outgrowth of cancer cells into specific metastatic foci.

The Blood-Brain Barrier (BBB)

The brain provides an environment that differs from other organs. The brain microvasculature is bounded by a continuous, non-fenestrated endothelium with tight junctions and reduced pinocytic activity [28-30]. Known for its ability to limit the entrance of circulating macromolecules, the blood-brain barrier (BBB) protects the brain against the invasion of microorganisms and the entry of most therapeutic drugs [31]. The BBB consists of the brain vasculature where tightly adjoined endothelial cells are surrounded by pericytes, a basement membrane and astrocytes foot processes, all of which cooperatively functioning as a barrier [32]. The BBB structure is morphologically, biochemically and functionally heterogeneous throughout the brain [33, 34] and does not prevent the invasion of circulating tumor cells into the brain. In fact, tumor cells are able to survive and proliferate in the brain by exploiting the BBB biological protective features to their benefit [35]. When circulating tumor cells arrest in the vascular branch points of the capillary beds, reactive astrocytes closely interact with the cancer cells throughout their outgrowth into clinically detectable metastases [36]. Histological analysis of resected human brain metastases disclosed a neuro-inflammatory process in which activated microglia also play a prominent role. The chemoprotective nature of astrocytes towards cancer cells has been demonstrated in *in vitro* co-culture experiments of human melanomas [37], breast - and lung cancer cells [38]. Intricate vascular adaptations are evident along parenchymal colonization, and a rich supply of dilated and tortuous blood vessels are usually found to accompany brain metastases [39, 40]. Metastases not only induce structural changes in blood vessels, but also cause increased vessel density, particularly in metastases from melanoma and lung cancer [41].

Chemotherapy, resistance and brain metastasis

Resistance to chemotherapy is a major cause of death in patients with brain metastasis [42]. In end-stage malignant disease, metastatic relapse is often associated with resistance to systemic therapy. Cell intrinsic mechanisms, such as genetic alterations, provide drug resistance in lung cancers: epidermal growth factor receptor (EGFR) mutations respond to EGFR kinase inhibitors – but often the tumors relapse due to secondary EGFR mutations that confer resistance [43, 44]. Other resistance mechanisms, such as MET amplification, render subsets of EGFR-mutant lung carcinomas insensitive to gefitinib and erlotinib [45, 46]. Moreover, some systemic therapies, like trastuzumab (Herceptin) therapy, seem to induce brain metastases in ERBB2-positive breast cancer patients [47]. Aside from therapy-driven resistance and metastatic traits, cancer cells may acquire drug resistance by independent intrinsic mechanisms, which is of

particular relevance in rapidly evolving tumors such as melanomas and lung cancers, for which few effective treatments, other than surgery, are available [18].

OUTLINE OF THIS THESIS

The scope of this thesis lies on the identification of relevant molecules and pathways involved in the development of brain metastases. **Chapters two** and **three** summarizes the so far reported molecular mechanisms predictive and inherent to the development of brain metastases from two of the most common cancers to metastasize to the brain: breast cancer and lung cancer, respectively. In order to identify genes and molecular pathways involved in the formation of brain metastasis from ER negative primary breast cancers, in **Chapter four** we compared gene expression profiles of primary breast cancers that developed metastasis including and excluding brain. T lymphocytes were shown to play a role in the promotion of the formation of brain metastasis, which we further corroborated in an *in vitro* functional blood-brain barrier (BBB) model and in an *in vivo* mouse model. In addition, we show that interaction of breast cancer cells with activated T lymphocytes induces the overexpression of the protein Guanylate binding protein 1 (*GBP1*) in the breast cancer cells, a molecule that was found upregulated in the cancers of patients who developed brain metastases. Further *in vitro* studies, in which *GBP1* was silenced, showed the functional involvement of this protein in the tumor cells' ability to cross the BBB.

In order to further scrutinize the role of T lymphocytes in facilitating the formation of brain metastasis from ER negative breast cancers, in **Chapter five** we analysed which sub-types of activated T lymphocytes stimulated breast cancer cells most to pass the BBB. In addition, the levels of Interferon-gamma (IFN-γ) secreted in the media were measured and compared between the different settings. The involvement of the IFN-γ signaling pathway in breast cancer cells, for the development of brain metastasis, was validated using inhibitory antibodies and by blocking the IFN-γ receptor 1 (IFNGR1) in breast cancer cells. CD8+ T lymphocytes was shown to be the subset with the strongest stimulatory effect on the BBB transmigration of breast cancer cells, and IFNGR1 blocking in breast cancer cells was demonstrated to impair the BBB transmigration of these cells. From the comparison of the initial gene expression profiles, between ER negative breast cancers that developed brain metastasis and metastasis to organs other than brain, three genes that are not involved in the T cell response were significantly overexpressed in the brain metastasis group, and used for further validation in **Chapter six**. We analyzed the mRNA and protein level changes for BOC, GJD3 and SPOCK2 in an independent breast cancer cohort, from patients who have developed metastases to organs excluding and including brain, and our conclusions were corroborated by data mining from a publicly available mRNA expression primary breast cancer database.

In **Chapter seven** we evaluate the effects of T lymphocytes in co-culture with different types of cancers. Cell lines of breast-, prostate and lung cancers and melanomas were co-cultured with T lymphocytes and evaluated in *in vitro* BBB functional studies, followed by proteome comparisons of the tumor cells before and after their contact with T lymphocytes. Coronin-1A (CORO1A) was identified as a common overexpressed protein in cancers which ability to cross the *in vitro* BBB was increased by prior contact with activated T lymphocytes. siCORO1A was further shown to impair BBB trespassing of brain-trophic breast cancers. In addition, an increased Coronin-1A protein expression was validated in an independent primary breast cancer cohort of patients with and without brain metastasis, as well as CORO1A mRNA expression levels in a publicly available mRNA database of primary breast cancer samples from patients with various metastasized sites.

The work presented in this thesis is discussed in **Chapter eight** and summarized in **Chapter nine**.

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Chapter 2

Breast Cancer Brain Metastases: Molecular Mechanisms and Directions for Treatment

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ABSTRACT

The development of brain metastasis (BM) of breast cancer is usually a late event with deleterious effect on the prognosis. Treatment options for intracerebral seeding of breast cancer are limited and, so far, nonspecific. Molecular detailing of subsequent events of penetration, seeding, and outgrowth in brain is highly relevant for developing therapeutic strategies to treat, or prevent, BM.

We scrutinize recent literature for molecules and pathways that are operative in the formation of breast cancer BM. We also summarize current data on therapeutic efforts to specifically address BM of breast cancer. Data on molecular pathways underlying the formation of BM of breast cancer are sketchy and, to some extent, inconsistent. The molecular makeup of BM differs from that of the primary tumors, as well as from metastases at other sites. Current efforts to treat breast cancer BM are limited, and drugs used have proven effects on the primary tumors but lack specificity for the intracerebral tumors.

More basic research is necessary to better characterize BM of breast cancer. Apart from the identification of drug targets defined by the intracerebral tumors, also targets in the molecular pathways involved in passing the blood–brain barrier and intracerebral tumor cell growth should be revealed.

INTRODUCTION

Breast cancer is a histologically and genetically heterogeneous disease, categorized by the expression of estrogen and progesterone receptors (ER and PR, respectively) and the human epidermal growth factor receptor 2 (HER2/neu). There are six basic molecular subtypes of breast cancer. Based on the ER, PR and her2neu status only four categories are clinically distinguished [1]. Each subtype exhibits a particular natural history, metastatic potential and outcome. Breast cancer tends to metastasize to bone, lung, liver and brain. Seeding to brain is usually a late event. Tumor cell (TC) growth in the brain microenvironment is the result of genetic predisposition and cellular adaptation mechanisms, and is largely dependent on cross-talk between TCs and brain-resident cells. BM develop more frequently in triple-negative (TN) (25% - 27%) and HER2-positive breast cancers (11% - 20%), while the incidence of BM in luminal A and B is much lower (8% - 15% and 11%, respectively) [2]. TN breast cancer BM tend to develop earlier in the course of disease and are almost invariably associated with extracranial metastases [3]. BM in HER2+ tumors occur in up to 50% of patients receiving HER2+ targeted therapies (in particular trastuzumab). The TN subtype displays the worse (3 - 4 months), and luminal B the best (19 - 20 months) median survival in the presence of BM [4]. This review will focus on the presently known molecular mechanisms that grant breast circulating TCs (CTCs) extravasation into de brain parenchyma. Discovering specific gene mutations or expressional profiles in TCs prone to disseminate to brain, will open avenues to intervene with the rise of intracerebral dissemination, and offer a great step forward in the treatment of breast cancer.

GENETIC PREDICTORS OF BM

Over the last decades, improvements of microarray technology and the capacity to carry out massive parallel gene expression analysis have provided awareness of the complexity of breast cancer. Data on the breast cancer sub clones and molecular pathways involved in metastasis, in particular to brain, are sketchy and not univocal. Using next generation sequencing (NGS) data, of four DNA samples collected from one breast cancer patient, it was suggested that the development of metastases may be triggered by a minority of cells within the primary tumor [5], and the results of various studies point to large similarities between the parent tumors and their metastatic offspring. In a study where 15 primary breast cancer samples were compared with their intracerebral metastases by NGS, no significant differences in mutation profiles were detected, while actionable gene alterations in the breast cancer BM (such as TP53, PIK3CA, KIT, MLH-1 and RB1) had been preserved [6]. Similarly, in a study on 12 primary breast cancers and their BM, in which 19 oncogenes were scrutinized, all somatic mutations were found, in both primary and metastatic specimens, except for one EGFR mutation identified only in

the primary tumor [7]. However, whole-exome sequencing of 86 matched primary tumors and BM from patients with breast, lung and renal cancer, revealed that 53% of the BM harbored at least one clinically actionable alteration that was not detected in the matched primary tumor, while the BM had more unique mutations than the primary and lung metastasis combined [8]. Further assessment of different intracranial metastatic sites in the same patient, showed that almost all of the potentially clinically informative driver alterations were shared, suggesting that BM are homogeneous within an individual [8]. Clearly, differences in findings between studies depend on the extent of screenings applied.

Interestingly, between 16% and 22% of ERBB2/HER2 negative breast cancers have been reported to acquire ERBB2/HER2 amplifications and/or mutations in the BM. The discordance between primary tumors and their BM could be even greater regarding the ER status. Similarly, EGFR expression was increased in the brain-seeking, but not the bone-seeking, subline of a MDA-MB-231 model system [9]. The induction of Her2 in this cell line resulted in a 3-fold increase of brain macro-metastasis in a xenograft model [9]. A significant gain of EGFR copy number status was found in breast cancer BM, when compared to the primary breast cases. EGFR amplification is also more frequently found in brain metastatic adenocarcinomas from the lung, when compared to corresponding primary tumors. Furthermore, EGFR amplified primary lung tumors are significantly correlated with shorter time to BM development [10]. The identification of exclusive mutations in tumor cells (TCs) at different sites, is suggestive of a metastatic cascade-dependent evolution that is influenced by, or dependent on, the cross-talk between CTCs and their variable microenvironment. Some of these mutations may be necessary for the establishment of the initial metastatic seeding in the brain, but not for its continued growth or maintenance. While PTEN mutations are rarely found in primary breast [11] or lung [12] cancers, 21% of the brain metastatic breast tumors were reported to carry PTEN mutations and 31% were determined with loss of PTEN protein expression [11]. Results of comparative genetic hybridization (CGH) of arrays of breast tumors and BM, emphasized the relevance of PTEN loss and the role of EGFR gene in BM formation [13]. In a study on 119 primary TN breast tumors, it was shown that analysis of gene transcripts in the primary tumors is insufficient to predict the development of BM [14]. It is clear that BM of breast cancer do differ from their primary tumors in terms of mutations and expression. Importantly, some data indicate that therapeutic actionable mutations can be present in the BM while not in the primary tumors. This difference has consequences for choosing the right therapy for the BM, and therefore, intensified research aiming at the characterization of primary breast cancer and their BM is important for the development of targeted therapies.

Although Wnt/β-catenin signaling activation is associated with breast cancer BM, the significance of this pathway to predict BM remains unclear. Two potential Wnt ligands, Wnt5A and Wnt5B and their respective receptors, ROR1 and ROR2, were identified as signal-mediators

and potential therapeutic targets [15], and it has been suggested that Wnt signaling in breast tumor progression occurs independent of β-catenin. Microglia reportedly promotes colonization of brain tissue by breast cancer cells in a Wnt-dependent way. Moreover, the Wnt ligand Wnt5A was found to be constitutively expressed in microglia and is a critical mediator of CXCR4-induced T-cell migration [16].

INFLUENCE OF THE IMMUNE SYSTEM

In a recent study, in which gene expressions of breast cancers from women with metastasized disease, with and without cerebral metastases, were compared, T cell response to the primary tumors appeared crucial for the rise of BM [17]. The T cells appeared to change the TC proteome, and the expression of some of these crucial proteins was confirmed in those primary tumors that gave rise to BM. The findings were confirmed in a blood-brain-barrier (BBB) cell culture model, in which breast cancer cell lines were incubated with T cells. Moreover, injecting breast cancer cells, that were co-cultured with T cells, in mice, gave a brain phenotype as well. The results need further exploration in order to scrutinize current anti-cancer T cell therapies, and to find targets for therapeutic interference with the T cell response and the resulting upregulated proteins. The influence of the immune system on the formation of BM needs further scrutiny in order to find ways to prevent the formation of BM in breast cancer patients.

STAGES OF METASTASIZING TO BRAIN

Passage through the BBB

TCs mimic mechanisms used by immune cells, but the adhesion molecules and ligands they use for extravasation are different. It is unclear whether the attachment characteristics of leukocytes are a precondition for trans-endothelial migration (TEM) [18]. The migration into the brain occurs foremost by opening tight junctions[19]. In an *in vitro* experiment, melanoma cells disrupted the intercellular junctions (TJs) and adherens junctions (AJs) of the endothelial cells [19], and their sizes are more important for the initial arrest than the capacity to adhere to vascular walls [20]. It is not yet clear how and whether CTCs affect the function of the BBB. So far, apoptosis or hypoxia has not been recorded in the endothelial cells at the site of extravasation, suggesting that extravasation events do not directly correlate with damage of the vessel walls. To enter the brain parenchyma, cancer cells must pass through micro-capillary walls that constitute the BBB [21]. The TJs and the absence of extensive pinocytosis and fenestrae, account for protecting the brain from cellular and molecular intruders. The extravasation strategy of TCs comprises the arrest and adherence to the endothelium (docking), the establishment

of intercellular contacts (locking), TEM, adhesion to the subendothelial matrix (foothold) and subsequent modification of the surrounding host tissue microenvironment (colonization) (**Figure 1**).

Reciprocal interaction with stromal cells grants the successful formation of macrometastasis [22]. Initial investigations showed that the production of cytokines, resulting from inflammation, increase the ability of CTCs to adhere to the vascular endothelium, while the endothelium itself was thought to be a mere passive participant. However, later studies revealed that the attachment of TCs to brain endothelial cells is mediated by bilateral cell surface receptors and adhesion molecules, such as integrins, selectins and chemokines. Therefore, endothelial cells actively influence TC extravasation and proliferation [20] (**Figure 1**; **Table 1**). Only a minority of TCs, known as tumorigenic cells or cancer-initiating cells, trigger tumor proliferation and self-renewal [23]. Only a small proportion of breast cancer cells display a $CD44^{high}/CD24^{low}$ antigenic phenotype, contrasting with the predominant $CD44^{low}/CD24^{high}$ phenotype [24]. Breast cancer cells with a $CD44^{+}/CD24^{-}$ phenotype display highly invasive properties and elevated levels of pro-invasive genes, like interleukin (IL) 1α, IL-6, IL-8 and urokinase plasminogen activator (uPA) [25]. Stromal uPA and matrix metalloproteinase 2 (MMP-2) are

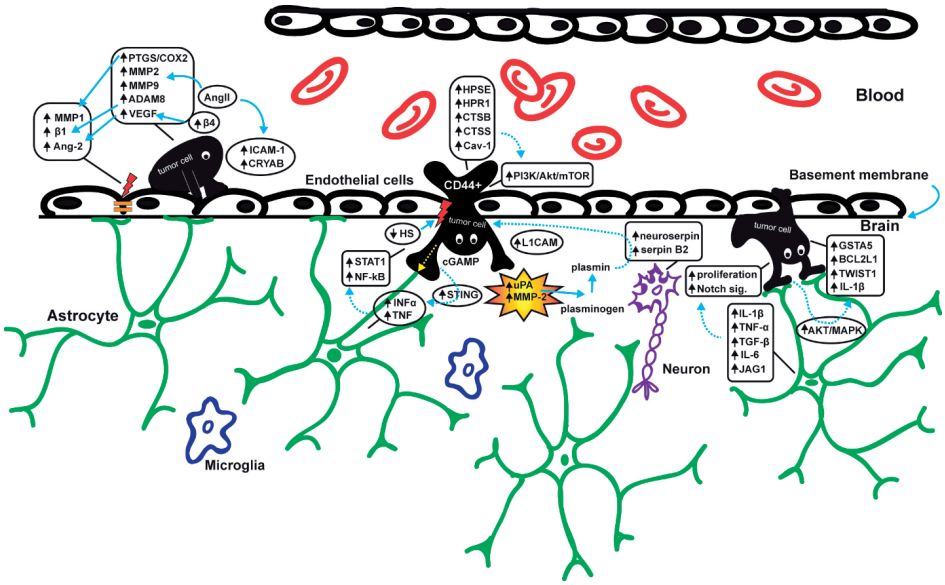

Figure 1 – Schematic representation of the stages of the formation of cerebral metastasis. From left to right, the processes of vascular adhesion, transgression through the BBB, extravasation, interaction with the brain microenvironment of the tumor cells and involved molecules are presented. MMP, matrix metalloproteinase; β1, beta 1 integrin subunit; PTGS2/COX-2, prostaglandin-endoperoxide synthase 2/cyclooxygenase-2; ADAM8, a disintegrin and metalloproteinase domain-containing protein 8; β4, beta 4 integrin subunit; CRYAB, αβ-crystallin; NFα, interferon alpha; STING, stimulator of interferon genes; L1CAM, L1 cell adhesion molecule; JAG1, jagged 1; GSTA5, glutathione S-transferase alpha 5; BCL2L1, BCL2 like 1; TWIST1, twist family BHLH transcription factor 1.

produced as non-active precursors and become responsive on the surface of the TCs, allowing the neoplastic cells to break through de basement membrane [26]. uPA converts plasminogen into plasmin. Suppression of plasmin is caused by overexpression of neuroserpin and serpin B2, which in turn enable the infiltration of metastatic breast cancer cells through the BBB. This mechanism is mediated by L1 cell adhesion molecule (L1CAM), which will empower the metastatic process [27]. Through NF-κB pathway stimulation, uPA activates MMPs, endopeptidases that can cleave any component of the extracellular matrix (ECM) [28].

In a mouse model of breast cancer metastasis, up-regulation of $\alpha_v\beta_3$ integrin in TCs appeared to interact with platelets, resulting in thrombus formation that facilitates the arrest of TCs within the vasculature [29]. Activation of $\alpha_v\beta_3$ integrins also assists in the intracerebral growth of TCs through continued up-regulation of vascular endothelial growth factor (VEGF) [30]. Enhanced β4 integrin signaling induces HER2-dependent expression of VEGF, that is responsible for the disruption of the inter-endothelial junctions [31]. VEGF is highly expressed in breast cancer cells, and is known as a vascular permeability factor and major regulator of angiogenesis during tumor development. The interaction between TCs and blood vessels relies on β1 integrin-mediated adhesion, and it has been suggested that antagonists of the β1 integrin subunit, expressed in the vascular basal membrane, would reduce the formation of CNS metastases [32]. Another role in the interaction between endothelial cells and TCs is played by the selectins. E-selectin is expressed on activated endothelial cells, and P-selectin is found on both endothelial cells and activated platelets [33]. P-selectin may directly assist the metastatic process through communication with arrested TCs, masking and protecting TCs from the immune response [34].

Other soluble factors affecting the formation of metastases are chemokines and their specific receptors. The chemokine stromal cell-derived factor 1α (SDF-1α or CXCL12) and its receptor CXCR4 play a role in multiple biological functions as homing, motility and progression of metastases [35]. CXCL12/CXCR4 signaling is involved in the migration of breast cancer cells through the BBB. SDF-1α induces blood vessel instability and is significantly highly expressed in breast cancer cells when compared to normal breast tissue [36]. CXCL12 was shown to be overexpressed in BM from breast and lung cancer [35]. CXCR4 is among the chemokine receptors that are most commonly expressed in cancer. SDF-1α expression is frequently observed at common metastatic sites of breast cancer [37]. The stimulation of SDF-1α increases adhesion and activates TEM, by activating the phosphatidylinositol-3 kinase pathway (PI-3K/AKT) [36]. Nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) signaling, mediated by CXCR4, plays a role in organ-specific metastasis of breast cancer, migration and tumor growth [38]. Taken together, these data suggest that the expression of chemokine receptors in primary breast cancers correlate with the appearance of BM. Whether any of these

Table 1 - Molecular mechanisms and potential therapeutic targets in breast cancer brain metastasis.

Mechanism	Potential Therapeutic Target	location	Regulated by	Regulates	Action	Reference
Passage through BBB	Wnt5A/ROR1; Wnt5B/ROR2	NA	Wnt signaling	NA	↑ invasion	15
	αvβ3 integrin	NA	NA	↑ tumor-platelet interaction	↑ arrest in vasculature	29
	αvβ3 integrin	NA	NA	↑ VEGF	↑ intracerebral growth	30
	β4 integrin signaling	NA	NA	↑ VEGF	↑ disruption of inter-endothelial junctions	31
	β1 integrin	NA	NA	NA	↑ adhesion	32
	P-selectin	endothelial cells and activated platelets	NA	communication / protection of arrested TC	↑ metastatic ability	34
	CXCL12/CXCR4 signaling	metastatic site/TC	NA	↑ PI-3K/AKT	↑ adhesion, ↑ TEM	36
	NF-κB signaling	NA	CXCR4	organ-specific metastasis of breast cancer	↑ migration, ↑ tumor growth	38
Intracerebral Progression	CD44+/CD24- phenotype	breast cancer cells	NA	IL-1α ↑, IL-6 ↑, IL-8 ↑, uPA ↑	↑ invasion	25
	uPA	breast cancer cells	NF-κB pathway	Plasmin production, MMPs	↑ ECM cleavage	28
	↓ Plasmin	NA	↑ neuroserpin, ↑ serpin B2, ↑ L1CAM	NA	↑ TEM	27
Interaction with Astrocytes	IL-1β, TNF-α, TGF-β, IL-6	Astrocytes	NA	NA	↑ proliferation (breast and lung cancer cells)	41
	cyclic GAMP	Astrocytes	TCs	↑ INFα, ↑ TNF -> ↑STAT1 and NF-κB pathways	↑ tumor growth, ↑ chemo-resistance	42
	AKT/MAPK pathways	TCs	astrocytes-TC interaction	↑ GSTA5, ↑ BCL2L1, ↑ TWIST1	↑ resistance, ↑ survival	43
	IL-1β	(secreted by) BM breast cancer	NA	↑ JAG1	↑ Notch signaling	44

Table 1 - Molecular mechanisms and potential therapeutic targets in breast cancer brain metastasis. (*continued*)

Mechanism	Potential Therapeutic Target	location	Regulated by	Regulates	Action	Reference
Interaction with ECM	MMP-14 (or MT1-MMP)	target site	COX-2 (or PTGS2)	MMP-2	↑ TEM	47
	MMP1	(secreted by) breast cancer cells	NA	degrades tight junctions of BBB	↑ TEM	48
	HPSE	BM breast cancer, endothelial cells and glial cells	EGFR/Her2 signaling	NA	↑ TEM	53
	HPR1	NA	↓ HS	NA	↑ metastatic ability	55
	CTSB	breast cancer cells	NA	↑ angiogenesis, ↑ MMPs	↑ ECM cleavage, ↑ invasion, ↑ tumor growth	57
	CTSS	NA	NA	proteolytic processing of JAM-B	↑ TEM	59
	Cav-1	NA	NA	↓ MMP-9 and MMP-2, ↓ Stat3 / (LSS exposure):↑ PI3K/ Akt/mTOR signaling	↓ tumor growth, ↓ invasion / (LSS exposure):↑ motility, ↑ invadopodia formation, ↑ invasion	60, 61
	MEK5	NA	Stat3	↑ EMT	↑ invasion	64
	exo-AnxA2	breast cancer cells	NA	↑ p38MAPK, ↑NF-κB, ↑ stat3 pathways; (secretion of) ↑ IL-6, ↑ TNF-α	↑ angiogenesis, ↑ proliferation	65
	TUBB3	breast cancer BM cell line	NA	NA	↑ metastatic ability	66
	AngII	vasoactive peptide	NA	↑ MMP-2, ↑ MMP-9 in breast cancer cells; ↑ICAM-1	↑ adhesion, ↑ TEM, ↑ motility	67
	CRYAB	(independent predictor of BM)	NA	NA	↑ adhesion, ↑ TEM	68, 69
	Ang-2	activated brain endothelial cells	VEGF secreted by TN breast cancer cells	impairment of TJ structures	↑ TEM	70

Akt, protein kinase B; TEM, transendothelial migration; L1CAM, L1 cell adhesion molecule; TNF, tumor necrosis factor; TGF, tumor growth factor; cGAMP, cyclic guanosine monophosphate–adenosine monophosphate; INF, interferon; GSTA5, glutathione S-transferase alpha 5; BCL2L1, BCL2 like 1; TWIST1, twist family BHLH transcription factor 1; JAG1, jagged 1; PTGS2, prostaglandin-endoperoxide synthase 2; JAM-B, junctional adhesion molecule B; Cav-1, caveolin-1; LSS, low shear stress; MEK5, mitogen extracellular-signal-regulated kinase 5; exo-AnxA2, exosomal-annexin A2; ICAM-1, intercellular adhesion molecule 1; CRYAB, αβ-crystallin; TN, triple negative; TJ, tight junction; NA, not available

receptors will become useful as biomarker predictive of the appearance of BM is an unanswered question.

Intracerebral Progression

Astrocytes are confronted by breast cancer cells that have breached the endothelial side of the BBB. Astrocytes have a pivotal role in the maintenance of the BBB, contributing to cerebral and extracellular homeostasis [39]. Direct contact between astrocytes and TCs induces calcium sequestration [40]. The production of IL-1β, tumor necrosis factor-α (TNF-α), tumor growth factor-β (TGF-β) and IL-6 by astrocytes, was shown to induce proliferation of breast and lung cancer cells [41]. TCs use astrocyte gap-junctions to transfer cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) to astrocytes, boosting the production of inflammatory cytokines such as interferon-α (INFα) and TNF (**Figure 1**). In turn, these factors activate the signal transducer and activator of transcription 1 (STAT1) and NF-κB pathways in brain metastatic cells, promoting tumor growth and chemo-resistance [42]. Direct contact between astrocytes and TCs, induce activation of the AKT/MAPK pathways, leading to the up-regulation of anti-apoptotic genes (GSTA5, BCL2L1 and TWIST1) in TCs that mediate resistance against cytotoxic drugs [43]. In addition, IL-1β secreted by the breast cancer cells, specifically up-regulates the Notch ligand JAG1 on primary rat astrocytes, which in turn promote Notch signaling in CTCs [44].

Matrix metalloproteinases (MMP) are produced by TCs or their environment and play a role in growth, angiogenesis and migration in multiple stages of tumor progression. MMPs are characterized according to the specificity of the degrading substrate; MMP-2 and MMP-9 degrade type IV collagen, which is the main component of the vascular basement membrane [45]. In a rat model, MMP-2, MMP-9 and MMP-3 have been proposed to play a role in the establishment of breast cancer BM [46]. MMP-2 is initially secreted in a pro-active form, and it is activated through interaction with MMP-14 (or MT1-MMP) on the cell surface at the target site, which expression is increased by cyclooxygenase-2 (COX-2, also known as PTGS2) [47]. MMP-1, secreted by 231BrM cells, degrades occludin and claudin-5 that are major components of the tight junctions of the BBB. MMP-1 expression strongly correlates with COX-2 expression, indicating its active role in BM of breast cancer patients [48]. COX-2, the epidermal growth factor receptor ligand HBEGF, and the α2,6-sialyltransferase ST6GALNAC5 genes were identified as mediators of breast cancer cell passage through the BBB. COX-2 changes the permeability of the BBB [49] and its overexpression is observed in about 40% of breast tumors. While COX-2 and HBEGF expression, in primary tumors, enhances TC extravasation both into brain and lungs, ST6GALNAC5 was found as a specific mediator of BM of breast cancer [50]. In contrast, upregulation of ST6GALNAC5 in brain-seeking breast cancer cells (MDA-MB-231 BrM2) decreased their adhesion to the endothelial component of a well-characterized human BBB *in vitro* model [51]. Various other genes involved in

transcription, translation and metabolism, were associated with BM [52]. Heparanase (HPSE) is a pro-tumorigenic, proangiogenic and pro-metastatic endoglycosidase overexpressed in brain metastatic breast cancer [53]. This downstream target of EGFR/HER2 signaling, is also produced by brain endothelial and glial cells, promoting TC invasion [54]. The expression of heparanase-1 (HPR1) reportedly is inversely correlated with heparin sulfate (HS) deposition, a component of proteoglycans, and constituent of the ECM and basement membrane, correlated with the metastatic potential of breast cancer [55]. The coordinated action of MMP-9 and HPSE contribute to breast cancer cell carcinogenesis and progression [56]. Angiogenesis and MMPs activity are enhanced by cathepsin B (CTSB) [57]. This cysteine protease is often overexpressed in breast cancer, increasing matrix degradation, invasiveness and tumor growth [58]. Cathepsin S (CTSS) was identified as a breast cancer BM-promoter, crucial for metastatic seeding and outgrowth; CTSS mediates BBB transmigration of breast CTCs by proteolytic processing of the junctional adhesion molecule B (JAM-B) [59]. Various studies have shown that caveolin-1 (Cav-1) acts as a tumor suppressor protein in human breast cancer. Cav-1 also inhibits discharge and expression of MMP-9 and MMP-2 [60]. Upregulation of Cav-1 *in vitro* and in nude mice was shown to mimic effects of STAT3 activation, suppressing tumor growth and attenuating the invasiveness of breast cancer [61]. Conversely, in another recent *in vivo* study, Cav-1 was shown mechanosensitive to low shear stress (LSS) exposure, and its activation-induced PI3K/Akt/mTOR signaling promoted motility, invadopodia formation and lung metastasis of breast carcinoma MDA-MB-231 cells [62]. STAT3 controls constitutive and inducible VEGF receptor 2 expression in tumor-associated brain endothelial cells, and its inhibition suppresses BM of breast cancer cells [63]. STAT3 intercedes with the upregulation of mitogen extracellular-signal-regulated kinase 5 (MEK5), which promotes breast cancer cell invasion through epithelial to mesenchymal transition (EMT). Ectopic expression of MEK5 could provide non-invasive breast cancer cells with invasive capacity [64]. Recent work on expressed proteins in tumor-derived exosomes, demonstrated an important role of exosomal-AnnexinA2 (exo-AnxA2) in breast cancer pathogenesis. Moreover, exo-AnxA2, overexpressed in malignant breast cells, was shown to mediate activation of the p38MAPK, NF-κB and STAT3 pathways, as well as the secretion of IL-6 and TNF-α, increasing angiogenesis and breast cancer proliferation. Furthermore, *in vivo* analysis revealed that priming with AnxA2-depleted exosomes reduced BM formation in ~4-fold [65]. Altogether, these findings highlight the critical role of the tumor microenvironment in breast cancer progression and metastatic behavior.

Several other genes were reported to be upregulated in breast cancer BM, being potential target of treatment. Hexokinase II (HK2) and βIII-tubulin (TUBB3) expression is significantly associated with distant metastases. Knockdown of TUBB3 in a breast cancer BM cell line, compromised its metastatic ability *in vivo*, improving survival in a BM model [66]. Angiotensin II (AngII), a potent vasoactive peptide, was shown to contribute to increased tumor-endothelial

cell adhesion, trans-endothelial migration and motility, accelerating metastatic progression in an experimental mouse model. Besides up-regulating MMP-2 and MMP-9 gene expression in breast cancer cells, AngII was also reported to up-regulate the expression of intercellular adhesion molecule 1 (ICAM-1) [67]. In the same line, αβ-crystallin (CRYAB) promoted adhesion of TN breast cancer cells to human brain microvascular endothelial cells, enhanced penetration through the BBB *in vivo* and was indicated as an independent predictor for the development of BM [68, 69]. Using *in vivo* mouse models for breast cancer BM, Angiopoietin-2 (Ang-2) was observed to be elevated in activated brain microvascular endothelial cells, due to the presence of VEGF secreted by TN breast cancer cells and by their "brain-seeking" variant. Secreted Ang-2 impaired TJ structures and increased BBB permeability, resulting in TN breast cancer colonization of the brain [70].

CLINICAL PERSPECTIVES

So far, only few clinical trials have allowed for the inclusion of patients with breast cancer and CNS metastases. There is a trend of initiating trials that explore the efficacy of new targeted therapies with specific focus on BM [71] (**Table 2**).

None of the agents used to treat BM in a clinical setting are directed against any of the molecules that are operative in crossing the BBB or the intracerebral propagation of the TCs. Conversely, the available studies have investigated, in BM from breast cancer, compounds with proven efficacy in the systemic disease. Their activity has been evaluated in terms of either intracranial response and/or progression-free survival (PFS) in patients with established BM, or prevention of the development of BM in patients with, or without, stable BM following local treatments (chemoprevention). So far, no targeted agents with the specific indication of BM are registered in the US or Europe [72]. Most investigations concern the HER2+ subtype of breast cancer. Monoclonal antibodies actionable against HER2, such as trastuzumab, pertuzumab and trastuzumab emtansine (TDM1), are considered to be too large to cross an intact BBB for an effective chemoprevention. However, the BBB may be damaged in case of macrometastases, or following radiotherapy, resulting in increased CSF levels of trastuzumab [73, 74]. HER2+ metastatic brain lesions can be visualized by 64Cu-DOTA-trastuzumab positron emission tomography (PET) [75]. Continuing treatment with trastuzumab, beyond the development of BM, may result in a survival benefit [76], and there is now renewed interest in studying high doses of trastuzumab as a treatment of HER2+ BM [77]. Following treatment with TDM1, response rates in the brain are reported to be similar to those observed in extracranial sites [78].

HER2 receptors enhance the EGFR signaling [79] and therefore, simultaneous inhibition of HER2 and EGFR receptors may be superior to HER2 inhibition alone [80]. Lapatinib, a

small molecule TKI with activity against HER2 and EGFR, is approved in combination with capecitabine for the treatment of metastatic breast cancer. The activity of lapatinib used as single agent in pretreated BM is modest (response rate of 6%) [81], but increases to 66% by adding capecitabine [82-84]. The potential chemopreventive activity of lapatinib was suggested by the results of a phase III randomized trial, in which the effects of lapatinib plus capecitabine vs. capecitabine alone were compared in patients with advanced breast cancer, who had progressed on trastuzumab: fewer patients with CNS involvement at first progression were in the lapatinib-containing arm (2% vs 6%) [85]. However, prospective validation of this finding was inconclusive in the CEREBEL [86] and EMILIA [87] trials. Recent preclinical studies have shown that lapatinib is a substrate of ATP-binding cassette (ABC) transporters (in particular ABCB1), potentially limiting the capacity of the drug to penetrate an intact BBB [88]. Therapeutic levels of lapatinib may be reached in established BM, but concentrations are by far lower than those reached for extracranial metastases [89]. A dual HER2 inhibition, for instance, by the combined use of pertuzumab and trastuzumab, could be active in the prevention of BM. A post hoc analysis of the Cleopatra trial suggested that the combination of pertuzumab, trastuzumab and docetaxel could delay the onset of CNS disease, compared to docetaxel alone [90].

Novel HER2 targeted TKIs, that are potentially active in BM, include neratinib and tucatinib (ONT-380; ARRY 380). Neratinib is an irreversible inhibitor of HER2, erb1 and erbB4, and preclinical data suggest that it may penetrate an intact BBB. In contrast to lapatinib, neratinib reverses ABCB1-mediated chemo resistance, and is unaltered by the presence of ABCB2 transporter [91]. Neratinib was reported to target activating HER2 mutations in HER2 gene amplification negative breast cancer, thereby overcoming resistance to trastuzumab or lapatinib [92, 93]. Thus far, the efficacy of neratinib on established BM is modest, and similar to that observed with lapatinib. A recent phase II study on HER2-positive BM, in patients who had progressed on at least one prior CNS-directed therapy, has shown an intracranial response rate of 8% and a median PFS of 1.9 months [94]. The association of neratinib and capecitabine is being investigated in an ongoing trial, with interesting preliminary results in terms of response rate (49%) and 12-months survival (63%) [95]. Neratinib may be more active in the prevention than in the treatment of BM. A randomized clinical trial in previously untreated metastatic HER2-positive breast cancers, showed that symptomatic or progressive CNS recurrences occurred in 8.3% of patients in the neratinib-paclitaxel group vs 17% of patients in the trastuzumab-paclitaxel group [96]. The estimated Kaplan Meier 2-year incidence of CNS recurrences was 16.3% in the neratinib-paclitaxel group vs 31.2% in the trastuzumab-paclitaxel group. ONT-380 selectively targets HER2, has the potential to cross the BBB, and a minimal activity against EGFR, leading to a more favorable toxicity profile. ONT-380 has shown antitumor activity in heavily pretreated HER2+ metastatic breast cancer patients in a phase I study [97]. In another phase I study, in which ONT-380 is combined with TDM1,

Table 2 - Overview of actionable targets and clinical studies on targeted therapies in established brain metastasis.

TARGET	TARGETED AGENT	PRETREATMENT WITH RADIOTHERAPY	RESPONSE RATE (%)	PROGRESSION-FREE SURVIVAL (months)	OVERALL SURVIVAL	TYPE OF STUDY	REFERENCE or Clinicaltrials.gov
Her2, EGFR	Lapatinib	Yes	6%	2.4	6.4	Phase II	81
	Lapatinib + Capecitabine	No	66%	5.5	>70% (1 year)	Phase II	83
Her2	Neratinib	Yes	8%	1.9	8.7	Phase II	94
	Neratinib + Capecitabine	Yes	49%	NA	63% (1 year)	Phase II	95 (preliminary results)
	Tucatinib (ONT-380) + (TDM1)	Yes	33%	6.5	NA	Phase I	97
PARP	Iniparib c	Yes	27%	2.14	NA	Phase II	102
Her2	Tucatinib (ONT-380) + Trastuzumab	Yes	NA	NA	NA	Phase I	NCT019221335
	Pertuzumab + High-dose Trastuzumab (intravenous)	Yes	NA	NA	NA	Phase II	NCT02536339
	Pertuzumab + Trastuzumab (intrathecal)	No	NA	NA	NA	Phase I	NCT02598427
CDK4/6	Abemaciclib	Yes	NA	NA	NA	Phase II	NCT02774681
	Palbociclib	No	NA	NA	NA	Phase II	NCT02308020
P13K/AKT	Everolimus	Yes	NA	NA	NA	Phase II	NCT01305941 a NCT01783756 b
PARP	Veliparib	Yes (in association)	NA	NA	NA	Phase II	NCT00649207

TDM1, trastuzumab emtansine; CDK4/6, cyclin-dependent kinase 4 and 6; Akt, protein kinase B; NA, not available.
aIn association with trastuzumab and vinorelbine. bIn association with lapatinib and capecitabine. cIn association with irinotecan.

a response rate of 33% and an intracranial PFS of 6.5 months, among 26 evaluable patients with BM, was reached [98]. Another phase I study on the association of escalation doses of ONT-380 in combination with trastuzumab is ongoing [99].

For the treatment of patients with BM in hormone receptor positive (HR+) breast cancer, CDK4/6 inhibitors (abemaciclib, palbociclib) are of interest. In particular abemaciclib, that has a good CNS penetration in preclinical models, can reach therapeutic levels in human BM [100]. Other potential therapeutic targets of patients with breast cancer BM include the PI3K/AKT mammalian target of rapamycin (m-TOR) pathway, and everolimus, an m-TOR inhibitor, is currently being investigated in trials on BM.

Given the frequency of BM in patients with TN breast cancer, and the limited efficacy of conventional chemotherapy, there is urgent need of new molecular approaches. PARP inhibitors are now being investigated, and preliminary results from a phase III trial (OlympiAD Study) suggest that olaparib, as compared with single-agent standard chemotherapy, could be an effective option, especially in patients with TN breast cancer and BRCA mutations [101]. Other PARP inhibitors under investigation in ongoing trials, in breast cancer, are veliparib and iniparib [102], allowing the inclusion of patients with BM. Alternative new avenues of treatments that are being investigated in TN breast cancer are inhibitors of the PI3/AKT pathway, selective androgen antagonists and newer antibody drug conjugates. VEGF plays an important role in angiogenesis of BM. Vascular normalization induced by bevacizumab, a monoclonal antibody targeting VEGF, delivered prior to chemotherapy, could enhance its efficacy, and trials have been launched to confirm these preliminary results [103].

There are several challenges for the development of new drugs with better activity in BM from breast cancer. Regarding established BM, there is the need to improve the quality of results of novel clinical trials by employing specific inclusion criteria, and more homogeneous and well defined end-points adapted for the evaluation of targeted agents, such as the recently proposed RANO Criteria [104, 105]. In particular, promising new agents should be tested upfront in clinical trials in patients with small and asymptomatic metastatic lesions, reserving radiotherapy (WBRT, stereotactic radiosurgery) at salvage. This is important as lesions that emerge after radiotherapy are often resistant to drugs. On the other hand, new compounds with radio potentiating properties should be investigated in clinical trials in association with radiotherapy, with careful monitoring of acute/early adverse effects and late cognitive function deterioration.

Phase 0 trials that investigate the distribution and activity of molecular compounds, given before neurosurgical resection of BM, are needed for monitoring potential intra- and/or interpatient heterogeneity [106]. Trials focusing on chemoprevention are important. In addition

to compounds that are active in preclinical models and able to penetrate an intact BBB, there is need to identify those patients who are at high risk of developing BM, particularly those at higher risk of first or isolated brain relapse, such as HER2+ patients [3]. The development of biomarkers associated with the risk of brain colonization in humans is another unmet need [107].

CONCLUDING REMARKS

BM is considered one of the major causes of mortality in breast cancer patients. TN breast cancer, basal-like subtype and Her2 enriched breast cancers are most associated with BM. Patients with active BM are often excluded from clinical trials, in part because systemic response and brain response do not correlate. The divergent therapeutic responses might be due to the molecular alterations that are specifically present in brain metastatic lesions, while not in the primary tumors. Results so far indicate that tumor tissue in regional lymph nodes or extracranial metastasis does not resemble BM. A more comprehensive characterization of the primary lesion might disclose sub clones that more closely feature intracranial disease. The identification of genomic and expressional alterations specific to BM is crucial to the development of BM specific therapies. In addition, following up on discoveries regarding the molecular pathways involved in crossing the BBB and entering the brain of TCs will yield targets for BM preventive strategies.

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Chapter 3

Potential Molecular Signatures Predictive of Lung Cancer Brain Metastasis

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ABSTRACT

Brain metastases are the most common tumors of the central nervous system (CNS). Incidence rates vary according to primary tumor origin, whereas the majority of the cerebral metastases arise from primary tumors in the lung (40-50%). Brain metastases from lung cancer can occur concurrently or within months after lung cancer diagnosis. Survival rates after lung cancer brain metastasis diagnosis remain poor, to an utmost of 10 months. Therefore, prevention of brain metastasis is a critical concern in order to improve survival among cancer patients. Although several studies have been made in order to disclose the genetic and molecular mechanisms associated with CNS metastasis, the precise mechanisms that govern the CNS metastasis from lung cancer are yet to be clarified. The ability to forecast which patients have a higher risk of brain metastasis occurrence, would aid cancer management approaches to diminish or prevent the development of brain metastasis, and improve the clinical outcome for such patients. In this work, we revise genetic and molecular targets suitable for prediction of lung cancer CNS disease.

INTRODUCTION

Brain metastases are the most common tumors of the central nervous system (CNS). Metastatic brain lesions outnumber primary brain tumors with a 10 fold [1], with incidence rates varying according to the primary tumor origin. The majority of the cerebral metastases arise from primary tumors in the lung (40-50%) and it is estimated that 50% of the patients with small cell lung cancer (SCLC) or non-small cell lung cancer (NSCLC) will develop brain metastasis [2, 3]. In contrast to cerebral metastases from other primary cancers, where generally a metastatic latency period takes place, brain metastasis from lung cancers often occur months after, or even concurrently, with the diagnosis of the primary tumor [4]. Metastatic brain lesions carry a clinical burden of morbidity and mortality, as well as significant neurological deficits, cognitive impairment and emotional difficulties [5]. Despite treatment, lung cancer brain metastases are usually fatal for 90% of patients within two years after the initial diagnosis, with a median survival of 7-10 months five years after diagnosis [2]. Previous efforts to characterize patients that are at high risk of developing brain metastasis have been fairly disappointing.

Currently, only clinical and pathologic variables are used to predict the risk of brain metastasis in patients with lung cancer. However, data on predictive parameters are diverse and not clinically usable (**Table 1**). Identifying patients at highest risk of developing brain metastases on the basis of standard clinical and pathological factors, such as status of primary tumor, tumor histology, nodal involvement and patient age, may not be reliable due to small hazard ratios and unknown prognostic factors [6]. Recently, Hung et al [7] demonstrated, in a study on 182 lung adenocarcinomas with distant metastases, that the micropapillary histology subtype was significantly associated with brain metastasis ($p = 0.01$). However, a more robust method to identify which patients are at risk of developing brain metastasis is urgently needed.

Molecular classification, by correlating distinct molecular markers with oncogenic mechanisms, has been practiced to improve risk stratification of the TNM staging system [8]. The potential of molecular biologic distinction would direct appropriate therapy, thereby improving patient outcome. Among early stage (I/II) NSCLC patients, the 5-year overall survival (OS) rate is only 45.1% [9]. Many clinical trials have confirmed that postoperative adjuvant therapy can prolong survival of NSCLC patients. In a recent meta-analysis of 3,923 patients, Chen et al [10] demonstrated the efficacy of postoperative chemotherapy – both cisplatin-based ($p < 0.0001$) and single tegafur-uracil (UFT, $p = 0.002$), in stage I-II, IA and IB NSCLC, and no significant benefit was found in stage IA patients ($p = 0.43$). In addition, cisplatin was shown to be better than single UFT chemotherapy in OS ($p = 0.0005$ and $p = 0.81$, respectively) [10]. More trials should be conducted in order to confirm the efficacy of disease-free survival (DFS) therapies in future clinical practice. In order to predict the rise of cerebral metastasis of lung cancer, we would need a measurable biomarker that correlates well with brain seeding

of the lung cancer cells. Molecular markers may be classified into subgroups, based on their mechanism of action, in the metastatic cascade to the brain [6]. The optimal marker to disclose concealed (brain) metastatic disease would be displayed in primary tumors while not detectable in serum of control subjects [24]. The capacity to identify metastatic disease based on proto-oncogenes like Kirsten rat sarcoma viral oncogene homolog (KRAS) and tumor suppressor p53 (TP53), present in only half of the lung cancer patients (47% and 50%, respectively) [25], demands a more broaden and deepened spectrum of investigation of primary lung cancers, the molecular interactions with other cells and the tumor microenvironment.

The process of metastasis is a selective and refined event called organotropism whereby, apart from an overall tendency to spread and invade, primary tumors show predilection for particular distinct organs [26]. Cancers that metastasize to brain need to take a number of anatomic, physiologic and molecular hurdles. The first requirement is intravasation into the blood stream, dependent upon a reversible epithelial to mesenchymal transition (EMT). The epithelial cell traits, such as cell polarity and E-cadherin mediated cell adhesion, are suppressed and replaced by mesenchymal cell characteristics. The cells become motile, invasive and resistant to apoptosis [27]. Through the EMT process, tumor cells acquire stem cell-like features such as self-renewal, differentiation and ability to seed, justifying the term "tumor-initiating cells" [27]. EMT molecular regulation is accomplished through an intricate network, arranged by different genes and molecules inducers of EMT [28-30]. AXL, a receptor tyrosine kinase belonging to the TAM family, and its ligand GAS6, growth arrest-specific gene 6, have been reported to downregulate several oncogenic signaling pathways [31], through activation of MAPK/ERK and PI3K/AKT signaling pathways [32, 33]. Recently, AXL-GAS6 signal axis has been reported to have a potential key role in NSCLC tumor progression and may be suitable as a prognostic biomarker for identifying high-risk NSCLC brain metastasis patients [34]. Tumor cell growth in the brain microenvironment is the result of genetic predisposition and cellular adaptation mechanisms, and is largely dependent on cross-talk between tumor and brain-resident cells.

Genomic instability and mutations are just two of the characteristics of cells associated with the transition from a pre-neoplastic lesion to an invasive tumor state, and consequent progression to metastatic disease. During tumorigenesis, a sequence of genetic modifications such as gene deletions, copy number alterations or chromosomal rearrangements occur. This review focuses on the use of molecular characteristics that are predictive of tumor progression and development of metastatic NSCLC brain metastasis in particular.

Table 1 - Conflicting clinical and pathological risk-factors associated with the development of brain metastases.

Reference	Analysis	Nr	Type Tumor	Pathologic Stage	Recurrence Site	Age	Tumor Status	Lymph-vascular Space Invasion	Nodal Status	Histologic Type
[11]	Multivariate	112	NSCLC	IIB-IIIB	Brain	<60, p = 0,03	ND	ND	p = 0,003*	Nonsquamous +
[12]	Multivariate	267	NSCLC	IIIN2	Brain	ND	-	ND	ND	Adenocarcinoma +
[13]	Multivariate	305	NSCLC	I-IIIB	Brain	<62, p = 0,004	T4, p = 0,0009	ND	N2-3, p = 0,0057	Adenocarcinoma, p = 0,0002
[14]	Multivariate	83	NSCLC	IIIB	Brain	<60, p = 0,022	ND	ND	ND	-
[15]	Kaplan-Meier	211	NSCLC	IIIA	Brain	-	-	ND	ND	Squamous vs nonsquamous, p = 0,02
[3]	Multivariate	975	NSCLC	I-II	Brain	<77, p < 0,01	Size, p < 0,01	p = 0,03	p = 0,04	-
[16]	Multivariate	78	NSCLC	II, III	Brain	-	-	ND	N1-2 vs N0, p < 0,02	ND
[17]	Hierarchical logistic regression	264	NSCLC	I-IV	Brain	-	Size, p < 0,001	ND	p < 0,017	Adenocarcinoma + undifferentiated vs squamous, p = 0,001
[18]	Multivariate	150	NSCLC	II, III	Brain	-	ND	ND	ND	IIIB nonsquamous +
[19]	Univariate	2724	div.	I-IV	Brain	<70 (breast and lung cancer)	ND	ND	ND	ND
[20]	Univariate	25	NSCLC	I-III	Brain	-	-	ND	Mediastinale vs hilar, p = 0,03	ND
[21]	Multivariate	292	NSCLC	ND	Brain	-	T2 vs T3-4, p = 0,005*	ND	N0-1 vs N2-3, p < 0,001*	-
[22]	Multivariate	322	NSCLC	IA	Brain and others	-	Size≥15mm, p = 0,038	ND	ND	Squamous, p = 0,002
[23]	Multivariate	192	NSCLC	I-IV	Brain and others	<61, p = 0,01	ND	ND	ND	ND

ND, not determined; "-", no predictive value (p > 0.05); "+", predictive value, no significance.

GENETIC ALTERATIONS

Due to the recent discoveries of targetable genetic alterations in the treatment of NSCLC, patients have been stratified according to genetic variations in the primary tumor, including epidermal growth factor receptor (EGFR), KRAS and anaplastic lymphoma kinase (ALK) [35]. A summary of all genetic alterations that will be addressed in this review are presented in **Figure 1**. There are considerable differences in reported incidence and time to development of brain metastases for these genetic alterations.

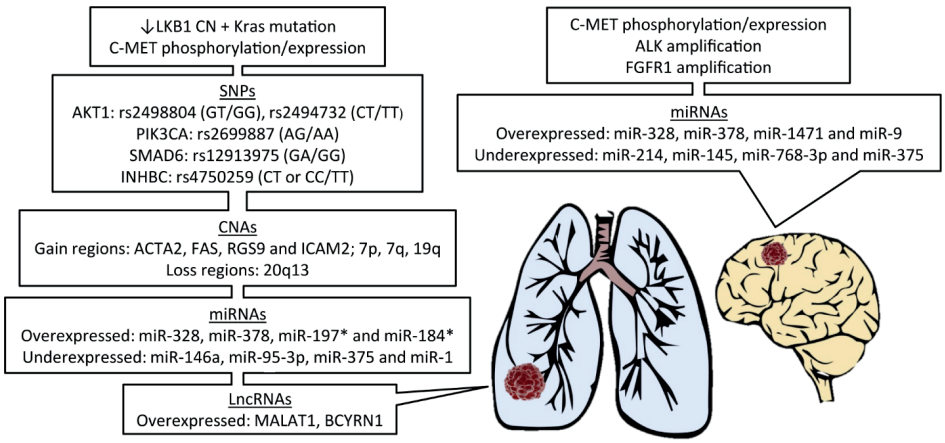

Figure 1 – Features of primary lung cancer from patients known to develop brain metastasis and potential biomarker candidates. LKB1, liver kinase B1; CN, copy number; SNPs, Single-nucleotide polymorphisms; CNAs, copy number alterations; miRNAs, microRNAs; LncRNAs, long non-coding RNAs; *in EGFR mutant patients

EGFR

In the Caucasian population, EGFR activating mutations are present in 10 to 15 % of adenocarcinomas and in less than 5 % of squamous cell carcinomas [36]. Roughly 90% of all known EGFR mutations reside in exon 19 (in-frame deletions) and in exon 21 (L858R, point mutation) [37, 38]. The prevalence of activating EGFR mutations appears to be dependent on gender, smoking status and ethnicity. In patients from East-Asia, EGFR mutation is reportedly up to five times higher than in Caucasian patients [39-41]. The relation between EGFR status, brain metastasis and survival, is complex and not fully understood. It has been shown that lung cancer patients suffering from tumors with particular EGFR mutations survive longer, probably due to effective treatment. However, data also suggest that brain metastases arise more frequently in patients with primary lung tumors bearing EGFR mutations [42, 43], and the development of brain metastases is relatively frequent during treatment. There are discordance

rates of EGFR mutational status, between primary tumors and their CNS metastases, that vary from 0 until 32% [44-50]. In a series of 55 NSCLC primary tumors with matched cerebral metastases, EGFR was found to be more frequently amplified in the metastatic adenocarcinomas than corresponding primary tumors, with 30% and 10%, respectively [50]. Discrepancies regarding the response of the brain metastases may well be due to the timing of adjuvant chemotherapy administration for the primary tumors, relative to the occurrence of brain metastases. The choice of the agents is currently based on the molecular characteristics of the primary, not the metastatic, tumors. Paradoxically, prolonging survival times due to successful response of the primary tumors would create more time for brain metastases to develop as late complication [51, 52]. Similarly to EGFR, the KRAS status may also be discordant between primary and metastatic tissues [44], and a KRAS mutation in a small subset of tumor cells may confer resistance to EGFR tyrosine-kinase inhibitors (TKI) therapy.

KRAS

EGFR and KRAS mutations are generally mutually exclusive [53, 54] but cases of EGFR and KRAS co-mutations have been identified [55-57]. Roughly 15-30% of NSCLCs harbor activating mutations in codons 12 and 13 of the KRAS gene [58]. KRAS mutations are associated with advanced tumor progression and clinical aggressiveness [59], forming a persistent risk of lung adenocarcinoma and implying to be an early event in the tumorigenesis process [53]. The correlation of the presence of KRAS mutations with a smoking history [60] suggests that KRAS mutations are a sequel of the actions of carcinogens of tobacco products [53]. However, in a cohort of 482 lung adenocarcinomas, it was demonstrated that KRAS mutations do occur in patients with lung adenocarcinomas without a smoking history [61], but the mutations are different. Significantly more transition mutations (G>A) are being found in non-smokers than the transversion mutations (G>T or G>C, $p < 0.0001$), which occur in former- or current smokers [61]. This observation supports the idea that the distinct transition profile – replacement of a purine for a purine or a pyrimidine for a pyrimidine [62], – in never-smokers is very unlikely to be caused by passive tobacco vulnerability. No specific KRAS targeting treatment has so far shown efficacy. There is little available data on the KRAS mutational status in primary lung cancers as compared to that in their brain metastases [44, 57]. In a relatively small series, Munfus-McCray et al. found 23.5% of brain metastatic lung adenocarcinomas with KRAS mutation exclusively in patients with a smoking history ($p < 0.01$) [59].

ALK

ALK rearrangements occur in 2-7% of all NSCLC, with predominance in non- or light smokers, younger age and adenocarcinomas [63, 64]. Fusion between EML4 (echinoderm microtubule associated protein-like 4) and ALK yields at least 15 molecular variants with different biological behavior and affected signaling pathways, and consequences for therapy choice [65]. ALK testing is particularly recommended for non-squamous lung cancers in the absence of EGFR

mutation, in patients with non- or light smoking history [66]. The recommended method for testing the presence of ALK translocation is fluorescent in situ hybridization (FISH), and immunohistochemistry (IHC) as confirmation [67, 68]. In a large Western cohort, functional ALK rearrangements appeared to be mutually exclusive with EGFR and KRAS mutations [69]. Although ALK translocations seem to be similar in primary tumors and their brain metastases, ALK amplifications are found more frequently in CNS metastasis with discordance rates of only 12.5% [70]. Similarly to EGFR, ALK rearrangements are predictive of response to TKIs, but the development of brain metastasis in patients with ALK translocations receiving ALK directed TKI is a major clinical problem [71, 72]. Recently, second generation TKI alectinib has shown to delay the development of brain metastases, compared to first generation TKI, and also demonstrated promising efficacy in the CNS for crizotinib-resistant ALK positive NSCLC patients [73, 74]. Similarly to EGFR and KRAS mutations and ALK rearrangements, several other molecules such as liver kinase B1 (LKB1, also known as STK11), proto-oncogene tyrosine-protein kinase ROS1 and C-MET, that encodes the hepatocyte growth factor receptor (HGFR), were found to be implicated in the development of lung cancer [75-79]. However, the connection of these molecules with the development of brain metastases is still under investigation and not yet implicated in clinical decision making. KRAS aberrations have a synergistic effect with LKB1 inactivation in lung cancer development and distant metastasis formation [80, 81]. In a cohort of 154 NSCLC patients, Zhao et al demonstrated that a lower LKB1 copy number (CN), along with KRAS mutation, were significantly associated with a higher number of brain metastasis. Moreover, the odds ratio of brain metastasis was ~20 times higher in patients with one decrease in LKB1 CN values [82]. LKB1 is observed to be inactivated in ~30% of all NSCLCs [83].

Other mutations

Although several potential targets may not regularly be expressed in a high number of lung cancer brain metastasis, their potential use for personalized treatment of selected lung cancer patients, harboring actionable mutations, should not be discarded. In a cohort of 874 brain metastases samples, of which 295 NSCLC, Capper et al showed that, although a total of 51/874 samples harbored a BRAF V600E mutation, only 1/295 NSCLC brain metastases (~0.3%) was BRAF-mutant [84]. Despite this low frequency of BRAF mutations in lung brain metastasis, regression of both visceral and brain metastases by BRAF inhibitor vemurafenib was reported in a patient with a BRAF V600E-mutated NSCLC [85]. While 3% of primary lung cancers harbor ROS1 alterations, only 1/99 adenocarcinomas bore ROS1 translocations, and 1/11 squamous cell carcinomas showed ROS1 amplifications [86].

Activating mutations in EGFR are associated with sensitivity to TKI therapy, but nearly 30% of EGFR positive patients show primary resistance to EGFR inhibitor therapy [87]. While C-MET amplification is one of the factors commonly associated with disease progression [88],

Benedettini et al, in a first cohort of 23 NSCLC samples of patients harboring an EGFR activating mutation, showed that both C-MET phosphorylation and expression were significantly associated with shorter time to progression, correlating with *de novo* resistance to EGFR TKI. In a second cohort of 40 patients, englobing 18 primary NSCLC from patients who later developed brain metastases and 22 NSCLC from patients that did not develop brain metastases, Benedettini et al demonstrated that both C-MET expression and phosphorylation – but not C-MET amplification, were significantly higher in the tumors from patients that developed brain metastasis. In 18 matched brain metastasis, amplification was demonstrated [89]. In addition, in a cohort of 196 NSCLC brain metastasis samples, Presseur et al. found C-MET gene amplification and overexpression in 21.6% and 44.4%, respectively, confirming that C-MET is commonly activated in brain metastasis manifestation [90]. Further, a significant correlation between C-MET and ALK amplification status was observed ($p = 0.039$). In another study, these authors demonstrated that fibroblast growth factor receptor 1 (FGFR1) amplification in brain metastases of adenocarcinomas – but not squamous cell carcinomas, is 5-fold more frequent than reported for primary tumors (~3%). Similarly to C-MET, a positive correlation of ALK and FGFR1 amplification status in brain metastasis was reported as significant ($p < 0.001$) [91]. In a recent study, Keap-1, Nrf2 and P300, key genes of the Keap1-Nrf2-ARE survival pathway, were found to be mutated in brain metastatic tissue of progressive NSCLC patients [92]. Moreover, mutations in Keap1-Nrf2-ARE pathway were also found in circulating tumor cells (CTCs), suggesting a role in the ability of CTCs to bear the rough environment in blood-circulation and attain distant organs [92].

CIRCULATING TUMOR DNA

An adequate characterization of somatic genetic modifications in human cancers is critical for an optimal diagnosis and subsequent therapy. In brain metastatic tissue, as for all other brain malignancies, repeated biopsies are not a feasible approach to portray the tumor clonal diversity. Several studies have shown that cell-free circulating tumor DNA (ctDNA) in the plasma could serve to characterize and monitor tumors [93-95]. Nevertheless, ctDNA analysis of plasma from patients with brain malignancies has disclosed very low levels of tumor DNA [96]. Recently, ctDNA analysis from cerebrospinal fluid (CSF) has been shown promising for brain cancer [97-99] and brain metastatic cancer patients [100, 101]. CSF is in direct contact with the brain and, therefore, with tumor cells of brain cancer patients. In a comparative study of ctDNA, derived from plasma and CSF of patients with primary or metastatic brain tumors, De Mattos-Arruda et al. showed ctDNA levels of brain malignancies to be more abundantly present in the CSF than in the plasma [100]. Moreover, ctDNA from CSF was shown to recapitulate the brain metastasis specific mutations – private mutations –, absent in extracranial tumors of a patient with Her2-positive metastatic breast cancer [100]. In addition, the CSF

ctDNA proficiency to monitor responses to systemic therapy and brain tumor progression [99, 100], i.e. the capacity of the CSF ctDNA to recapitulate the modulation of mutant allele frequency (MAF) over time in the brain tumor burden, suggests that genomic CSF analysis may be useful not only in facilitating diagnosis of tumor in the CNS, or as guidance to second-line agents choice, but possibly to pinpoint pathways intimate related with cancer spread to the CNS and predictive of brain metastases [99].

SINGLE-NUCLEOTIDE POLYMORPHISMS (SNPS) ASSOCIATED WITH BRAIN METASTASES

Studying SNPs in signaling pathways that regulate cell proliferation and migration, as well as assessing the relationship between multiple SNPs, can be used to estimate the risk of brain metastasis. The PI3K-PTEN-AKT-mTOR pathway, important in the control of cell growth, tumorigenesis and cell invasion, has been shown to be abnormally activated in several cancer types, including NSCLC [102, 103]. In a study of genetic variations in the PI3K-AKT-mTOR pathway, to predict brain metastasis in NSCLC patients, Quianxia et al. identified three SNPs that were shown to be exclusively associated with higher risk of brain metastasis: the GT/GG (p = 0.006) and CT/TT (p = 0.002) genotypes of AKT1, variant alleles rs2498804 and rs2494732, respectively and AG/AA (p = 0.010) genotype of PIK3CA, variant allele rs2699887 [102]. Furthermore, patients carrying at least one variant allele in PIK3CA, had roughly twice the risk of brain metastasis as those without those variants [102]. Multiple mechanisms of PI3K activation may be responsible for activation of the PI3K pathway [104], and increased PI3K activity would result in increased metastases. In concordance, Paik et al reported that patients with aberrant PI3K squamous lung carcinomas (n=9) had worse survival (median OS: 8.6 vs 19.1 months, $p < 0.001$), higher metastatic burden (>3 organs, 18% vs 3%, p = 0.025) and higher incidence of brain metastases (27% vs 0%, $p < 0.001$) [105]. Similarly to PIK3K-AKT-mTOR pathway, it was hypothesized that common genetic variants in the TGF-β pathway would be associated with the risk of brain metastasis [106]. TGF-β pathway has been demonstrated to suppress early-stage tumor development and to stimulate tumor cell growth and invasiveness at later stages of tumorigenesis [107]. Quianxia et al found the GG genotype of SMAD6: rs12913975 (p = 0.014) and the TT genotype of INHBC: rs4750259 (p = 0.024) to be associated with risk of brain metastasis in a cohort of 161 blood samples from NSCLC patients. Furthermore, de combination of both genetic variants was shown to be higher for prediction of brain metastasis (p = 0.001) [106].

COPY NUMBER ALTERATIONS (CNA) ASSOCIATED WITH BRAIN METASTASIS

Activation or inhibition of a gene occurs through a variety of mechanisms such as, for example, activating mutations and deletions. Gene deletion can be evaluated by CNA. Animal models have given clear evidence that LKB1 haploinsufficiency stimulates KRAS driven lung cancer in mice [80], and a single copy inactivation of LKB1 can considerably ease brain recurrence [82]. Although EGFR CN status is still controversial and some of the available data do not support EGFR CN as a prognostic factor [108, 109], Bonanno et al. have shown, despite the less predictive accuracy of FISH analysis compared to EGFR mutation analysis, that patients with EGFR-FISH-positive tumors have better outcomes (median OS: 177 weeks vs 57 weeks, $p = 0.048$) [110]. Considering that primary lung adenocarcinomas with early development of brain metastasis would contain more CNAs predictive of metastatic potential, Lee et al. compared the CN changes of 4 lung adenocarcinomas with coexistent brain metastasis, with 8 lung adenocarcinomas with metachronous brain metastasis [111]. Amplification in 5q35.1-2 and 17q23.3-24.1 was detected in 100% and 10q23.31 and 17q24.1 in 75% of the cases with synchronous brain metastasis. On the other hand, and in a less frequent ratio, only 5q35.1-2 and 17q24.1 amplification status was found in 12.5% of the metachronous brain metastasis. Moreover, gained regions specific for early (simultaneous) brain metastasis, were found to contain ACTA2, FAS, RGS9 and ICAM2 as putative metastasis promoting genes, the latter being most significant ($p = 0.002$) [111]. In the same line, another study compared CNAs of primary NSCLC tumor and matched brain metastasis from one single patient [112]. Brain metastatic tissue exhibited a higher degree of genetic heterogeneity when compared with the primary tumor with common regions of gain, including 7p, 7q and 19q, and common regions of loss, including 20q13 [112]. In a stage IV SQCLCs study, 4 brain metastases and matched archived FFPE primary cancers were shown to have complete loss of PTEN by IHC and whole exome sequencing [105]. In an early-stage NSCLC report, thirty (24%) of the total of 125 specimens, analyzed for PTEN-IHC, showed a lack of staining [113]. Although genetic alterations of the PTEN gene are unusual in NSCLC, loss of PTEN protein is not a unique event in early-stage NSCLC, and Soria et al. demonstrated that, besides being a reversible event, PTEN loss may be partially explained by promotor methylation, in addition to point mutations and homozygous deletions [113].

MICRORNAS (MIRNAS) ASSOCIATED WITH BRAIN METASTASIS

Recently, molecular studies have stressed the role of miRNAs, which are small non-coding endogenous RNAs containing 18-24 nucleotides, that regulate gene expression at the post-

transcriptional level, thereby acting as negative regulators of mRNA translation and/or stability [114]. MiRNAs appear to regulate several hundred genes and could serve as a better classifier than gene expression profiling [115]. miRNAs are known to play a crucial role in normal development, proliferation, differentiation and apoptosis, and dysregulation of miRNAs has been linked to various pathological conditions, including cancer [116]. The role of miRNAs in the development of brain metastases has been recently explored [117, 118].

Several studies have addressed the miRNA expression as biomarkers to predict the occurrence of brain metastases in lung cancer. MiRNA-328 appeared to be significantly overexpressed in both primary tumor samples and cerebral metastases of patients with NSCLC, when compared to NSCLC patients without brain metastasis. Moreover, miRNA-328 overexpression has been found to promote migration and subsequent brain metastasis formation of NSCLC cells through PRKCA deregulation [119]. PRKCA mediates the expression of urokinase plasminogen activator (uPA), leading to migration of tumor cells [120]. Similarly to miRNA-328, miRNA-378 has also been demonstrated as a potential biomarker to assist clinicians in stratifying patients for high-risk of brain metastasis, because miRNA-378 was also found to be overexpressed in NSCLC primary tumor samples and matched brain metastasis of NSCLC patients [121]. Also miRNA-378 promotes cell migration, invasion, tumor growth and angiogenesis, *in vitro* and *in vivo* [121]. Recently, Remon et al. have identified miRNA-197 and miRNA-184 as two significantly overexpressed miRNAs in EGFR-mutant patients with brain metastases, when compared to EGFR-mutant patients with no brain metastasis [122]. However, because of lack of patients with EGFR wild type (EGFRwt) tumors without BM, no comparison between patients with EGFRwt tumors, with and without BM, could be made. Therefore, the effects of these micro-RNAs, irrespective of the EGFR status, need further scrutiny.

MiRNAs expression status varies according to their targeted genes. Zhao et al. has reported the significant upregulation of miRNA-1471 and miRNA-9 and downregulation of miRNA-214 and miRNA-145 in 11 brain metastatic lung cancer samples, when compared to 40 primary lung adenocarcinomas ($p < 0.001$ for all four miRNAs) [123]. The upregulation of miRNA-145 in primary lung adenocarcinomas was shown to suppress proliferation of tumor cells [123], consistent with other reports that show inhibition of cell proliferation in human lung adenocarcinomas through miRNA-145 targeting c-Myc, EGFR and NUDT1 [124, 125]. Subramani et al. have shown the miRNA-768-3p to be underexpressed in several brain metastases, compared to matched primary tumors [126]. MiRNA-768-3p was found to be underexpressed in *in vitro* lung cancer cells after co-culture with astrocytes, driving to increased KRAS protein and downstream effectors ERK1/2 and BRAF, thereby boosting tumor cell viability and promoting metastasis. From various studies, it appears that miRNAs regulate the growth of metastases either by under- or overexpression, within the tumor tissue or in the tumor environment. The brain microenvironment negatively regulates miRNA-768-3p to enhance KRAS expres-

sion, which promotes the propagation of lung cancer brain metastasis [126]. miRNA-146 was shown to be significantly up-regulated in NSCLC tissue, when compared to healthy adjacent lung tissue ($p < 0.05$) [127]. In another study, miRNA-146a expression in primary NSCLC was correlated with advanced clinical TNM stages and distant metastasis ($p < 0.05$). The patients with a high miRNA-146a expression showed longer progression-free-survival times than those with a low expression of miRNA-146a (25.6 weeks and 4.8 weeks, respectively, $p < 0.05$) [128]. In the same line with these findings, in a xenograft model, Hwang et al. showed high expression of miRNA-146a in parental cells, while diminished expression in the brain-seeking cells. Moreover, miRNA-146a overexpression in the brain-seeking cancer cells suppressed their metastatic potential, which was correlated to the upregulation of β-catenin and downregulation of heterogeneous nuclear ribonucleoprotein C1/C2 (hnRNPC) [129]. Taken together, these findings suggest that miRNA-146a serve as a valid clinical biomarker for prediction of brain metastasis in lung cancer patients. However, validation of miRNA-146a expression levels in a large cohort of human matched primary and brain metastatic lung tumors is essential to confirm this finding. Similarly to miRNA-146a, overexpression of miRNA-95-3p suppresses brain metastasis of lung adenocarcinoma through downregulation of cyclin D1 [130]. MiRNA-95-3p is decreased in brain metastases of lung cancers as compared to the primary tumors, and higher cyclin D1 expression correlates with poorer prognosis [130]. In a recent study, Chen et al. reported miRNA-375 deregulation to be associated with NSCLC brain metastasis [131]. MiRNA-375 is another miRNA documented to be downregulated in primary tumors of NSCLC patients with brain metastasis. MiRNA-375 expression was significantly decreased in matched brain metastatic NSCLC tissues ($p < 0.05$) and significantly correlates with total number of brain metastasis ($p < 0.001$). In addition, VEGF and MMP-9 – which roles have been extensively studied in the development of brain metastasis –, were overexpressed in downregulated miRNA-375 tumors [131].

MiRNAs are linked with several molecular pathways. Several studies have correlated the overexpression of ADAM9 in NSCLC patients with brain metastases [4, 132]. ADAM9 has been demonstrated to enhance the ability of tissue plasminogen activator (tPA) to cleave and stimulate the function of CUB domain containing protein 1 (CDCP1) – promigratory protein –, to promote brain metastasis [4]. Recently, Chiu et al. reported that ADAM9 downregulates miRNA-1 via EGFR signaling pathways activation, enhancing CDCP1 expression to promote lung cancer progression [133]. MiRNA-1 expression was shown to be downregulated in primary lung tumors, but increased in ADAM9-knockdown lung cancer cells. Moreover, miRNA-1 negatively correlates with CDCP1 expression and with migration ability of lung cancer cells [133]. Another study has identified miRNA-21 as a target of signal transducers and activators of transcription 3 (STAT3) pathway activity in lung-derived brain metastasis initiating cells (BMICs) [134]. STAT3 is admitted as a central regulator in the metastatic process [135] and STAT3-knockdown has been demonstrated to reduce expression of known

downstream targets of miRNA-21, while STAT3 and miRNA-21 act as cooperative regulators of stemness, migration and tumor initiation in lung-derived brain metastasis [134]. MiRNAs appear very promising as diagnostics, prognostics and therapeutics to improve cancer patient outcome, however, the clinical use of miRNA therapeutics to treat brain metastases has yet to be achieved. Advances in pre-clinical and translational studies to identify miRNAs that change after growth in the brain microenvironment have been made, but validation of large cohorts from patient tumor samples are required.

LONG NON-CODING RNAS (LNCRNAS) ASSOCIATED WITH BRAIN METASTASIS

Long non-coding RNAs (lncRNAs) have been recently identified as effective players in tumorigenesis. LncRNAs represent a class of non-protein coding transcripts longer than 200 nucleotides [136] that covers a broad spectrum of physiological and pathological functions by implementing different modes of action [137]. Similarly to miRNAs – that regulate several hundred genes –, lncRNAs are involved in the regulation of multiple miRNAs, impacting the expression of thousands of genes [136]. Besides performing a single function, some lncRNAs, act at multiple functional levels in different types of cells. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), localized in nuclear speckles and highly conserved among mammals, regulates alternative splicing [138] and gene expression through additional splicing-independent mechanisms in lung cancer metastasis [139]. In a recent study, Shen et al. have shown lncRNA-MALAT1 levels to be significantly higher in primary NSCLC from patients who developed brain metastasis, when compared with primary NSCLC from patients without brain metastasis ($p < 0.001$) [140]. Additional *in vitro* functional studies showed overexpression of vimentin in a highly invasive subline of brain metastasis lung cancer cells overexpressing MALAT1, while overexpression of E-cadherin was observed when MALAT1 was silenced, indicating that MALAT1 overexpression promotes lung cancer brain metastasis by inducing EMT [140]. Accordingly, RNAi-mediated suppression of MALAT1-RNA, negatively influenced migration and clonogenic growth in established human NSCLC cell lines. Forced expression of MALAT1 in mouse NIH 3T3 fibroblasts significantly increased migration [141]. Concordantly, long noncoding MALAT1 expression was found to enhance cell motility through transcriptional and post-transcriptional regulation of motility related gene expression [142], displaying the strongest association with genes involved in cancer, like cellular growth, movement, proliferation, signaling and immune regulation genes [141]. MALAT1 and thymosin $\beta 4$ expression levels were identified as prognostic parameters for patient survival in stage I NSCLC that are at high risk to develop metastasis ($p = 0.04$ and $p = 0.01$, respectively) [143]. Tumorigenesis and metastases may be driven by tumor suppressive and oncogenic pathways deregulation, through aberrant expression of cancer metastasis-associated lncRNA

[144]. In a recent *in vitro* study, the lncRNA brain cytoplasmatic RNA 1 (BCYRN1) was found upregulated and targeted by c-MYC in human NSCLC cell lines [145]. c-MYC is a commonly inhibited oncogene which becomes activated in oncogenic pathways, and correlates with metastasis of NSCLC [146]. Besides demonstrating that IncRNA BCYRN1 is essential in the c-MYC-regulated cell migration and invasion, BCYRN1 positively correlates with the expression levels of MMP9 and MMP13 [145]. MMP9 and MMP13, two members of the matrixin subfamily of the metzincin superfamily of Zn-dependent metalloproteinases [147], are extracellular matrix-degrading proteins proven to induce migration and invasion of tumor cells [147, 148], thereby regulating cancer cell metastasis [149].

CONCLUDING REMARKS

Lung adenocarcinoma establishes distant clinical detectable metastasis within months of initial diagnosis [26, 150]. This short abeyance indicate that metastatic ability would arise from early oncogenic events that stimulate primary tumor growth rather than late-arising, scarce genomic alterations specific for metastasis [151]. Thus, monitoring persistent chromosomal changes in the primary NSCLC, alongside with prospective multicenter studies of patient-matched primary and CNS metastatic lesions, could help identify targetable approaches for brain metastasis-specific signatures.

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Chapter 4

T lymphocytes facilitate Brain Metastasis of Breast Cancer by inducing Guanylate-Binding Protein 1 expression

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ABSTRACT

The discovery of genes and molecular pathways involved in the formation of brain metastasis would direct the development of therapeutic strategies to prevent this deadly complication of cancer. By comparing gene expression profiles of Estrogen Receptor negative (ER-) primary breast tumors, between patients who developed metastasis to brain and to organs other than brain, we found that T lymphocytes promote the formation of brain metastases. To functionally test the ability of T cells to promote brain metastasis, we used an *in vitro* blood–brain barrier (BBB) model. By co-culturing T lymphocytes with breast cancer cells, we confirmed that T cells increase the ability of breast cancer cells to cross the BBB. Proteomics analysis of the tumor cells revealed Guanylate-Binding Protein 1 (*GBP1*) as a key T lymphocyte-induced protein that enables breast cancer cells to cross the BBB. The *GBP1* gene was shown to be up-regulated in breast cancer of patients who developed brain metastasis. Silencing of *GBP1* reduced the ability of breast cancer cells to cross the *in vitro* BBB model. In addition, the findings were confirmed *in vivo* in an immunocompetent syngeneic mouse model. Co-culturing of ErbB2 tumor cells with activated T cells induced a significant increase in *GBP1* expression by the cancer cells. Intracardiac inoculation of the co-cultured tumor cells resulted in preferential seeding to brain. Moreover, intracerebral outgrowth of the tumor cells was demonstrated. The findings point to a role of T cells in the formation of brain metastases in ER- breast cancers, and provide potential targets for intervention to prevent the development of cerebral metastases.

INTRODUCTION

Breast cancer is among the tumors that most frequently metastasize to brain [1-7]. The appearance of metastases in the brain invariably defines the terminal stage of disease for women suffering from disseminated breast cancer, and the prevention of cerebral spread would significantly improve their survival [8]. The formation of cerebral metastases depends on the capability of circulating tumor cells to successfully penetrate the blood-brain barrier (BBB). Various genes and pathways have been associated with seeding of breast cancer cells to various organs and the rise of brain metastases in particular [9-13]. Several associations between innate features of the primary tumors and their propensity to intracerebral seeding have been reported. HER2-enriched (HER2+) and triple-negative (TNBC) primary tumors are at higher risk for developing brain metastases relative to the hormone receptor-positive tumors [14-18]. So far, *ST6GALNAC5* is the only specific gene that was found to mediate the formation of brain metastases of a human breast cancer-derived cell line when injected in mice. Moreover, its expression in human breast cancer samples appeared to be associated with the occurrence of cerebral metastases [19]. However, the identification of pathways associated with brain metastasis is necessary to elucidate the mechanisms of crossing the BBB and developing strategies to prevent the formation of brain metastasis.

Here we sought pathways specifically involved in the formation of cerebral metastases of breast cancer by comparing RNA expression profiles of primary ER- breast cancer samples of patients who developed cerebral metastases, with those who developed metastasis to other organs but brain. We discovered that the T cell response is crucial for the development of brain metastases. In both *in vitro* studies using a BBB model and *in vivo* studies using a mouse model, T cells appear to change the expressional profiles of the breast cancer cells and facilitate their passage through the BBB. Guanylate binding protein 1 (*GBP1*) is prominent among the involved proteins and its expression was shown to be upregulated in the primary tumor specimens. Silencing of *GBP1* significantly decreased the ability of breast cancer cells to cross the BBB. The involvement and specific action of T lymphocytes in the process of cerebral metastasis is novel, and opens new therapeutic opportunities for preventing tumor cells to enter the brain.

METHODS

Tissue sample selection

To identify genes and pathways involved in the formation of brain metastasis, we exclusively used specimens of primary tumors, and did not use specimens of metastatic sites. Fresh frozen (FF) tissue specimens of 22 primary breast cancer patients who developed metastasis to brain and/or to other organs were selected. Two groups of samples were compared; those from

patients who had developed brain metastasis (exclusively or in addition to a maximum of 2 organs; n= 13) and those from women who developed metastases to a maximum of three organs (n = 9). None of the 22 patients had received adjuvant therapy (chemo- or hormonal therapy) prior to developing metastases, all samples were ER- and none of the patients had more than three metastatic sites. The relevant clinical data is provided in **Table 1**. In addition, we used 20 primary breast cancer samples for independent validations from patients of whom 13 developed brain metastasis. This study was approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, The Netherlands (MEC 02·953) and performed in adherence to the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (www.fmwv.nl).

Morphological assessment

Five µm thick H&E sections, from each sample, were prepared before and after sectioning for RNA isolation. To ascertain the origin of the tumor, the percentages of invasive tumor cells, inflammatory infiltrates and the presence of necrosis were taken into consideration (JMK).

RNA extraction, purification and quality control

Total RNA from FF tissue samples was extracted from 20 - 30 sections of 30 µm (depending on the size of the sample) using RNABee reagent according the supplier's instructions (Campro Scientific, Veenendaal, The Netherlands) [20]. Following isolation, RNA was stored in RNase/DNase-free water at -80°C. The quantity and quality of the isolated RNA was assessed by nanodrop. Samples were excluded if the yield did not reach the minimum requirement of 1000 ng.

Gene expression profiling

Illumina Whole-Genome cDNA-mediated Annealing, Selection, extension and Ligation (WG-DASL) assay was used to profile the samples. In the assay, 24,526 annotated transcripts, corresponding to 18,391 unique genes, are measured. The WG-DASL assay was performed according to the manufacturer's instructions with an input of 500 ng total RNA. To monitor the assay performance and to evaluate the inter-assay BeadChip variability between the experiments, an inner-assay control consisting of 500 ng total RNA pooled from RNA isolated from several cultured breast cancer cell lines was used in each experiment [21].

Data analysis

Scanned data were uploaded into GenomeStudio software version 2011.1 via the WG-DASL gene expression module for further analysis. The average signal, detection *p*-value, bead standard error and average beads were used to quantile normalize the data in the statistical language R (www.r-project.org) using the "Lumi" package [22].

Table 1 – Clinical information

#	Age at diagnosis (years)	ER	PR	HER2/neu	Metastasis-free period (months)	First metastatic sites	Subsequent metastatic sites	follow-up (months)
					Discovery Set			
1	41	0	0	0	9	brain	-	14
2	48	0	0	0	51	brain	-	55
3	62	0	0	0	7	brain	-	8
4	51	0	0	0	16	brain + lung + pleura	-	23
5	51	0	0	1	12	brain	-	18
6	53	0	0	0	16	brain + liver	-	18
7	41	0	0	0	25	brain + lung	liver	54
8	49	0	0	0	37	brain + lung	skin	39
13	52	0	0	1	12	bone + brain	-	13
9	38	0	0	0	23	skin + lung	brain + bone	41
10	58	0	0	0	8	lung	brain	20
11	58	0	0	0	30	liver	brain	45
12	35	0	0	0	29	bone	brain	63
14	45	0	0	0	4	skin	-	9
15	44	0	0	0	22	bone	-	35
16	35	0	0	0	7	liver + lung	-	18
17	40	0	0	0	33	bone + lung	-	65
18	66	0	0	0	5	pleura	-	12
19	57	0	0	0	14	bone + lung + pleura	-	22
20	45	0	0	1	17	liver + bone	-	29
21	70	0	0	0	17	liver + bone + lung	-	20
22	44	0	0	0	9	bone + lung	-	22
					Validation Set			
1	49	0	0	1	19	brain	-	19
2	39	0	0	0	51	brain	-	60
3	53	0	0	1	63	brain	-	69
4	56	0	0	0	6	brain	-	7
5	35	0	0	0	23	brain	-	118
6	39	0	0	0	59	brain	-	88
7	74	0	0	0	8	brain	-	14
8	29	0	0	0	116	brain	-	154
9	49	0	0	1	13	brain	bone	50
10	38	0	0	0	12	brain	lung + skin	16
11	53	0	0	1	35	brain	other breast	169
12	59	0	0	1	19	brain	lung + liver	52
13	47	0	0	1	16	brain + lung	-	18
14	69	0	0	1	14	skin	-	39
15	56	0	0	0	13	bone	-	28
16	57	0	0	0	61	bone + lung	-	61
17	69	0	0	1	7	liver	-	7
18	58	0	0	0	9	pleura	-	12
19	31	0	0	0	12	pleura	liver + bone	12
20	36	0	0	0	159	lung	kidney	241

For ER, PR and HER2/neu status: 0 = negative; 1 = positive

To identify significantly differentially expressed genes, three steps were followed: sample exclusion criterion, reliable probe selection and gene expression comparisons. Sample exclusion criterion and probe selection method were described previously [21]. For the gene expression comparison, Biometric Research Branch ArrayTools (BRB-array tool (V4.3.1)) was used [23]. Within BRB, the 4,150 most reliable probes for FF samples were exposed to the class comparison algorithm to identify differentially expressed genes with a maximum *p*-value of 0.05 after a 10,000 permutations-multiple correction to determine significance.

Pathway analysis

Pathway analysis was done by two different methods. Firstly, the differentially expressed genes (resulted from the gene expression comparisons of samples with brain metastasis compared to samples with metastasis to other organs) was submitted to Ingenuity Pathway Analysis (Ingenuity, Mountain View, CA). Secondly, all reliably profiled genes were submitted to the Global Test [24] (version 4.4.0) to associate Biocarta pathways (www.biocarta.com) to the groups of samples metastasizing to brain or to other organs. The R version 2.4.1 (www.r-project.org) was used to run the Global Test package. A *p*-value for a pathway was considered significant when the univariate *p*-value of the test as well as the *p*-value calculated by 1,000 re-samplings were both <0.05. In addition, the lists of differentially expressed probes were uploaded into the function annotation tool DAVID version 6.7 [25] to functionally annotate the differentially expressed genes. DAVID was used with the data bases and settings that are preselected by default. Statistical analyses and multiple testing correction procedures are those included in the DAVID analyses [26].

In vitro BBB model

Tissue culture procedures

Human umbilical vein endothelial cells (HUVECs, ScienCell) were cultured in endothelial cell medium (ECM, ScienCell) supplemented with endothelial cell growth factors, 5% fetal bovine serum and Penicillin/Streptomycin. HUVECs were used between passage 2 and 5. Human astrocytes (ScienCell) were cultured in astrocytes medium (AM, ScienCell) supplemented with astrocyte growth factors, 2% fetal bovine serum and Penicillin/Streptomycin. Human astrocytes were used between passage 2 and 5. No further authentication was performed for this study for HUVECs and human astrocytes. Three breast cancer cell lines, which had proven to be able to cross the BBB, were used: MDA-MB-231, MDA-MB-231-BM (a breast cancer cell line that metastasize specifically to brain) [19] and SUM159PT [27]. The characterization of the breast cancer cell lines are summarized (Supplementary Table 1). The tumor cell lines and normal fibroblasts (isolated from non-cancer tissue) were previously characterized [28, 29], and were cultured in RPMI medium supplemented with L-glutamine, 10% fetal bovine serum and Penicillin/Streptomycin. T cells were isolated, activated and transduced as described previously [30, 31]. Two types of T cells were used: T cells that were transduced with an empty

vector, referred to as "T cells", and T cells that were transduced with a T cell receptor specific for MAGE-C2/HLA-A2 vector, referred to as "antigen-specific T cells". The later T cells allowed studies into the role of antigen-specific activation of T cells, to which end SUM159PT cells were used, as these cells express the cognate antigen (MAGE-C2/HLA-A2). Both types of T cells were cultured in RPMI media, supplemented with 10% fetal bovine serum, IL-2 (360 IU/mL, Chiron, Amsterdam, the Netherlands) and Penicillin/Streptomycin. The T cell transduction process, validation of TCR expression by flow cytometry and particular culture conditions by allogeneic feeder cells have been previosuly described [30, 31].

Construction of the in vitro blood-brain barrier (BBB) assays

To develop a BBB model, HUVECs were co-cultured with human astrocytes on opposite sides of a transwell insert [32]. Twenty-four-wells transwell polycarbonate inserts (surface area 0.33 cm^2, pore size three µm, Becton Drive, Franklin Lakes, NJ, USA) were coated with 2% gelatin (Sigma) for 45 min. The transwell inserts were placed upside-down and ~10^5 human astrocytes were seeded at the bottom side of each of the inserts. The cells were allowed to adhere for 3 h at 37°C in a 5 % CO_2 incubator, and were fed every 15-30 min. After 3 hours, inserts were flipped and placed in 24-well plates. One mL of astrocyte media was added to the lower chamber and astrocytes were allowed to grow for one day. 5 x 10^4 endothelial cells were plated on the upper chamber of the inserts and the cultures were placed in the incubator for three days. The permeability of the BBB model was verified by adding trypan blue dye to the upper chamber and incubating the model for 30 min at 37°C. Medium from the lower chamber was collected and absorbance was measured at 595 nm. The permeability of the BBB model by trypan blue was included in duplicate in each experiment.

In vitro BBB-T cell response functional studies

To investigate the influence of T lymphocytes on the ability of tumor cells to cross the BBB, MDA-MB-231, MDA-MB-231-BM and SUM159PT breast cancer cells were co-cultured with T cells (no specific binding occurred between T cells and breast cancer cells). In addition, SUM159PT breast cancer cells were co-cultured with antigen-specific T cells (specific binding occurred between T cells and breast cancer cells). The optimal ratio of tumor cells and T cells was achieved following a titration to reach the best balance between cell killing and migration. The used co-culture ratio of breast cancer cells over T cells was 3:1. After co-culturing for 3 days, T cells were removed by three washes using PBS. The breast cancer cells were harvested and labelled with 5 µM CFMDA cell tracker green (Invitrogen) for 45 min in serum-free medium. The breast cancer cells were collected and re-suspended in RPMI medium supplemented with L-glutamine, 10% fetal bovine serum and Penicillin/Streptomycin. 10^4 cells were seeded in the upper chamber of the BBB model and incubated overnight at 37°C. The cells which successfully passed through the BBB and adhered on the bottom from the lower chamber, were recorded by confocal microscopy after removing the transwell inserts.

To investigate whether cytokines secreted by T cells are responsible for the changes of breast cancer cells, the three breast cancer cell lines were incubated with 5 ml T cell-free conditioned media, collected from activated T cells, for three days. Then, breast cancer cells were washed, harvested and labelled using the same method as described above. 10^4 cells were seeded on the upper chamber of the insert and incubated overnight at 37°C. As a control, 10^4 breast cancer cells of the three breast cancer cell lines, without exposure to either T cells or to conditioned media from T cells, were used. These experiments were repeated 10 times. As a negative control, fibroblasts isolated from healthy tissue were used.

To study the effect of IL-2 (which is essential in culturing T cells) and IFN-γ (a cytokine produced by T cells), the three types of breast cancer cell lines were incubated with IL-2 (360 IU/ml) or IFN-γ (10 and 20 µg, Bio-Connect, Huissen, the Netherlands) for 3 days. Subsequently, breast cancer cells were washed, labelled with cell tracker green and seeded in the upper chamber of the BBB model. These experiments were repeated three times. Moreover, to study the effects of T cells on the permeability of the BBB model, conditioned media of T cells, IL-2 or IFN-γ were added to the upper chamber of the BBB model and incubated overnight. Subsequently, the permeability of the BBB model was investigated by adding trypan-blue dye (20% in RPMI media) to the upper chamber and incubated for 30 min at 37°C. Medium from the lower chamber was collected and absorbance was measured at 595 nm. In addition, the effect of the mentioned factors on the permeability of the BBB model was checked by seeding breast cancer cells in the upper chamber of the BBB model overnight. These experiments were repeated three times.

Confocal laser scanning microscopy and quantification of migrated cells

Confocal images were obtained using a Zeiss LSM510 confocal laser-scanning microscope equipped with a 488 nm Argon-laser and a Plan-Neofluar 20x objective with NA 0.5 (Zeiss, Oberkochen, Germany). Images were made with a pixel-size of 0.9 µm. Pictures were submitted to ImageJ software version 1.49S (www.fiji.sc) and used to calculate the number of cells/mm^2.

Proteomics measurements

MDA-MB-231 and MDA-MB-231-BM breast cancer cells were co-cultured with T cells, in a 3: 1 ratio, and SUM159PT was co-cultured with antigen-specific T cells for three days (following the same method described earlier). As controls, MDA-MB-231, MDA-MB-231-BM and SUM159PT cells were cultured without T cells for three days. All cell cultures were washed three times with PBS, to remove T cells, before they were scraped and collected in 1.5 mL Eppendorf tubes. Three additional washing steps with PBS were performed. After removing the supernatant, cell pellets were immediately snap-frozen on dry-ice and stored at -80 °C until the time of preparation. After thawing, samples were prepared and measured on nano LC as described previously [33].

Proteomics data analysis

From the raw data files of the Orbitrap Fusion mass spectrometer, MS/MS spectra were extracted and converted into .mgf files by using MSConvert of ProteoWizard1 (version 3.0.06245). All .mgf files were analyzed using Mascot (version 2.3.02; the Matrix Science, London, UK), which was used to perform searches against the Uniprot_sprot_2014_09 database; Homo sapiens species restriction; 66,244 sequences. For the database search, the following settings were used: a maximum of two miss cleavages, oxidation as a variable modification of methionine, carbamidomethylation as a fixed modification of cysteine and trypsin was set as enzyme. A peptide mass tolerance of 10 ppm and a fragment mass tolerance of 0.5 Da were allowed. An ion score of 40 was used as cut-off value.

Scaffold software (version 4.4.3, Portland, OR) was used to summarize and filter MS/MS based peptides and protein identifications. Protein identifications were accepted if they could be established at > 99.0% probability and contained at least two identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped. Using these criteria, Scaffold generated a list of identified proteins (Minimum: 6% coverage and 2 peptides), including the number of sequenced peptides that corresponded to these proteins. The identified breast cancer cell proteins (of the three cell lines) that were co-cultured with T cells, were compared to those of breast cancer cells cultured without the T cells. The comparison was done based on 2 sample T-test, and the p-values of all proteins were calculated and corrected for multiple variants using Benjamini-Hochberg. A protein was considered as a differentially expressed protein if $p < 0.05$, and all three breast cancer cell lines in one group showed the same direction of expression.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [34] partner repository with the dataset identifier PXD006750.

GBP1 ***silencing***

To study the role of *GBP1* in crossing the BBB by the tumor cells, silencing transfection experiments were performed. A mix of four siRNA sequences that target *GBP1* mRNA and non-targeting siRNA (referred to as siSham) were obtained from Dharmacon (GE health care, Netherland). Silencing experiments were performed using transfection buffer 1, following the manufacturer's instructions (Dharmacon, GE health care, Netherland). MDA-MB-231 and MDA-MB-231-BM breast cancer cell lines were transfected for 24 hours. RNA was isolated using RNeasy Micro Kit (Qiagen), and the silencing efficiency was evaluated by reverse transcriptase quantitative PCR (RT-qPCR) using TaqMan gene expression assay (Applied Biosystems).

When si*GBP1* proved to be efficient, breast cancer cells were co-cultured with T cells (3:1 ratio) for an additional 24 hours. Afterwards, T cells were washed away with PBS, and breast cancer cells were harvested and labelled with green tracker as described above. 10^4 cells were seeded on the upper chamber of the BBB model as previously described.

Immunohistochemistry

Formalin fixed, paraffin embedded (FFPE) tissue samples, that morphologically matched the primary FF tissues, were used to perform immunohistochemistry. We retrieved 11 FFPE samples of the 22 FF samples enclosed in the discovery set. The T cell markers were stained with an automated IHC staining system (Ventana BenchMark ULTRA; Ventana Medical System Inc. Tucson, AZ). CD3 (0.4 µg/mL, clone 2GV6, Ventana, Tuscan, AZ) antibody was used as general marker for T cells; CD4 (2.5 µg/mL, clone PS35, Ventana, Tuscan, AZ) and CD8 (1:100 dilution, clone C8/144B, Dako, Heverlee, Belgium) were used as markers for T helpers and T cytotoxic subsets, respectively. The staining was performed according to the manufacturer's protocol. In addition, *GBP1* (1:250 dilution, Santa Cruz Biotechnology, Heidelberg Germany) was used according to the manufacturer's instructions. In addition to the 11 FFPE samples of the discovery set, we used 20 independent samples for extra validation. The clinical information of the samples is summarized in **Table 1**.

In vivo mouse model

T cell isolation and culture

T cells were obtained from the spleen of 4–6 week-old FVB mice weeks old. Splenocytes were sorted using anti-mouse CD4-Pe (BD Pharmigen, catalog 553048) and activated with anti-mouse CD3e clone 145-2C11 (BD Pharmigen catalog 553066) coated plates, soluble anti-mouse CD28 (37.51, Tonbo Biosciences, catalog 70-0281-U500) and mouse IL-2 (Miltenyi Biotec catalog 130-094-054). The ErbB2-P cell line was established from MMTV driven-NeuNT transgenic mammary tumors in mice [35]. They express Luciferase and gfp. ErbB2-P are cultured *in vitro* in DME media supplemented with 10% fetal bovine serum, 2 mM l-Glutamine, 100 IU/ml penicillin/streptomycin, and 1 mg/ml amphotericin B. The ErbB2-P was co-cultured with T cells in RPMI medium supplemented with 10% fetal bovine serum and penicillin–streptomycin for three days and then sorted out and injected in syngeneic animals into the heart. Three weeks later, metastasis burden was analyzed by bioluminescence imaging (BLI).

qRT-PCR

RNA (QIAGEN) from sorted gfp + ErbB2-P cancer cells was used to generate cDNA (iScript cDNA Synthesis Kit Bio-Rad catalog. 1708890). Gene expression was analysed using SYBR green gene expression assays (GoTaq® qPCR Master Mix Promega catalog. A6002). Primers:

GBP1 (Sequence of the primer 5–3): 5'-3'GGG CAG CTG TCT TTG GGT AGAC, 3'-5'AGC ATG AGG CCC TAG GAG CTGT. Quantitative PCR reaction was performed on QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) and analysed using the software QuantStudio 6 and 7 Flex Software.

Flow cytometry

ErbB2-P and T cells were isolated from the co-culture based on gfp expression using FACS ARIA Ilu sorter.

Animal experiments

All animal experiments were done in accordance with a protocol approved by the CNIO, Instituto de Salud Carlos III and Comunidad de Madrid Institutional Animal Care and Use Committee. ErbB2-P cells were injected intracardiacally. Briefly, a cell suspension containing 10^5 ErbB2-P cells in a volume of 100 µl was injected in the left cardiac ventricle of anesthetized 4–6 week-old FVB mice. Tumor burden was evaluated by bioluminescence imaging using the IVIS-200 imaging system from Xenogen as previously described. Metastases were defined with BLI as those with signal equal or above $2x10^3$ photon flux.

Statistics

P-value was calculated using two-tailed *t* test.

Free floating immunofluorescence

Tissue for immunofluorescence was obtained after overnight fixation with PFA 4% at 4 C. Slicing of the brain was done using a sliding microtome (Fisher). Brain slices (80 µm) were blocked in NGS 10%, BSA 2%, Triton 0.25% in PBS for 2 hours at room temperature (RT). Primary antibodies were incubated overnight at 4°C in the blocking solution and the following day for 30 min at RT. After extensive washing in PBS-Triton 0.25%, the secondary antibody was added in the blocking solution and incubated for 2 hours. After extensive washing in PBS-Triton 0.25%, nuclei were stained with Bis-Benzamide for 7 min at RT. Primary antibody GFP (Aves Labs, ref. GFP-1020, 1:1,000). Secondary antibody is Alexa-Fluor anti-chicken 488 (Invitrogen).

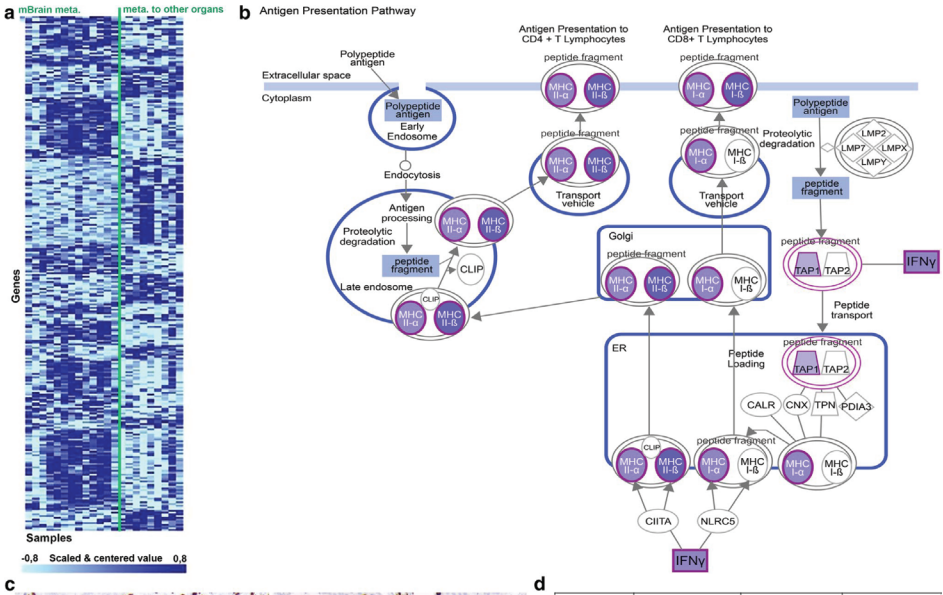
Quantification of brain metastases histology

80 µm sections were obtained from each brain generating 10 series. One series containing 10-12 slices representative of the whole organ was used for immunofluorescence analysis. The staining of *GBP1* was performed and positive lesions were quantified under a fluorescence microscope. Total number of metastases per series was obtained and plotted.

RESULTS

Breast cancers associated with brain metastasis express genes involved in the T lymphocyte response.

Comparing the gene expression profiles of primary breast cancer samples that developed brain metastasis, with those that developed metastasis to other organs, resulted in 298 differentially regulated genes at $p < 0.05$ (**Figure 1a**). Among the significant genes, 176 were up-regulated



Samples	Metastasis site	% of CD3+ cells Stroma	% of CD3+ cells Tumor islands
1	brain met	40	10
2	brain met	80	40
3	brain met	80	20
4	brain met	80	40
5	brain met	80	20
6	brain met	60	10
7	brain met	60	20
8	no brain met	75	5
9	no brain met	60	1
10	no brain met	60	1
11	no brain met	80	1

Figure 1 – Breast cancer samples associated with brain metastasis express genes involved in the T lymphocyte response. a) Heat map of the 298 differentially expressed probes between the primary breast cancer samples that developed metastases, with or without brain metastasis. **b)** Pathway analysis revealed the involvement of the T cells response in the formation of brain metastasis. In this "Antigen Presenting Pathway", the up-regulated genes in the samples that developed brain metastasis are shown in purple. **c)** (I) In the primary breast cancer samples associated with brain metastasis, T cells invade the tumor tissue. (II) In the samples associated with metastasis to other sites, the T cells remain in the tumor stroma. (CD3, 100×). **d)** Percentages of TILs in the primary breast cancer samples of patients who developed metastasis to brain or to other organs.

in the group associated with brain metastasis, while 122 genes were up-regulated in the group associated with metastasis to other organs. To prioritize these genes, we ran functional and pathway analyses. The function annotation tool DAVID revealed that "regulation of T cell activation" was most prominent among the samples associated with brain metastasis (p-value < 0.00002; enrichment score of 6.02). Pathway analysis pointed to a prominent involvement of the "T lymphocyte response" based on both methods (Ingenuity and Global testing) (**Tables 2**, **3**). Moreover, all involved genes in the pathway were up-regulated in the group associated with the brain metastases (**Figure 1b**, **Table 2**).

Table 2 – Top ten canonical pathways (Ingenuity).

Ingenuity Canonical Pathways	log (p-value)	Ratio *	Genes
Communication between Innate and Adaptive Immune Cells	$1.65e^{-08}$	$9.82e^{-02}$	CXCL10, IFNG, HLA-C, HLA-DRB3, TLR8, CCL3L1/ CCL3L3, CD83, CD8A, TLR9, HLA-DRB5, TNFSF13B
Antigen Presentation Pathway	$1.44e^{-06}$	$1.67e^{-01}$	IFNG, HLA-DOA, HLA-DQB2, HLA-C, HLA-DRB3, TAP1, HLA-DRB5
Allograft Rejection Signaling	$1.77e^{-06}$	$7.22e^{-02}$	IFNG, PRF1, HLA-DOA, HLA-DQB2, HLA-C, HLA-DRB3, HLA-DRB5
Type I Diabetes Mellitus Signaling	$4.71e^{-06}$	$8.26e^{-02}$	CD247, IFNG, PRF1, HLA-DOA, HLA-C, IFNGR2, STAT1, CASP8, CD3D, HLA-DRB5
Pathogenesis of Multiple Sclerosis	$4.92e^{-06}$	$4.00e^{-01}$	CXCL10, CXCL11, CXCL9, CXCR3
T Helper Cell Differentiation	$7.10e^{-06}$	$1.11e^{-01}$	IFNG, HLA-DOA, IL12RB1, ICOS, IL10RA, IFNGR2, STAT1, HLA-DRB5
OX40 Signaling Pathway	$9.83e^{-06}$	$7.22e^{-02}$	CD247, HLA-DOA, HLA-DQB2, HLA-C, HLA-DRB3, CD3D, HLA-DRB5
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	$4.90e^{-05}$	$1.28e^{-01}$	CD247, PRF1, HLA-C, CASP8, CDd3D
Crosstalk between Dendritic Cells and Natural Killer Cells	$8.95e^{-05}$	$7.55e^{-02}$	IFNG, PRF1, HLA-C, HLA-DRB3, CD83, TLR9, ITGAL, HLA-DRB5
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	$2.42e^{-04}$	$7.00e^{-02}$	IFNG, HLA-DOA, CXCL13, TLR8, TLR9, HLA-DRB5, TNFSF13B

Red – up-regulated, green – down-regulated genes in primary breast cancers samples associated with metastasis to brain. *Ratio of genes found over their total number in a particular pathway.

We screened 11 tumors for the presence of tumor infiltrating lymphocytes (TILs), according to the criteria of the TILs working group 2014 [36], and found that TILs were confined to the stroma surrounding tumor islands, in the primary breast cancer samples of patients who had developed metastasis to other organs (**Figure 1c**, **d**). In contrast, TILs had invaded the tumor island in patients who had developed cerebral metastases. This observation suggested that T cells may play a role in increasing the ability of breast cancer patients to develop brain metastasis.

Table 3 – Top ten canonical pathways resulted from global testing.

Global Test Canonical Pathways	Comparative *p*-value *	*p*-value
The 41BBdependent immune response	0.004	0.0016
Chaperones modulate interferon Signaling Pathway	0.004	0.0043
Th1 Th2 Differentiation	0.008	0.0045
Roles of arrest independent Recruitment of Src Kinases in GPCR Signaling	0.012	0.0055
X arrest ins in GPCR Desensitization	0.012	0.0101
B Lymphocyte Cell Surface Molecules	0.011	0.0117
Role of Tob in T cell activation	0.006	0.0118
Dendritic cells in regulating TH1 and TH2 Development	0.009	0.0144
Activation of Csk by cAMP dependent Protein Kinase Inhibits Signaling through the T Cell Receptor	0.012	0.0180
NO2 dependent IL-12 Pathway in NK cells	0.015	0.0187

**p*-value calculated by 1000 re-samplings

T lymphocytes increase the ability of breast cancer cells to cross an in vitro BBB model.

To functionally validate the importance of T cells to stimulate the development of brain metastasis, we developed an *in vitro* BBB model and performed functional analysis with three breast cancer cell lines (MDA-MB-231, MDA-MB-231-BM, and SUM159PT). These cell lines are known for their ability to cross the BBB [19, 27], and they all were able to cross the BBB of our *in vitro* model. However, the number of cells that crossed the artificial BBB overnight was very limited (< 10 cells). We co-cultured breast cancer cells with activated T cells, isolated from healthy donors, for three days, removed the T cells and added the breast cancer cells to the BBB model (**Figure 2a**). The number of breast cancer cells that crossed the BBB increased significantly (> 300–650 cells; **Figure 2b**, **c**). To investigate if the interaction between breast cancer cells and T cells was necessary for the dramatic effect that we observed, we run two experiments. First, we incubated breast cancer cells with conditioned media from activated T lymphocytes (in the absence of T cells) and found an increased ability of breast cancer cells to cross the BBB, albeit less significantly (> 80–250 cells; **Figure 2b**, **c**). Second, we co-cultured SUM159PT breast cancer cells (that express the cognate antigen MAGE-C2/HLA-A2) with antigen-specific T cells (CD3+ T lymphocytes transfected with MAGE-C2/HLA-A2 vector). The specific interaction between the breast cancer cells and the T lymphocytes increased the ability of the breast cancer cells to cross the BBB significantly (> 400 cells, Supp. Figure 1). To investigate if T cells have the same effect on normal cell, we replaced the tumor cells by healthy fibroblasts. Co-culturing fibroblasts with activated T lymphocytes, or with conditioned media from activated T lymphocytes, did not change their ability to cross the BBB (**Figure 2b**, **c**). To investigate if T lymphocytes have a direct influence on the permeability of the BBB in the model, we added T cells, antigen-specific T cells or T cell-conditioned media to the upper chamber of the model. T lymphocytes were able to cross the BBB, but neither the T

cells nor their conditioned media changed the permeability of the BBB (data not shown). We also tested a possible direct effect of interferon gamma (IFNγ), secreted by the T cells, on the tumor cells, by incubating the breast cancer cells with several concentrations of IFNγ, but no effect on the ability to cross the BBB model was noticeable. In addition, we tested the effect of IL-2 (a required growth factor for culturing T lymphocytes) on tumor cells, but no facilitating effect was observed either (**Figure 2d**). Both IFNγ and IL-2 did not change the permeability of the BBB (data not shown). These experiments confirmed that T lymphocytes and their secreted factors increase the ability of breast cancer cells to cross the *in vitro* BBB model, and

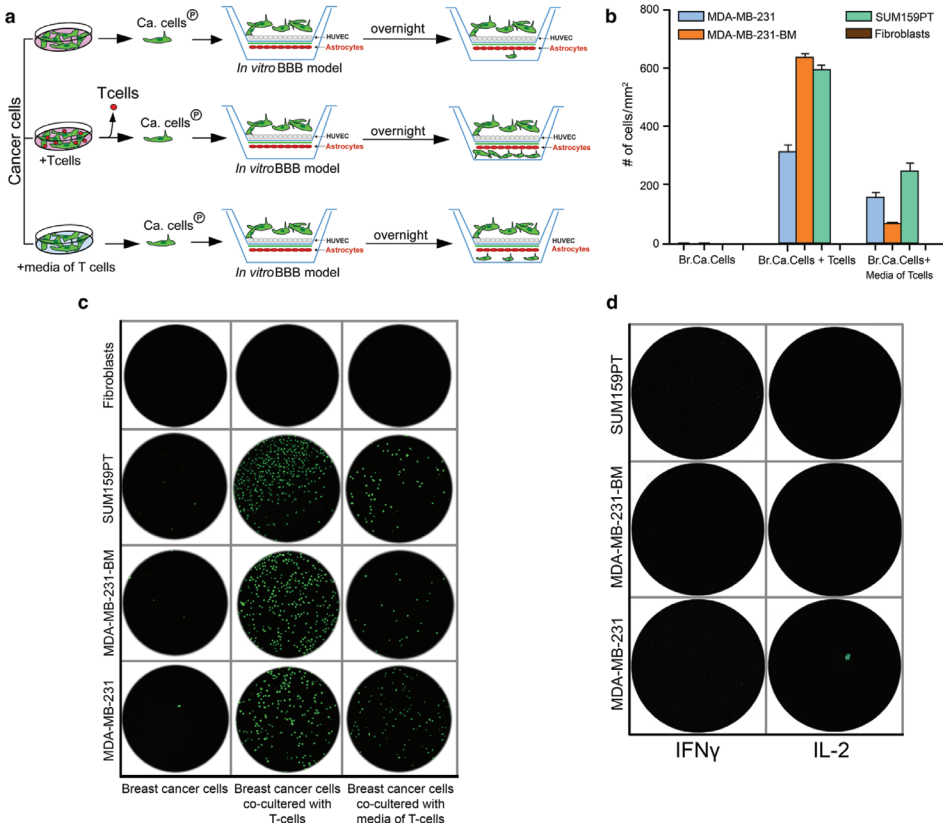
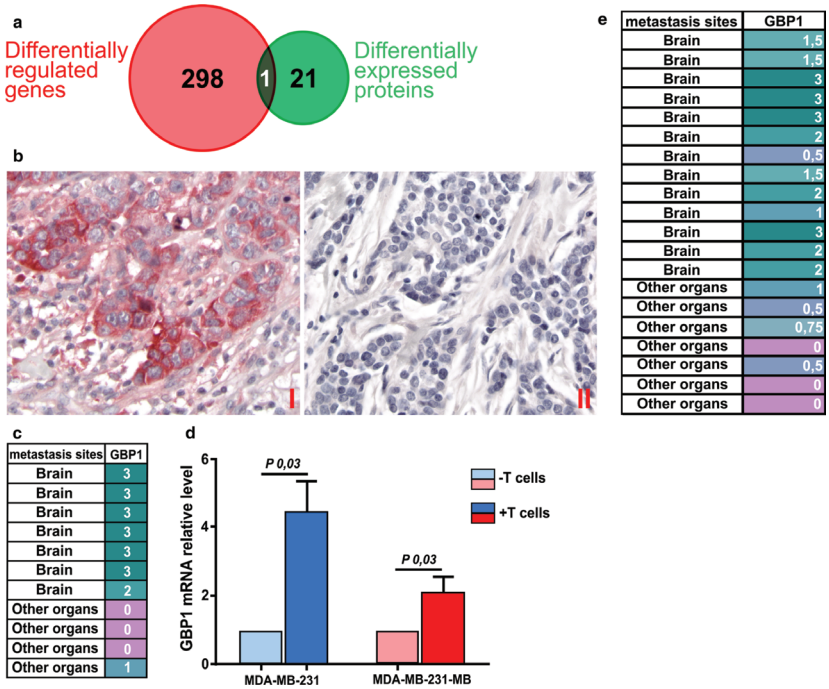


Figure 2 – T lymphocytes increase the ability of breast cancer cells to cross the BBB in the *in vitro* model. **a)** Schema of experimental design. **b)** Three breast cancer cell lines (MDA-MB-231, MDA-MB- 231-BM, and SUM159PT) showed a limited ability to cross the *in vitro* BBB (left column, lower three fields). Following co-culture with T cells, the ability of breast cancer cells to cross the BBB increased significantly (middle column, lower three fields). Conditioned media of T cells also increased the ability of breast cancer cells to cross the BBB, but to a lesser extent (right column, lower three fields). Neither T cells, nor their media, facilitated fibroblasts to cross the BBB (upper row). All experiments were repeated ten times. **c)** Quantitative representation of B (error bars indicate standard deviation) (Br. Ca. = breast cancer). **d)** IFNγ and IL-2 did not change the ability of breast cancer cells to cross the BBB. These experiments were repeated three times.

also proved that antigen-specific interaction between breast cancer cells and T lymphocytes is not necessary to promote the discovered promoting effect.

GBP1 *protein is involved in changing the ability of breast cancer cells in crossing the BBB.*

Next, we aimed to identify the changes that occur in breast cancer cells, after co-culturing with T lymphocytes, responsible for their increased capacity to pass through the BBB. Therefore, we measured the proteome of breast cancer cells before and after co-culturing with T cells, by mass spectrometry. The proteomics comparisons lead to the identification of 21 differentially regulated proteins between the two groups (out of over 2500) at $p < 0.05$, 12 of which up-regulated in the cells exposed to the T cells (**Tables 4**, **5**). The 21 differentially regulated proteins were compared to the 298 differentially expressed genes identified in the mRNA gene expression



metastasis sites	GBP1
Brain	3
Brain	3
Brain	3
Brain	3
Brain	3
Brain	3
Brain	2
Other organs	0
Other organs	0
Other organs	0
Other organs	1

metastasis sites	GBP1
Brain	1,5
Brain	1,5
Brain	3
Brain	3
Brain	3
Brain	2
Brain	0,5
Brain	1,5
Brain	2
Brain	1
Brain	3
Brain	2
Brain	2
Other organs	1
Other organs	0,5
Other organs	0,75
Other organs	0
Other organs	0,5
Other organs	0
Other organs	0

Figure 3 – Immunohistochemistry for *GBP1* in primary breast cancer samples. a) Venn diagram illustrating the overlap of differentially regulated genes and proteins. **b)** (I) Expression of *GBP1* protein in primary breast cancers with brain metastasis. (II) *GBP1* protein in primary breast cancers with metastases to organs other than brain. **c)** Semi-quantitative results of immunohistochemistry for *GBP1* using the discovery sample set. The color scale represents the scores of the immunohistochemical staining, ranging from 0 = no expression to 3 = highest expression. **d)** RT-PCR results of the *GBP1* expression in MDA-MB-231 and MDA-MB-231-BM before and after co-culturing with T lymphocytes. For both cell lines, the *GBP1* expression before and after co-culturing with T cells differ significantly. Bars indicate mean values ± SEM, from three independent experiments. **e)** Semi-quantitative results of immunohistochemistry for *GBP1* using 20 independent primary breast cancer samples. The color scale represents the scores of the immunohistochemical staining, ranging from 0 = no expression to 3 = highest expression.

Table 4 - Differentially expressed proteins of breast cancer cells before and after co-culturing with T cells.

						Breast cancer cells co-cultured with T cells			Pure breast cancer cells		
	Identified Proteins	Gene	Accession # (Uniprot)	Molecular Weight	*t*-test (*p*-value)	MDA-MM-231	MDA-MM-231 BM	SUM159PT	MDA-MM-231	MDA-MM-231 BM	SUM159PT
1	Rab11 family-interacting protein 5	RAB11FIP5	Q9BXF6	70 kDa	< 0.00010	2	2	2	0	0	0
2	AP-1 complex subunit gamma-1	AP1G1	O43747	91 kDa	< 0.00010	2	2	2	0	0	0
3	WD repeat-containing protein 43	WDR43	Q15061	75 kDa	< 0.00010	2	2	2	0	0	0
4	Eukaryotic translation initiation factor 4E	EIF4E	P06730	25 kDa	< 0.00010	2	2	2	0	0	0
5	NADH-cytochrome b5 reductase 1	CYB5R1	Q9UHQ9	34 kDa	< 0.00010	2	2	2	0	0	0
6	SAFB-like transcription modulator	SLTM	Q9NWH9	117 kDa	0.001	3	3	2	0	0	0
7	Guanylate-binding protein 1	*GBP1*	P32455	68 kDa	0.0013	3	2	3	0	0	0
8	Transmembrane emp24 domain-containing protein 2	TMED2	Q15363	23 kDa	0.0016	3	2	2	0	0	0
9	Serotransferrin	TF	P02787	77 kDa	0.0029	4	4	6	0	0	0
10	Arf-GAP domain and FG repeat-containing protein 1	AGFG1	P52594	58 kDa	0.0029	2	2	3	0	0	0
11	DnaJ homolog subfamily C member 11	DNAJC11	Q9NVH1	63 kDa	0.0029	2	2	3	0	0	0
12	60S acidic ribosomal protein P1	PLP1	P05386	12 kDa	0.019	2	2	2	0	0	0

Detected proteins in the three breast cancer cell lines after co-culturing with T cells. Numbers represent the number of unique peptides belonging to a specific identified protein in each sample.

Table 5 - Detected proteins in the three breast cancer cell lines that had not been co-culturing with T cells.

						Breast cancer cells co-cultured with T cells			Pure breast cancer cells		
#	Identified Proteins	Gene	Accession # (Uniprot)	Molecular Weight	*t*-test (*p*-value)	MDA-MM-231	MDA-MM-231 BM	SUM159PT	MDA-MM-231	MDA-MM-231 BM	SUM159PT
1	Charged multivesicular body protein 4b	CHMP4B	Q9H444	25 kDa	< 0.00010	0	0	0	2	2	2
2	Sjoegren syndrome/scleroderma autoantigen 1	SSSCA1	O60232	21 kDa	0.00098	0	0	0	3	3	2
3	C-terminal-binding protein 2	CTBP2	P56545	49 kDa	0.0015	0	0	0	2	3	2
4	Programmed cell death protein 6	PDCD6	O75340	22 kDa	0.0018	0	0	0	3	2	3
5	Transcription elongation factor A protein-like 3	TCEAL3	Q969E4	23 kDa	0.0023	0	0	0	3	2	2
6	UBX domain-containing protein 1	UBXN1	Q04323	33 kDa	0.003	0	0	0	2	2	3
7	ER membrane protein complex subunit 2	EMC2	Q15006	35 kDa	0.003	0	0	0	2	2	2
8	U4/U6 small nuclear ribonucleoprotein Prp31	PRPF31	Q8WWY3	55 kDa	0.0051	0	0	0	3	4	2
9	ATPase ASNA1	ASNA1	O43681	39 kDa	0.016	0	0	0	4	2	2

arrays (**Figure 3a**) and GBP1 was the only protein that matched at the mRNA level. GBP1 protein was measured and exclusively detected in all three breast cancer cell lines that were co-cultured with T lymphocytes ($p < 0.001$), and *GBP1* gene was up-regulated in the primary breast cancer samples that developed brain metastasis ($p < 0.05$, FOC = 1.5). Furthermore, staining for GBP1 was positive in the samples of the primary breast cancers of the patients who developed brain metastases and negative in the samples of patients who developed metastasis to other organs (**Figure 3b**, **c**). Moreover, the tumor areas with and without GBP1 positivity were morphologically indistinguishable, except for the presence of TILs in the former. To further confirm our findings, we validated the upregulation of *GBP1,* after co-culturing breast cancer cells with T lymphocytes, by RT-PCR (**Figure 3d**). In addition, immunohistochemical staining for GBP1 of 20 independent samples, of which 13 developed brain metastasis, showed positivity only in the ER- breast cancer samples of patients who developed brain metastasis (**Figure 3e**).

The prominent effect of *GBP1,* on the capacity of tumor cells to cross the modelled BBB, was examined by silencing experiments. *GBP1* was silenced in MDA-MB-231 and in MDA-MB-231-BM breast cancer cell lines using pooled probes against *GBP1*. However, the expression of *GBP1* was not affected when using siSham (negative control; **Figure 4a**). Subsequently, the cancer cells were co-cultured with activated T cells. The *GBP1*-silenced breast cancer cells showed a significant decrease in the ability to cross the BBB, following co-cultured with T cells, as compared to siSham cells or to breast cancer cells that were not affected by silencing. A 30 – 70 fold decline in crossing of the BBB was reached following silencing of *GBP1* (**Figure 4b**, **c**).

T lymphocytes increase the ability of breast cancer cells to induce brain metastases in a mouse model.

To functionally test the ability of T cells to promote brain metastasis, we used a cancer cell line (ErbB2-P) established from a spontaneous ErbB2 + mammary tumor, derived from MMTV driven- NeuNT transgenic mice [35]. This cell line does not have the ability to target the brain when injected in the systemic circulation [37]. The ErbB2-P cells were co-cultured with T cells, which were previously activated *in vitro* (**Figure 5a**). Sorted cancer cells were initially interrogated by qRT-PCR to analyse *GBP1* levels. Analysis of three independent experiments showed that co-culture of ErbB2-P with activated T cells induces a significant increase in *GBP1* expression levels in the cancer cells (**Figure 5b**). To interrogate the influence of T cells on metastasis, ErbB2-P cells were intracardially inoculated in immunocompetent syngeneic mice (**Figure 5a**). Twenty-one days after inoculation, most of the clones of the ErbB2-P cell line were not able to generate brain metastasis, with only one animal out of 12 (8.3%) showing bioluminescence in the brain (**Figure 5c**, **d**, **f**, **h**). This limited potential, of the parental cell line, to grow in the brain, dramatically increased when cancer cells inoculated had previously been in contact with T cells. Seven out of thirteen (53.8%) animals, inoculated with ErbB2-P

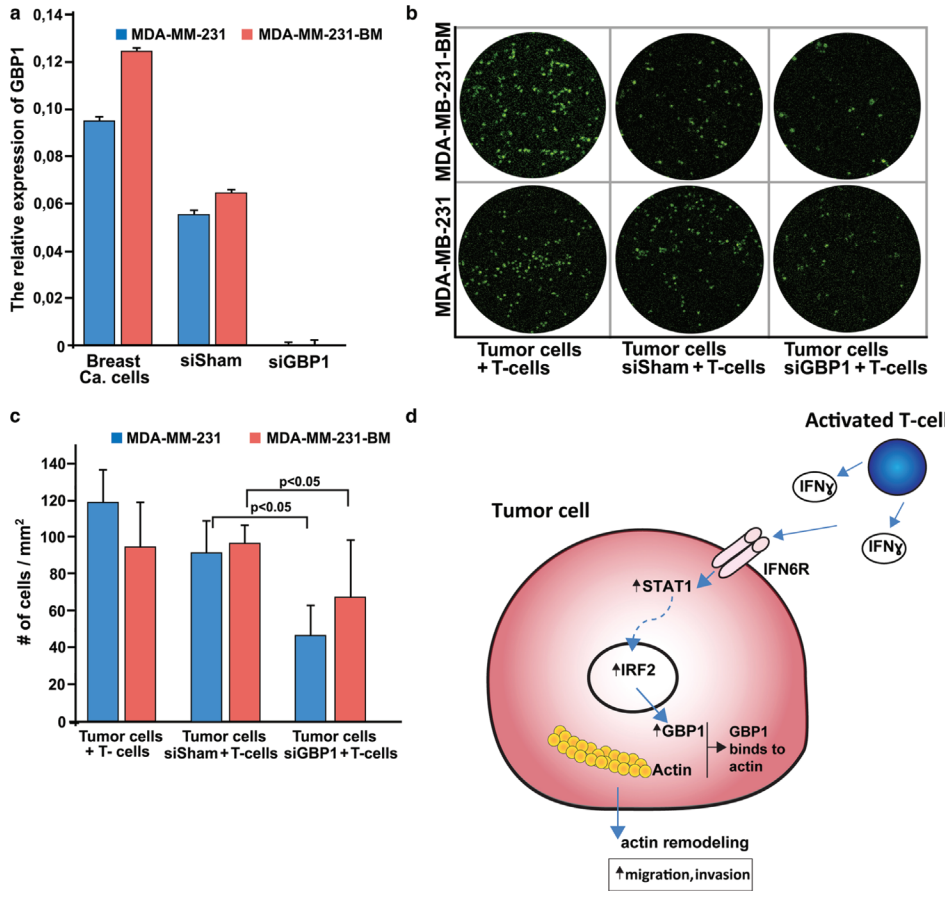
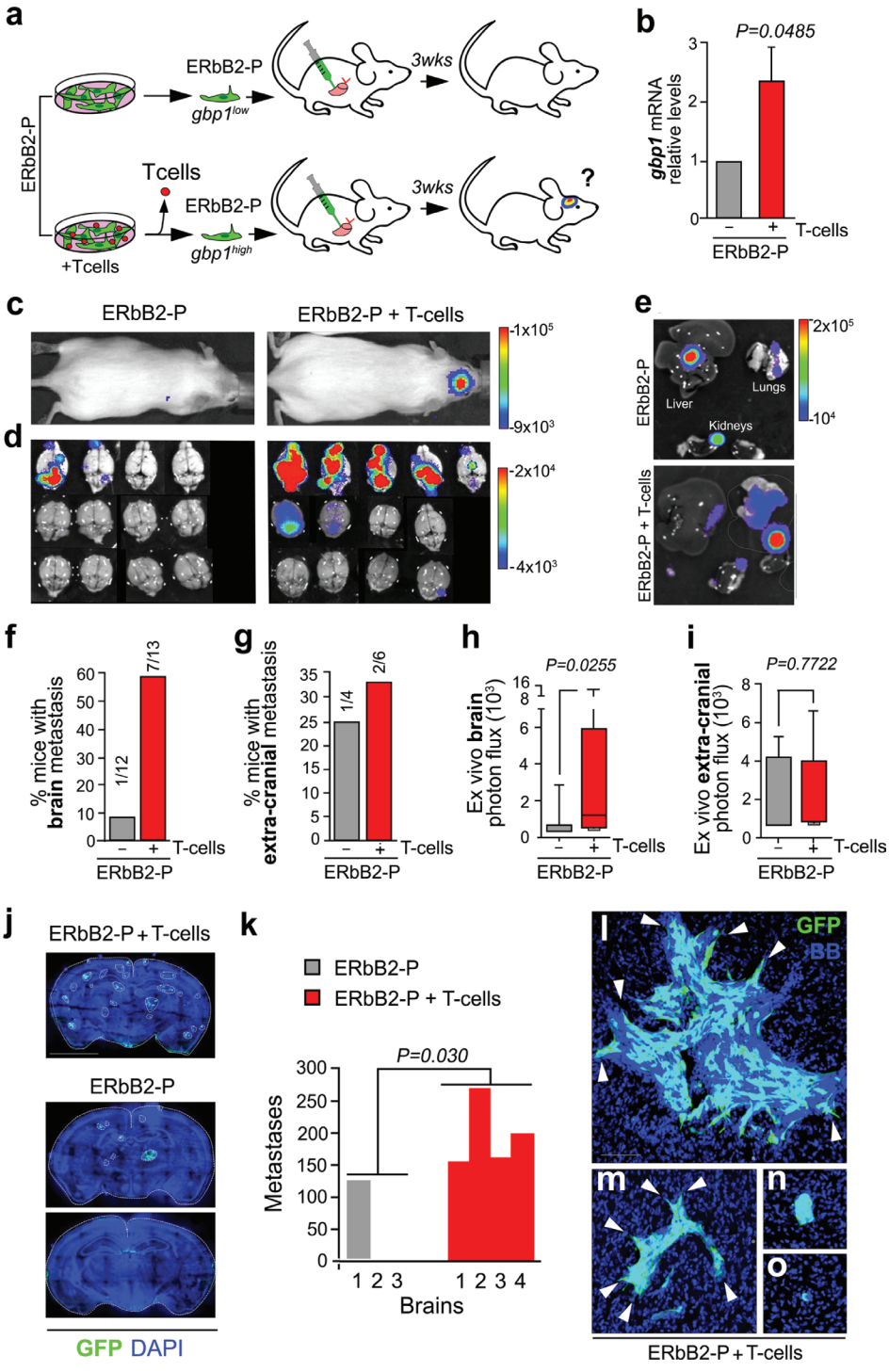


Figure 4 – *GBP1* affects crossing of breast cancer cells through the BBB. a) RT-PCR results of GBP1 expression showing successful silencing. The expression of *GBP1* in breast cancer cells was compared to that of the non-targeting siRNA (siSham) ($n = 3$; bars indicate standard deviation). These experiments were repeated twice, and the results were reproducible. **b)** Breast cancer cells silenced for *GBP1* and co-cultured with T cells show a reduction in their ability to pass the BBB (right column) as compared to the breast cancer cells that were silenced for siSham (middle column). As a control, breast cancer cells not silenced for *GBP1*, were co-cultured with T cells, showed their ability to cross the BBB (left column). These experiments were repeated twice, and the results were reproducible. **c)** Quantitative results of B; error bars indicate standard deviation). The number of breast cancer cells that were able to cross the BBB had decreased significantly after silencing *GBP1*. **d)** Cartoon illustrating the function of *GBP1*.

co-cultured with T cells, developed brain metastasis (**Figure 5c**, **d**, **f**, **h**). This sixfold increase in the ability to generate secondary tumors in the brain was not mimicked in other organs, since ex vivo analysis of lungs, liver, kidneys, adrenal glands, and bones (data not shown) did not show any difference in the percentage of animals affected (ErbB2-P: 25% mice with extra-cranial disease; ErbB2-P + T cells: 33.3%) nor in the bioluminescence signal from ex vivo analysis (**Figure 5e**, **g**, **i**). We conclude that activated T cells are sufficient to increase the ability of breast cancer cells to develop brain metastasis. To investigate the brain metastatic phenotype in more detail, in particular if there was increased access of cancer cell clones in the brain parenchyma, or if there is also an increased ability to colonize the brain, we microscopically examined the brains. Most brains inoculated with ErbB2-P cells did not show any cancer cell, even at the single cell level with the exception of the BLI + one (**Figure 5j**, **k**). The ErbB2-P cells that were co-cultured with T cells consistently showed multiple cancer cell clones within the perivascular spaces (indicative of the ability to get access to the brain parenchyma; **Figure 5j**, **k**), suggesting an increased ability to cross the BBB. In addition, increased size and invasive fronts at the metastatic deposits was observed, indicative of increased capability of parenchyma invasion (**Figure 5l**, **m**, vs **Figure 5n**, **o**). This observation suggests an additional influence of T cells providing cancer cells with further capabilities to advance from the first extravasation compartment. We conclude that the influence of the T cells extends beyond making the breast cancer cells pass the BBB, but also makes them invade the brain tissue.


a
ERbB2-P
gbp1low
3wks
Tcells
ERbB2-P
gbp1high
3wks
?
+Tcells
b
P=0.0485
gbp1 mRNA relative levels
T-cells
ERbB2-P
c
ERbB2-P
ERbB2-P + T-cells
1x10^5
9x10^3
d
2x10^4
4x10^3
e
ERbB2-P
Liver
Lungs
Kidneys
2x10^5
10^4
ERbB2-P + T-cells
f
% mice with brain metastasis
1/12
7/13
T-cells
ERbB2-P
g
% mice with extra-cranial metastasis
1/4
2/6
T-cells
ERbB2-P
h
P=0.0255
Ex vivo brain photon flux (10^3)
T-cells
ERbB2-P
i
P=0.7722
Ex vivo extra-cranial photon flux (10^3)
T-cells
ERbB2-P
j
ERbB2-P + T-cells
ERbB2-P
GFP DAPI
k
ERbB2-P
ERbB2-P + T-cells
P=0.030
Metastases
1 2 3
1 2 3 4
Brains
l
GFP
BB
m
n
o
ERbB2-P + T-cells

← **Figure 5 - T lymphocytes facilitate brain metastasis of breast cancer in mice. a)** Schema of experimental design. **b)** qRT-PCR of sorted ErbB2-P cells after being co-cultured with T cells. Values indicate mean values ± SEM, from three independent experiments. **c)** Representative bioluminescence images (BLI) from mice 21 days after intracardiac injection. **d)** Ex vivo BLI of brains from injected animals. **e)** Representative BLI of extra-cranial metastases. **f)** Graph showing the percentage of mice affected with brain metastasis. Numbers in bars indicate the absolute values g. Graph showing the percentage of mice affected with extra-cranial metastases. Numbers in bars indicate the absolute values. **g)** Quantification of bioluminescence signal emitted by brains ex vivo. Error bars, minimum and maximum values reached by brains. Line in each bar indicates mean BLI value. (n = 12 brains, ErbB2-P; 13 brains, ErbB2-P previously co-cultured with T cells (ErbB2-P + T cells), from 2 independent experiments. P value is calculated using two-tailed t test. **h)** Quantification of bioluminescence signal emitted by extra-cranial metastases (liver, lungs and kidneys) ex vivo. Error bars, minimum and maximum values reached. Line in each bar indicates mean BLI value. (n = 4 mice, ErbB2-P; 6 mice, ErbB2-P + T cells, from one experiment. P value is calculated using two-tailed t test. **i)** Confocal scans of representative slices from different brains. Two brains from mice previously inoculated with ErbB2-P are shown to illustrate the limited seeding (upper panel) and complete absence (lower panel) of metastatic cells. Scale bar 300 mm. White line demarcates brain slices. Dotted white line surrounds gfp + metastases. **j)** Quantification of mean number of metastases in representative brains from each condition. Individual brains are plotted in each experimental condition (n = 3 brains from ErbB2-P injected mice; 4 brains from ErbB2-P + T cells injected mice). P value is calculated using two-tailed t test. **k)** L–O. Representative images to show the heterogeneity present in brains from mice injected with ErbB2-P + T cells. (l-m) Large and medium size metastases with invasive fronts (arrows) co-exist with less abundant (n) well circumscribed metastases and (o) abundant single cell events. Scale bar 75 μm.

DISCUSSION

The identification of the mechanisms and underlying molecular pathways that cancer cells use to cross the BBB is important for the development of strategies to prevent cerebral dissemination. In this study, we found the T lymphocyte response to be most prominently involved in the formation of cerebral metastases of ER- breast cancer patients. The involvement of T lymphocytes in the metastatic potential of breast cancer has been noticed previously, particularly implicating induction of immune tolerance by regulatory T lymphocytes [38-41]. The present results however, reveal an entirely different effect of T cells, namely that T lymphocytes and their secreted factors change the expressional profiles of tumor cells, thereby increasing their ability to cross the BBB. It is known that the immune system mediates primary tumors, regarding their proliferation and invasion, by secretion of inflammatory cytokines, chemokines, autoantibodies, proteases, and more. The role of the immune system in the formation of metastases is complex and far from understood [42-45]. To some extent, the present findings are reminiscent of the T cell reported ability to induce receptor activator of nuclear factor-κB (RANK) signaling, causing pulmonary metastases in a mouse breast cancer model [46]. However, the results obtained using human breast cancer samples show that the presence of T cells correlates with the formation of cerebral metastasis. This finding has not been reported previously. The discovery of T cell involvement in the formation of cerebral metastasis was based on a relatively small series of samples of breast cancer patients. Therefore, we confirmed our findings at the functional level using *in vivo* and *in vitro* models. Injecting mice with breast

cancer cells that were co-cultured with activated T cells, proved that T cells play an important role in increasing the ability of breast cancer cells to cross the BBB, and in the development of brain metastasis. Interestingly, co-culturing breast cancer and T cells did not increase the tendency to metastasis in organs other than brain. The *in vitro* BBB model we used is composed of human endothelial cells and astrocytes, closely reflecting the normal barrier function of the human BBB. The ability of breast cancer cells to cross the BBB improved significantly when the tumor cells were co-cultured with T cells. The facilitating effect was observed after co-culturing with T cells isolated from healthy donors, as well as with antigen-specific T cells, indicative of antigen independency. Incubation of the breast cancer cells with the conditioned media of the T cells showed similar results, pointing to the importance of particular factors secreted by T lymphocytes. In an effort to identify the factors that caused this promoting effect, we incubated breast cancer cells with IFNγ, which is secreted almost exclusively by T cells. Interestingly, IFNγ did not change the ability of breast cancer cells to cross the BBB, nor did it change the permeability of the BBB itself. Similar results were obtained when incubating breast cancer cells with IL-2. It could be argued that instead of single proteins, several secreted proteins and cytokines are necessary to induce the observed effect. The complex interplay between T cells and the humoral immune system was demonstrated in the MMTV-PyMT mouse model, where IL-4-expressing CD4 + T lymphocytes indirectly promote invasion and subsequent metastasis of mammary adenocarcinomas [43]. In another mouse breast cancer model, the effects of interleukin (IL)-1β on the IL-17 expression of gamma delta (γδ) T cells were shown, affecting neutrophils and suppression of CD8 + T cells, also leading to the formation of metastases [47].

Among the 21 differentially expressed proteins, in the three breast cancer cell lines following co-culturing with T cells, only the *GBP1* gene was found to be overexpressed in the set of primary breast cancer samples that developed brain metastasis. RT-PCR results confirmed that *GBP1* expression is significantly upregulated in breast cancer cells after co-culturing with T lymphocytes. Further confirmation of the GBP1 expression was confirmed by immunohistochemistry in the discovery sample cohort and in an additional 20 independent samples. GBP1 positive cells were detected in tumor areas, where T lymphocytes invaded among the tumor cells. Human *GBP1* is a secreted GTPase that is induced by IFNγ and mediate the antibacterial and antiviral activities of IFNγ [48]. The GBP1 protein binds to actin and plays a role in remodeling the fibrous actin structure, thereby influencing cellular motility [48-50]. The regulation of the cytoskeleton and remodeling of actin by *GBP1* is of great relevance in processes like migration, invasion, proliferation and defence against barrier function, a possible link with the increased passage through the BBB [29, 48, 51]. However, the relation with brain invasion seems more complex, since *GBP1*-mediated actin remodeling also contributes to the regulation of the innate and adaptive immune defence [48]. Moreover, mutations in the *GBP1* gene are among those related to the tumorigenesis of breast cancer [52] and the aggressive hormone-negative inflammatory subtype in particular [53]. *In vitro* studies revealed a role of

GBP1 in tamoxifen resistance [54]. The *GBP1* protein is also involved in resistance to docetaxel of prostate cancer [55] and, in a recent study, it was shown that it is one of the key molecules contributing to cancer radioresistance [56]. With respect to tumors other than breast cancer, *GBP1* is considered to act as tumor suppressor gene in colorectal cancer [57], and as an effector of EGFR-driven tumor cell invasion in glioblastomas [58]. In addition, *GBP1* was found to promote lymph node metastasis in oesophageal squamosal cell carcinoma [59]. So far, *GBP1* was not associated with brain metastasis of breast cancer. However, a recent study showed that the overexpression of GBP1 protein, among others, was associated with metastasis in TNBC [60]. Obviously, its expression sites, specific action and possible partners involved in brain metastasis, need further exploration. Most importantly, investigations of the effects of blocking its expression *in vivo* are needed to develop therapeutic strategies in preventing metastases to brain.

Our results highlight the importance of T lymphocytes and their secreted cytokines for the formation of brain metastasis originating from ER- breast cancers. This is new to current knowledge of the complex interplay between T lymphocytes and cancer cells. T lymphocytes change the expressional repertoire of breast cancer cells, which promotes their ability to cross the BBB. The up-regulation of the *GBP1* gene and the overexpression of GBP1 protein seem to be crucial to this effect. The predictive value of this protein in the rise of cerebral metastases should be evaluated in prospective settings.

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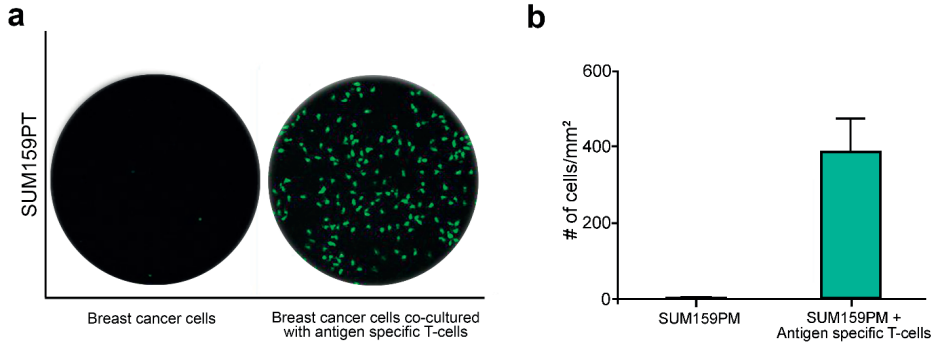
SUPPLEMENTARY INFORMATION

Supplementary Table 1 – Characterization of cell lines.

Cell lines	Classification	ER	PR	HER2	Invasiveness	Down-regulation	Up-regulation	Mutations
MDA-MB-231 [28]	Basal-type	neg	neg	neg	High	Claudin-3 Claudin-4 E-cadherin	CD44+ EGFR	KRAS BRAF P53
MDA-MB-231-BM	Basal-type	neg	neg	neg	High	Claudin-3 Claudin-4 E-cadherin	CD44+ EGFR	KRAS BRAF P53
SUM159PT [28]	Basal-type	neg	neg	neg	High	E-cadherin	CD44 EGFR N-cadherin P-cadherin	HRAS PIK3CA P53



Supplementary Figure 1 – T cells facilitate breast cancer cells to cross the BBB in an antigen-independent fashion. a) SUM159PT breast cancer cell line that express the cognate antigen MAGE-C2/HLA-A2 was co-cultured with antigen-specific T cells ($CD3^+$ T lymphocytes transfected with MAGE-C2/HLA-A2 vector). A similar facilitating effect was observed. **b)** Quantitative results of A. Error bars indicate standard deviation.

Chapter 5

T lymphocyte *IFN*-γ enhances the ability of Breast Cancer cells to pass the Bood-Brain Barrier

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ABSTRACT

Background: The appearance of brain metastasis is the most serious complication of breast cancer, with mostly fatal outcome. In order to reach the brain, tumor cells need to pass the blood-brain barrier (BBB), which underlying molecular mechanisms are largely unknown. Previously, we found that tumor infiltrating T lymphocytes enhance the development of brain metastasis of estrogen receptor-negative (ER-) breast cancer, inducing the expression of Guanylate-Binding Protein 1 (*GBP1*) in primary tumor cells. *GBP1* is a downstream target of IFN-γ, which is a product of T lymphocytes. In the current study, we investigate the molecular contribution of the IFN-γ pathway in enabling MDA-MB-231 breast cancer cells to pass the *in vitro* BBB. Methods: *In vitro* BBB transmigration ability of MDA-MB-231 breast cancer cells was examined following co-culture with different subsets of activated T lymphocytes and IFN-γ concentration was measured by ELISA. The influence of IFNGR1 blocking in MDA-MB-231 breast cancer cells, prior to incubation with medium conditioned by activated T lymphocytes, was examined through an *in vitro* BBB model and validated by Western blot. The endogenous expression of IFN-γ in MDA-MB-231, following co-culture with activated T lymphocytes, was validated by RT-PCR. The effect of IFN-γ in primary breast cancer cells was assessed in a previously generated RNA expression profiles of primary breast cancer samples from patients who developed brain metastases. Results: We identified CD8+ T lymphocytes as the subset with the strongest stimulatory effect on the breast cancer cell transmigration. Importantly, we showed that inhibition of the IFN-γ receptor in MDA-MB-231 breast cancer cells, or neutralization of soluble IFN-γ, impairs the *in vitro* trespassing of breast cancer cells. Moreover, the CXCL-9,-10,-11/CXCR3 axis, dependent on IFN-γ signaling activity, was overexpressed in primary breast cancer samples of patients who developed brain metastasis. Conclusion: Our results show a prominent role of the IFN-γ in the formation of brain metastasis of ER- breast cancer, and offer targets to design future therapies for preventing breast cancer cells to cross the BBB.

INTRODUCTION

The complication of brain metastasis occurs in 10-16% of breast cancer patients and is usually a late event in the natural course of the disease. The median survival following cerebral seeding is very limited, ranging from only a few months to ultimately two years [1, 2]. Among all breast cancer subtypes, human epidermal growth factor receptor 2 (HER2)-positive and triple-negative (estrogen- and progesterone receptors- and HER2 protein-negative) breast cancers have an increased risk for the development of brain metastases, along with decreased survival rates [3-6]. The formation of cerebral metastases depends on the ability of circulating tumor cells to successfully penetrate the blood-brain barrier (BBB). Essential underlying mechanisms and molecular pathways utilized by breast cancer cells to cross the BBB are largely unknown, albeit several genes and pathways have been associated with this event [7].

In our previous work, we compared the gene expression profiles of primary breast cancer samples of patients with and without brain metastasis. Genes related to T lymphocytes, as well as an increased number of T lymphocytes within the tumor parenchyma, have been reported in primary breast samples of patients who developed brain metastasis [8]. We showed, by co-culturing estrogen receptor-negative (ER-) breast cancer cells with activated T lymphocytes, that the ability of breast cancer cells to cross the BBB increased significantly [8]. Moreover, by measuring the proteome of the breast cancer cells, we identified that the expression of guanylate-binding protein 1 (GBP1) was induced upon co-culture with activated T lymphocytes, and functionally involved with BBB crossing in an *in vitro* model. We demonstrated a similar effect of T lymphocytes on breast cancer cells to preferentially seed to the brain in an *in vivo* mouse model [8]. *GBP1* is a GTPase in the dynamin superfamily and a known downstream target of the interferon-gamma (IFN-γ) pathway [9, 10]. Reported an actin cytoskeleton remodeling factor [11, 12] and an invasion-promoter [13, 14], *GBP1* was confirmed to be overexpressed in the primary breast cancer samples of patients who developed brain metastasis [8].

The subtype of T lymphocytes that mainly contributes to enhance the transmigration of breast cancer cells across the BBB was not yet identified. T lymphocytes are known to secrete various cytokines upon activation and interaction with cancer cells, including IFN-γ. Nevertheless, the specific mechanism of how T lymphocytes increase the ability of breast cancer cells to cross the BBB is unknown. Moreover, the main secreted cytokines, that facilitate breast cancer cells to reach the brain, are unidentified. In addition, the downstream effect of these cytokines are not described in the context of breast cancer brain metastasis, nor the BBB transmigration. The aim of this study was to reveal the mechanism of how T lymphocytes facilitate brain metastasis of MDA-MB-231 breast cancer. Understanding such a mechanism will assist in designing preventive strategies against brain metastasis, not only from breast cancer but also from various

other types of cancers that are assumed to utilize similar mechanisms in the formation of brain metastasis.

MATERIALS AND METHODS

T lymphocyte fluorescence-activated flowcytometric cell sorting

Total T lymphocytes (CD45+ CD3+), CD4+ T lymphocytes (CD45+ CD3+ CD4+) and CD8+ T lymphocytes (CD45+ CD3+ CD8+) were sorted using FACSAria III™ (BD Biosciences) from peripheral blood mononuclear cells (PBMC) collected from three healthy donors, as approved by the local medical ethical committee (MEC-2016-202). Peripheral blood samples were collected in lithium heparin tubes (BD Biosciences) and the PBMCs were isolated by using Ficoll-based density separation. PBMCs were incubated for 15 minutes at room temperature (RT) with an antibody mixture consisting of CD45-PerCP (clone: 2D1; BD Biosciences), CD3-FITC (SK7; BD Biosciences), CD4-PB (RPA-T4; BD Biosciences), CD8-PE-Cy7 (SFCI21Thy2D3; Beckman Coulter). The different T lymphocyte populations were isolated with a purity of > 95%. Sorted T lymphocyte subpopulations were transferred to RPMI-Hepes media, supplemented with 6% human serum albumin (HSA, Sigma-Aldrich), 1% penicillin-streptomycin (P/S) and 1 µg/ml IL-2 (360 IU/mL, Chiron, Amsterdam, The Netherlands) within 1 hour.

Activation and Expansion of T lymphocytes

The sorted T lymphocyte fractions were cultured in RPMI supplemented with 10% fetal bovine serum (FBS, ScienCell) and 1% P/S and stimulated overnight with a commercially available phorbol 12-myristate 13-acetate (PMA) and ionomycin cell stimulation cocktail (CSC, eBioscience™, Invitrogen), according to manufacturer's instructions.

Bulk T lymphocytes were activated as previously described [8]. In short, PBMCs were co-cultured with gamma-irradiated (40 Gy) allogeneic PBMCs, Epstein-Barr Virus (EBV)-transformed B-lymphoblast cell lines BSM (also known as GM06821, GLCneg/HLA-A2pos) and APD (also known as GM06817, EADneg/HLA-A1pos) cells, in combination with Phytohemagglutinin-L (PHA-L, Sigma) and IL-2 in a 96-well flat-bottomed plate for 6-7 days at 37°C in a humidified incubator with 5% CO_2. After incubation, cells were harvested, centrifuged, and cultured in RPMI-Hepes medium supplemented with HSA and IL-2.

All T lymphocyte activations were performed prior to co-culturing experiments.

Cell line culture procedure

Human astrocytes (ScienCell) were cultured in astrocyte medium (AM, ScienCell) supplemented with 1% astrocyte growth factors (AGS, ScienCell), 2% FBS and 1% P/S. Human umbilical vein endothelial cells (HUVECs, ScienCell) were cultured in endothelial cell medium (ECM, ScienCell) supplemented with 1% endothelial cell growth factors (ECGS, ScienCell), 5% FBS and 1% P/S. Human astrocytes and HUVECs were used between passage 2 and 5. MDA-MB-231 breast cancer cell line (ER-, human breast cancer cell line known to metastasize to brain and other organs [15]), was used and cultured in RPMI-1640 with L-glutamine (BioWhittaker®) medium, supplemented with 10% FBS and 1% P/S.

Construction of the in vitro blood-brain-barrier (BBB) assays

Methodological specifications of the BBB *in vitro* model were described previously in detail [8, 16]. In short, 24-well polyethylene terephthalate (PET) hanging cell culture inserts (membrane surface area 0.3 cm2, pore size 3.0 μm, Merck Millipore) were coated with 2% gelatin, placed upside-down and ~$1x10^5$ human astrocytes were seeded at the bottom side of the inserts. The cells were allowed to adhere for three hours at 37°C, in a humidified incubator with 5% CO_2, and were supplemented with new AM every 15 to 30 minutes. After three hours, inserts were flipped and placed in 24-well plates. One mL of AM was added to the lower chamber. ~$5x10^4$ endothelial cells in 500 μL of ECM were plated to the upper chamber of the inserts and the cultures were given three days to grow, at 37°C in a humidified incubator with 5% CO_2.

Measuring the transmigration of breast cancer cells through the in vitro BBB

To investigate the a) influence of T lymphocytes on the ability of breast cancer cells to cross the BBB, MDA-MB-231 cells were co-cultured for three consecutive days with previously activated T lymphocytes (different sorted subsets or CD3+ bulk T lymphocytes), at 37°C in a humidified incubator with 5% CO_2. As control, MDA-MB-231 cells were used alone, or in the presence of cell stimulation cocktail, when using CD3+ bulk T lymphocytes or sorted subsets, respectively. To investigate the b) influence of the T-lymphocyte-secreted factors, in the absence of T lymphocytes, freshly collected conditioned medium (CM) of activated T lymphocytes was added to MDA-MB-231 cells for three consecutive days. The co-culture ratios of MDA-MB-231 breast cancer cells to T lymphocytes, and of volume RPMI to CM of T lymphocytes, was 3:1. After three days of co-culture, T lymphocytes were removed from the adherent layer of breast cancer cells by a thorough three-step PBS washing. Then, MDA-MB-231 breast cancer cells were trypsinized and labelled with 5 μM CFMDA cell tracker green (Invitrogen) in serum-free medium, for 45 minutes, at 37°C. The breast cancer cells were collected, PBS-washed and re-suspended in full-serum medium. ~$1.5x10^5$ MDA-MB-231 breast cancer cells were seeded in the upper chamber of the BBB model and incubated overnight at 37°C in a humidified incubator with 5% CO_2. After incubation, the transwells were removed and the MDA-MB-231 breast cancer cells which passed through the BBB and adhered the bottom of

the 24-well chamber (living cells), were nuclear-stained with Hoechst 33342 (Invitrogen) and recorded by confocal microscopy (**Figure 1a**). Unless otherwise stated, tested MDA-MB-231 breast cancer cell trespassing numbers are presented as a fraction of the positive control, set as 100%.

Confocal images were obtained using a Zeiss LSM510 confocal laser-scanning microscope, equipped with a 488 nm argon-laser, a 405 nm Diode and a Plan-Neofluar 20× objective with NA 0.5 (Zeiss, Oberkochen, Germany). Images were made with a pixel size of 1 µm. Pictures were submitted to ImageJ software version 1.49S (http ://www.fiji .sc) and used to calculate the number of cells/mm^2.

The effect of recombinant IFN-γ and/or TNF-α on breast cancer cell migration

MDA-MB-231 cells (~$5x10^5$) were plated in 6-well plates and left overnight to adhere. The next day, the medium was renewed and a titration series (0.01-50 ng/mL) of recombinant human IFN-γ (rIFN-γ, 300-02, Bio-Connect, Huissen, The Netherlands) was added to the breast cancer cells for 72 hours. Afterwards, MDA-MB-231 breast cancer cells were washed and seeded on the BBB. For tumor necrosis factor alpha (TNF-α) incubation purposes, breast cancer cells were incubated with 10 ng/ml of recombinant human TNF-α, with or without equal amounts of rIFN-γ, for 30 minutes or 24h. MDA-MB-231 cells were harvested, washed three times with PBS and labelled with CFMDA green tracker, as described above, before addition to the upper chamber of the BBB model.

IFN-γ signaling pathway blocking and inhibition experiments

The human IFN-γ receptor consists of IFNGR1 and IFNGR2 subunits [17]. To block the IFN-γ receptor, MDA-MB-231 breast cancer cells were pre-incubated for 1h with a concentration range (0.01-10 µg/ml) of human IFNGR1/CD119 monoclonal antibody (clone 92101; R&D Systems) or 10 ug/ml Mouse IgG1 isotype control (clone 11711; R&D Systems). Subsequently, the MDA-MB-231 breast cancer cells were incubated overnight with CM from activated bulk T lymphocytes. In order to study the effect of soluble IFN-γ (sIFN-γ), anti-hIFN-γ-IgA (clone H7WM120; InvivoGen, San Diego, CA, USA) or human IgA2 isotype control (anti-βGal, clone T9C6; InvivoGen, San Diego, CA, USA) were added to the CM of activated bulk T lymphocytes for 1h, prior to overnight incubation with MDA-MB-231 breast cancer cells. Culture supernatants were collected and MDA-MB-231 breast cancer cells were harvested for functional studies and protein Western blot analysis.

IFN-γ and TNF-α measurements

Supernatants from: a) non-activated and overnight activated T lymphocyte subsets, before and after 72h incubation with MDA-MB-231 breast cancer cells, b) five, 10, 20 and 30 minutes and one, two, four, six and 24h MDA-MB-231 breast cancer cell culture, alone or incubated

with CM from activated bulk T lymphocytes, or after co-culture with T lymphocyte, and c) five, 15, 30 and 60 minutes, and 24h MDA-MB-231 breast cancer cell culture, alone or incubated with 10 ng/ml rIFN-γ only or in combination with 10 ng/ml rTNF-α, were collected and IFN-γ and TNF-α levels were measured (DuoSet ELISA, R&D Systems, Minneapolis, MN, USA). The ELISA assays were performed following the manufacturer's instructions, and each sample was measured in duplo.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from previously snap-frozen cell pellets using the RNeasy Plus Micro kit (Qiagen), quantitated using NanoDrop 1000 (NanoDrop Technologies) and reverse transcribed into cDNA, using the RevertAid H-Minus first-strand cDNA synthesis kit (Thermo Scientific), according to the manufacturer's protocol. Quantitative real-time PCR was performed using TaqMan Master Mix (Applied Biosystems) on the 7500 Real-Time PCR system, v.2.3 (Applied Biosystems). The following commercially available exon-spanning TaqMan Gene Expression Assays (Applied Biosystems) were used: IFN-γ, exon 3-4 (Hs00989291_m1), HPRT1, exon 2-3 (Hs02800695_m1) and HMBS, exon 13-14 (Hs00609296_g1). HPRT1 and HMBS were used as reference genes. The relative quantification of target gene expression was performed using the 2-ΔΔCt comparative method and the threshold cycle value was defined by the point at which there was a statistically significant detectable increase in fluorescence.

Western blot

Cells were washed twice with ice-cold PBS and scraped in RIPA Buffer (ThermoScientific, Rockford, USA). A protease and phosphatase inhibitor cocktail (Halt™, ThermoScientific, Rockford, USA) was added to RIPA lysis buffer before use. Cell lysates were centrifuged (1000 g for 15 minutes). The protein content of the cleared lysates was determined (Pierce™ BCA Protein Assay Kit; ThermoScientific, Rockford, USA). Protein lysates were boiled in SDS-sample buffer and separated by SDS-PAGE (12.5% acrylamide). Proteins were blotted onto nitrocellulose membranes (BIO- RAD; Bio- Rad Laboratories, Hercules, CA, USA) and probed with the following antibodies: phospho-STAT1 (Ser727, PSM1, 1:1000, Thermo Fisher Scientific), total-STAT1 (SM1, 1:1000; Thermo Fisher Scientific), IFN-γ R2 (1:2000, R&D Systems), *GBP1* (1B1, 1:200, Santa Cruz) and actin (C-2, 1:1000, Santa Cruz). Visualization was conducted with a chemiluminescence kit (BM Chemiluminescence Western Blotting Substrate (POD), Roche) and a maximum sensitivity substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific), according to the manufacturers' instructions. Western blot images were acquired and analyzed through a digital Western blot scanner, Amersham™ Imager 600 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Densities of protein of interest were measured and normalized towards the structural control protein actin or, in the case of phosphorylated proteins, to its matched total protein.

Re-analysis of the gene expression profile data

To scrutinize additional genes involved in the IFN-γ pathway, the previously generated gene expression data of primary breast cancers from patients with (n=13) and without (n=9) brain metastasis were re-analyzed. Morphological assessment, RNA expression profiling and relevant clinical data regarding this discovery cohort were previously provided [8].

Statistical analysis

Prism 5.0 (GraphPad Software) was used to perform statistical tests. A two-tailed Student's t-test was used to determine differences in the sample means. Data are presented as means ± SD. In all statistical analysis, a p-value < 0.05 was considered statistically significant. Unless otherwise stated, all *in vitro* experiments were repeated independently three times.

RESULTS

Activated CD8+ T lymphocytes most strongly stimulate the trespassing of MDA-MB-231 breast cancer cells through the BBB.

All cell stimulation cocktail (CSC)-activated T lymphocyte fractions increased the ability of MDA-MB-231 breast cancer cells to cross the BBB. However, CD8+ T lymphocytes induced a more potent BBB-passage of MDA-MB-231 breast cancer cells than CD4+ T lymphocytes (p > 0.05, **Figure 1b**, **c**). Addition of CSC to MDA-MB-231 breast cancer cells directly, without activated T lymphocytes, did not result in an increased BBB transmigration.

T lymphocyte-secreted IFN-γ rapidly disappears from the MDA-MB-231 breast cancer cell-conditioned medium.

Culture media from non-activated T lymphocytes or from CSC-exposed MDA-MB-231 breast cancer cells did not contain detectable levels of IFN-γ (**Figure 2a**). On the contrary, the three fractions of CSC-activated T lymphocytes secreted significant amounts of IFN-γ. CD8+ T lymphocytes secreted approximately four-fold higher levels of IFN-γ than CD4+ cells (p < 0.001; **Figure 2a**). Following 72h incubation with MDA-MB-231 breast cancer cells, IFN-γ levels were significantly reduced in all conditions, showing no significant differences between the three T lymphocyte populations (**Figure 2a**). In order to monitor sIFN-γ concentrations in CM over time, activated bulk T lymphocytes were used. The supernatant was collected at different time points following co-culture of MDA-MB-231 breast cancer cells with activated T lymphocytes (MDA+T), or after MDA-MB-231 breast cancer cells incubation with media conditioned by activated T lymphocytes (MDA+CM). Strikingly, sIFN-γ was no longer detected after five minutes of co-culture time in MDA+T. In MDA+CM, only approximately 20% of the initial amount of sIFN-γ was left detectable after five minutes incubation (**Figure 2b**). The sIFN-γ concentration in all subsequent time-points of MDA+CM remained similarly

low (**Figure 2c**), suggesting that the vast majority of sIFN-γ molecules is immediately associated to the tumor cells, but otherwise remains stable in the culture medium.

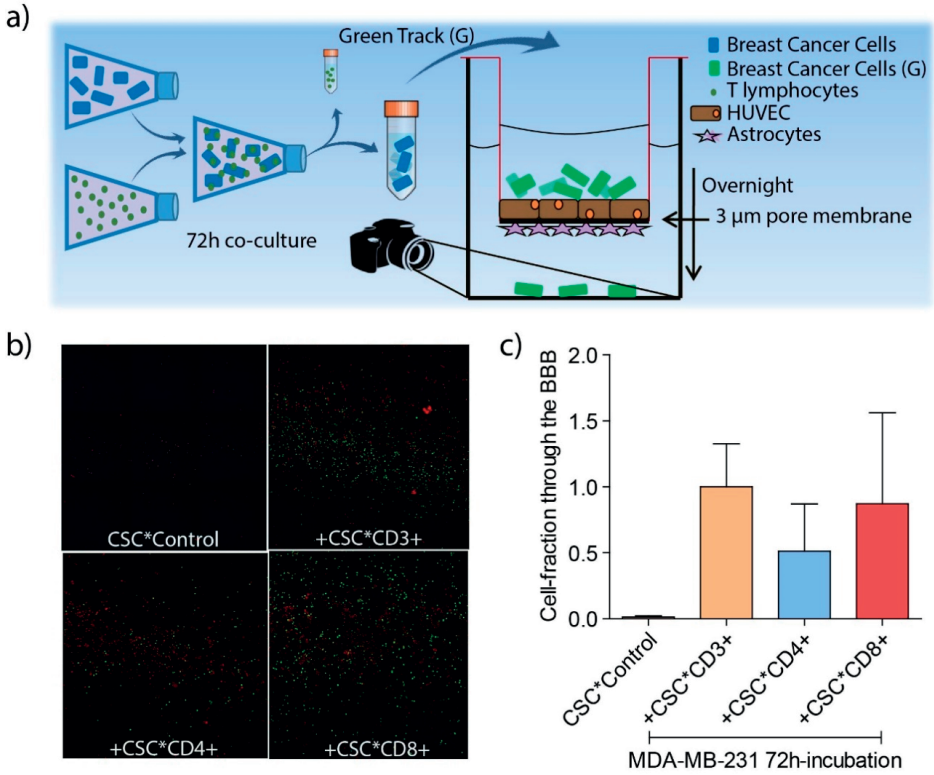

Figure 1 - The effect of T lymphocytes on the transmigration of MDA-MB-231 breast cancer cells in the *in vitro* BBB model. a) Scheme of *in vitro* experimental design; MDA-MB-231 breast cancer cells were co-cultured with activated T lymphocytes. Upon T lymphocyte removal, breast cancer cells were washed, trypsinized, green-tracked, and added to the upper chamber of the BBB model. Transmigration of the cells was monitored using a confocal laser-scanning microscope. **b)** Confocal images of MDA-MB-231 breast cancer cells that crossed the BBB model. MDA-MB-231 breast cancer cells incubated with cell stimulation cocktail (CSC) did not cross the BBB. Co-culture with CD8+ T lymphocytes yielded a larger fraction of cells passing the BBB as compared to CD4+ T lymphocytes ($p > 0.05$). Green dots, breast cancer cells; red dots, nuclei of cells other than breast cancer cells. **c)** Quantitative representation of the observation made (b)). Bars represent the mean percentages transmigrated MDA-MB-231 breast cancer cells imaged in ± SD.

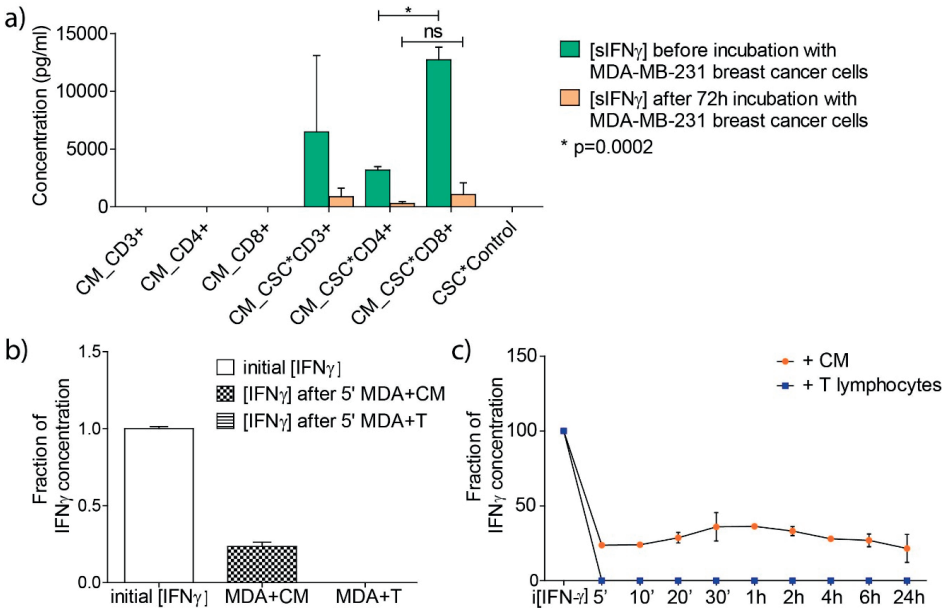

Figure 2 - T-lymphocyte secreted IFN-γ concentrations rapidly drop following incubation with MDA-MB-231 breast cancer cells. a) Concentrations of sIFN-γ in the CM before and after 72 hours incubation with MDA-MB-231 breast cancer cells measured by ELISA. CD8+ lymphocytes secrete higher concentrations of sIFN-γ compared to CD4+ ($p < 0.001$; green bars). There is a dramatic decrease in concentrations of sIFN-γ following 72 hours of incubation with MDA-MB-231 breast cancer cells. No significant differences were observed between CD8+ and CD4+ lymphocytes after 72 hours incubation (orange bars). Neither unstimulated T lymphocytes, nor MDA-MB-231 breast cancer cells incubated with cell stimulation cocktail secreted IFN-γ. Graph representative of two independent experiments (CM, conditioned medium; CSC, cell stimulation cocktail). **b)** sIFN-γ concentration in the media conditioned by activated bulk T lymphocytes, before or after five minutes incubation of MDA-MB-231 breast cancer cells with CM of T lymphocytes, or co-culture with activated CD3+ T lymphocytes. Graph representative of two independent experiments (MDA, MDA-MB-231 breast cancer cells; CM, conditioned medium; T, T lymphocytes). **c)** Concentration curve of sIFN-γ during MDA-MB-231 breast cancer cells incubation with CM of activated bulk T lymphocytes (+ CM), or co-culture with activated bulk T lymphocytes (+ T lymphocytes). Graph representative of two independent experiments.

IFN-γ-receptor blocking in MDA-MB-231 breast cancer cells impairs their transmigration through the BBB model.

In order to investigate whether IFN-γ is critical in the enhanced transmigration of the MDA-MB-231 breast cancer cells through the *in vitro* BBB, we impaired its activity using a monoclonal antibody inhibitor of the IFNGR1 or using a IFN-γ neutralizing monoclonal antibody. Blocking IFNGR1 in MDA-MB-231 breast cancer cells, prior to overnight incubation with CM of activated bulk T lymphocytes, resulted in a concentration-dependent decrease of breast cancer cell passage through the BBB. Moreover, blocking IFNGR1 with a concentration ≥ 0.5 μg/ml, showed a significantly reduced passage-extent through the BBB ($p < 0.001$; **Figure 3a**). Similarly, neutralization of IFN-γ in the media of the activated bulk T lymphocytes, with the anti-hIFN-γ mAb (10 μg/ml), prior to overnight incubation with MDA-MB-231 breast cancer

cells, significantly reduced the BBB-passage numbers of MDA-MB-231 breast cancer cells ($p < 0.001$; **Figure 3b**).

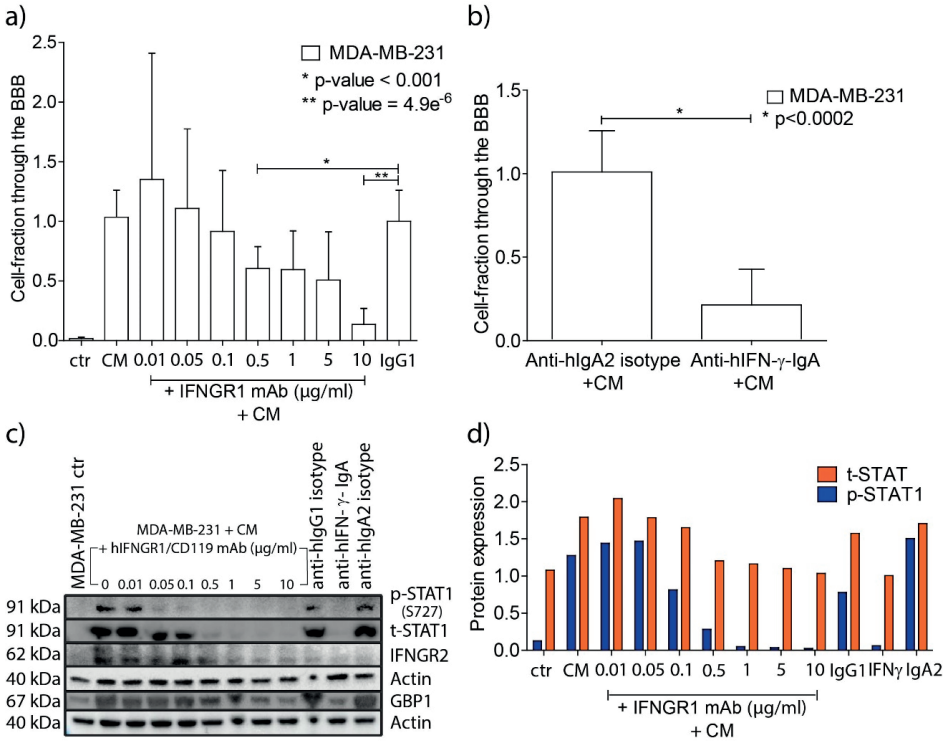

Figure 3 - Blocking of the IFN-γ pathway in MDA-MB-231 breast cancer cells impairs passage through the BBB model. a) Fractions of transmigrated MDA-MB-231 breast cancer cells through the *in vitro* BBB model after one hour incubation with increasing concentrations of human IFNGR1/CD119 mAb and subsequent overnight incubation with equal amounts of CM of activated bulk T lymphocytes. The fractions are relative to 10 µg/ml IgG1 isotype control. ctr, MDA-MB-231 control cells; CM, conditioned medium; IgG1, hIgG1 isotype control. Graph representative of three independent functional experiments. **b)** Fractions of transmigrated MDA-MB-231 breast cancer cells though the *in vitro* BBB after neutralizing the sIFN-γ in the media of activated T lymphocytes. The fractions are relative to 10 µg/ml anti-IgA2 control. Graph representative of two independent experiments. **c)** Western blots of phospho-STAT1, total-STAT1, IFNGR2 and *GBP1* protein expression levels in MDA-MB-231 samples after blocking IFNGR1 (experiment described in a) or after neutralizing the sIFN-γ in the media of activated T lymphocytes. Actin was used as loading control for both membranes used. A concentration-dependent reduction of p-STAT1, t-STAT1, IFNGR2 and *GBP1* proteins was obtained by using increased concentrations of human IFNGR1/CD119 monoclonal antibody. Western blots representative of two independent experiments. **d)** Normalized expression of t-STAT1 and p-STAT1 in MDA-MB-231 breast cancer cells following concentration-dependent IFNGR1 blocking in MDA-MB-231 breast cancer cells or after neutralizing the sIFN-γ in the media of activated T lymphocytes. The level of p-STAT1 is increased by the addition of CM to the breast cancer cells and step-wise curtailed until it matches the p-STAT1 level observed in MDA-MB-231 control cells, synchronous with the fully IFN-γ pathway blocking. t-STAT1 and p-STAT1 expression levels were calculated in MDA-MB-231 breast cancer cells after background deduction and normalization against actin and normalized t-STAT, respectively. ctr, MDA-MB-231 control cells; CM, conditioned medium; IgG1, anti hIgG1 isotype control; IFN-γ, anti-hIFN-γ-IgA; IgA2, anti hIgA2 isotype control. Graph representative of two independent W.B. analyses.

Nevertheless, with the used concentrations of IFNGR1 or IFN-γ neutralizing antibodies, complete prevention of transmigration was not achieved (86% and 80% of accomplished inhibition, respectively; **Figure 3a**, **b**). The same results were obtained by repeating the experiments twice.

In order to ascertain the effects of blocking the IFN-γ pathway in MDA-MB-231 cells, phosphorylation of its downstream signaling molecule STAT1 (p-STAT1) was determined. The concentration-dependent inhibition of STAT1 phosphorylation confirmed the successful inhibition of the IFN-γ signaling pathway in MDA-MB-231 breast cancer cells; along with the increasing blockage of IFNGR1 in MDA-MB-231 breast cancer cells, the detection of p-STAT1 decreased (**Figure 3c**, **d**). Because only subunit R2 of the IFNγ receptor – IFNGR2, is regulated by external stimuli, the protein expression of IFNGR2, after IFNGR1 blocking, was also measured in MDA-MB-231 breast cancer cells. In addition, the previously identified protein GBP1, upregulated in breast cancer cells upon co-culturing with activated T lymphocytes, was also examined [8]. The presence of both proteins, IFNGR2 and GBP1, was reduced by the blocking of IFNGR1 (**Figure 3c**, Supplementary figure 1).

Stimulation of MDA-MB-231 breast cancer cells with rIFN-γ, rTNF-α, or both cytokines, does not increase transmigration through the BBB.

In order to evaluate the effect of recombinant human IFN-γ, and a putative synergistic effect in combination with recombinant human TNF-α, breast cancer cells were stimulated with these cytokines. Stimulation with various concentrations of IFN-γ did not increase the transmigration of the breast cancer cells through the BBB (data not shown). Stimulation of the breast cancer cells with TNF-α alone, or in combination with IFN-γ, did not significantly increase the transmigration abilities of breast cancer cells either (**Figure 4a**). However, a reduction of the concentrations of rIFN-γ was measured in the culturing media of MDA-MB-231 cells. The concentration of rIFN-γ was measured immediately after the addition of this cytokine to the MDA-MB-231 breast cancer cells (0' incubation time). Following the abrupt reduction observed after the first measurement (**Figure 4b**), the concentration of the rIFN-γ stabilized for the following hour, and was reduced to less than a tenth of the initial amount after 24h. Same results were obtained for rIFN-γ alone or in combination with rTNF-α (**Figure 4b**). These results are in line with our previous results showing an abrupt decrease of sIFN-γ after five minutes incubation of MDA-MB-231 breast cancer cells with CM (**Figure 2c**). No IFN-γ was detected in the media of MDA-MB-231 breast cancer cells only. The same results were obtained by repeating the experiments twice.

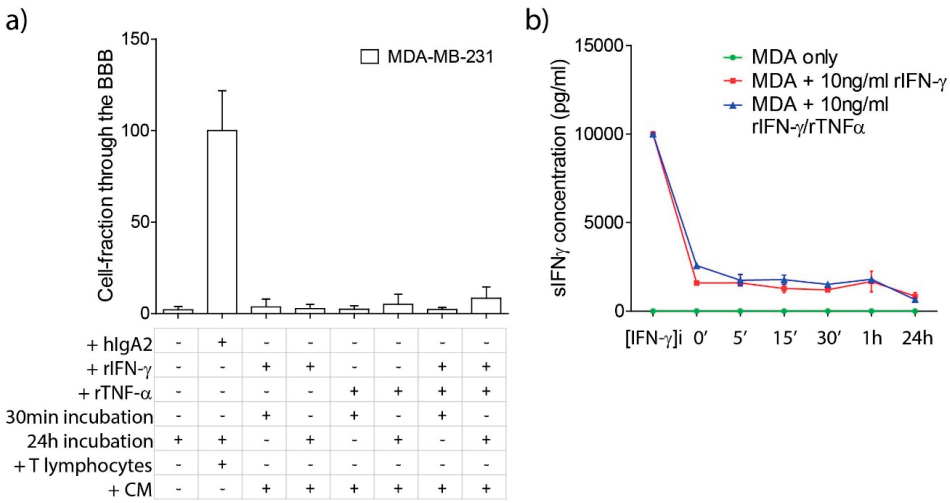

Figure 4 - Recombinant IFN-γ or TNF-α, alone or in combination, did not increase the crossing ability of MDA-MB-231 breast cancer cells through the BBB. a) Incubating MDA-MB-231 breast cancer cells with 10ng/ml of rIFN-γ or rTNF-α, individually or in combination, for 30 min or 24 hours, did not increase the crossing of MDA-MB-231 breast cancer cells. As control, MDA-MB-231 breast cancer cell were co-cultured with activated bulk T lymphocytes and hIgA2 isotype. Graph representative of two independent functional analysis. **b)** rIFN-γ concentration in the conditioned media of MDA-MB-231 breast cancer cells incubated with 10ng/ml rIFN-γ or in combination with equal amounts of rTNF-α, significantly decreased during the first seconds of incubation. Graph representative of two independent experiments.

T lymphocytes stimulate the endogenous expression of IFN-γ in MDA-MB-231 breast cancer cells.

Co-culture of activated bulk T lymphocytes with MDA-MB-231 breast cancer cells resulted in ~35 times increased expression of IFN-γ in the breast cancer cells as compared to incubation with CM (1.6x10-3 vs 4.6x10-5, respectively). No IFN-γ mRNA levels were detected in the MDA-MB-231 control cells (**Figure 5**).

IFN-γ, CXCL9, -10, -11 and CXCR3 are overexpressed in primary breast cancer samples of patients who developed brain metastasis.

By re-analyzing the previously obtained gene expression data of primary breast cancer samples of patients who developed brain metastasis (n=13) or metastasis to organs other than the brain (n=9), the expression of the IFN-γ gene was found to be significantly higher in the former (1.86-fold, $p < 0.001$; **Figure 6d**). In addition, the three chemokines CXCL9, -10, -11, induced by IFN-γ, and their receptor CXCR3, were found to be significantly overexpressed in the primary breast cancers from patients who developed brain metastasis (**Figure 6**).

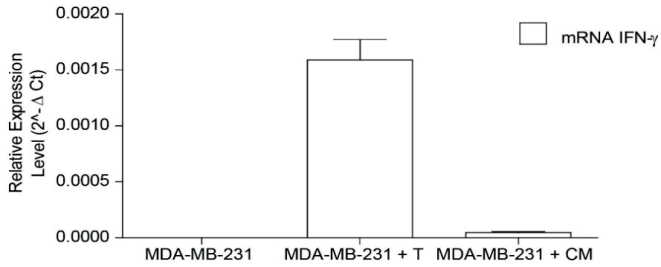

Figura 5 - T lymphocytes stimulate the endogenous expression of IFN-γ in MDA-MD-231 breast cancer cells. Co-culture of activated bulk T lymphocytes with MDA-MB-231 breast cancer cells resulted in a 34.8x higher increased expression of IFN-γ mRNA levels in the breast cancer cells, when compared to incubation with CM (1.6x10-3 vs 4.6x10-5, respectively); No IFN-γ mRNA levels were detected in the control MDA-MB-231 breast cancer cells. Graph representative of three independent experiments (T, T lymphocytes; CM, conditioned medium).

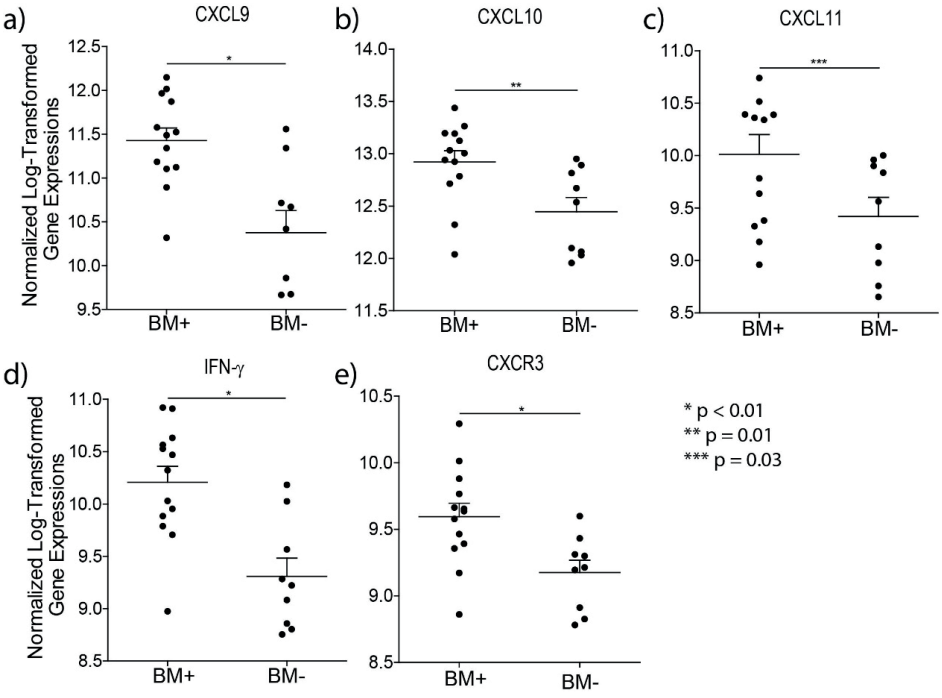

Figure 6 - Overexpression of IFN-γ-inducible chemokines in primary breast cancer samples of patients who developed brain metastasis. CXCL9, -10, -11/CXCR3 axis and IFN-γ are overexpressed in primary breast cancer samples of patients who developed brain metastasis (a-e). Solid lines indicate the mean gene expression and the error bars the SEM. Normalized log 2-transformed gene expression data were used to calculate significance by the two-tailed unpaired Student's *t*-test. (BM+, primary breast cancer sample of patients who developed brain metastasis; BM-, primary breast cancer sample of patients who developed metastasis at sites other than brain).

DISCUSSION

T lymphocytes enhance the formation of brain metastases of ER- breast cancer. T lymphocytes facilitate the transmigration of breast cancer cells through the BBB, both in an *in vitro* model and in an *in vivo* mouse model [8]. In our current study, we demonstrate the central role of T-lymphocyte-derived IFN-γ in enhancing the transmigration of MDA-MB-231 breast cancer cells across the BBB.

We found that especially CD3+ CD8+ T lymphocytes enhance the transmigration of MDA-MB-231 breast cancer cells through the *in vitro* BBB. It has long been recognized that CD8+ T lymphocytes may not necessarily function purely as cytotoxic killer cells, but also fulfill important regulatory functions through differential cytokine production [18-20]. The infiltrating CD8+ T lymphocytes in progressive breast cancer are known for their anti-tumor activity [21-23]. However, it has become clear that the effects of an inflammatory infiltrate, on tumor progression and prognosis of the cancer patients, are numerous and complex [24-26]. The identification of CD3+ CD8+ lymphocytes as the subset that most enhances the transmigration of breast cancer cells, matches the observation that this fraction generally outnumbers CD3+ CD4+ lymphocytes in the infiltrates of primary breast cancer samples [27]. Moreover, CD3+ CD8+ lymphocytes are more efficient producers of IFN-γ than CD3+ CD4+ T lymphocytes [28]. Accordingly, the sIFN-γ in the media conditioned by matched numbers of the different T lymphocyte subsets revealed higher IFN-γ secretion by the CD3+ CD8+ T lymphocytes (**Figure 2a**). When MDA-MB-231 breast cancer cells were incubated with CM of activated bulk T lymphocytes, nearly 80% of the secreted IFN-γ became non-detectable within the first five minutes. The tumor cells are regarded as the major target of IFN-γ action [29] and its lack of detection, observed following co-culture of breast cancer cells with activated T lymphocytes (MDA+T), may well mirror the *in vivo* situation [30]. This is further corroborated by the rapid decline of detectable IFN-γ upon recombinant IFN-γ addition to the breast cancer cells (**Figure 4b**).

IFN-γ signaling pathway reportedly plays a role in growth and metastasis of triple-negative breast cancer [31]. In addition, IFN-γ blocking is beneficial to reduce tumor metastasis formation of tumors of various lineages [32, 33]. IFN-γ is a potent molecule that regulates the expression of many genes, influencing many pathways in different cell types. Microarray analysis has demonstrated that over 100 genes in a single cell type are regulated by IFN-γ [34]. A major cluster of those genes comprise chemokines and their receptors, which are involved in the recruitment and directional migration of specific cell types, antiviral responses and tumor surveillance [35].

To test whether an active IFN-γ signaling pathway in MDA-MB-231 breast cancer cells is crucial for their successful passage across the *in vitro* BBB, we impaired the IFN-γ pathway in breast cancer cells in two different and independent manners: 1) by blocking the IFN-γ receptor on the MDA-MB-231 breast cancer cells and 2) by neutralizing the sIFN-γ in the CM of activated T lymphocytes. Both interventions resulted in a reduced ability of breast cancer cells to cross the *in vitro* BBB (**Figure 3a**). However, for both inhibition assays, the MDA-MB-231 BBB transmigration ability was not completely prevented, indicative of the importance of other players present in the CM of activated T lymphocytes.

The INF-γ receptor consists of two subunits: IFNGR1 (CD119 or subunit α) responsible for binding ligand in a species-specific manner, and IFNGR2, (AF-1 or subunit β) required for induction of biologic responses. In contrast to IFNGR1, IFNGR2 is constitutively expressed at low levels and is up-regulated by external stimuli [17, 30]. Therefore, to confirm the successful blockade of the IFN-γ pathway, the expression of IFNGR2 was monitored [17, 30]. Accordingly, IFNGR2 expression in MDA-MB-231 breast cancer cells showed a significant protein reduction, concurrent with the increased concentration of IFN-γ receptor inhibitor (**Figure 3c**, Supplementary Figure 1b).

In order to validate the IFN-γ pathway blockade in MDA-MB-231 breast cancer cells, we monitored the downstream protein phospho-STAT1. IFN-γ signaling induces phosphorylation of two STAT1 residues: tyrosine 701 (Y701), which facilitates dimerization, nuclear translocation and DNA binding, and Serine 727 (S727), which enables maximal STAT1 transcriptional activity [36]. Serine 727 phosphorylation grants nearly 80% of IFN-γ-induced transcriptional activity [37]. Our data show that at a concentration of ≥ 0.5 μg/ml IFNGR1 mAb, both total STAT1 and p-STAT1 protein expressions decreased to levels comparable to that observed in unstimulated MDA-MB-231 breast cancer cells (**Figure 3c**, **d**). Nevertheless, breast cancer cells incubated with 0.5-5 μg/ml IFNGR1 mAb still crossed the *in vitro* BBB with a rate up to nearly 50 percent higher than their negative control counterparts (**Figure 3a**).

In previous work, we found upregulation of *GBP1* in breast cancer cells upon co-culturing with activated T lymphocytes [8]. The expression of *GBP1* is under control of IFN-γ [38, 39] and blocking the IFN-γ pathway became evident on *GBP1* expression with inhibitor doses ≥ 5 μg/ml (Supplemental figure 1a). MDA-MB-231 breast cancer cells blocked with high concentrations of IFNGR1 mAb (10 ug/ml) showed a significant decrease of transmigration through the BBB. These data are in line with the assumption that *GBP1* expression, dependent on IFN-γ, is linked with an increased motility of the cells [14, 40] resulting in penetration through the BBB. Because blocking the IFN-γ pathway did not result in a complete transmigration arrest (~80% maximal achieved inhibition), we conclude that, although necessary for an effective BBB-passage, IFN-γ pathway is not exclusively responsible for that action. Clearly, pathways

other than IFN-γ play a role in the transmigration of MDA-MB-231 breast cancer cells, which identification is thus still needed for the successful prevention of brain metastasis in patients.

TNF-α is another factor secreted by activated T lymphocytes [41]. Despite the reported increased transwell migration of breast cancer cells incubated with TNF-α [42], incubation of MDA-MB-231 breast cancer cells with IFN-γ, TNF-α or both cytokines did not enhance the transmigration ability of the cells through the *in vitro* BBB (**Figure 4a**). These results show that, in the absence of other factors, rTNF-α does not have a synergistic effect with rIFN-γ to enhance the BBB transmigration of breast cancer cells. These results indicate the importance of other molecules present in the CM of activated T lymphocytes, operative in the transmigration process of breast cancer cells. At this point, it remains to be seen if the biological TNF-α, as well as other T lymphocyte-secreted factors, increase the ability of breast cancer cell to cross the BBB.

CXCL9, also referred to as monokine induced by IFN-γ (MIG) and exclusively induced by IFN-γ [43], was the most significantly overexpressed ligand from the CXCL9, -10, -11/CXCR3 axis in the primary breast cancer samples that developed brain metastasis ($p < 0.001$; **Figure 6**). CXCL9 behaves as a homing chemokine and is constitutively present in the brain [43]. In addition, the CXCL9,-10,-11/CXCR3 signaling axis takes part in intracellular communication and plays a crucial role during the pathogenesis of various CNS diseases [44-47]. Human brain endothelium and astrocytes express and modulate IFN-γ-inducible chemokines, revealing a possible role for the invasion of the CNS by immune cells [43], and possibly by metastasized breast cancer cells as well. CXCL9 is overexpressed in human astrocytes compared to human brain endothelial cells [43], suggestive of a continuous overexpression of IFN-γ-inducible chemokines on the brain-side of the BBB and a concentration gradient supporting the development of brain metastasis. The cross-talk between tumor cells and brain cells was not considered in this work and warrants further studies.

In conclusion, we highlight the importance of T lymphocyte-mediated activation of IFN-γ signaling in breast cancer cells to facilitate their migration across an *in vitro* BBB. Our results offer directions for further investigations on the mechanisms underlying the formation of brain metastasis, which will be helpful in future therapy design for the prevention of cancer cells crossing the BBB in patients with breast cancers.

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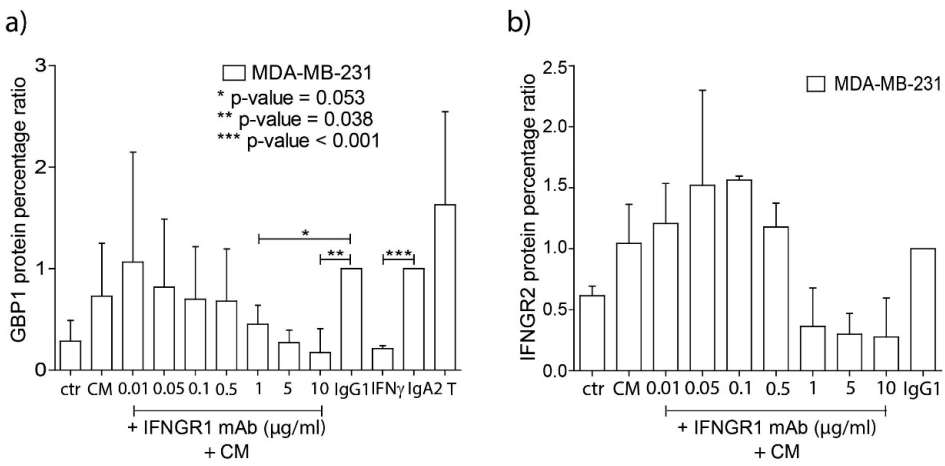
SUPPLEMENTARY INFORMATION



Supplementary Figure 1 - *GBP1* and IFNGR2 protein expressions are reduced in MDA-MB-231 breast cancer cells after IFNGR1 blocking. a) *GBP1* expression levels after blocking IFNGR1 in MDA-MB-231 breast cancer cells, in a dose dependent manner, or after neutralizing the sIFN-γ in the media of activated T lymphocytes. Significant protein reduction is evident when using concentrations of IFNGR1 ≥ 5 μg/ml or 10 μg/ml hIFN-γ mAb. The *GBP1* expression was calculated after background deduction and normalization against actin. *GBP1* level-fractions are relative to 10 μg/ml matched hIgG1 or hIgA2 isotype controls. ctr, MDA-MB-231 control cells; CM, conditioned medium; IgG1, anti hIgG1 isotype control; IFN-γ, anti-hIFN-γ-IgA; IgA2, anti hIgA2 isotype control; T, T lymphocytes. Graph is representative of two independent W.B. analyses. **b)** IFNGR2 expression levels after blocking IFNGR1 in MDA-MB-231 breast cancer cells in a dose dependent manner. Significant protein reduction is evident when using concentrations of IFNGR1 ≥ 1 μg/ml. IFNGR2 expression was calculated after background deduction and normalization against actin. IFNGR2 level-fractions are relative to 10 μg/ml matched hIgG1 isotype control. ctr, MDA-MB-231 control cells; CM, conditioned medium; IgG1, anti hIgG1 isotype control. Graph is representative of two independent W.B. analyses.

Chapter 6

Differential expression of *BOC*, *SPOCK* and *GJD3* is associated with Brain Metastasis of ER-negative Breast Cancers

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ABSTRACT

Background: Brain metastasis is considered one of the major causes of mortality in breast cancer patients. To invade the brain, tumor cells need to pass the blood-brain barrier by mechanisms that are only partially understood. In primary ER-negative breast cancers that developed brain metastases, we found that some of the differentially expressed genes play roles in the T cell response. The present study aimed to identify genes involved in the formation of brain metastasis independently from the T cell response. Method: A previously profiled primary breast cancer samples were re-analyzed. Genes that were found to be differentially expressed were confirmed by RT-PCR and by immunohistochemistry using an independent cohort of samples. Results: *BOC, SPOCK2,* and *GJD3* were overexpressed in the primary breast tumors that developed brain metastasis. *BOC* expression was successfully validated at the protein level. *SPOCK2* was validated at both mRNA and protein levels. *SPOCK2* and *GJD3* mRNA overexpression were also found to be associated with cerebral metastasis in an external online database consisting of 204 primary breast cancers. Conclusion: the overexpression of *BOC, SPOCK2*, and *GJD3* is associated with the invasion of breast cancer into the brain. Further studies to determine their specific function and potential value as brain metastasis biomarkers are required.

INTRODUCTION

Breast cancer is one of the most notorious cancers associated with brain metastasis [1]. The appearance of brain metastasis invariably heralds the terminal stage of the disease and therefore, prevention of cerebral metastases would be a major step in improving the outlook of patients suffering from breast cancer [2]. Various parameters such as early-onset breast cancer, human epidermal growth factor receptor 2 (HER-2) upregulation, high tumor grade, and estrogen receptor negativity (ER-) were identified as independent risk factors for the development of brain metastasis [3, 4]. The spread of tumor cells to the brain is a complicated process consisting of a series of subsequent and interrelated events. One essential step is the penetration of the blood-brain-barrier (BBB) by circulating tumor cells. The molecules involved and pathways used by tumor cells to pass through the BBB remain largely unknown [5]. In a previous study, we identified genes involved in the T cell response to play important roles in the trespassing of the BBB of ER- breast cancer [6]. The aim of the present study was to identify genes, other than those related to the T cell response, that are involved in the formation of brain metastasis of ER- breast cancers. The differences in gene expression between specimens of primary ER- breast cancers that were associated with systemic metastases, with and without brain involvement, were identified. Since we were interested in the genes specifically acting in the formation of brain metastases, we compared only these two groups and did not include primary tumors from patients without systemic metastases. Based on fold-of-changes (FOC) and *p*-values, 55 differentially expressed genes were identified. We validated the highest ranked genes by RT-PCR and immunohistochemistry in primary ER- breast cancer specimens of patients with metastatic disease, with or without cerebral metastases.

MATERIALS AND METHODS

Tissue sample selection

All tissue samples included and analyzed in this study were primary breast cancer samples. To identify genes involved in the formation of cerebral metastasis, we reanalyzed a previously generated RNA expression database [6]. Fresh-Frozen (FF) samples of primary breast cancers from patients with systemic metastases, with and without brain involvement, were used (n=13 and n=9, respectively). Morphological assessment, RNA expression profiling and relevant clinical data regarding this discovery cohort were provided previously [6]. Based on similar selection criteria, a validation cohort consisting of 30 FFPE primary ER- breast cancer samples was assembled: ten samples from patients who had developed brain metastasis and 20 samples from patients with metastasized cancer without brain involvement. The relevant clinical data of this cohort is provided in **Table 1**. This study was approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, The Netherlands and performed in adherence

to the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.fmwv.nl/).

Morphological assessment

Haematoxilin-Eosine (H&E) stained 5 µm-thick sections from each sample, prepared before and after sectioning for RNA isolation, were evaluated by an experienced pathologist, whereas a tumor cell area was selected for RNA isolation.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from FFPE patient material was extracted from 10-15 5 µm-thick sections (depending on the size of the sample) using the RNeasy FFPE Micro kit (Qiagen, Hilden, Germany). The quantity and quality of the isolated RNA were assessed by the 2100 Bioanalyser (Agilent Technologies, Santa Clara, USA). Samples were excluded if the yield did not reach a minimum of 100ng/µl, with a minimum template size of 150 nucleotides. Reverse transcription was performed using the RevertAid H-Minus first-strand cDNA synthesis kit (Thermo Scientific, Vilnius, Lithuania), according to the manufacturer's protocol. Quantitative real-time PCR (RT-PCR) was performed using TaqMan Master Mix (Applied Biosystems, Austin, USA) on the 7500 RT-PCR system, v.2.3 (Applied Biosystems, Foster City, CA, USA). The following commercially available exon-spanning TaqMan Gene Expression Assays (Applied Biosystems, California, USA) were used: *BOC*, exon 4-5 (Hs00264408_m1), *GJD3*/CX30.2, exon 1-1 (Hs00987388-s1), *SPOCK2*, exon 4-5 (Hs00360339_m1), HPRT1, exon 2-3 (Hs02800695_m1) and HMBS, exon 13-14 (Hs00609296_g1). HPRT1 and HMBS were used as reference genes. The relative quantification of target gene expression was performed using the 2-ΔΔCt comparative method and the threshold cycle value was defined by the point at which there was a statistically significant detectable increase in fluorescence.

Immunohistochemistry

Anti-*BOC* (1:1000, bs-12322R, Bio-Connect, Huissen, The Netherlands), Anti-*SPOCK2* (1:800, HPA044605, Merck, Darmstadt, Germany) and Anti-*GJD3* (1:100, 40-7400, ThermoFisher, Rockford, USA) antibodies were used according to the manufacture instructions. For semi-quantitative determination of protein expression, the protocol of Crowe et al. was followed [7]. The immunostained slides were scanned by Nanozoomer 2.0HT scanner (40x magnification, Hamamatsu Photonics, Hamamatsu, Japan) and four tumor regions of interest (ROIs) per slide were selected. ROIs were evaluated with a semi-quantitative IHC method [7]. ImageJ Fiji 1.52p software (USA) was used to deconvolute the selected immunostained ROI's and convert the slides into gray shades. The shades are related to the counterstained nuclei and thresholds set accordingly. Final intensity scores are given in units and are based on the mean grey values and area intensity ratios of each specific IHC staining. All four ROI's per slide were implemented in the evaluation.

Table 1 – Relevant clinical information regarding 30 primary breast cancer samples with systemic metastases from the validation cohort set.

#	Age at diagnosis (years)	ER	PR	Her2/neu	Lymph node status	Neoadjuvant Therapy	Adjuvant Therapy	Metastasis free-period (months)	Time to BM (months)	1st metastatic site	Other metastatic sites
1	32	neg	neg	neg	pos	-	CT + RT	6	11	liver	lung, brain
2	47	neg	neg	neg	pos	-	CT + RT	10	-	skin	-
3	53	neg	neg	neg	pos	-	CT + RT	48	-	skin	-
4	44	neg	neg	neg	pos	-	CT + RT	33	-	lung	-
5	42	neg	neg	neg	pos	-	CT + RT	24	-	liver	-
6	53	neg	neg	neg	pos	-	CT + RT	42	48	skin	brain
7	52	neg	neg	neg	pos	-	CT + RT	48	34	lung	brain, lung
8	59	neg	neg	neg	pos	-	CT + RT	35	-	pleura	bone, liver, lung, meninges
9	42	neg	neg	neg	pos	-	CT + RT	84	-	bone	liver
10	39	neg	neg	neg	pos	-	CT + RT	21	19	skin	brain
11	55	neg	neg	neg	neg	-	CT + RT	14	-	liver	bone
12	44	neg	neg	neg	pos	-	CT + RT	14	11	brain	meninges, pleura
13	61	neg	neg	neg	pos	-	CT + RT	26	56	bone, lung	liver, brain
14	64	neg	neg	neg	pos	-	CT + RT	7	-	lung	liver, bone
15	54	neg	neg	neg	pos	-	CT + RT	17	-	liver	skin, bone, leptomengieal
16	37	neg	neg	neg	neg	-	CT + RT	18	-	lung, liver, bone	
17	51	neg	neg	neg	neg	-	CT + RT	15	-	bone	lung, skin, liver
18	49	neg	neg	neg	neg	-	CT + RT	24	-	bone	lung
19	61	neg	neg	neg	neg	-	CT + RT	24	37	lung	brain
20	39	neg	neg	pos	neg	-	CT	51	95	lung	brain, bone
21	33	neg	neg	neg	pos	-	CT + RT	14	-	lung,	liver, adrenal
22	46	neg	neg	neg	pos	-	CT + RT	17	-	bone	bone, lung, liver
23	42	neg	neg	neg	pos	-	CT	51	-	lung	liver

Table 1 – Relevant clinical information regarding 30 primary breast cancer samples with systemic metastases from the validation cohort set. (*continued*)

#	Age at diagnosis (years)	ER	PR	Her2/neu	Lymph node status	Neoadjuvant Therapy	Adjuvant Therapy	Metastasis free-period (months)	Time to BM (months)	1st metastatic site	Other metastatic sites
24	63	neg	neg	neg	pos	-	CT + RT	42	-	bone	-
25	40	neg	neg	neg	neg	-	CT + RT	4	66	lung	brain
26	34	neg	neg	neg	pos	-	CT + RT	9	-	liver	bone
27	32	neg	neg	neg	pos	N.A.	N.A.	12	-	skin	-
28	25	neg	neg	-	neg	-	CT + RT	115	130	bone	liver, brain
29	53	neg	neg	neg	pos	-	CT + RT	15	-	pleura	-
30	33	neg	neg	neg	pos	-	CT + RT	34	-	lung	liver, adrenal

The clinical information provided includes age at diagnosis, Estrogen Receptor (ER), Progesterone Receptor (PR) and Her2/neu status of the primary tumor, lymph node status, neoadjuvant therapy, therapy post-breast cancer surgery (adjuvant therapy), the period from breast cancer diagnosis to detection of the first metastatic site (metastasis-free period in months), the period from breast cancer diagnosis to detection of brain metastasis (time to BM), 1st and other metastatic sites. 10/32 primary breast cancers from patients who developed brain metastasis are underlined. Neg = negative; pos = positive; N.A. – information not available; CT = chemotherapy; RT = radiotherapy.

Statistics

Statistical comparisons of immunostaining results and online mRNA data were performed using Mann-Whitney U test. RT-PCR data were analyzed using unpaired two-tailed student's t-test (significance levels $p < 0.05$). Data were analyzed and graphs were made by using GraphPad Prism (GraphPad Software 5.0, San Diego, CA, USA).

RESULTS

Identification of genes involved in brain metastasis of primary breast cancer.

Analysis of the expression arrays revealed that 55 genes were differentially expressed between the groups with (BM+) and without (BM–) brain metastasis ($p < 0.01$, **Figure 1a**, **b**). Out of the 55 genes, 38 were excluded because they presented a FOC < 1.5. Eight out of 17 differentially expressed genes, with a FOC > 1.5, were associated with the regulation of the immune system and were reported on in a previous study [6]. From the other genes, three were identified with either highest FOC, or most significant difference in expression level: *BOC*

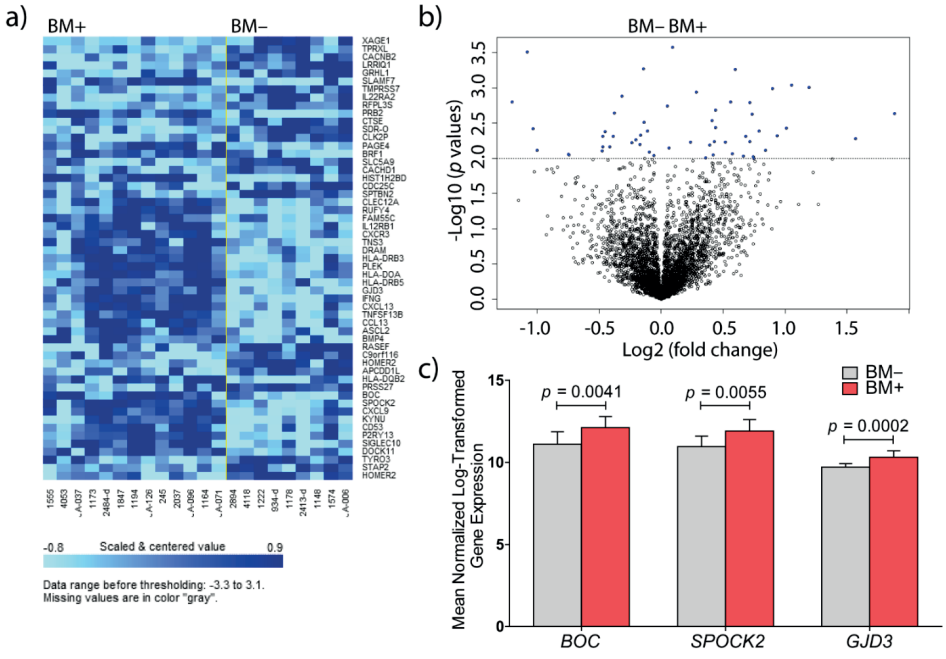


Figure 1 – Differentially expressed genes in primary breast cancer samples with or without brain metastasis. **a)** Clustered heatmap of the 55 significantly expressed genes ($p < 0{,}01$, univariate test) in fresh-frozen primary human breast cancer samples associated with the development of brain metastases (left) and breast cancer samples associated with metastasis to other organs (right); **b)** Volcano plot representation of the significantly expressed genes ($p < 0{,}01$) clustered in a); **c)** Mean normalized Log 2-transformed *BOC, SPOCK2* and *GJD3* differential expression between BM+ and BM– groups from the discovery sample set.

(FOC = 2.01; p = 0.0041); *SPOCK2* (FOC = 1.91; p = 0.006) and *GJD3* (FOC=1.52; p < 0.001). All three genes were upregulated in the BM+ group (**Figure 1c**).

Validations at mRNA and protein levels.

BOC mRNA expression and immunohistochemistry.

BOC mRNA levels were successfully evaluated in 60% of the BM+ samples and in 60% of the BM– samples. *BOC* mRNA expression levels presented a trend towards higher expression levels in the BM+ group (**Figure 2**), trend confirmed by data mining in a publicly available online dataset of 204 primary breast cancers (Supplementary Figure 1a). Therefore, mRNA levels of *BOC* were consistent with the RNA expression profiles data.

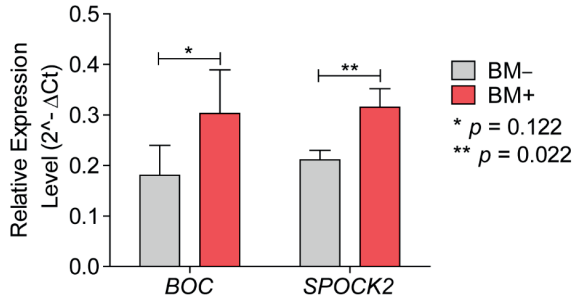


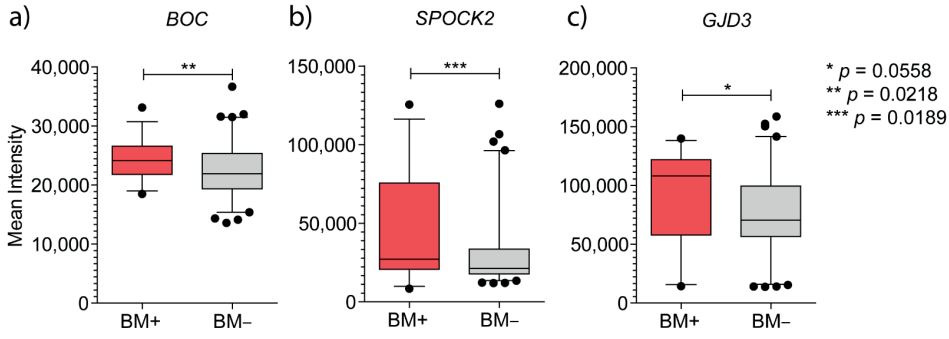
Figure 2 – Relative mRNA expression of *BOC* and *SPOCK2* in primary breast cancers of patients who developed metastasis to organs excluding brain (BM–) and including brain (BM+). Bars indicate Mean ± SD.

Immunohistochemistry was successfully carried out for 9/10 BM+ and for 20/20 BM– samples. The immunohistochemistry results of BOC revealed a significantly higher expression in the BM+ than in the BM– group (median intensity of 24,143 and 21,925 units in BM+ and BM– groups, respectively, p = 0.022; **Figure 3a**). BOC expression in both groups revealed a pale homogeneous cytoplasmic staining throughout tumor areas. Roughly 70% of BM+ samples showed higher mean intensity values than those in the BM– group (**Figures 3**, **4a**, **b**).

SPOCK2 mRNA expression and immunohistochemistry.

SPOCK2 mRNA was significantly overexpressed in the BM+ group (0.21 ± 0.02 vs. 0.32 ± 0.04, p = 0.022; **Figure 2**). The overexpression of *SPOCK2* mRNA in primary breast cancers that metastasized to the brain was further corroborated by data mining, in a publicly available online dataset of 204 primary breast cancers (Supplementary Figure 1b, p = 0.0026). Herein, the p-value becomes significant as the group narrows into exclusively brain metastasis (BM+O vs BM, Supplemental Figure 1b). The immunohistochemistry results of SPOCK2 revealed a significant differential expression level between both groups (p = 0.02; **Figures 3b**, **4c**, **d**). The mean intensity in the BM+ group was 47,500 units, with the 25-75 percentiles ranging over

54,834 units, as compared to a mean of 33,100 units and a 25-75 percentile-range of 15,750 units in the BM– group (**Figure 3b**). SPOCK2 staining displayed considerable heterogeneity in the BM+ tumor areas (**Figure 4c**).



	BOC		SPOCK2		GJD3	
ROI's (total *n*)	36	80	32	80	36	80
Median	24,143	21,925	27,130	21,293	108,042	70,443
Mean	24,183	22,518	47,500	33,100	89,636	78,332

Figure 3 – Box-whisker plots of a) *BOC*-, b) *SPOCK2*- and c) *GJD3*-IHC mean intensities, with tabular statistics summary for both BM+ and BM– groups of ER- primary breast cancers. The lowest and highest boundaries of the box represent the 25 and 75 percentiles, respectively. The solid line across the box indicates the median value. Error bars indicate the 5-95 percentile.

GJD3 mRNA expression and immunohistochemistry.

All quality-approved 30 breast cancer samples, measured with different dilutions, delivered a Ct value below the threshold of detection of the RT-PCR method used. Nevertheless, the overexpression of *GJD3* mRNA in primary breast cancers that developed brain metastasis was successfully confirmed in a publicly available online dataset of 204 primary breast cancers, when considering only breast cancers which metastasized exclusively to the brain (Supplementary Figure 1c, $p = 0.0149$). There was a strong heterogeneous cytoplasmic staining, as measured by immunohistochemistry, ranging from strong to weak (**Figure 4e**, **f**), with a median difference of 37,599 units between BM+ and BM– groups (108,042 and 70,443 units, respectively). Mean GJD3 protein expression levels, between the BM+ and BM– groups, were 89,636 vs. 78,332, respectively (FOC = 1.14), with a higher trend towards the BM+ group, though not significant ($p = 0.06$; **Figure 3c**).

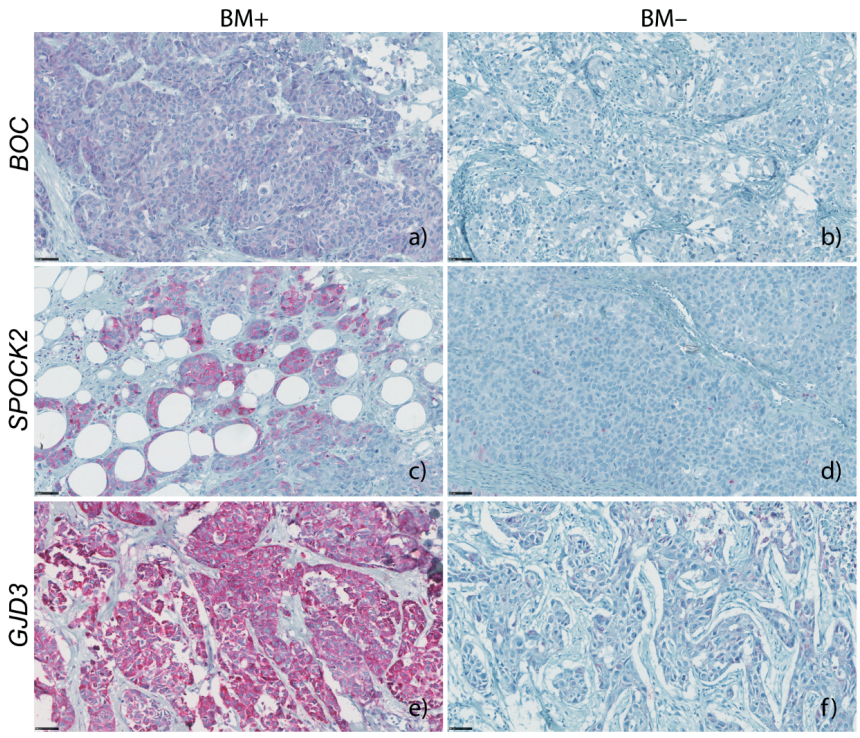


Figure 4 – Representative *BOC*, *SPOCK2* and *GJD3* positive and negative stainings of FFPE primary breast cancer samples. On the left column (a), c) and e)) are representative positive stainings from BM+ group; on the right column (b), d) and f)), representative negative stainings from BM– group. Scale bars = 50 µm.

DISCUSSION

In the current study, we found that the expression of *BOC*, *SPOCK2* and *GJD3* is upregulated in the ER- primary breast cancer samples of patients who developed brain metastases.

The immunohistochemistry results for BOC confirmed the results of the gene expression profiles: BOC was found to be a significantly overexpressed protein in our validation cohort, associated with cerebral metastasis of ER- breast cancer (**Figure 3a**) and has not been reported previously. *BOC* mRNA levels in the independent cohort (**Figures 2**) presented a trend towards overexpression in the breast cancers that metastasized to the brain, which was corroborated, in a similar fashion, in the online dataset of 204 primary breast cancers ($p > 0.05$; Supplemental Figure 1a, [8]). This lack of significance in the *BOC* mRNA levels between breast cancers with and without brain metastasis might be reasoned by the combination of different factors: a narrow low intensity level-difference between both groups (**Figures 3a**, **4a** vs **b**) and a low number of successfully evaluated mRNA samples, from a group of primary breast cancers which lack brain metastases exclusivity (**Table 1**). *BOC* is a member of the Ig/FNIII repeat family of

receptor-like proteins [9]. *BOC* is an essential component of the Hedgehog (Hh) pathway that promotes Sonic HH (SHH) signaling [10, 11]. SHH is essential for the normal development of the nervous system [12] and plays a role in the maintenance and regeneration of adult tissues [13]. All mammalian SHH proteins interact with BOC and CDO, another component of SSH signaling [14]. Abnormal SHH signaling is not only associated with developmental defects, but also with cancer [15, 16]. Since CDO and *BOC* join the cadherin-β-catenin complexes to contribute to cell signaling at sites of cell-cell adhesion [17], it is tempting to speculate that signaling activities from these complexes are involved in cytoskeletal structural-changes, analogous to the formation of protrusions enabled by Cdon in zebrafish neural crest migration [18]. In chick embryos, *BOC* and CDO were found spatially linked in long filopodial extensions that need SHH signaling [19]. The relation between *BOC* expression and metastasis has been described in non-small-cell lung cancer and cancers of the pancreas and prostate. In addition, there is data supporting the contribution of SHH signaling to increased proliferation, invasion, and migration of breast cancer cells [20-22]. Inhibition of SHH signaling was shown to reduce these metastatic traits [22-24].

SPOCK2 was significantly upregulated in breast cancer cells that metastasized to the brain. The expression of *SPOCK2* in this context was successfully validated both at mRNA (**Figure 2**) and protein levels (**Figures 3b**, **4c**). Moreover, we were able to show a strong association of *SPOCK2* expression at the mRNA level in breast cancers with exclusive cerebral metastasis in an independent online cohort of 204 primary breast cancers (Supplemental Figure 1b). *SPOCK2* is a member of the Ca2+-binding proteoglycan family and binds with glycosaminoglycans to form part of the extracellular matrix (ECM) [25]. Cross-talk with ECM proteins exists throughout development and the misexpression of matrix molecules is a cause of many developmental defects [26]. The tumor microenvironment plays an essential role in cell proliferation and differentiation, and is key to the invasive and migratory capacity of tumor cells. A splice variant of the SPOCK3 gene has been found to inhibit the expression of matrix metalloproteinase 2 (MMP-2), which is mediated by membrane-type (MT)-MMPs [27]. Activated MMP-2 was observed in various tumor tissues, suggestive of pro-MMP-2 activator(s) present in the tumor microenvironment [28, 29]. MT1-MMP, an activator of pro-MMP-2 expressed on the surface of tumor cells, closely correlates with the invasive phenotype of human tumors [30, 31]. All members of the SPOCK family, with the exception of *SPOCK2*, interfere with pro-MMP-2 activation mediated by MT1-MMP or MT3-MMP [27]. *SPOCK2* abolishes the inactivation of MT-MMPs by other SPOCK family members, causing ECM remodeling and allowing the migration of glioma cells expressing MT1-MMP [32]. Therefore, it is tempting to speculate that the expression of *SPOCK2* results in changes of the microenvironment that facilitate tumor cell migration. How this would translate in the specific passage through the BBB of the tumor cells remains to be elucidated.

Based on our previous RNA expression data, *GJD3* (also termed connexin 30.2 or Cx30.2) was identified as the most significant gene (p = 0.0002, permutation p-value ≤ 0.001; **Figure 1**) out of the 55 significantly differentially expressed genes, albeit with a narrow FOC between BM+ and BM– groups. Further analysis on the GJD3 protein status in 30 additional breast cancer samples showed a higher trend of GJD3 protein overexpression in the BM+ group. More than 50 percent of the BM+ breast cancer samples presented a higher mean intensity values than those detected in the BM– samples (**Figure 4e** vs **f**). Unfortunately, validation of *GJD3* at the mRNA expression level was not successful. This may be due to technical limitations: RNA from FFPE tissues may miss out in quantitative RT-PCR assays [33]. In addition, there may be significantly different median Ct values between RNAs derived from fresh-frozen samples, as compared to FFPE samples [34]. This is further supported by the significantly overexpressed *GJD3* mRNA levels in the fresh-frozen breast cancer samples that metastasize to the brain in the independent online cohort of 204 primary breast cancers (Supplemental Figure 1c). GJD3 is a protein member of the large family of connexins apt to form functional intercellular channels linking the cytoplasm of two adjacent cells, and is localized to the plasma membrane, in areas of cell-cell contact [35, 36]. Connexins are the sole proteins required for the assembly of gap-junctions [37]; this way of communication is thought to be essential to coordinate tissue growth, development, and physiological activities [36, 38]. Differential regulation of connexin isotypes is fairly common and in some cancers connexins may display tumor suppressor potential [39], or oncogenic activity [40, 41]. Dysfunctional connexins affect the growth control of cells, resulting in the rise of tumors [42]. Although deregulation of connexins and their gap-junctions has been implicated in breast carcinogenesis and tumor progression, specific correlations with tumor progression have only incidentally been reported [43, 44], restricted to connexin isotypes from sub-family groups I [45, 46], II [47] and III [48]. *GJD3* was never before associated with breast cancer and it was believed that ancient connexins (Groups IV and V) are not expressed in cancers in the first place [42]. The role of *GJD3* expression in the formation of cerebral seeding of ER- breast cancer requires, at this point, further ratification.

Only a few patients developed brain metastasis shortly after the diagnosis of the primary tumor (2/10 in < 12 months or 3/10 in <24 months) (**Table 1**). Therefore, the association of high expression of one or more of the three identified genes with early metastatic spread to the brain could not be calculated. The recent guidelines to treat patients with breast cancer include the use of neoadjuvant treatments. As a result, the availability of treatment-naïve primary breast cancer samples is very limited. For future aspects, it is important to investigate the effect of the various types of neoadjuvant treatments on the expression of *BOC*, *SPOCK2*, and *GJD3*. In addition, identification of genes involved with brain metastasis of patients who received neoadjuvant treatment is still needed.

CONCLUSION

The ability to identify breast cancer patients at risk for developing brain metastases may head to new prophylactic intervention that will diminish morbidity and mortality. Here we found the genes *BOC*, *SPOCK2* and *GJD3* to be associated with metastatic potential of ER- breast cancer. These genes warrant further analysis as predictive biomarkers for the risk of brain metastases in patients with ER- breast cancers, and as targets for preventive strategies.

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Chapter 7

T Lymphocytes Induce the Expression of Coronin-1A in Various types of Cancer cells Enabling the Transmigration Through a Blood-Brain-Barrier model.

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Submitted

ABSTRACT

In previous work, we found a prominent role of T lymphocytes in the development of brain metastases of ER-negative breast cancers. The present study, investigates if T lymphocytes affect the transmigration through a blood-brain barrier (BBB) model of other cancer cells lineages (lung and prostate cancers and melanoma), and aims to identify common proteins involved in the transmigration through the BBB.

All (n=9) but one cancer cell line (H2030BrM3) showed increased transmigration through the BBB following co-culture with activated T lymphocytes. Using liquid chromatography-mass spectrometry (LC-MS), Coronin-1A was shown to be upregulated in all breast and prostate cancer cells and melanomas with increased BBB transmigration, following co-culture with activated T lymphocytes. *CORO1A* knockdown in breast cancer cell lines significantly impaired transmigration rates ($p < 0.0001$). Coronin-1A overexpression was validated at the protein and mRNA levels, in tissue samples of primary breast cancers that had metastasized to brain, and *CORO1A* upregulation was confirmed in tumor samples derived from patients with brain metastasis, in a publicly available mRNA expression database from 204 primary breast cancers.

This is the first report on a commonly expressed protein that plays a role in the formation of brain metastasis from various cancers.

INTRODUCTION

The incidence of brain metastases is increasing and outnumbers that of primary brain tumors [1]. Up to 30% of patients suffering from common cancer will develop brain metastasis [2, 3]. The most common primary tumor types to metastasize to the brain are carcinomas of the lung, breast, kidney, GI and urogenital tracts, and melanomas. Interestingly, some malignant tumors, like prostate cancer and skin cancers other than melanomas, rarely seed to brain. Incidences of brain metastases differ considerably between tumors of different origins. While cerebral metastasis of lung cancer is relatively frequent (incidence rates of 16-20%) and occurs relatively early in the course of the disease, brain seeding of breast cancer is less common (incidence rate of 5% to 21% in autopsy series [4]) and is usually a late complication [2, 5]. In all instances, the rise of brain metastases is a serious complication that significantly worsens the clinical outlook.

In order to identify mechanisms used by tumor cells to pass the blood-brain barrier (BBB), we previously compared gene expression patterns of breast cancers from patients with and without cerebral metastases. The differentially expressed genes pointed to a promoting role of the T cell response in the development of brain metastases [6]. Subsequent *in vitro* studies, in which an artificial BBB model was used, followed by liquid chromatography-mass spectrometry (LC-MS) analysis, showed changes in the proteome of the breast cancer cells following co-culturing with activated T lymphocytes. We found upregulation of GBP1 in the cancer cells that showed an increased ability to cross the BBB. Importantly, we also found GBP1 overexpression in the primary tumor samples from patients with breast cancer brain metastases [6]. The findings were confirmed using a mouse model for brain metastasis. In these *in vivo* experiments, we found that mice injected with breast cancer cells, which were co-cultured with activated T lymphocytes, developed brain metastasis more readily than those who were injected with breast cancer without prior co-culturing with T lymphocytes [6].

The aim of this study was to investigate if the promoting effect of T lymphocytes, on the development of brain metastasis, also takes place in cancers other than breast cancer. To that aim, we examined cells from cancers known to develop brain metastasis, like lung- and breast cancers and melanomas. In addition, we used cells derived from prostate cancer – a tumor type that rarely metastasizes to the brain. The various cancer cells were co-cultured with activated T lymphocytes, and their ability to cross the BBB was mimicked by using our *in vitro* BBB model. The effect of co-culturing with T lymphocytes was measured using LC-MS. The protein of interest was validated at the mRNA and protein levels, and its function was confirmed by gene silencing experiments.

MATERIALS AND METHODS

Cell line culture procedure

Human astrocytes (ScienCell) were cultured in astrocyte medium (AM, ScienCell) supplemented with 1% astrocyte growth factors (AGS, ScienCell), 2% fetal bovine serum (FBS, ScienCell) and 1% penicillin and streptomycin (P/S, ScienCell). Human umbilical vein endothelial cells (HUVECs, ScienCell) were cultured in endothelial cell medium (ECM, ScienCell) supplemented with 1% endothelial cell growth factors (ECGS, ScienCell), 5% FBS and 1% P/S. Human astrocytes and HUVECs were used between passage 2 and 5. Three breast cancer, three lung cancer, two melanoma and two prostate cancer cell lines were used (**Table 1**). Breast and lung cancer cell lines were cultured in RPMI-1640 with L-glutamine (BioWhittaker®) medium supplemented with 10% FBS and 1% P/S, and 1% gentamicin was added to the breast cancer cells. Melanoma's and prostate cancer cell lines were cultured in DMEM (Dulbecco's Modified Eagle's Medium) with 4.5 g/L Glucose and L-glutamine (BioWhittaker®), supplemented with 10% FBS and 1% P/S. All cell lines we cultured at 37°C, in a humidified incubator with 5% CO_2.

Table 1 – Cell-specifications of cell lines used in this study.

Cell Line	Cancer Type	Metastatic propensity/observations
MDA-MB-231	Breast cancer	Metastasizes to the brain and other organs. Parental cell line of MDA-MB-231-B2M2 [7].
MDA-MB-231-B2M2		Specifically metastasizes to the brain* [8].
SK-BR-7		No registration of metastasis capability [8].
H2030	Lung cancer	Metastasizes to the brain and lymph nodes. Parental cell line of H2030BrM3* [9]
H2030BrM3		Specifically metastasizes to the brain* [10].
H1299		Metastasizes to lymph nodes [11, 12]
H1-DL2	Melanoma	Brainmetastasis-derived melanoma.** [13, 14].
SK-MEL-28		Metastatic human skin melanoma cell line [15].
Du 145	Prostate cancer	Human prostate cancer cell line that grew out of brain tumor tissue [16].
PC-3		Human prostate adenocarcinoma which metastasizes to the bone [17].

* lung cell lines purchased from Memorial Sloan Kettering Cancer Center; ** kind gift from Prof. F. Thorsen, University of Bergen, Bergen, Norway

Activation and Expansion of T lymphocytes

Human bulk T lymphocytes, isolated from healthy donors, were expanded on irradiated allogenic feeder cells, consisting of a mixture of 40-Gy gamma-irradiated peripheral blood mononuclear cells, Epstein-Barr Virus (EBV)-transformed B-lymphoblast cell lines BSM (also known as GM06821, GLC^{neg}/$HLA\text{-}A2^{pos}$) and APD (also known as GM06817, EAD^{neg}/$HLA\text{-}A1^{pos}$) cells. Cells were cultured in RPMI-Hepes (Gibco) with 1% L-glutamine (BioWhittaker®),

1% P/S and 6% human serum albumin (HSA), in combination with Phytohemagglutinin-L (PHA-L, Sigma) and IL-2 in a 96-well flat-bottomed plate for 6-7 days at 37°C in a humidified incubator with 5% CO_2. After incubation, cells were harvested, centrifuged, and cultured in RPMI-Hepes medium supplemented with 6% HSA and IL-2 (360 IU/mL, Chiron, Amsterdam, The Netherlands).

Construction of the in vitro blood-brain barrier (BBB) assays

The specifications of our BBB *in vitro* model were published previously [6, 18]. HUVECs were co-cultured with human astrocytes on the opposite sides of a transwell insert [18]. Inserts with pore sizes of 3.0 µm were used for the breast cancer cells, while inserts with pore sizes of 5.0 µm were used for all other cells.

Functional studies using the in vitro BBB

To investigate the influence of T lymphocytes on the ability of the various cancer cells to cross the BBB, three parallel sets of experiments were conducted: 1) cancer cells were co-cultured with activated T lymphocytes, in a 3:1 ratio, to investigate the effects of direct cell-cell contacts; 2) cancer cells were cultured with cell-free conditioned media (CM) from activated T lymphocytes, in a 3:1 volume-ratio, to investigate the effects of activated T lymphocyte-derived soluble factors; and 3) cancer cells cultured without the addition of T lymphocytes or CM were used as controls. Co-culturing was performed for five consecutive days, at 37°C, 5% CO_2. T lymphocytes were removed from the adherent layer of cancer cells by three consecutive PBS washing steps. The cancer cells were trypsinized and labelled with five µM CFMDA cell tracker green (Invitrogen), in serum-free medium, for 45 minutes, at 37°C. The cancer cells were collected, washed in PBS and re-suspended in conditioned media, as described previously [6]. A total of ~$1.5x10^5$ cancer cells were seeded in the upper chamber of the BBB model and incubated overnight at 37°C with 5% CO_2. After incubation, inserts were removed and cancer cells which passed through the BBB and adhered to the bottom of the 24-well chamber were nuclear-stained with Hoechst 33342 (Invitrogen) and detected by confocal microscopy (schematic experimental assay has been previously provided [19]).

Confocal images were obtained using a Zeiss LSM510 confocal laser-scanning microscope equipped with a 488 nm argon-laser, a 405 nm Diode and a Plan-Neofluar 20× objective with NA 0.5 (Zeiss, Oberkochen, Germany). Images were made with a pixel size of 1 µm. Pictures were submitted to ImageJ software version 1.49S (www.fiji.sc) and used to calculate the number of cells/mm^2.

Cell pellet preparation for LC-MS and RT-PCR measurements

Co-culturing experiments were repeated twice for proteomics and genomics measurements. Following the first five days of the co-culture procedure, T lymphocytes were removed by

three consecutive PBS-wash steps. Cancer cells were trypsinized, collected into sterile tubes and washed with PBS six consecutive times, to remove serum traces. During the last wash step, cells were collected in 1.5 ml Eppendorf tubes, centrifuged for five minutes at 1000 g, the supernatant was removed and cell pellets were snap-frozen in dry ice and stored at -80˚C until further use.

Protein digestion and proteomics measurements

Frozen cell pellets were re-suspended in 50 µL of 0.1% RapiGest buffer (Waters Corporation, Milford, MA) and cells were disrupted by external sonification for one minute at 70% amplitude at a maximum temperature of 25˚C (Branson Ultrasonics, Danbury, CT). A reducing agent, 0.5 M DL-Dithiothreitol (Sigma-Aldrich), was added to a final concentration of 5 mM and the samples were incubated at 60ºC for 30 minutes. Iodoacetamide (Sigma-Aldrich) alkylating reagent was added to a final concentration of 15 mM and samples were kept in the dark for 30 minutes. To each sample, 0.5 µg trypsin gold, mass spectrometry grade (Promega, Madison, WI), was added and incubated overnight at 37 ˚C for protein digestion. To stop the reaction, the pH of the solution was adjusted to < 2 with trifluoroacetic acid (TFA, Sigma-Aldrich) to a final percentage of 0.5%. Protein samples were incubated at 37ºC for 30 to 45 minutes. Subsequently, these samples were spun at maximum speed for 40 minutes at 4ºC. The supernatant was transferred into sterile LC-vials to be measured with LC-MS, as previously described [6].

Proteomics data analysis

Samples were measured on LC-MS and raw data files from the Orbitrap Fusion mass spectrometer were treated as described before [6]. Scaffold software (version 4.8.3, Portland, OR) was used to summarize and filter MS/MS based peptides and protein identifications, as previously characterized [6]. Identified proteins, of each type of cancer cells that were co-cultured with activated T lymphocytes, were compared to those of cancer cells cultured without activated T lymphocytes. Comparison was done based on a two-sample t test, and the *p*-values of all proteins were calculated and corrected for multiple testing, using Benjamini-Hochberg false-discovery rate procedure. A protein was considered differentially expressed if $p < 0.05$ and all cell lines within one group showed the same direction of expression.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [20] partner repository with the dataset identifier PXD027123 and 10.6019/PXD027123.

Protein educational selection

Overexpressed proteins, in all cancer types investigated in this study, were attained as above described. Protein of interest was selected following analysis of overlapped proteins among

cancer types, following co-culture with activated T lymphocytes, which were shown to acquire an enhanced transmigration rate through the transwell membrane of an *in vitro* BBB model (**Figure 2a**), and which function disclosed a role in migration and/or cell locomotion (Table S3).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from frozen cell pellets using the RNeasy Plus Micro kit (Qiagen, Hilden, Germany). The quantity and quality of the isolated RNA were assessed (2100 Bioanalyser, Agilent Technologies, Santa Clara, USA). Samples were excluded if the yield did not reach a minimum of 100 ng/µl. Reverse transcription was performed using the RevertAid H-Minus first-strand cDNA synthesis kit (Thermo Scientific, Vilnius, Lithuania) according to the manufacturer's protocol. Quantitative real-time PCR (RT-PCR) was performed using TaqMan Master Mix (Applied Biosystems, Austin, USA) on the 7500 RT-PCR system, v.2.3 (Applied Biosystems, Foster City, USA). The following commercially available exon-spanning TaqMan Gene Expression Assays (Applied Biosystems, California, USA) were used: *CORO1A* (Hs00200039_m1), *HPRT1*, exon 2-3 (Hs02800695_m1) and *HMBS*, exon 13-14 (Hs00609296_g1). *HPRT1* and *HMBS* were used as reference genes. The relative quantification of target gene expression was performed using the $2^{-\Delta\Delta Ct}$ comparative method and the threshold cycle value was defined by the point at which there was a statistically significant detectable increase in fluorescence.

siRNA Knockdown

A mix of four siRNA sequences that target *CORO1A* mRNA (5'-CCUCAAGGAUGGCUACGUA-3', 5'-CCAUGACAGUGCCUCGAAA-3', 5'-CCGCAAAGGCACUGUCGUA-3' and 5'-GUGCAGUGUUCGUGUCGGA-3') and scrambled non-targeting siRNAs (5'-UGGUUUACAUGUCGACUAA-3', 5'-UGGUUUACAUGUUGUGUGA -3', 5'-UGGUUUACAUGUUUUCUGA-3' and 5'-UGGUUUACAUGUUUUCCUA-3') were obtained from Dharmacon (GE health care, Netherland). Breast cancer cells were transfected following the manufacture's protocol (using Lipofectamine® RNAiMAX Reagent, Invitrogen). The efficiency of *CORO1A* knockdown was assessed after 24 and 72 hours, at mRNA, protein and functional levels.

Immunofluorescence microscopy

Breast cancer cells were trypsinized and ~$5x10^4$ cells were fixed in formaldehyde for 15 minutes and kept in ethanol 100% until cytocentrifuged and stained with an antibody against Coronin-1A (1:250, sc-100925, Santa Cruz Biotechnology). Cells from two independent experiments were analyzed by confocal microscopy using a LSM700 Zeiss microscope (Zeiss, Oberkochen, Germany). Images were captured with a 20X lens and a 4x4 tile was made from each slide.

Each image was divided in 16 individual fields of view from which signal-intensity as a mean ± SD were determined using ImageJ software [21].

Tissue sample selection

Primary ER- breast cancer samples from patients who developed brain metastasis (n = 23) and from patients who developed metastases to other organs, excluding brain metastases (n = 29), were collected. None of the breast cancer patients received neoadjuvant therapy (clinical information has been previously provided in [6] - n=20 samples from Validation Set and n=32 in [22], respectively). This study was approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, The Netherlands (MEC 02·953) and performed in adherence to the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.fmwv.nl/).

Immunohistochemistry

Anti-CORO1A (1:2000, sc-100925, Santa Cruz Biotechnology) antibody was used according to the manufacturer's instructions. All IHC slides were scanned on a Nanozoomer 2.0HT scanner (40x magnification, Hamamatsu Photonics, Japan) and four tumor regions of interest (ROI's), per slide, were randomly selected. ROI's were evaluated with a semi-quantitative IHC method [23]. In short, ImageJ Fiji 1.52p software (USA) was used to deconvolute the selected immunostained ROI's and convert the slides into gray shades. Finally, intensity scores are based on the mean grey value per ROI's and total area intensity ratio of each specific IHC Coronin-1A staining. All four ROI's per slide were implemented in the evaluation.

Publicly-available mRNA expression data assessment

Validation of mRNA *CORO1A* levels was performed using a primary breast cancer dataset in the publicly available repository Human Cancer Metastasis Database (HCMDB; http://hcmdb.i-sanger.com/index). HCMDB consists of 124 previously published transcriptome datasets collected from Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA). RNA expression status of *CORO1A* was evaluated in 204 GEO primary breast cancer samples available in the National Centre for Biotechnology Information (NCBI) under GEO accession number GSE12276. The Log2 Median-Centered ratio was used to evaluate the differential expression of *CORO1A* in primary breast cancer of patients who developed brain metastasis, compared to patients who developed metastasis to other organs.

RESULTS

Activated T lymphocytes facilitate BBB transmigration of various cancer cells.

All cell lines, except for one lung cancer cell line, showed enhanced passage through the BBB model upon five days of co-culturing with activated T lymphocytes, or with medium conditioned by activated T lymphocytes (**Figure 1a-d**). The strongest effects of T lymphocytes were monitored on MDA-MB-231 and SK-BR-7 cells (p = 10^{-4} and p = $4x10^{-5}$, respectively;

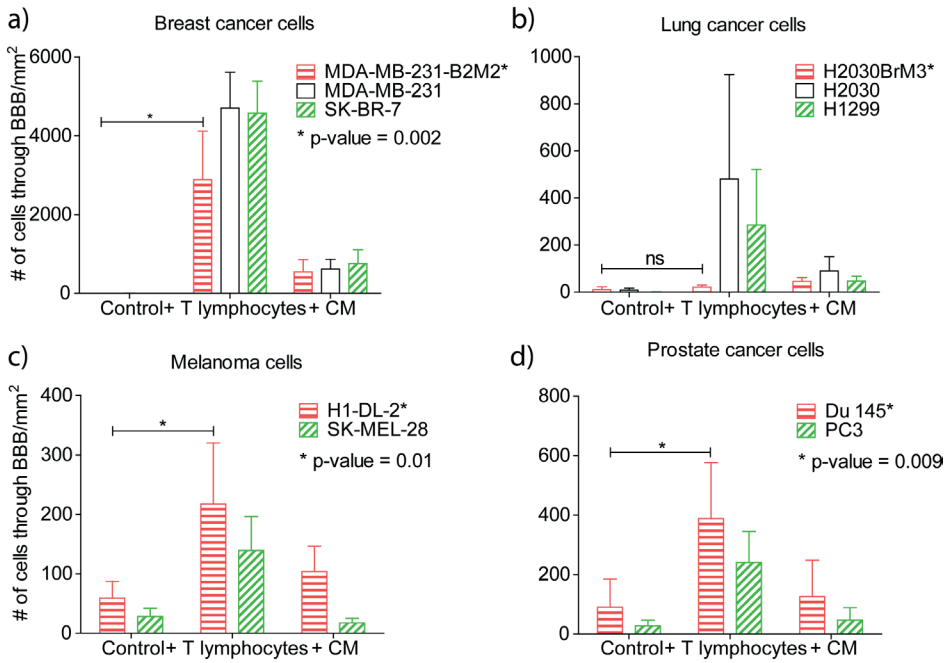

Figure 1 – T lymphocyte co-culture enhances the *in vitro* BBB transmigration ability of different types of cancer cell lines. a) Quantification of successfully BBB-passaged breast cancer cells, **b)** lung cancer cells, **c)** melanomas and **d)** prostate cancer cells. Control = matched cancer cells without pre-treatment; + T lymphocytes = cancer cells following five days of co-culture with activated T lymphocytes; + CM = cancer cells following five days incubation with cell-free media conditioned by activated T lymphocytes. Data are from two independent experiments with three replicates each. For abbreviated cell lines see **Table 1**. * Cell lines derived from cancers with predilection for brain.

Figure 1a, Table S1). Only the H2030-BrM3 lung cancer cell line remained unaffected by the activated T lymphocytes (p = 0.11; **Figure 1b**). The melanoma and prostate cancer cell lines, derived from tumors with a predilection for the brain, showed higher BBB transmigration before and after T lymphocyte co-culture, when compared to tissue-matched cell lines without brain metastasis.

Coronin-1A protein is overexpressed by cancer cells that display increased BBB transmigration capacity following T lymphocyte co-culture.

To identify common functional changes in cancer cells that showed increased transmigration abilities following co-culture with activated T lymphocytes, the proteome of all cancer cells before and after co-culture with activated T lymphocytes was measured. Proteomics comparisons of all cancer cell lines, before and after co-culturing with T lymphocytes, are summarized in Table S2.

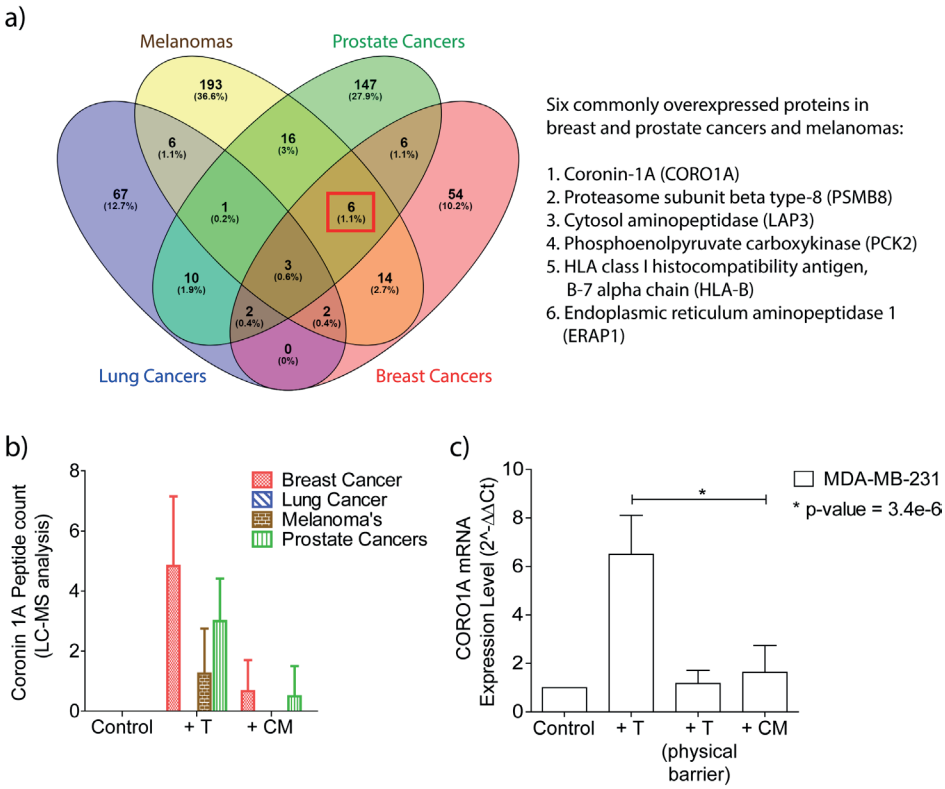

Figure 2 – Coronin-1A is highly regulated in breast cancer cells after contact with T lymphocytes. a) Venn diagram [24] illustrating the overlapped proteins, measured by LC-MS, among all overexpressed proteins in all cancer types after co-culture with activated T lymphocytes (breast cancer cells n = 87, lung cancer cells n = 91, melanomas n = 241 and prostate cancer cells n = 191); commonly overexpressed proteins in cancer cells with enhanced transmigration ratio following co-culture with activated T lymphocytes (n = 6, red square, common to breast and prostate cancers and melanomas). **b)** Coronin-1A peptide counts before and after five days co-culture of the various cell lines with activated T lymphocytes, or incubation with CM. **c)** *CORO1A* mRNA expression levels in MDA-MB-231 cells before and after 24h contact with activated T lymphocytes; or indirect co-culture with activated T lymphocytes; or after incubation with CM of activated T lymphocytes. Control = matched cancer cells without pre-treatment; + T = cancer cells after five days of co-culture with activated T lymphocytes; + CM = cancer cells after five days incubation with cell-free media conditioned by activated T lymphocytes.

Because breast cancer cells showed the highest transmigration abilities following co-culture with activated T lymphocytes, their proteome analysis was prioritized over the proteome analysis of the other cancer cells. Eighty-seven proteins were significantly overexpressed in all breast cancer cells following co-culture with T lymphocytes (Table S3). Further analysis on the overexpressed proteins, common to the cancer types which transmigration through the BBB model was enhanced following co-culture with activated T lymphocytes (**Figure 2a**), and educated selection on the different protein functions, lead to the identification of Coronin-1A (p = 0.002, Table S3). Specifically, all cancer cells, except lung cancers, showed an overexpression of Coronin-1A after co-culturing with T lymphocytes (**Figure 2a, b**, Table S4).

Coronin-1A protein is significantly overexpressed in breast cancer cells following direct contact with activated T lymphocytes.

The number of Coronin-1A peptides was significantly higher after co-culture with T lymphocytes, as compared to those following incubation with CM (**Figure 2b**, Table S4). Therefore, we postulated that direct contact with activated T lymphocytes would enhance the expression of *CORO1A* mRNA levels. The RT-PCR results confirmed this hypothesis (**Figure 2b**); direct contact between T lymphocytes and MDA-MB-231 cells, brain-trophic breast cancer cells with the strongest transmigration effect following co-culture with activated T lymphocytes, increased the expression of *CORO1A* mRNA significantly ($p < 10^{-5}$). Furthermore, *CORO1A* mRNA levels of the MDA-MB-231 breast cancer cells, in the presence of a 0.4 μm pore membrane physical barrier during co-culture with activated T lymphocytes, was not significantly different from the *CORO1A* mRNA levels of MDA-MB-231 following CM incubation, or control cells (**Figure 2b**).

CORO1A knockdown in breast cancer cells impairs BBB transmigration ability

To validate the function of Coronin-1A in breast cancer cells, RNA knockdown (siRNA) experiments were conducted prior to BBB functional studies. Following silencing, *CORO1A* mRNA levels in breast cancer cells were successfully reduced in all three breast cancer cell lines (**Figure 3a**). *CORO1A* knockdown significantly reduced (50-60% reduction) the capacity of both brain-trophic breast cancer cell lines to transmigrate through the *in vitro* BBB ($p < 0.001$; **Figure 3b**). Silencing *CORO1A in* SK-BR-7 cells resulted in a reduced ability to cross the BBB, however not significantly.

Immunofluorescence analysis (IF) of Coronin-1A expression levels in breast cancer cells, after T lymphocyte co-culture, revealed overexpression of Coronin-1A in all three breast cancer cell lines, although the increased expression for the brain-specific MDA-MB-231-B2M2 cells remained marginal (**Figure 3c**, **d**). *CORO1A* siRNA IF intensity levels were reduced to half the intensity levels of matched control cells, for all three breast cancer types (**Figure 3c**, **e**).

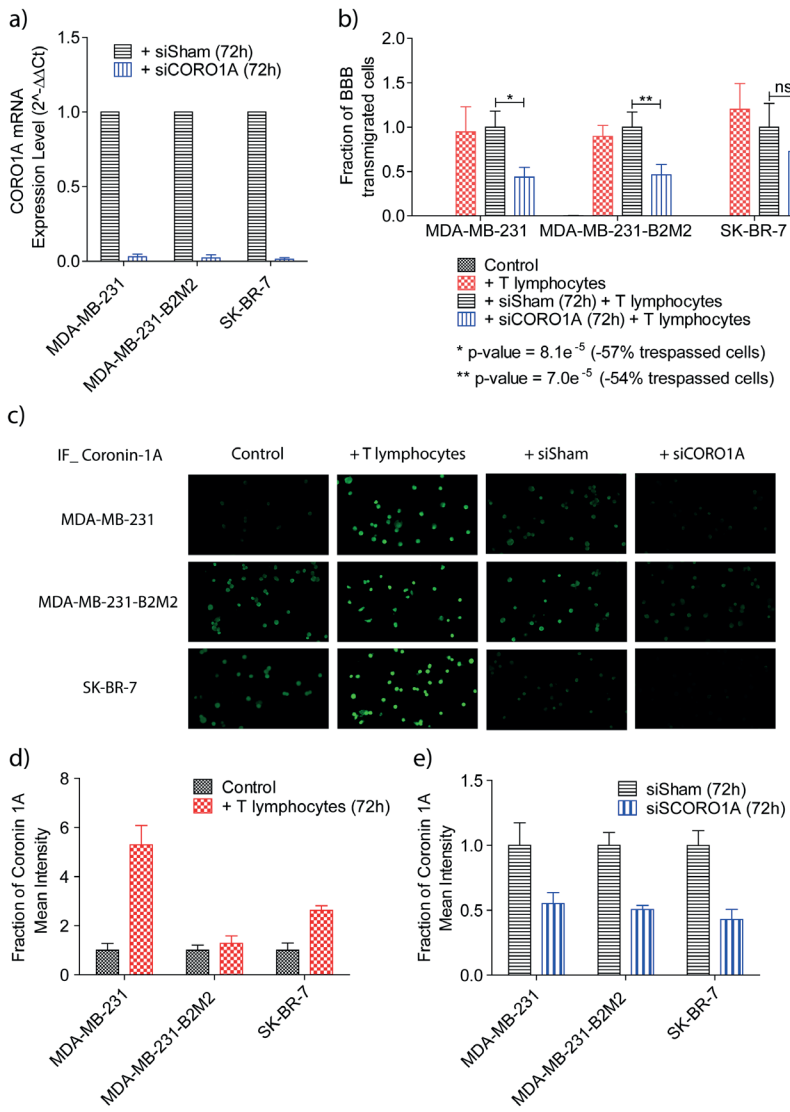

Figure 3 – 72h *CORO1A* siRNA in three breast cancer cell lines impairs BBB transmigration extent. a) *CORO1A* mRNA fold of change levels in the three breast cancer cell lines before and after 72h *CORO1A* siRNA. Data are of two independent experiments. **b)** Fraction of successfully migrated breast cancer cells before and after 72h incubation with siRNA *CORO1A* or non-targeting siSham, and subsequent co-culture with activated T lymphocytes. The extent of BBB crossing was significantly reduced for the cancer cell lines MDA-MB-231 and MDA-MB-231-B2M2. For the SK-BR-7 cells, migration was not significantly reduced. Data are of two independent experiments of three replicates each. **c)** Coronin-1A immunofluorescence (IF) (from left to right) of all breast cancer cells before (control) and following co-culture with activated T lymphocytes, following siSham incubation or si*CORO1A* knockdown. **d)** Coronin-1A-IF FOC intensity-levels in all three breast cancer cell lines before and after 72h of co-culture with activated T lymphocytes. Data representative of three independent experiments. **e)** Coronin-1A protein fraction levels before and after 72h incubation with non-targeting siSham or siRNA *CORO1A*. Data representative of three independent experiments.

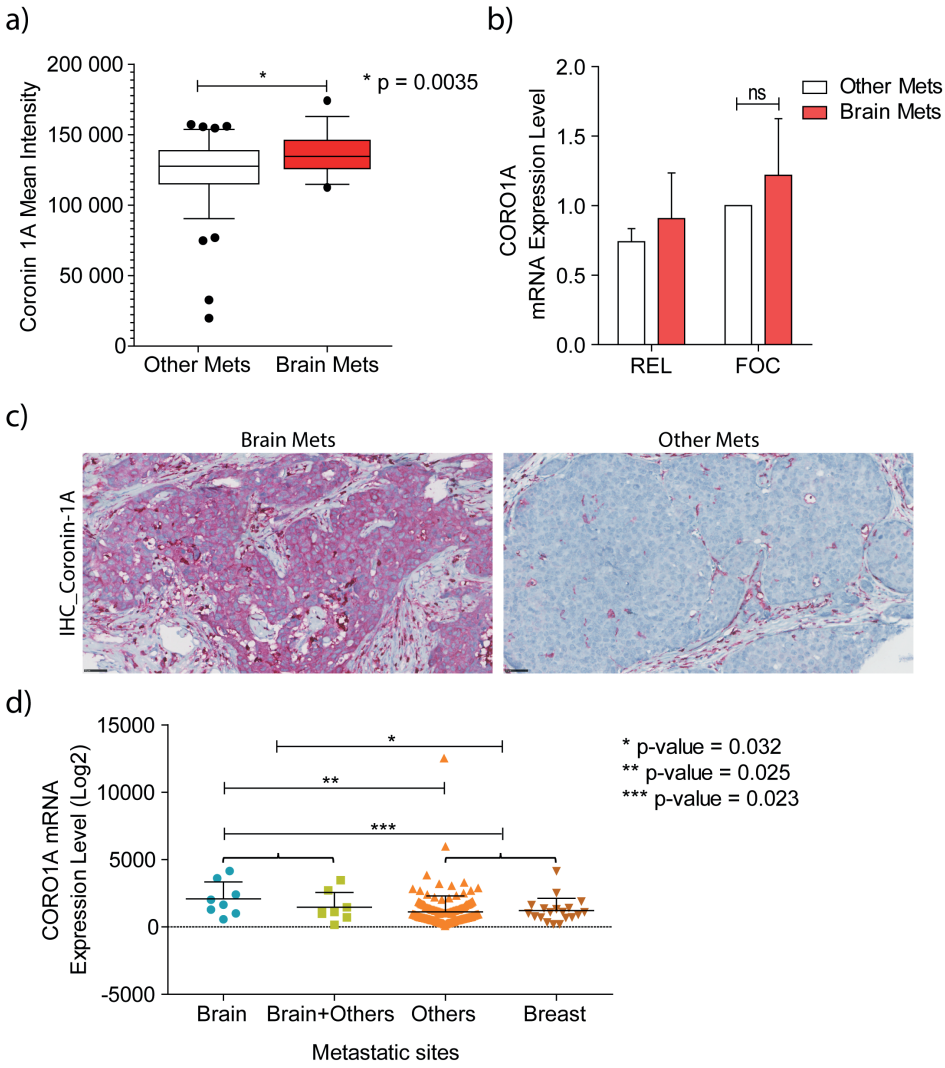

Figure 4 – Coronin-1A is highly expressed in primary breast cancers from patients who developed brain metastases. a) Coronin-1A immunohistochemistry mean-intensity levels are significantly higher in the group of breast cancers from patients who developed brain metastasis (n = 10, Brain Mets) when compared with breast cancers from patients with metastases in organs other than brain (n = 22, Other Mets), p = 0.0035. The lowest and highest boundaries of the box represent the 25 and 75 percentiles, respectively. The solid line across the box indicates the median value. Error bars indicate the 5-95 percentile. **b)** *CORO1A* mRNA relative (REL) and fold of change (FOC) expression levels in the two groups described in a), $p > 0.05$. **c)** Example of Coronin-1A IHC of primary breast cancers from patients who developed brain metastasis (left, tumor Coronin-1A cytoplasmic positive staining) or metastasis to other organs than brain (right, tumor Coronin-1A negative staining). **d)** *CORO1A* mRNA expression levels of 204 primary breast cancers associated with different sites of metastasis: brain (n=8); brain and other organs (n=8); other metastatic organs (n=169) and metastasis to the contralateral breast (n=19). Dataset information is publicly available under GEO accession number GSE12276, EXP00013 [7].

Primary breast cancers from patients with brain metastasis overexpress Coronin-1A

Coronin-1A protein expression levels in primary breast cancer samples of patients who developed brain metastasis were significantly higher (p = 0.004) compared to Coronin-1A expression levels in the cancers of patients who developed metastasis to other organs (**Figure 4a**, **c**). *CORO1A* mRNA expression levels showed a positive trend in the tumors associated with brain metastases (p > 0.05; **Figure 4b**). The finding was validated in an online mRNA expression database of 204 primary breast cancers. Of note, *CORO1A* mRNA levels were significantly higher in tumors that specifically and only metastasized to the brain (p = 0.023; **Figure 4d**).

DISCUSSION

Genes and mechanisms that drive cerebral seeding of metastatic cancers are largely undiscovered. Understanding the molecular features of primary tumors contributing to the development of brain metastasis, will ultimately pave the way to develop preventive strategies. In our previous work, we identified the involvement of T lymphocytes in the formation of cerebral metastases of breast cancer. We found that *GBP1* protein plays a role in the passage of tumor cells through the BBB [6]. *GBP1* is a downstream molecule in the interferon gamma (IFNγ) pathway. The aim of the present study was to investigate if T lymphocytes have similar effects on tumors other than breast cancer. In addition, we investigated what other molecules facilitate BBB passage following contact with T lymphocytes. We found that the effect of T lymphocytes on promoting an enhanced passage through the BBB is not restricted to breast cancer cells. T lymphocytes also stimulate the passage of lung - and prostate cancer cells and melanomas, albeit to a lesser extent than of breast cancers (**Figure 1a, b, c, d**). Remarkably, the transmigration rates of prostate cancer cells grown from tumors with high affinity for brain, were highly affected by the T lymphocytes. The same was true for melanoma cell lines derived from tumors with metastatic preference for brain. In contrast, the lung cancer cell line H2030BrM3, derived from a tumor that had metastasized to brain very early on, was not significantly affected by the T lymphocytes. These contrasting data suggest differences between tumors regarding the operative mechanisms to migrate to the brain. It may be that either the molecular cascades triggered by T lymphocytes are defective in the brain-trophic lung cancer cell lines, or that other molecular pathways are concerned. Interestingly, while the IFN-γ signaling pathway is involved in the formation of metastasis in various organs of lung carcinomas [25], some IFN-γ-insensitive lung tumors were shown to carry mutations in IFNγ signaling proteins, such as IFN-γR1, IFN-γR2, Jak1, Jak2 or STAT1 [26]. Such differences may reflect disparity between tumors, regarding seeding to the brain in the clinical setting [27, 28].

In our previous work, we found that T lymphocyte contact with breast cancer cells resulted in the upregulation of *GBP1*. In the present study we did not detect *GBP1* peptides in the lung cancer cell line H2030BrM3 following co-culture with T lymphocytes (Table S5). *GBP1* is one of the many STAT1-dependent IFNγ-regulated genes [29] and missing out on any of the upstream molecules will affect the function of this pathway [19, 26, 30]. The lineage-specific differences in response to T lymphocyte contact also hint to the involvement of other pathways in bringing the tumor cells across the BBB.

By comparing the proteomes of the tumor cells, following co-culture with activated T lymphocytes, Coronin-1A was identified as a migration-related protein (Table S3) commonly upregulated in all studied cancer cell lines but lung cancer (**Figure 2a, b**). Coronin-1A peptide count was not-detectable in all cancer cells before T lymphocyte co-culture, but present upon activated T lymphocyte contact (**Figure 2b**, Table S4) and subsequent increased transmigration numbers across the *in vitro* BBB model. The Coronin-1A peptide levels were higher in breast cancer cells than in other cancer types (4.8±2.3 vs ≤ 3±1.4; **Figure 2b**). Levels of *CORO1A* were not elevated after 24h indirect co-culture or incubation with CM from activated T lymphocytes, suggesting that either direct contact between cancer cells and T lymphocytes, or T lymphocyte-secreted factors, are necessary for an effective BBB transmigration. The *CORO1A* mRNA levels were reduced and close to control levels following five days co-culture with activated T lymphocytes in the various cancer cell lines, except for the breast cancer lines (Figure S1). This suggests that breast cancer cells exploit the T lymphocytes and T lymphocyte-secreted factors differently than other cancer types, which correlates with a more efficient passage-rate through the BBB. It remains to be investigated how these differences affect the clinical experienced variations in time to brain relapse of the various tumors [27, 28, 31]. Coronins are actin-branching regulators which bind to F-actin and the ARP2/3 complex, required for dimerization and filament bundling [32, 33]. Type I coronins (1A, 1B and 1C) localize to actin-rich cellular structures that serve a wide variety of cellular processes, among which cell motility [34]. By modelling F-actin, coronins alter lamellipodiakomma by which they steer cellular migration [35, 36]. In the *in vitro* BBB invasion model, 24 hours of *CORO1A* knockdown significantly reduced the BBB transmigration of breast cancer cells (Figure S2). Longer incubation with *CORO1A* siRNA reduced the BBB transmigration of the brain trophic breast cancer cell lines in more than 50% (**Figure 3b**), while 72 hours si*CORO1A* in SK-BR-7 cells did not significantly impair the transmigration ability of these cancer cells (**Figure 3b**). These results suggest that Coronin-1A overexpression contributes to an increased BBB transmigration, which is dependent on the particular tumor cells used. So far, we identified effects of Coronin-1A, in addition to activation of the IFNγ pathway, taking part in the trespassing of the tumor cells [19], and probably more molecules and pathways may be operative. As predicted, Coronin-1A knockdown was not sufficient to completely block the passage through the *in vitro* BBB.

Coronins inactivate the ARP2/3 complex [33, 37], thereby ceasing actin-branching and conferring a more temperate phenotype to invasive cells. The overexpression of type I coronin in cancers, reportedly correlates with increased biological aggressiveness of tumors [38-42]. An explanation may be that the debranching capabilities restocks available pools of G-actin and ARP2/3 complexes for continuous and sustained cell movements [43]. The speed and length of an actin tail is determined by the balance between the rate of new actin polymerization and filament assembly in the tail [44]. Significantly higher Coronin-1A expression levels were found in the group of breast cancers associated with brain metastases, as compared to those which had metastasized to other organs (p = 0.004; **Figure 4a**), suggestive of higher efficiency of actin filament turnover in the former. By using a publicly available database of primary breast cancers, we could confirm higher *CORO1A* mRNA expression levels in primary breast cancers from patients who had developed brain metastases. Of note, *CORO1A* mRNA levels were significantly higher in tumors that exclusively metastasized to brain (p = 0.032 vs p = 0.023, respectively; **Figure 4c**) [7].

In conclusion, activated T lymphocytes increase the transmigration ability through an *in vitro* BBB model for breast, lung and prostate cancers and melanomas. Activated T lymphocytes induce the expression of Coronin-1A in breast- and prostate cancer cells and melanomas and increase the BBB trespassing ability of these cancer types. The effects are to some extent cell lineage-specific and may well mirror the clinical differences in brain metastatic potential of different tumors.

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Supplementary Table 1 – Statistics summary of number of BBB transmigrated cells before and after co-culture or incubation with activated T lymphocytes or medium conditioned by activated T lymphocytes (+CM), respectively.

		# cells ± SD			% increase		T-test (*p*-value)	
cancer type	Cell line	Control	+ T lymphocytes	+ CM	+ T lymphocytes	+ CM	+ T lymphocytes	+ CM
Breast	MDA-MB-231-B2M2*	2.3 ± 0.6	2,884 ± 1230.8	545.2 ± 311.4	1236.0	233.6	0.002	0.008
	MDA-MB-231	2.7 ± 2.9	4,697 ± 910.9	618.2 ± 247.3	1761.5	231.8	0.0001	0.002
	SK-BR-7	2.0 ± 1.0	4,568 ± 820.0	758.0 ± 349.5	2283.8	379.0	0.00004	0.003
Lung	H2030BrM3*	10.2 ± 12.9	21.3 ± 7.8	45.5 ± 16.3	2.1	4.5	0.11	0.002
	H2030	7.8 ± 8.7	480.5 ± 444.0	89.5 ± 60.9	61.3	11.4	0.048	0.02
	H1299	0.5 ± 0.8	284.7 ± 236.7	46.8 ± 20.7	569.3	93.7	0.03	0.003
Melanoma	H1-DL-2*	59.0 ± 28.3	217.7 ± 102.4	103.7 ± 42.8	3.7	1.8	0.01	0.06
	SK-MEL-28	28.3 ± 13.8	139.2 ± 57.2	17.5 ± 7.6	4.9	0.6	0.004	0.13
Prostate	Du 145*	89.7 ± 94.6	387.8 ± 188.5	125.7 ± 122.0	4.3	1.4	0.01	0.58
	PC3	27.2 ± 19.2	240.5 ± 104.7	46.7 ± 41.6	8.9	1.7	0.004	0.33

Average number of two independent experiments of three replicates each with ± SD, percentage of increased BBB transmigrated cell numbers for cancer cells regarding control and correspondent *p*-value for each of the cell types used. For abbreviated cell lines see **Table 1**. * Cell lines derived from cancers with predilection for brain.

Supplementary Table 2 – Number of proteins identified by LC-MS measurements.

	Total # detected proteins		
Cancer Type	**Control**	**+ T lymphocytes**	**# overlapped**
Breast cancer cells*	1,416	1,350	1,233
MDA-MB-231-B2M2	1,925	1,915	1,692
MDA-MB-231	2,062	1,961	1,781
SK-BR-7	1,864	1,746	1,586
Lung cancer cells*	1,690	1,629	1,498
H2030BrM3	2,287	2,242	2,061
H2030	2,076	1,981	1,808
H1299	2,264	2,219	2,022
Melanoma cells*	1,279	1,843	1,220
H1-DL-2	2,434	2,354	2,174
SK-MEL-28	1,347	2,134	1,281
Prostate cancer cells*	1,568	1,534	1,371
Du 145	1,993	1,953	1,726
PC3	1,966	1,931	1,708

Number of identified proteins present in all replicates (n=2) before (control) and after co-culture with activated T lymphocytes (+ T lymphocytes) common to all cell lines within a cancer group (*) or to individual cell lines. # Overlapped represents the number of proteins commonly identified in all replicates before and after co-culture with activated T lymphocytes.

Supplementary Table 3 – Eighty-seven differentially overexpressed proteins in breast cancer cells following co-culture with activated T lymphocytes.

#	Identified Proteins	Gene	Accession #	Molecular Weight	t-test (*p*-value)	Protein function*
1	40S ribosomal protein S12	RPS12	RS12_HUMAN	15 kDa	0.0069	Ribonucleoprotein, Ribosomal protein.
2	40S ribosomal protein S9	RPS9	RS9_HUMAN	23 kDa	0.033	
3	60S ribosomal protein L24	RPL24	RL24_HUMAN	18 kDa	0.014	
4	Aconitate hydratase, mitochondrial	ACO2	ACON_HUMAN	85 kDa	0.0091	Catalyzes the isomerization of citrate to isocitrate via cis-aconitate.
5	Annexin A4	ANXA4	ANXA4_HUMAN	36 kDa	0.023	Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis.
6	Antigen peptide transporter 1	TAP1	TAP1_HUMAN	87 kDa	0.00075	Involved in the transport of antigens from the cytoplasm to the endoplasmic reticulum for association with MHC class I molecules.
7	Antigen peptide transporter 2	TAP2	TAP2_HUMAN	76 kDa	0.0017	
8	Arf-GAP with GTPase, ANK repeat and PH domain-containing protein 3	AGAP3	AGAP3_HUMAN	95 kDa	0.013	GTPase-activating protein for the ADP ribosylation factor family.
9	Asparagine--tRNA ligase, cytoplasmic	NARS	SYNC_HUMAN	63 kDa	0.022	Catalyzes the attachment of asparagine to tRNA(Asn); acts as a signaling molecule that induced migration of CCR3-expressing cells.
10	HLA class I histocompatibility antigen, Cw-15 alpha chain	HLA-C	1C15_HUMAN	41 kDa	0.022	Antigen-presenting major histocompatibility complex class I (MHCI) molecule.
11	ATP-dependent Clp protease proteolytic subunit, mitochondrial	CLPP	CLPP_HUMAN	30 kDa	0.037	Protease component of the Clp complex that cleaves peptides and various proteins in an ATP-dependent process.
12	Beta-2-microglobulin	B2M	B2MG_HUMAN	14 kDa	0.00073	Component of the class I major histocompatibility complex (MHC). Involved in the presentation of peptide antigens to the immune system.
13	EF-hand domain-containing protein D2	EFHD2	EFHD2_HUMAN	27 kDa	0.017	May regulate B-cell receptor (BCR)-induced immature and primary B-cell apoptosis. Plays a role as negative regulator of the canonical NF-kappa-B-activating branch.

Supplementary Table 3 – Eighty-seven differentially overexpressed proteins in breast cancer cells following co-culture with activated T lymphocytes. (*continued*)

#	Identified Proteins	Gene	Accession #	Molecular Weight	t-test (*p*-value)	Protein function*
14	Guanylate-binding protein 1	*GBP1*	*GBP1*_ HUMAN	68 kDa	0.018	Hydrolyzes GTP to GMP in 2 consecutive cleavage reactions. Promotes oxidative killing and delivers antimicrobial peptides to autophagolysosomes.
15	Cluster of HLA class I histocompatibility antigen, A-2 alpha chain	HLA-A	1A02_ HUMAN	41 kDa	0.0059	Antigen-presenting major histocompatibility complex class I (MHCI) molecule.
16	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	PCK2	PCKGM_ HUMAN	71 kDa	0.0066	Catalyzes the conversion of oxaloacetate (OAA) to phosphoenolpyruvate (PEP).
17	Proteasome subunit alpha type-7	PSMA7	PSA7_ HUMAN	28 kDa	0.049	Component of the 20S core proteasome complex involved in the proteolytic degradation of most intracellular proteins.
18	Cluster of Zinc finger protein 236	ZNF236	ZN236_ HUMAN	204 kDa	0.015	May be involved in transcriptional regulation.
19	Coronin-1A	*CORO1A*	COR1A_ HUMAN	51 kDa	0.0022	May be a crucial component of the cytoskeleton of highly motile cells, functioning both in the invagination of large pieces of plasma membrane, as well as in forming protrusions of the plasma membrane involved in cell locomotion.
20	Cysteine-rich with EGF-like domain protein 1	CRELD1	CREL1_ HUMAN	45 kDa	0.012	Protein disulfide isomerase (By similarity). Promotes the localization of acetylcholine receptors (AChRs) to the plasma membrane .
21	Cytochrome c	CYCS	CYC_ HUMAN	12 kDa	0.012	Electron carrier protein.
22	Cytosol aminopeptidase	LAP3	AMPL_ HUMAN	56 kDa	0.029	Presumably involved in the processing and regular turnover of intracellular proteins.
23	Polyadenylate-binding protein 3	PABPC3	PABP3_ HUMAN	70 kDa	0.03	Binds the poly(A) tail of mRNA. May be involved in cytoplasmic regulatory processes of mRNA metabolism.
24	DDB1- and CUL4-associated factor 13	DCAF13	DCA13_ HUMAN	51 kDa	0.013	Possible role in ribosomal RNA processing
25	DNA ligase 3	LIG3	DNLI3_ HUMAN	113 kDa	0.049	Isoform 3 functions as heterodimer with DNA-repair protein XRCC1 in the nucleus and can correct defective DNA strand-break repair.

Supplementary Table 3 – Eighty-seven differentially overexpressed proteins in breast cancer cells following co-culture with activated T lymphocytes. (*continued*)

#	Identified Proteins	Gene	Accession #	Molecular Weight	t-test (*p*-value)	Protein function*
26	DnaJ homolog subfamily B member 11	DNAJB11	DJB11_HUMAN	41 kDa	0.0068	As a co-chaperone for HSPA5 it is required for proper folding, trafficking or degradation of proteins.
27	DnaJ homolog subfamily C member 10	DNAJC10	DJC10_HUMAN	91 kDa	0.024	
28	Heat shock 70 kDa protein 6	HSPA6	HSP76_HUMAN	71 kDa	0.024	Molecular chaperone implicated in a wide variety of cellular processes, including protection of the proteome from stress, folding and transport of newly synthesized polypeptides, activation of proteolysis of misfolded proteins and the formation and dissociation of protein complexes.
29	Ras-related protein Rab-10	RAB10	RAB10_HUMAN	23 kDa	0.025	The small GTPases Rab are key regulators of intracellular membrane trafficking, from the formation of transport vesicles to their fusion with membranes
30	E3 ubiquitin-protein ligase RNF213	RNF213	RN213_HUMAN	591 kDa	0.041	E3 ubiquitin-protein ligase involved in angiogenesis. Involved in the non-canonical Wnt signaling pathway in vascular development
31	Endoplasmic reticulum aminopeptidase 1	ERAP1	ERAP1_HUMAN	107 kDa	0.0017	Aminopeptidase that plays a central role in peptide trimming, a step required for the generation of most HLA class I-binding peptides.
32	Putative HLA class I histocompatibility antigen, alpha chain H	HLA-H	HLAH_HUMAN	41 kDa	0.0021	Antigen-presenting major histocompatibility complex class I (MHCI) molecule.
33	Endoplasmic reticulum resident protein 44	ERP44	ERP44_HUMAN	47 kDa	0.031	Mediates thiol-dependent retention in the early secretory pathway, forming mixed disulfides with substrate proteins through its conserved CRFS motif.
34	Exosome complex exonuclease RRP44	DIS3	RRP44_HUMAN	109 kDa	0.018	Putative catalytic component of the RNA exosome complex which has 3'->5' exoribonuclease activity and participates in a multitude of cellular RNA processing and degradation events.
35	HLA class I histocompatibility antigen, Cw-18 alpha chain	HLA-C	1C18_HUMAN	41 kDa	0.018	Antigen-presenting major histocompatibility complex class I (MHCI) molecule.
36	Golgin subfamily B member 1	GOLGB1	GOGB1_HUMAN	376 kDa	0.0014	May participate in forming intercisternal cross-bridges of the Golgi complex.

Supplementary Table 3 – Eighty-seven differentially overexpressed proteins in breast cancer cells following co-culture with activated T lymphocytes. (*continued*)

#	Identified Proteins	Gene	Accession #	Molecular Weight	t-test (*p*-value)	Protein function*
37	Growth arrest and DNA damage-inducible proteins-interacting protein 1	GADD45GIP1	G45IP_ HUMAN	25 kDa	0.049	Acts as a negative regulator of G1 to S cell cycle phase progression by inhibiting cyclin-dependent kinases.
38	H/ACA ribonucleoprotein complex subunit 4	DKC1	DKC1_ HUMAN	58 kDa	0.021	Catalytic subunit of H/ACA small nucleolar ribonucleoprotein (H/ACA snoRNP) complex, which catalyzes pseudouridylation of rRNA.
39	Heterogeneous nuclear ribonucleoprotein U-like protein 2	HNRNPUL2	HNRL2_ HUMAN	85 kDa	0.033	-
40	Histone H1x	H1FX	H1X_ HUMAN	22 kDa	0.0073	Histones H1 are necessary for the condensation of nucleosome chains into higher-order structures.
41	HLA class II histocompatibility antigen, DR alpha chain	HLA-DRA	DRA_ HUMAN	29 kDa	0.015	Binds peptides derived from antigens that access the endocytic route of antigen presenting cells (APC) and presents them on the cell surface for recognition by the CD4 T-cells.
42	Intercellular adhesion molecule 1	ICAM1	ICAM1_ HUMAN	58 kDa	0.0001	ICAM proteins are ligands for the leukocyte adhesion protein LFA-1.
43	Interferon-induced 35 kDa protein	IFI35	IN35_ HUMAN	32 kDa	0.013	Not yet known.
44	Kinectin	KTN1	KTN1_ HUMAN	156 kDa	0.0062	Receptor for kinesin involved in kinesin-driven vesicle motility.
45	Kynurenine--oxoglutarate transaminase 1	CCBL1	KAT1_ HUMAN	48 kDa	0.049	Catalyzes the irreversible transamination of the L-tryptophan metabolite L-kynurenine to form kynurenic acid (KA).
46	Leukocyte elastase inhibitor	SERPINB1	ILEU_ HUMAN	43 kDa	0.041	Neutrophil serine protease inhibitor that plays an essential role in the regulation of the innate immune response, inflammation and cellular homeostasis.
47	Zinc finger protein 233	ZNF233	ZN233_ HUMAN	77 kDa	0.049	May be involved in transcriptional regulation.
48	Major vault protein	MVP	MVP_ HUMAN	99 kDa	0.021	Required for normal vault structure, which are multi-subunit structures that may act as scaffolds for proteins involved in signal transduction.

Supplementary Table 3 – Eighty-seven differentially overexpressed proteins in breast cancer cells following co-culture with activated T lymphocytes. (*continued*)

#	Identified Proteins	Gene	Accession #	Molecular Weight	t-test (*p*-value)	Protein function*
49	Microfibrillar-associated protein 1	MFAP1	MFAP1_ HUMAN	52 kDa	0.012	Involved in pre-mRNA splicing as a component of the spliceosome.
50	NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial	NDUFS7	NDUS7_ HUMAN	24 kDa	0.00018	Core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase.
51	NHP2-like protein 1	SNU13	NH2L1_ HUMAN	14 kDa	0.0024	Involved in pre-mRNA splicing as component of the spliceosome.
52	Nicotinamide phosphoribosyltransferase	NAMPT	NAMPT_ HUMAN	56 kDa	0.0001	Catalyzes the condensation of nicotinamide with 5-phosphoribosyl-1-pyrophosphate to yield nicotinamide mononucleotide, an intermediate in the biosynthesis of NAD.
53	Nuclear factor NF-kappa-B p100 subunit	NFKB2	NFKB2_ HUMAN	97 kDa	0.00066	NF-kappa-B is a pleiotropic transcription factor present in almost all cell types and is the endpoint of a series of signal transduction events that are initiated by a vast array of stimuli related to many biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis.
54	HLA class I histocompatibility antigen, Cw-2 alpha chain	HLA-C	1C02_ HUMAN	41 kDa	0.00069	Antigen-presenting major histocompatibility complex class I (MHCI) molecule.
55	Peptidyl-prolyl cis-trans isomerase B	PPIB	PPIB_ HUMAN	24 kDa	0.00043	PPIase that catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides and may therefore assist protein folding.
56	PEST proteolytic signal-containing nuclear protein	PCNP	PCNP_ HUMAN	19 kDa	0.013	May be involved in cell cycle regulation.
57	HLA class I histocompatibility antigen, A-34 alpha chain	HLA-A	1A34_ HUMAN	41 kDa	0.014	Antigen-presenting major histocompatibility complex class I (MHCI) molecule.
58	Phosphatidate cytidylyltransferase 2	CDS2	CDS2_ HUMAN	51 kDa	0.049	Catalyzes the conversion of phosphatidic acid (PA) to CDP-diacylglycerol (CDP-DAG), an essential intermediate in the synthesis of phosphatidylglycerol, cardiolipin and phosphatidylinositol.
59	Plastin-2	LCP1	PLSL_ HUMAN	70 kDa	0.0091	Actin-binding protein. Plays a role in the activation of T-cells in response to costimulation through TCR/CD3 and CD2 or CD28.

Supplementary Table 3 – Eighty-seven differentially overexpressed proteins in breast cancer cells following co-culture with activated T lymphocytes. (*continued*)

#	Identified Proteins	Gene	Accession #	Molecular Weight	t-test (*p*-value)	Protein function*
60	Proteasome activator complex subunit 2	PSME2	PSME2_HUMAN	27 kDa	0.0074	Implicated in immunoproteasome assembly and required for efficient antigen processing.
61	HLA class I histocompatibility antigen, Cw-17 alpha chain	HLA-C	1C17_HUMAN	41 kDa	0.0089	Antigen-presenting major histocompatibility complex class I (MHCI) molecule.
62	Proteasome subunit alpha type-3	PSMA3	PSA3_HUMAN	28 kDa	0.031	Component of the 20S core proteasome complex involved in the proteolytic degradation of most intracellular proteins.
63	Proteasome subunit alpha type-4	PSMA4	PSA4_HUMAN	29 kDa	0.016	
64	Proteasome subunit beta type-1	PSMB1	PSB1_HUMAN	26 kDa	0.023	
65	Proteasome subunit beta type-10	PSMB10	PSB10_HUMAN	29 kDa	0.0001	
66	Proteasome subunit beta type-3	PSMB3	PSB3_HUMAN	23 kDa	0.012	
67	Proteasome subunit beta type-4	PSMB4	PSB4_HUMAN	29 kDa	0.021	
68	Proteasome subunit beta type-8	PSMB8	PSB8_HUMAN	30 kDa	0.00088	
69	Proteasome subunit beta type-9	PSMB9	PSB9_HUMAN	23 kDa	0.0021	
70	Serine/threonine-protein phosphatase 4 regulatory subunit 1	PPP4R1	PP4R1_HUMAN	107 kDa	0.012	Regulatory subunit of serine/threonine-protein phosphatase 4.
71	Sideroflexin-1	SFXN1	SFXN1_HUMAN	36 kDa	0.021	Mitochondrial serine transporter that mediates transport of serine into mitochondria, an important step of the one-carbon metabolism pathway.
72	Signal transducer and activator of transcription 1-alpha/beta	STAT1	STAT1_HUMAN	87 kDa	0.0001	Signal transducer and transcription activator that mediates cellular responses to interferons (IFNs), cytokine KITLG/SCF and other cytokines and other growth factors.

Supplementary Table 3 – Eighty-seven differentially overexpressed proteins in breast cancer cells following co-culture with activated T lymphocytes. (*continued*)

#	Identified Proteins	Gene	Accession #	Molecular Weight	t-test (*p*-value)	Protein function*
73	Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial	SUCLG2	SUCB2_ HUMAN	47 kDa	0.032	GTP-specific succinyl-CoA synthetase functions in the citric acid cycle (TCA), coupling the hydrolysis of succinyl-CoA to the synthesis of GTP; represents the only step of substrate-level phosphorylation in the TCA.
74	Sulfide:quinone oxidoreductase, mitochondrial	SQRDL	SQRD_ HUMAN	50 kDa	0.0051	Catalyzes the oxidation of hydrogen sulfide with the help of a quinone, such as ubiquinone-10, giving rise to thiosulfate and ultimately to sulfane (molecular sulfur) atoms.
75	Superoxide dismutase [Mn], mitochondrial	SOD2	SODM_ HUMAN	25 kDa	0.039	This protein binds to the superoxide byproducts of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen.
76	SURP and G-patch domain-containing protein 2	SUGP2	SUGP2_ HUMAN	120 kDa	0.049	May play a role in mRNA splicing.
77	Tapasin	TAPBP	TPSN_ HUMAN	48 kDa	0.00045	This gene encodes a transmembrane glycoprotein which mediates interaction between newly assembled major histocompatibility complex (MHC) class I molecules and the transporter associated with antigen processing (TAP), which is required for the transport of antigenic peptides across the endoplasmic reticulum membrane.
78	T-complex protein 1 subunit delta	CCT4	TCPD_ HUMAN	58 kDa	0.014	Component of the chaperonin-containing T-complex (TRiC), a molecular chaperone complex that assists the folding of proteins upon ATP hydrolysis.
79	Translocon-associated protein subunit alpha	SSR1	SSRA_ HUMAN	32 kDa	0.023	TRAP proteins are part of a complex whose function is to bind calcium to the ER membrane and thereby regulate the retention of ER resident proteins.
80	Tryptophan--tRNA ligase, cytoplasmic	WARS	SYWC_ HUMAN	53 kDa	0.0015	Regulates ERK, Akt, and eNOS activation pathways that are associated with angiogenesis, cytoskeletal reorganization and shear stress-responsive gene expression.
81	Type-1 angiotensin II receptor-associated protein	AGTRAP	ATRAP_ HUMAN	17 kDa	0.049	Appears to be a negative regulator of type-1 angiotensin II receptor-mediated signaling by regulating receptor internalisation as well as mechanism of receptor desensitization such as phosphorylation.
82	Ubiquitin/ISG15-conjugating enzyme E2 L6	UBE2L6	UB2L6_ HUMAN	18 kDa	0.0013	Catalyzes the covalent attachment of ubiquitin or ISG15 to other proteins.
83	UPF0587 protein C1orf123	C1orf123	CA123_ HUMAN	18 kDa	0.049	-

Supplementary Table 3 – Eighty-seven differentially overexpressed proteins in breast cancer cells following co-culture with activated T lymphocytes. (*continued*)

#	Identified Proteins	Gene	Accession #	Molecular Weight	t-test (*p*-value)	Protein function*
84	Valine--tRNA ligase	VARS	SYVC_HUMAN	140 kDa	0.032	Aminoacyl-tRNA synthetase, Ligase.
85	Voltage-dependent anion-selective channel protein 1	VDAC1	VDAC1_HUMAN	31 kDa	0.039	Forms a channel through the mitochondrial outer membrane and also the plasma membrane.
86	V-type proton ATPase subunit H	ATP6V1H	VATH_HUMAN	56 kDa	0.0096	Subunit of the peripheral V1 complex of vacuolar ATPase. Subunit H activates the ATPase activity of the enzyme and couples ATPase activity to proton flow.
87	HLA class I histocompatibility antigen, B-7 alpha chain	HLA-B	1B07_HUMAN	40 kDa	0.011	Antigen-presenting major histocompatibility complex class I (MHCI) molecule.

* Protein function provided by UniProt; (-) function not specified.

Supplementary Table 4 – Average number of Coronin-1A peptides detected by LC-MS measurements.

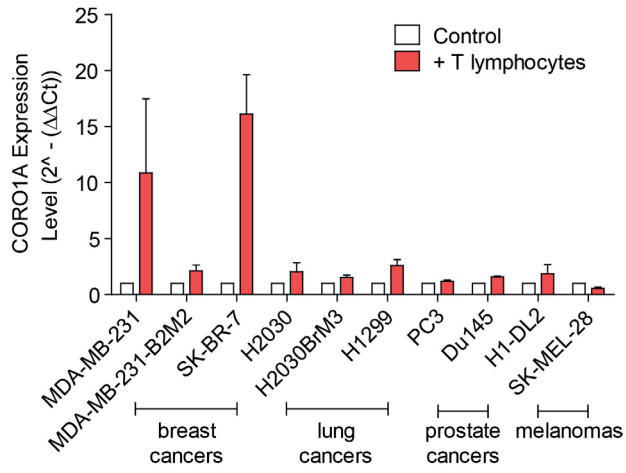
Cell Line	Coronin-1A (mean±SD) # peptides		
	Control	+T lymphocytes	+CM
MDA-MB-231	ND	6.0±2.8	1.0±1.4
MDA-MB-231-B2M2	ND	3.5±2.1	ND
SK-BR-7	ND	5.0±2.8	1.0±1.4
H2030	ND	ND	ND
H2030BrM3	ND	ND	ND
H1299	ND	ND	ND
PC3	ND	3.5±2.1	ND
Du145	ND	2.5±0.7	1.0±1.4
H1-DL2	ND	1.5±2.1	ND
SK-MEL-28	ND	1.0±1.4	ND

Average number of Coronin-1A peptides in all cell lines used in this study before (Control) and following co-culture with activated T lymphocytes (+T lymphocytes) or following incubation with media conditioned by activated T lymphocytes (+CM). Data representative of two independent measurements. ND, not detected.

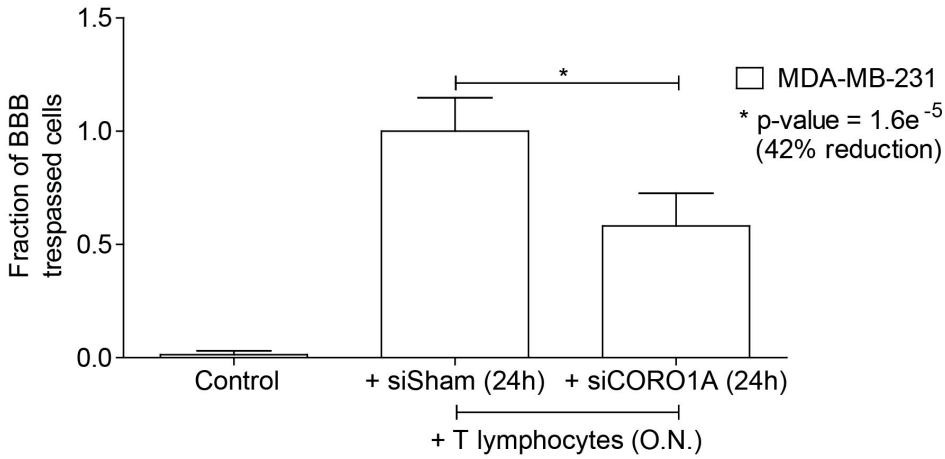
Supplementary Table 5 – Average number of *GBP1* peptides detected by LC-MS analysis in all cell lines.

Cell Line	*GBP1* (Mean±SD) # peptides*
MDA-MB-231	12±2.8
MDA-MB-231-B2M2	3.5±4.9
SK-BR-7	4.5±3.5
H2030	4±5.7
H2030BrM3	ND
H1299	ND
PC3	9±5.7
Du145	ND
H1-DL2	4±5.7
SK-MEL-28	7.5±10.6

*following co-culture with activated T lymphocytes. Data representative of two independent measurements. ND, not detected.



Supplementary Figure 1 – *CORO1A* mRNA FOC levels of all studied cancer cells, before and after five days co-culture with activated T lymphocytes. Data representative of two independent experiments.



Supplementary Figure 2 – Fraction of MDA-MB-231 cells which successfully crossed the *in vitro* BBB before and after 24h *CORO1A* siRNA or non-targeting siSham. Data representative of three independent experiments of three replicates each.

Chapter 8

General Discussion

GENERAL DISCUSSION

Brain metastasis is a deadly complication of cancer. The formation of cerebral metastases relies upon a series of steps, starting from tumor cells leaving the primary tumor and entering the blood stream to become circulating tumor cells (CTCs), and subsequent penetration of the BBB and outgrowth in their new brain microenvironment. The aim of this thesis was to provide a comprehensive understanding of the genes and pathways required for the development of brain metastasis. The first question to be answered was which genes are differentially upregulated in the primary tumors that are associated with brain metastases. Subsequent questions involved the complex interactions between the primary breast cancer cells and their microenvironment. Comparison of gene expression profiles of ER- primary breast cancers between patients who developed metastases, including and excluding the brain, revealed the involvement of the 'T lymphocyte response' in primary breast cancers that gave rise to cerebral metastases (**Chapter four**). This finding shed light on the T lymphocyte infiltration (TILs) in the tumor parenchyma of breast cancers that had, and had not, metastasized to brain: the T lymphocytes change the expressional patterns of tumor cells, a finding that was not reported previously [1-3]. We further explored the role of T lymphocytes in the development of brain metastases from breast cancer, both *in vitro* and *in vivo*.

Using an *in vitro* BBB model, we compared the invasion rate of different breast cancer cell lines, before and after co-culture with *in vitro*-activated T lymphocytes, isolated from healthy donors. A dramatic increase in cell numbers crossing the BBB, after priming with activated T lymphocytes, was observed and not replicated when replacing breast cancer cells by non-cancer cells, such as fibroblasts. Proteome comparisons by mass spectrometry (MS) of breast cancer cells, before and after co-culture with activated T lymphocytes, led to the identification of Guanylate binding protein 1 (GBP1) as an up-regulated protein after co-culture with activated T lymphocytes. Interestingly, the expression of GBP1 was specifically detected by immunohistochemistry in primary breast cancers that had metastasized to brain. Moreover, we observed an increased number of TILs in these specimens, as well as invasion of the tumor parenchyma by T lymphocytes, to directly make contact with the tumor cells.

The increased transmigration through the BBB by the cancer cells was also observed when breast cancer cells were incubated with media conditioned by activated T lymphocytes, in the absence of the latter – albeit to a lesser extent. Subsequent MS analysis, revealed only cell-intrinsic proteins, due to the sole use of cell lysates in MS measurements. Other T lymphocyte derived factors, that might have enhanced the passage through the BBB, are easily missed in the analysis. Additional strategies could entail harvesting proteins available in the culture medium, followed by MS, in order to identify T lymphocyte-derived secreted cytokines that are produced upon breast cancer cell co-culture with T lymphocytes. Although 2D transwell

BBB models are relatively easy to create and use, they fail in faithfully recapitulate the genetic and anatomical characteristics of the human brain barrier, whereas the cell-cell and cell-matrix interactions are a crucial part of the real human BBB. Therefore, in future studies, the use of a dynamic, *in vivo*-like 3D BBB system, such as microfluidic chips [4, 5], that replicate the key structural, functional and mechanical properties of the blood-brain barrier *in vivo,* would be a significant improvement.

The *in vitro* findings were reproduced in our mouse model, where tumor cells need to cross a real BBB. Intra-cordial inoculation of breast cancer cells, that had been in prior contact with activated T lymphocytes, specifically led to the formation of brain metastases, as compared to the non-primed parental cell line (incidences 53.8% vs. 8.3%, respectively). Importantly, this six-fold increase in the ability to generate brain metastatic tumours, after contact with activated T lymphocytes, was not mimicked in other organs. The increased sizes of the metastases and larger invasive fronts highlights three critical results of T lymphocyte contact: increased ability to advance from the first extravasation compartment, increased capability to cross the BBB and increased competence to invade the brain. It remains to be investigated which of the upregulated proteins are taking part in these subsequent steps of metastasis.

Because GBP1 is one of the many secreted GTPases induced by IFN-γ [6], in **Chapter five** we scrutinized the extent to which the IFN-γ pathway plays a role in promoting brain metastases of breast cancer. CD8+ T lymphocytes tend to predominate in isolated infiltrates, outnumbering CD4+ cells [7]. The CD8+ T lymphocytes have, aside from their cytotoxic functions, a regulatory role, relying on differential cytokine secretion [8-10] and higher efficiency in IFN-γ production [11]. Our results showed that co-culturing breast cancer cells with activated CD8+ T lymphocytes, enhanced the BBB passage-rate of breast cancer cells significantly more than co-culturing with other subtypes of T lymphocytes. No IFN-γ was detected after five minutes of co-culture of breast cancer cells with T lymphocytes. This can be easily justified by the known positive feedback loop of IFN-γ production, uptake and expression by T lymphocytes [12]. Eighty % of the total soluble IFN-γ (sIFNγ), initially boosted in the culture media of breast cancer cells by the addition of media conditioned by activated T lymphocytes (IFN-γ-rich medium), was no longer detectable after five minutes incubation-time, suggestive of an effective IFN-γ absorption and/or uptake by the breast cancer cells. To investigate whether IFN-γ removal from the media is linked and relevant to an increased BBB invasion, we impaired the IFN-γ pathway by using a sIFN-γ neutralizing antibody or an IFN-γ receptor 1 (IFNGR1) blocker. These results elegantly showed a concentration-dependent reduction of BBB-invading breast cancer cells, in addition to a matched concentration-dependent reduction of GBP1 protein expression in breast cancer cells. However, the numbers of tumor cells crossing the BBB were still significantly higher than the nearly zero numbers of control cells that crossed the BBB. This finding suggests that the STAT1-dependent IFN-γ signaling pathway is not the

only pathway involved and triggered by contact with T lymphocytes – there seems to be more pathways and molecules upregulated by this contact.

GBP1 is an IFN-γ-regulated actin-binding protein which plays a role in cytoskeleton remodelling [6, 13] and plays roles in processes such as invasion or barrier function [14]. IFN-γ also regulates many other genes, of which chemokines and their receptors, such as the CXCL-9, -10, -11 and CXCR3 axis, found up-regulated in our brain metastatic breast cancer cohort. This axis is known to have a crucial role in directional migration [15]. Moreover, it is constitutionally active in the brain, having a crucial role during CNS pathogenesis [16-18]. It would be interesting to use a breast cancer-specific inducible IFN-γ-knockout mouse model to study the effects of breast cancer specific IFN-γ-knockout in *in vivo* brain metastases formation, and further study the activation status of the CXCL-9,-10,-11/CXCR3 axis.

In **Chapter seven** we addressed the extent to which activated T lymphocytes affect the trespassing ability of other brain-trophic tumor types. We showed that, although the ability of T lymphocytes to change the proteome of cancer cells is not breast cancer-exclusive, the effect is most prominent in breast cancer. The same was true for the trespassing-rates through the BBB. Comparison of the proteome changes induced by T lymphocyte contact with all tested cancer cell types, led to the identification of one commonly overexpressed protein, namely Coronin-1A (CORO1A). Similar to GBP1, CORO1A is a type I coronin known to bind F-actin *in vitro* and to localize to actin-rich cellular structures, thereby playing a wide variety of actin-based motility processes [19]. In contrast to GBP1, CORO1A was exclusively upregulated after direct contact with activated T lymphocytes. This is suggestive of the existence of a point in time where cell-cell contact does occur, sustaining the upregulation of GJD3-connexin required for gap junction channel formation, allowing direct physical communication between two cells [20, 21] in primary breast cancers which develop brain metastases (**Chapter six**). In addition, it highlights the idea that other non-IFNγ dependent pathways are likely to assist in the development of brain metastases. *CORO1A* knockdown experiments on breast cancer cells showed significant decrease in crossing the BBB, supporting the impairment of cell migration as a consequence of coronin depletion [22, 23]. By searching mRNA expression databases of primary breast cancers from patients who developed brain metastases, we found that *CORO1A* mRNA is significantly overexpressed and that levels are higher in patients who exclusively developed brain metastases, validating the upregulation of *CORO1A* in two independent brain metastatic breast cancer datasets.

FUTURE PERSPECTIVES

The work presented in this thesis, highlights the relevance of T lymphocyte contact with primary breast cancer cells at the primary tumor site, in the development of brain metastasis. These studies used an *in vitro* allogeneic stimulation of T lymphocytes isolated from healthy patients, which were subsequently co-cultured with allogeneic human-derived breast cancer cell lines. In order to better understand whether an *in vivo* T lymphocyte activation enhances the natural development of brain metastasis from breast cancer, it is imperative to repeat the experimental work presented in this thesis with autologous T lymphocytes and breast cancer cells isolated from breast cancer patients.

The contact with activated T lymphocytes, at the primary tumor site, most likely involves the synchronous activation of several signaling pathways enabled by the panoply of secreted cytokines and chemokines. The latter mediate their function by activating G-protein-coupled receptors. Chemokine receptor activation triggers several downstream effector molecules in a cascade of signaling events within the cytoplasm [24]. CXCR3 is a G-protein-coupled receptor and an IFN-γ-inducible chemokine receptor linked to the Src, PI3K and MAPK signaling pathways [25]. c-Src promotes other signalling proteins, coordinating a variety of normal and oncogenic processes, such as proliferation, angiogenesis, survival and metastasis. c-Src interacts with cell surface receptors, steroid hormone receptors, heterotrimeric G-protein-regulated molecules, STATs and many others (reviewed in [26]). The drug Dasatinib (BMS-354825 or Sprycel) is an ATP-competitive tyrosine kinase inhibitor which inhibits all members of the Src family, including c-Src, Lck, Fyn and Yes [27, 28]. Dasatinib not only blocks *in vitro* migration and invasion in MDA-MB-231 breast cancer cells [29, 30], but also supresses T lymphocyte functions [31]. The effects on the formation of brain metastases are unknown. Although Dasatinib proved unsuccessful against triple negative (TN) breast cancer in a Phase 2 clinical trial [32], synergistic effects of different drugs may eventually lower the chance of resistance and toxicity [33]. Defactinib (VS-6063 or PF-04554878) is another ATP-competitive small molecule kinase inhibitor which effectively inhibits Focal Adhesion Kinase (FAK) [34]. FAK induces increased cell motility and invasiveness, and its role in both functions is distinct. FAK regulates actin assembly by interacting with the ARP2/3 complex [35] and regulates invadopodia formation by controlling localization of Src [36]. It promotes integrin-stimulated cell motility [37] through independent signalling pathways. Both Dasatinib and Defactinib drugs independently inhibit stimulus-specific migration of breast cancer cells [29, 38, 39]. So far, no data on results of combination therapy with these two drugs, or required concentrations, are available. Dasatinib concentration necessary to inhibit Fyn and Lck in T lymphocytes has been shown to be higher than those required for inhibition of Src activity in healthy cells [40]. The application of different drugs will lower the chance of resistance [33] and possibly of healthy-cell toxicity, frequently seen with the high demanding doses of one single drug.

The rationale for molecularly targeted drug-combinations may be elusive, unaccounted for or yet counter-intuitive. However, taking into account that no drugs specifically aimed at the prevention of brain metastasis formation have been introduced, it seems opportune to start therapeutic programs that include drugs that would specifically aim at the prevention of this deadly complication.

CONCLUSION

The investigations reported in this thesis were focused on the crosstalk between primary cancer cells and T lymphocytes, in the development of brain metastasis.

Thus far, studies have mainly focused on the management and therapeutic purposes of brain metastases, neglecting the need of basic insights into the reasons why brain metastases develop in the first place. The work in this thesis strongly suggests the eminent role of T lymphocyte contact at the primary tumor site to entrust breast cancer cells the ability to colonize the brain, surmising the involvement of common molecules inherent to trivial pathways, most likely tumor-type- and context-dependent, in the T lymphocyte-induced outline of a primary tumor-to-brain metastatic relapse.

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Chapter 9

Summary
Samenvatting
Resumo/Sumário

SUMMARY

The spread of cells from a primary tumor locus though the blood circulation (haematogenous seeding) into the brain microvasculature, is the first step towards the development of brain metastases. The brain is a unique target organ for metastatic outgrowth due to its lack of lymphatic drainage, and its surrounding blood-brain barrier (BBB), the latter rendering the brain a sanctuary site for exploitation and survival of neoplastic cells. **Chapter one** englobes a brief introduction to brain metastasis, its pathogenesis and historical perspectives on organotropism; the BBB is morphologically and functionally characterized, and resistance mechanisms known to support brain metastatic relapse, from lung and breast cancers' systemic therapies, are broached. Brain metastases from breast cancers are often single, whereas lung cancers brain metastases have a higher tendency to develop multiple colonies; **Chapters two** and **three** provide reviews of the biology and molecular mechanisms predictive of the spread of cancer cells into the brain, for breast and lung cancers, respectively; furthermore, the implication for current and potential future treatment strategies are discussed.

The involvement of the T-cell response in the promotion of brain metastasis from ER- breast cancers was identified in **chapter four**. Here, we compared gene expression profiles of ER- primary breast cancers between patients who developed metastases, including and excluding brain. The involvement of the T-cell response in promoting brain metastasis was further demonstrated by co-culture experiments with ER- breast cancer cells and activated T lymphocytes, followed by *in vitro* BBB trespassing assays of breast cancer cells only, and in an *in vivo* immunocompetent syngeneic mouse model, whereas the six-fold increase in the ability to generate secondary tumors in the brain was not mimicked in other organs. Comparison of mass spectrometry results from breast cancer cells co-cultured with T lymphocytes, matched against the gene expression profiles of ER- breast cancers from patients with brain metastasized disease, led to the identification of a common upregulated protein, Guanylate-Binding Protein 1 (*GBP1*), which binds to and remodels the actin cytoskeleton, of relevance in processes like migration and invasion; *GBP1* was shown to be upregulated in primary breast cancers of patients who developed brain metastases, which have been in direct contact with T lymphocytes. Furthermore, *GBP1* silencing experiments showed a reduced ability of breast cancer cells to cross the *in vitro* BBB model.

GBP1 is a secreted GTPase that is induced by IFNγ and therefore, in **chapter five**, we sought to scrutinize the extent in which IFN-γ pathway is involved in facilitating brain metastasis of ER- breast cancer. Co-culture of breast cancer cells with different subtypes of activated T lymphocytes, disclosed an enhanced influence of CD8+ T lymphocytes, which translated in a higher *in vitro* BBB invasion by breast cancer cells. The contribution of the IFN-γ signaling pathway in promoting brain metastasis development from ER- breast cancer cells, was confirmed by inhibition experiments on breast cancer cells, prior to the co-culture with activated

T lymphocytes. Moreover, using a neutralizing monoclonal antibody of soluble human IFN-γ, or using a monoclonal antibody blocker of the IFN-γ receptor 1 (IFNGR1) in breast cancer cells, we showed that concentration-dependent blocking of the IFN-γ pathway impairs both *GBP1* protein expression in breast cancer cells and invasion through the BBB. Furthermore, we showed that an active IFN-γ pathway in breast cancer cells grants the upregulation of CXCL9, -10, -11/CXCR3 axis, which has been shown to be an axis constitutively present in the brain and to take part in the pathogenesis of CNS diseases. Importantly, priming breast cancer cells with publicly available recombinant IFN-γ did not increase their trespassing ability.

Further validation of non-immune related overexpressed genes in the ER- primary breast cancers of patients with brain metastatic disease, was performed in **chapter six**. Here, Brother of CDO precursor (*BOC*), Gap junction protein delta 3 (*GJD3*) and SPARC/osteonectin, CWCV, and Kazal-like domains of the proteoglycan 2 (*SPOCK2*) were the most significant identified genes, and protein level expression for BOC and SPOCK2 was further validated on an independent group of FFPE ER- primary breast cancers. mRNA validation from the same sample cohort was successful only for *SPOCK2*, and a publicly available database of 204 primary breast cancers with different metastatic sites was used to validate the mRNA expression of both *SPOCK2* and *GJD3*.

Finally, and because the reduction of trespassed breast cancer cells achieved by the above mentioned neutralizing and blocking assays did not match the nearly-absent trespassing rates of non-primed breast cancer cells, observed in the control situation, in **chapter seven** we aimed to disclose other signaling pathways entangled in assisting the formation of brain metastasis enabled by T lymphocyte crosstalk, as well as to study the level of extent in which activated T lymphocytes are able to change the proteome of other brain-trophic tumor types. Here, we showed that although an increased BBB transmigration after the co-culture with activated T lymphocytes is not a breast cancer exclusive feature, activated T lymphocytes do enhance the transmigration abilities of breast cancer cells through an *in vitro* BBB with a higher significance than other cancer types. MS measurements of all cell lines, before and after T lymphocyte co-culture, revealed a common overexpressed protein in all cells that showed an enhanced transmigration after T lymphocyte priming (except lung cancers). Accordingly, Coronin-1A (*CORO1A*) was overexpressed in all breast and prostate cancers and melanoma cells after T lymphocyte co-culture. Furthermore, *CORO1A* silencing experiments on breast cancer cells showed a significant decreased invasion through the *in vitro* BBB of exclusively brain-trophic breast cancer cells. Finally, using an independent primary breast cancer cohort, we showed that *CORO1A* protein levels are significantly upregulated in primary breast cancers from patients who developed brain metastasis, whereas mRNA level upregulation of *CORO1A* was significantly validated in a publicly available mRNA expression database of primary breast cancers with different metastatic sites.

SAMENVATTING (DUTCH SUMMARY)

De verspreiding van cellen vanuit een primaire tumor via de bloedcirculatie naar de microvasculatuur van de hersenen is de eerste stap op weg naar de ontwikkeling van hersenmetastasen. De bloed-hersenbarrière (blood-brain barrier, BBB) speelt in dit proces meer dan één rol: de tumorcellen moeten deze specifieke vasculaire barrière doorkruisen, en de immuunrespons die met metastasering gepaard gaat, verloopt door aanwezigheid van de BBB anders dan bij andere organen.

Hoofdstuk 1 omvat een korte inleiding over hersenmetastasen, de pathogenese ervan en historische perspectieven van organotropisme, alsmede de morfologische en functionele gekarakterisering van de BBB. Tevens worden resistentiemechanismen van systemische therapieën van long- en borstkanker (waarvan bekend is dat ze hersenuitzaaiingen kunnen bevorderen), besproken. Hersenmetastasen van borstkanker komen vaak laat en zijn vaak enkelvoudig, terwijl hersenmetastasen van longkanker vroeg kunnen komen en de neiging hebben om meerdere kolonies te ontwikkelen. In de **Hoofdstukken 2** en **3** wordt een overzicht van de biologie en moleculaire mechanismen die voorspellend zijn voor de verspreiding van kankercellen naar de hersenen, voor respectievelijk borst- en longkanker, gepresenteerd. Daarbij worden de implicaties voor huidige, en mogelijke toekomstige, behandelingsstrategieën genoemd.

De betrokkenheid van de T-cel respons bij de bevordering van hersenmetastasen van borstkanker wordt in **hoofdstuk 4** behandeld. Hier hebben we de genexpressieprofielen van oestrogeenreceptor-negatieve (ER-) primaire borstkankers van patiënten met, en zonder, hersenmetastasen vergeleken. De betrokkenheid van de T-celrespons bij de vorming van hersenmetastasen werd experimenteel uitgewerkt door co-kweekexperimenten met ER-borstkankercellen en geactiveerde T-lymfocyten, gevolgd door onze *in vitro* BBB-invasie-assays. Tevens werd een *in vivo* immuuncompetent syngeen muismodel ontwikkeld, waarin de toename van hersenenmetastasering na T-lymphocyten interacties van de tumor cellen, kon worden nagebootst. Vergelijking van de *in vitro* geïdentificeerde eiwitten die tot expressie kwamen na T-lymphocyten interactie van de tumorcellen met de genexpressie profielen in humane borstkankers van patiënten die hersenmetastasen hadden ontwikkeld, leidde tot de identificatie van het Guanylate Binding Protein 1 (GBP1). GBP1 bindt aan, en remodelleert, het actine-cytoskelet van cellen, hetgeen van belang is voor processen als migratie en invasie. In GBP1-knockdown-experimenten vonden we inderdaad een verminderd vermogen van de borstkankercellen om het *in vitro* BBB-model te passeren.

GBP1 is een GTPase waarvan de expressie door IFN-γ wordt geïnduceerd en daarom hebben we in **hoofdstuk 5** onderzocht in hoeverre de IFN-γ-route betrokken is bij het faciliteren van de vorming van hersenmetastasen van ER- borstkankers. Allereerst wilden we weten welke van

de T-lymphocyten subpopulaties het meeste effect op de passage van de tumorcellen sorteert. Na kweken van de borstkankercellen met verschillende T lymfocyten subpopulaties bleek dat de invloed van CD8+ T-lymfocyten op de BBB passage van de tumorcellen het sterkst was. De rol van IFN-γ op de passage van de borstkankercellen werd getest in experimenten waarbij de invloed van IFN-γ op twee niveaus werd uitgeschakeld. Met behulp van een neutraliserend monoklonaal antilichaam van oplosbaar humaan IFN-γ enerzijds, en door middel van een monoklonale antilichaamblokker van de IFN-γ-receptor 1 (IFNGR1) anderzijds, konden we aantonen dat blokkade van de IFN-γ-signaleringsroute zowel de expressie van GBP1, als de passage door de BBB van de tumorcellen, vermindert. Verder toonden we aan dat de IFN-γ-signaleringsroute in borstkankercellen de expressie van de CXCL9,-10,-11/CXCR3-as stimuleert. Tot slot toonden we aan dat synthetisch geproduceerd (recombinant) IFN-γ geen positief effect heeft op de passage van tumorcellen door de BBB.

Naast de in Hoofdstuk 4 geïdentificeerde T cel respons en invloed van IFN-γ werden nog andere differentieel getransleerde eiwitten gevonden, die geassocieerd lijken te zijn met de vorming van hersenmetastasen bij patiënten met borstkanker. In **hoofdstuk 6** worden deze gevalideerd met behulp van tumorweefsels van patiënten met borstkanker, die wel en niet hersenmetastasen ontwikkelden. De meest significante verschil-genen waren Brother of CDO precursor (BOC); Gap junction protein delta 3 (GJD3) en SPARC / osteonectine; CWCV en Kazal-like domains of the proteoglycan 2 (SPOCK2). De expressie van BOC en SPOCK2 werd verder gevalideerd in een onafhankelijke groep ER- primaire borsttumoren. Op het niveau van mRNA, was de validatie van SPOCK2 het meest succesvol, zowel op het onafhankelijke FFPE-cohort van borsttumoren als op een mRNA-expressie openbaar beschikbare database van 204 primaire borstkankers met verschillende systemische metastases, waar GJD3 ook succesvol gevalideerd werd.

In **hoofdstuk 7** vergeleken we het effect van T-lymphocyten op de passage door de *in vitro* BBB van tumorcellijnen die werden afgeleid van andere primaire tumoren dan borstkanker, zoals longkanker, prostaatkanker en melanoom. We toonden aan dat T-lymphocyten niet uitsluitend effect hebben op de passage van de borstkanker cellijnen, maar ook op die welke afgeleid zijn van de andere tumoren. Door vergelijking van de eiwitexpressie van deze cellijnen, voor en na kweek met T lymfocyten, vonden we dat Coronin-1A (CORO1A) in alle borst- en prostaatkankers en melanoomcellen tot expressie wordt gebracht. *CORO1A*-knockdown in borstkankercellen veroorzaakte een significant verminderde passage door de *in vitro* BBB van die borstkankercellen, die waren afgeleid van tumoren die een predilectie voor hersenen hadden. Ten slotte konden we in een onafhankelijk primair borstkankercohort aantonen dat de CORO1A-eiwitniveaus significant verhoogd waren in de tumoren van de patiënten die hersenmetastasen ontwikkelden. Tevens vonden we in een openbaar beschikbare mRNA-expressiedatabase van primaire borstkankers met verschillende metastatische sites stijging van het mRNA-niveau van CORO1A in die tumoren, die naar de hersenen waren gemetastaseerd.

SUMÁRIO/RESUMO (PORTUGUESE SUMMARY)

A disseminação de células cancerígenas desde o local do tumor primário, através da circulação sanguínea (disseminação hematogénica) até à microvasculatura cerebral, é o primeiro passo para o desenvolvimento de metástases cerebrais. Devido à sua falta de drenagem linfática e à sua barreira hemato-encefálica (blood-brain barrier, BBB), o cérebro é um órgão-alvo único para crescimento metastático, tornando-o num santuário para exploração e sobrevivência de células neoplásicas.

O **Capítulo 1** desta tese aborda uma breve introdução a metástases cerebrais, a sua patogénese e perspetivas históricas sobre o organotropismo; a BBB é morfológica e funcionalmente caracterizada, assim como os mecanismos de resistência que apoiam recidivas cerebrais de cancro de pulmão e mama. Metástases cerebrais do cancro da mama são geralmente solitárias, enquanto metástases cerebrais do cancro do pulmão são mais comummente abundantes em número.

Os **Capítulos 2** e **3** fornecem revisões da biologia e dos mecanismos moleculares preditivos para disseminação cerebral de células do cancro da mama e do pulmão, respectivamente. Além disso, as implicações para estratégias de tratamento atuais e futuras são discutidas.

O envolvimento de células sanguíneas, nomeadamente linfócitos T, na promoção de metástases cerebrais do cancro da mama, negativo para o receptor de estrogéneo (ER-), foi identificado no **Capítulo 4**. Aqui, comparámos os perfis de expressão genética (p-value < 0.05) de tumores da mama primários ER- entre pacientes que desenvolveram metástases, incluindo e excluindo o cérebro. O envolvimento de linfócitos T na promoção de metástases cerebrais foi ainda demonstrado por experiências prévias de co-cultura com células cancerígenas da mama ER- e linfócitos T ativados, efectuadas previamente a experiências funcionais de invasão através de uma BBB *in vitro*, exclusivamente com células cancerígenas do cancro da mama.

Adicionalmente, o desenvolvimento de metástases cerebrais do cancro da mama foi demonstrado ser preferível (6:1) em prol de metástases em outros orgãos, usando um modelo singénico *in vivo* de ratinhos imunocompetentes. Comparação de análises de espectrometria de massa (MS) de células do cancro da mama que foram previamente co-cultivadas com linfócitos T activados, com os perfis de expressão genética de tumores da mama ER- de pacientes com doença metastática cerebral, levou à identificação de uma proteína, guanylate binding protein 1 (*GBP1*) , que se associa e remodela o citoesqueleto de actina, que tem relevância em processos como migração e invasão de células; *GBP1* demonstrou ser positivamente regulado em células de tumores da mama primários que se apresentam em contacto directo com linfócitos T, em pacientes que desenvolveram metástases cerebrais. Além disso, experiências funcionais usando um modelo *in vitro* de BBB, com células do cancro da mama, cujo RNA mensageiro (mRNA)

de *GBP1* foi previamente silenciado e posteriormente co-cultivadas com linfócitos T activados, mostraram uma subsequente capacidade reduzida de invasão.

Devido ao facto de *GBP1* ser uma GTPase secretada e induzida pelo Interferão Gamma (IFN-γ), no **Capítulo 5** procurámos examinar a extensão na qual o IFN-γ está envolvido em facilitar metástases cerebrais do cancro da mama ER-. A co-cultura de células de cancro da mama com diferentes sub-tipos de linfócitos T ativados (CD4+, CD8+ ou uma mistura de ambos, CD3+) revelou uma maior influência da co-cultura de linfócitos T CD8+, que se traduziu numa maior invasão de células do cancro da mama através da BBB *in vitro*.

A contribuição da via de sinalização do IFN-γ em facilitar o desenvolvimento de metástases cerebrais de células do cancro da mama ER- foi confirmada por experiências de inibição em células do cancro da mama, previamente à co-cultura com linfócitos T ativados. Aqui, usando um anticorpo monoclonal neutralizante do IFN-γ humano solúvel (sIFN-γ), ou por meio de um anticorpo monoclonal bloqueador do receptor 1 do IFN-γ (IFNGR1) – receptor responsável pela ligação/conecção/ associação do sIFN-γ –, em células do cancro da mama, mostrámos que o bloqueio da via de sinalização do IFN-γ, dependentemente da concentração usada, prejudica tanto a expressão da proteína *GBP1*, quanto a invasão através da BBB *in vitro*. Além disso, mostrámos que a activação da via de sinalização do IFN-γ em células do cancro da mama concede uma regulação positiva do eixo CXCL9,-10,-11/CXCR3 em células cancerígenas mamárias, eixo demonstrado ser constitutivamente presente no cérebro e activo na patogénese de doenças do sistema nervoso cerebral. É importante ressaltar que a cultura de células cancerígenas mamárias com IFN-γ recombinante, publicamente disponível, não influencia a sua capacidade de invasão através da BBB *in vitro*.

Validação adicional de genes não-imunes, positivamente expressos em tumores da mama primários de pacientes com doença metastática cerebral, foi realizada no **Capítulo 6**. Aqui, três genes, Brother of CDO precursor (*BOC*), Gap junction delta 3 (*GJD3*) e SPARC/ osteonectin, CWCV and Kazal-like domains of the proteoglycan 2 (*SPOCK2*), foram identificados como os genes mais significativamente expressos ($p < 0.01$), e a sua expressão proteica de BOC e SPOCK2 foi posteriormente validada num grupo independente de tumores primários de mama ER-, fixados e embebidos em parafina (FFPE). Em contrapartida, validação ao nível de mRNA foi bem-sucedida apenas para *SPOCK2*, usando o mesmo grupo independente de tumores primários da mama FFPE e usando um banco de dados, publicamente disponível, de 204 tumores da mama primários com diferentes locais metastáticos, usado igualmente para a validação de *GJD3*.

Finalmente, e porque a redução do número de células cancerígenas mamárias através da BBB, alcançada pelas experiências de neutralização e bloqueio da via de sinalização do IFN-γ men-

cionados acima, não coincidiu com as taxas de invasão, quase ausentes, das células controlo (células do cancro da mama que não foram submetidas a co-cultura com linfócitos T activados), no **Capítulo 7** pretendemos divulgar outras vias de sinalização envolvidas no auxílio à formação de metástases cerebrais possibilitadas pelo contacto prévio com linfócitos T, assim como avaliar a extensão de impacto que linfócitos T ativados têm no proteoma de outros tipos de tumores com preferência para metastizar para o cérebro (cancro do pulmão e melanomas). Apesar de não metastizar com frequência para o cérebro, a capacidade de invasão do tumor da próstata foi igualmente avaliado. Aqui, apesar do impacto dos linfócitos T activados, traduzido num aumento de invasão através da BBB *in vitro*, ter sido demostrado não ser uma característica exclusiva de células do cancro da mama, mostrámos que os linfócitos T ativados têm um impacto mais poderoso (7:1 e 13:1) na invasão de células cancerígenas mamárias, em detrimento de tumores da próstata ou melanomas, respectivamente.

Subsequentes análises de MS de todas as linhas celulares testadas, antes e depois da co-cultura com linfócitos T activados, revelaram uma proteína – Coronin-1A (*CORO1A*) –, comummente expressa em todos os tumores que mostraram um aumento de invasão através da BBB *in vitro* após a incubação com linfócitos T (com excepção do cancro do pulmão). Além disso, experiências de silenciamento do gene *CORO1A* em três tipos distintos de células cancerígenas mamárias (duas com preferência para metastizar para o cérebro e uma sem registo de formação de metástases) mostraram uma redução significativa do número de células invasoras através da BBB, exclusivamente observada em células cancerígenas mamárias com preferência para metastizar para o cérebro.

Finalmente, usando um grupo independente de tumores primários de mama ER-, FFPE, mostrámos que os níveis proteicos de *CORO1A* são significativos e positivamente regulados em tumores primários da mama oriundos de pacientes que desenvolveram metástases cerebrais; adicionalmente, uma regulação positiva do nível de mRNA de *CORO1A* foi validada significativamente num banco de dados, publicamente disponível, de expressão de níveis de mRNA de tumores primários da mama com diferentes locais metastáticos.

Appendices

Curriculum Vitae auctoris
PhD portfolio
List of Publications

CURRICULUM VITAE AUCTORIS

Rute Marisa dos Santos Moreira Pedrosa, daughter of Júlio Moreira Pedrosa and Marilinda dos Santos Pedrosa, and sister of Cláudia Sofia dos Santos Moreira Pedrosa, was born on the 19th of May 1984 in Leiria, Portugal.

In 2004, Rute started a bachelor in Laboratory Biomedical Sciences at the Egas Moniz Higher School of Health (ESSEM), in Lisbon, where she completed the first three years, finalizing her last graduation year at Higher School of Health and Technology of Lisbon (ESTeSL) in 2008, in Lisbon, Portugal. After graduation, Rute decided to start her full-time professional activity abroad. Having England in mind as her initial goal, a job interview opportunity in The Netherlands changed the scenario. In December of 2008 she started as a laboratory technician at the Pathology department of Erasmus Medical Center, in Rotterdam. In September 2010, she embraced a new challenge as an analyst of Molecular Diagnostics and in July 2016 she was recruited as a PhD student in the same department of Erasmus MC, in Rotterdam. The results of the work performed in these 5 years underlie the contents of this thesis.

PHD PORTFOLIO

Name PhD student: **Rute Marisa dos Santos Moreira Pedrosa**
Erasmus MC Department: **Pathology**
Research School: **Postgraduate School Molecular Medicine (MolMed)**
PhD Period: **2016 – 2021** Promotor: **Prof. dr. J.M. Kros** Co-Promotor: **Dr. D.A. Mustafa**

PhD training	Year	Workload (ECTS)
General Courses		
Basic and Translational Oncology Course	**2016**	**1.80**
Research Integrity Course	**2016**	**0.30**
Photoshop and Illustrator CS5 Workshop for PhD-students	**2016**	**0.30**
Follow-up Workshop on Photoshop and Illustrator CS6 for PhD students	**2017**	**0.30**
Biomedical English Writing Course	**2017**	**2.00**
Basic Introduction Course on SPSS	**2017**	**1.00**
Basic Course on R	**2017**	**2.00**
Workshop Ingenuity Pathway Analysis (IPA) Workshop	**2017**	**0.40**
Gene Expression data analysis using R: How to make sense out of your RNA-Seq/microarray data	**2018**	**2.00**
The Workshop Career Development for PhD Candidates	**2018**	**0.15**
The Introduction in GraphPad Prism Version 6	**2018**	**0.30**
The Microscopic Image Analysis: From Theory to Practice	**2019**	**0.80**
Seminars		
BBBNedwork meeting – Barriers of the Brain in Ageing – Leiden, The Netherlands	**2017**	**0.30**
(Inter) National Conferences and Presentations		
21st Molecular Medicine Day – Rotterdam, The Netherlands 21st March (1 poster presentation + Pitch)	**2017**	**0.40**
29th European Congress of Pathology – Amsterdam, The Netherlands 3rd – 6th September (2 oral presentations)	**2017**	**2.00**
7th Annual Brain Metastases Research and Emerging Therapy Conference – Marseille, France 6th and 7th October (1 oral presentation)	**2017**	**1.70**
6th Erasmus MC Cancer Institute Research Day – Rotterdam, The Netherlands 30th November (1 oral presentation)	**2017**	**0.30**
11th Joint Meeting of the British Division of the International Academy of Pathology and the Pathological Society of Great Britain & Ireland – Maastricht Pathology 2018 – Maastricht, The Netherlands 19th – 22nd June (1 poster presentation)	**2018**	**1.70**
30th European Congress of Pathology – Path to Precision medicine – European Society of Pathology, Bilbao, Spain 8th – 12th September (1 poster presentation)	**2018**	**2.00**

2nd EACR-MRS Conference, Berlin, Germany 16th and 17th August	**2019**	**2.00**
Teaching Activity		
Mentored and supervised a bachelor trainee in the lab (6 months)	**2018**	**4.00**
Teaching assistance for Pathology: - Histology and Histopathology for endocrine organs - Pathology between SCLC and NSCLC	**2016-2021**	 **2.50** **2.50**
Awards		
- Best Oral Presentation – 29th European Congress of Pathology – Amsterdam, The Netherlands. “T lymphocytes induce the expression of GBP1 and facilitate brain metastasis of breast cancer”	**2017**	
	Total	**30.75**

LIST OF PUBLICATIONS

This Thesis

- **Pedrosa, R.M.S.M.**, Mustafa, D.A., Soffietti, R. and Kros, J.M. *Breast cancer brain metastasis: molecular mechanisms and directions for treatment.* Neuro Oncol, 2018. **20**(11): p. 1439-1449.
- **Pedrosa, R.M.S.M.**, Mustafa, D.A., Aerts, J.G.J.V. and Kros, J.M. *Potential Molecular Signatures Predictive of Lung Cancer Brain Metastasis.* Frontiers in Oncology, 2018. **8**. p. 159.
- Mustafa, D.A.M., **Pedrosa, R.M.S.M**., Smid, M., Weiden, M., Weerd, V., Nigg, A., Berrevoets, C., Zeneyedpour, L., Priego, N., Valiente, M., Luider, T.M., Debets, R., Martens, J.W.M., Foekens, J.A., Sieuwerts, A.M. and Kros, J.M. T lymphocytes facilitate brain metastasis of breast cancer by inducing Guanylate-Binding Protein 1 expression. Acta Neuropathol, 2018. **135**(4): 581-599.
- **Pedrosa, R.M.S.M.**, Leonoor L. V. Wismans, Renata Sinke, Marcel van der Weiden, Casper H. J. van Eijck, Johan M. Kros and Dana A. M. Mustafa (2021). "Differential Expression of BOC, SPOCK2, and GJD3 Is Associated with Brain Metastasis of ER-Negative Breast Cancers." Cancers, 2021. **13**(12): 2982.

o T lymphocyte IFN-γ enhances the ability of breast cancer cells to pass the blood-brain barrier (*submitted to Advanced Science*)
Authors: **Rute M.S.M. Pedrosa**, Johan M. Kros, Benjamin Schrijver, Cor Berrevoets, Rute B. Marques, Casper C.H.J van Eijck, Reno Debets, Pieter J. M. Leenen, Willem A. Dik, Dana A. M. Mustafa

o T lymphocytes induce the expression of Coronin-1A in various types of cancer cells enhancing the transmigration through the blood-brain barrier (s*ubmitted to Genomics, Proteomics & Bioinformatics*)
Authors: **Rute M.S.M. Pedrosa**, Lona Zeneyedpour, Idries Habib, Cor Berrevoets, Reno Debets, W.A. van Cappellen, Casper C.H.J van Eijck, Theo M. Luider, Willem A. Dik, Johan M. Kros, Dana A.M. Mustafa

Other Publications

- Geurts-Giele, W.R., Verschuer, V.M.T., Deurzen, C.H.M., Diest, P.J., **Pedrosa, R.M.S.M.**, Collée, J.M., Koppert, L.B., Seynaeve, C. and Dinjens, W.N.M. *Molecular determination of the clonal relationships between multiple tumors in BRCA1/2-associated breast and/or ovarian cancer patients is clinically relevant.* Mod Pathol, 2017. **30**(1): p. 15-25.
- van der Leest, C., Wagner, A., **Pedrosa, R.M.S.M.**, Aerts, J.G., Dinjens, W.N.M. and Dubbink, H.J. *Novel EGFR V834L Germline Mutation Associated With Familial Lung Adenocarcinoma.* Jco Precision Oncology, 2018. **2**.

- Mustafa, D. A., **Pedrosa, R.** and Kros, J.M. (2020). Pathology of Brain Metastasis. Central Nervous System Metastases. M. Ahluwalia, P. Metellus and R. Soffietti. Cham, Springer International Publishing**:** 15-29.

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