Universidade de Lisboa

Faculdade de Medicina da Universidade de Lisboa

Brain Metastases: immune characterisation and the impact over prognosis

Catarina Pinheiro Barracha Pinto de Abreu

Orientadora: Doutora Maria Rita Dionísio, HSM-CHULN
Co-Orientadora: Profª. Doutora Sandra Casimiro, iMM, FMUL

Dissertação especialmente elaborada para obtenção do grau de Mestre em Oncobiologia

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A impressão desta dissertação foi aprovada pelo Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 16 de Abril de 2019.

“When you change the way you look at things,
the things you look at change.”

Max Planck

AMDG
Para a minha Família.

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Muito obrigada a todos,

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RESUMO

O cancro é uma das principais causas de morte em todo o mundo. Estima-se que em 2018 existam mais de 18.1 milhões de casos de cancro e 0.5 milhões de novos casos em todo o mundo. As metástases cerebrais são o tumor intracraniano com maior incidência na idade adulta, ocorrendo mesmo com mais frequência que os tumores primários do cérebro. Estima-se que cerca de 20% dos doentes por cancro desenvolverão metástases cerebrais, com significativo aumento de morbilidade e mortalidade associada a cancro. A principal causa de morte por cancro está relacionada com a recaída à distância e, embora o sistema nervoso central não seja o local primário de recaída mais frequente, no caso do tumor da mama – HER2-amplificado e triplo negativo – existe tropismo para o cérebro, sendo frequente o sistema nervoso central o local primário de recaída. Historicamente, o prognóstico da doença metastática cerebral é considerado mau e as abordagens clássicas de radioterapia, nomeadamente radiocirurgia e radioterapia à totalidade do cérebro (*Whole-Brain Radiotherapy,* WBRT), são tratamento paliativo. Contudo, as alternativas terapêuticas que têm surgido nas últimas décadas têm vindo a alterar este paradigma. Actualmente, os pacientes vivem mais tempo após o diagnóstico e tratamento de metástases cerebrais, o que levanta novas preocupações, nomeadamente quanto à toxicidade neurocognitiva associada à terapêutica mencionada, e constitui um desafio na prática clínica. Mais ainda, a barreira hematoencefálica impede a livre passagem de diversas moléculas e de agentes quimioterápicos. No entanto, sofre disrupção ao ser lesada aquando do desenvolvimento de metástases cerebrais, o que torna muito relevante a investigação de terapêuticas eficazes que a consigam penetrar.

Em cancro do pulmão, carcinoma de células renais, melanoma e cancro da mama, a incidência de metástases cerebrais revela-se elevada, correspondendo a cerca de 16-20%, 6-10%, 6-8% e 5-6%, respectivamente. A prevalência é maior nos cancros do pulmão e da mama, mas o maior risco de desenvolvimento de lesões secundárias no sistema nervoso central é registado em melanoma.

O cancro da mama é tradicionalmente considerado não-imunogénico, no entanto, diversos estudos têm vindo a sugerir que o sistema imunitário, incluindo os linfócitos que infiltram o tumor (*tumour-infiltrating lymphocytes*, TILs), tem um papel na interacção hospedeiro-tumor. Os TILs, leucócitos que migram para o tumor

através da corrente sanguínea, são genericamente considerados indicadores de bom prognóstico e preditivos de resposta, nomeadamente em pacientes com cancro da mama tratados em neoadjuvância. Existem diferentes subpopulações de TILs com funções distintas no microambiente tumoral, incluindo os TILs CD4+ e CD8+, biomarcadores de bom prognóstico, particularmente em carcinoma ductal invasivo da mama.

Deste modo, o nosso principal objectivo foi aferir o valor prognóstico de TILs e das subpopulações TILs CD4+ e CD8+ no estroma tumoral de metástases cerebrais de cancro da mama, no contexto clínico. Os resultados obtidos em cancro da mama foram comparados com os resultados obtidos num grupo de doentes com cancros do pulmão, do rim, do cólon e melanoma, tumores considerados imunogénicos. Como objectivos secundários, propusemo-nos avaliar a presença de astrócitos reactivos, células caracterizadas por fenótipo hipertrófico e aumento de densidade e rearranjo de GFAP (proteína específica dos filamentos intermédios do citoesqueleto de elementos gliais) e que têm sido associadas a metastização cerebral, bem como verificar uma possível associação com sobrevida global; e avaliar a expressão de PD-L1, uma proteína envolvida na resposta imunitária, em metástases cerebrais. Finalmente pretendíamos verificar se o subtipo molecular se alterava entre tumor primário da mama e metástase, já que muitas vezes se observa a perda ou ganho de expressão de receptores hormonais ou mesmo HER2 no tecido metastático, com consequências clínicas importantes sobretudo ao nível da terapêutica.

A coorte utilizada para este estudo incluía 56 doentes – 34 (60.7%) do sexo feminino e 22 (39.3%) do sexo masculino – diagnosticados com metástases cerebrais entre 2009 e 2013 e seguidos no Serviço de Oncologia Médica do HSM – CHULN. As metástases cerebrais provinham de cancro da mama (n=25, 44.7%), cancro do pulmão (n=15, 26.8%), cancro colo-rectal (n=6, 10.7%), cancro do rim (n=6, 10.7%) e melanoma (n=4, 7.1%). Todos os doentes foram submetidos a radioterapia holocraniana após ressecção cirúrgica. A idade mediana à data do diagnóstico era de 58.50 anos e variava entre 28 e 90 anos. Por forma a estudar esta coorte, procedemos à análise da coloração hematoxilina e eosina e imunohistoquímica dos marcadores acima mencionados na sub-coorte de doentes de cancro da mama e, no caso de TILs, CD4 e CD8, comparámos com os resultados dos doentes com metástases cerebrais de outras origens.

A análise de sobrevivência mostrou que existe uma associação entre níveis elevados de TILs CD8+ no estroma do tumor e uma maior sobrevida no grupo de doentes que inclui todos os tipos de tumor primário (Log-rank (Mantel Cox) test: HR 2.127, 95% CI 1.002-4.518; *p*=0.0495). O mesmo se observou no grupo que exclui os casos com origem em cancro da mama (Log-rank (Mantel Cox) test: HR 0.4290, 95% CI 0.1370-0.8486; *p*=0.0296). Observou-se ainda uma tendência para uma maior sobrevida, quando os níveis de TILs são elevados. No entanto, em nenhum dos grupos – metástases com origem em cancro da mama, incluindo todos os tipos de tumor primário ou excluindo cancro da mama – se obteve significância estatística.

Neste estudo, as amostras com TILs CD4+ ou expressão de PD-L1 foram raras. No entanto, num estudo *in vitro* num painel de linhas celulares tumorais, observou-se uma sobre-expressão de PD-L1 (*PD-L1*) nas linhas com tropismo para o cérebro e na linha com tropismo para o osso, quando comparadas com as restantes e com células epiteliais mamárias normais. A ligação de PD-L1 ao receptor PD-1 liberta um sinal inibitório que reduz a proliferação de células T com especificidade para antigénio e, simultaneamente, diminui a apoptose em células T regulatórias. No contexto tumoral, é um marcador preditivo de resposta à imunoterapia, permitindo identificar os subgrupos de doentes que beneficiarão mais da terapêutica com agentes anti-PD-1 ou anti-PD-L1, já que a sua sobre-expressão no tumor foi associada a maior agressividade do mesmo, nomeadamente em carcinoma de células renais. Finalmente, verificou-se uma alteração de subtipo molecular em 52.6% dos casos, incluindo perda ou ganho de receptores hormonais, e perda de expressão de HER2 num caso. No contexto dos objectivos secundários deste estudo, não foi possível verificar a associação com a densidade linfocitária devido ao tamanho reduzido da amostra.

Em suma, os resultados obtidos neste projecto mostram uma correlação entre elevada densidade de TILs CD8+ e maior sobrevida global, quando analisando todos os tipos de tumor primário em conjunto ou excepto cancro da mama e uma tendência para maior sobrevida global, nos casos de cancro da mama. Assim, a população linfocitária que infiltra o tumor tem relevância no contexto clínico e o estudo das suas várias subpopulações será sempre mais útil e informativo do que o da população total apenas, uma vez que o valor prognóstico varia entre subpopulações (e também é diferente comparando subpopulações e TILs totais). Os resultados apontam também para a relevância de estudar o subtipo molecular da

metástase cerebral, visto que pode ter impacto na progressão da doença, já que algumas linhas terapêuticas não eficazes aquando do diagnóstico do tumor primário podem revelar-se eficazes em contexto metastático, como a alteração do *status* do receptor de estrogénio para positivo que se registou neste estudo. Todas as análises referentes à densidade de TILs deverão ser replicadas numa coorte alargada e mais subpopulações, bem como outras populações imunitárias – como células T reguladoras ou linfócitos T gama-delta – deverão ser acrescentadas ao painel. A amostra de doentes com metástases cerebrais com origem em cancro da mama também deverá ser aumentada e o número de casos deverá ser semelhante entre subtipos, de forma a aprofundar o estudo da alteração de *status* de cada marcador e de subtipo molecular.

PALAVRAS-CHAVE

Metástases cerebrais, Microambiente tumoral, Infiltrado linfocitário (TILs), Infiltrado linfocitário CD8+ (TILs CD8+), Subtipo molecular.

ABSTRACT

Brain metastases are more frequent than primary tumours of the brain and breast cancer is one of the tumour types with more brain metastization propensity. Approximately 15% of women with newly diagnosed metastatic breast cancer will develop secondary lesions in the brain. In HER2-amplified and TNBC the brain can be first site of metastasis. Tumour-infiltrating lymphocytes, TILs, promising prognostic biomarkers in breast cancer, were described in brain metastases, although the brain has been considered an immune-privileged site for metastases.

Therefore, our main goal was to study the prognostic value of total, CD4+ and CD8+ stromal TILs in patients with breast cancer brain metastases, using brain metastases from immunogenic solid tumours, such as lung cancer, kidney cancer, colon cancer and melanoma, as comparators. As secondary objectives, we aimed to evaluate the presence of reactive astrocytes; and to assess the expression of PD-L1, as it is a predictive marker of benefit from immunotherapy. In parallel, we compared breast cancer and respective brain metastases patient tissue samples to assess a possible molecular subtype switch.

A positive and significant correlation between CD8+ TILs in the stroma and overall survival was found when analysing all tumours together. CD4 and PD-L1 staining were rare events in this study. However, PD-L1 was particularly up-regulated in brain tropic TNBC clones, amongst a panel of different breast cancer cell lines. Reactive astrocytes were not observed. Alterations in molecular subtype between primary tumours and secondary lesions were observed (10/19, 52.6%).

Overall, this project gives some input on T-cell infiltrates and their relevance in brain metastases from various types of tumours. It reinforces the importance of studying TILs subsets pointed out by several studies as crucial to understand their different associations with prognosis and progression in brain metastases and other tumour types. Immune microenvironment research is at the forefront of cancer research and this project raises awareness on the importance of studying immune profiles even when the immune system is thought not to have impact on the tumour and its secondary lesions, like in breast cancer.

KEYWORDS

Brain Metastases, Immune microenvironment, Tumour-infiltrating lymphocytes (TILs), CD8+ TILs, Molecular subtype.

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ºC	Degree Celsius
$2^{-\Delta\Delta Ct}$	Fold difference
µg	Microgram
µg/mL	Microgram/millilitre
µL	Microlitre
µm	Micrometre
Units/mL	Units/millilitre
v/v	Volume/volume percent
%	Percentage
B7-H1	B7-Homolog 1
BBB	Blood-Brain Barrier
BC	Breast Cancer
BCBrMets	Breast Cancer Brain Metastases
BrMets	Brain Metastases
BT-474	B-Type-474 (breast cancer cell line)
CCLE	Cancer Cell Line Encyclopedia
CD274	Cluster of Differentiation 274
CD279	Cluster of Differentiation 279
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
cDNA	complementary DNA
CNS	Central Nervous System
CO_2	Carbon dioxide
COX2	Cycloxygenase-2
Ct	Cycle threshold
CUP	Cancer of Unknown Primary
DAB	3,3'-diaminobenzidine
DFS	Disease-Free Survival
DMEM	Dulbecco's Modified Eagle's Medium
EGFP	Enhanced Green Fluorescence Protein
ER	Oestrogen Receptor
FBS	Fetal Bovine Serum
FFPE	Formalin-Fixed, Paraffin-Embedded
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GCO	Global Cancer Observatory
GFAP	Glial Fibrillary Acidic Protein

h	Hour
H&E	Haematoxylin and Eosin staining
HER2	Human Epidermal Growth factor Receptor type-2
HER2+	HER2-amplified
HR	Hormone Receptor
H-score	Histo-score
HSM – CHULN	*Hospital de Santa Maria – Centro Hospitalar Universitário Lisboa Norte*
IARC	International Agency for Research on Cancer
IHC	Immunohistochemistry
INSERM	*Institut National de la Santé et de la Recherche Médicale*
IPATIMUP	*Instituto de Patologia e Imunologia Molecular da Universidade do Porto*
IQR	Interquartile Range
MCF10A	Michigan Cancer Foundation-10A (mammary epithelial cell line)
MCF7	Michigan Cancer Foundation-7 (breast cancer cell line)
MDA-MB-231	M.D. Anderson – Metastatic Breast 231 (breast cancer cell line)
MDA-MB-231-BO2	M.D. Anderson – Metastatic Breast 231 – Bone tropic (breast cancer cell line)
MDA-MB-231-BR	M.D. Anderson – Metastatic Breast 231 – Brain tropic (breast cancer cell line)
MDA-MB-231-BR HER2+	M.D. Anderson – Metastatic Breast 231 – Brain tropic HER2-amplified (breast cancer cell line)
MDA-MB-231-BR HER2-	Brain tropic M.D. Anderson – Metastatic Breast 231 – Brain tropic HER2-negative (breast cancer cell line)
MDA-MB-361	M.D. Anderson – Metastatic Breast 361 – Brain tropic (breast cancer cell line)
MDA-MB-435S	M.D. Anderson – Metastatic Breast 435 spindle shaped variant (breast cancer cell line)
min	Minute(s)
NA	Not Applicable
NSCLC	Non-Small Cell Lung Cancer
ON	Overnight
OS	Overall Survival
PBS-T	Phosphate Buffered Saline with 0.05% Tween 20
PR	Progesterone Receptor
PD-1	Programmed Cell Death-Protein 1

PD-L1	Programmed Cell Death-Ligand 1
PD-L2	Programmed Cell Death-Ligand 2
PC-3	Prostate Cancer-3 (Prostate cancer cell line)
RNA	Ribonucleic Acid
RT	Room Temperature
RT-qPCR	Reverse Transcription - Quantitative Polymerase Chain Reaction
sec	Seconds
ST6GALNAC5	α2, 6-sialyltransferase
Th	Helper T cell
TILs	Tumour-Infiltrating Lymphocytes
TMAs	Tissue Microarrays
TNBC	Triple Negative Breast Cancer
Treg	Regulatory T cell
WBRT	Whole-Brain Radiation Therapy
WHO	World Health Organization

1 INTRODUCTION

Cancer is one of the main causes of death worldwide and the number of new cases is on the rise globally, which represents a major impact on society. The World Health Organization (WHO) estimates a number of incident cases of 18.1 million in 2018 for all cancers, all ages and both sexes, with 0.5 million new cases across the world this year. According to WHO, incidence will increase to 29.5 million in 2040. These data were obtained from the Global Cancer Observatory (GCO) web-based platform (https://gco.iarc.fr/tomorrow/home), consulted in 10 November 2018.

Cancer cells can spread from the primary cancer locally, regionally and to distant sites. The formation of new tumours in parts of the body other than the site where they first formed is called metastases. Although metastases can form in most any part of the body, different types of cancer are more likely to spread to certain areas than others. Brain metastases (BrMets), or secondary brain tumours, are thought to have an incidence higher than 9% to 17% based on various studies, and the tumour types that more frequently metastasize to the brain are breast cancer (BC), lung cancer, melanoma, colon cancer and kidney cancer[1]. Metastatic cancer to the brain has a poor prognosis, with an average survival of less than 6 months[2,3].

1.1 Brain Metastases

BrMets are one of the most frequent and devastating neurological complications related to systemic cancer, thus representing a significant cause of morbidity and mortality[1,4,5]. They are the commonest intracranial tumours, surpassing primary brain tumours. Despite that, there are no available data from the International Agency for Research on Cancer (IARC) neither other large-scale studies examining the incidence and prevalence of BrMets[6]. In Portugal these epidemiological data are also lacking. Most BrMets originate from lung cancer (36-64%), BC (15-25%) and melanoma (5-20%), which together account for 67% to 80% of all cancers[7]. Also, in up to 15% of patients with presentation of BrMets, these will correspond to a diagnosis of cancer of unknown primary (CUP)[5].

BrMets have traditionally been managed with whole-brain radiation therapy (WBRT) or stereotactic surgery, and are typically associated with a poor prognosis[3]. Still, early detection and improvements in the treatment of primary tumour and systemic disease over the past decades resulted in the increase of survival of

patients with BrMets[8,9]. However, the frequency of the diagnosis of BrMets appears to be increasing also, occurring in 20% to 40% of patients with cancer in the United States of America[1,10]. The increased rate of diagnosis and lack of effective therapies are of significant concern and turn BrMets in a burgeoning clinical challenge. In view of these facts and in order to improve patients' outcomes, it is clear that there is a need to better understand the brain's *milieu* in the context of metastatic disease.

1.1.1 Breast Cancer Brain Metastases

BC is the most prevalent cancer in women worldwide, and approximately 15% of women with newly diagnosed metastatic BC will develop BrMets. However, this percentage is referring only to clinically apparent cases, since autopsy studies reveal that the incidence may be higher[11].

BC is a heterogeneous disease divided into different molecular subtypes with clinical implications, according to the status of molecular markers such as oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor type-2 (HER2) and Ki67, a proliferation marker[12]. It is classified as Luminal A (ER+, HER2-, PR high, Ki67 low), Luminal B HER2- (ER+, HER2-, PR low or Ki67 high), Luminal B HER2+ (ER+, HER2+, any PR, any Ki67), HER2-Amplified (HER2+) (HER2+, ER and PR absent) or Triple Negative Breast Cancer (TNBC) (ER and PR absent, HER2-).

Patients with TNBC and HER2+ BC are at highest risk for central nervous system (CNS) recurrence, and incidences of breast cancer brain metastases (BCBrMets) as high as 30% to 40% have been reported for these molecular subtypes[13,14,15,16]. Retrospective studies have shown that BC subtype impacts survival in patients with BCBrMets, being TNBC the subtype with the poorest prognosis[17,18]. Median survival from the diagnosis of BCBrMets historically ranges between 2 and 16 months, depending on the involvement of the CNS, the extent of extracranial metastatic disease, performance status and administration of local or systemic therapy[19,20]. Differences in disease progression in patients with BCBrMets, which translate into different outcomes, highlight the need for a tailored approach in the care of these patients.

Despite the report of controversial results in published studies, the small size of many of the cohorts used and the little known about the following topic, compelling evidence has shown that there are molecular changes occurring in extracranial BC

metastases that could make a difference to treatment[21,22]. The selection of HER2+ clones in 24% to 48% of the cases, of ER negative clones in 7% to 13% of the cases or of PR negative ones into positive in the metastasis are alterations that could enable the prescription of treatments in the metastatic setting that were not an option for the primary tumour[23,24,25].

As previously mentioned, there is a need to know the brain microenvironment and to unravel the crosstalk between this particular metastatic niche and tumour cells. In the context of BC and other brain tropic tumours, this could provide new and more efficient therapeutic strategies in the future.

1.2 Brain Microenvironment

Organotropism or the propensity of tumour types to disseminate to specific organs, such as the brain, has always been a hot topic in cancer research[26]. It is consensual that tumour cells will grow in congenial environments and that cancer cells acquire specialised functions to overtake specific organs, but is still not well understood how the metastatic niche is pre-conditioned by the tumour cells in the micro- to macrometastases progression. However, it is known that for the brain metastatic process there is the formation of perivascular niche, at least at early stages, which has been associated with stem-like and resistant cellular phenotype *per se* in brain tumours[27,28]. The blood-brain barrier (BBB) components are the most likely candidates to provide this supportive niche for cancer cells[29]. The composition of tumour microenvironment depends on the tumour site, meaning that the brain microenvironment is different from that of extracranial sites[30]. It consists of various specialised cell types, such as brain endothelial cells, astrocytes and glial cells that may influence tumour growth (Figure 1). Finally, the presence of immune cells has been described in metastatic solid tumours to the brain, such as BC, lung cancer and melanoma, although their prognostic significance remains unclear[31].

Overall, it seems that the dissemination of cancer cells to the brain results from a symbiotic relationship between the tumour cells and the host, thus it is critical to elucidate the underlying molecular features of the metastatic cascade to further study and develop more effective therapy modalities that may interfere in this relationship, such as immunotherapy[5,7]. As the tumour microenvironment may deeply impact on the efficacy of cancer immunotherapies, the histologic assessment of

tumours and their immune contexture is becoming decisive. Quantifying and characterising the immune infiltrate, and assessing the presence of prognostically relevant cell subsets, such as CD8+ T cells, as well as determining the existence of actionable immunotherapy targets, as programmed cell death-ligand 1 (PD-L1), represent a new era in the clinical management of BrMets, namely in melanoma.

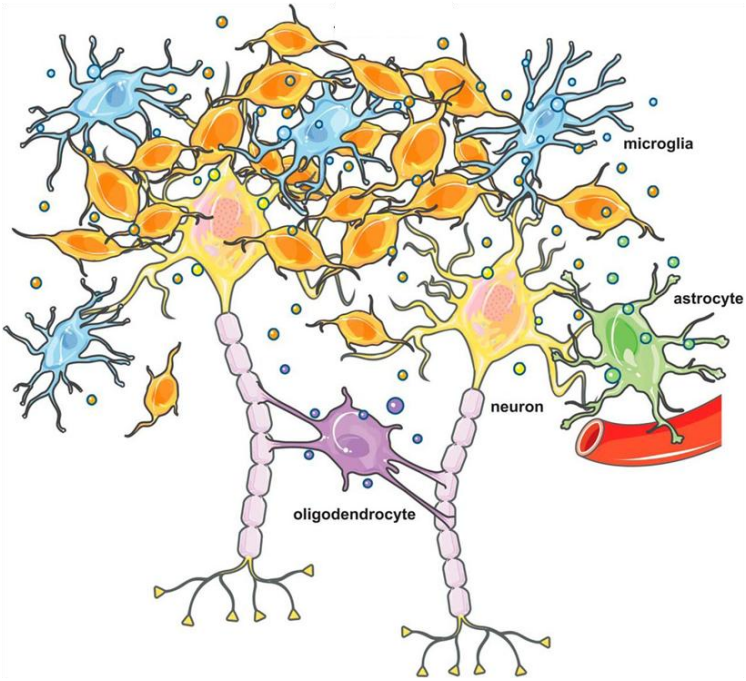


Figure 1 | Representation of the brain metastatic microenvironment. The tumour cells interact with different cell types, such as astrocytes, neurons, microglia, and oligodendrocytes. Adapted from [102].

1.2.1 Blood-Brain Barrier

The brain has been considered an immune-privileged site for metastases, a 'sanctuary' where cancer cells can lay dormant in the CNS, behind the BBB[10,32]. The BBB has a protective role of the brain and is formed by three main types of cells – endothelial cells, astrocytes, and pericytes – that limit the invasion of the brain parenchyma by circulating molecules, immune cells and antibodies[33,34].

However, any disseminated tumour may be able to subvert the BBB and reach the brain, mainly the brain parenchyma[1]. Gene expression analysis revealed that specific genes, like α2, 6-sialyltransferase (*ST6GALNAC5*), mediate cancer cell passage through it[35,36]. The expression of *ST6GALNAC5* is normally restricted to the brain, but it was found to enhance BC cells adhesion to brain endothelial cells. Along

with cycloxygenase-2 (*COX2*) and other genes, *ST6GALNAC5* mediates BC cells infiltration through the BBB.

Furthermore, debate continues as to what extent therapeutic resistance is related to inadequate delivery of drugs to the brain versus intrinsic tumour resistance and/or stromal protective effects[37].

1.2.2 Astrocytes

Astrocytes are non-proliferative cells in the normal adult brain that control homeostatic functions in health and disease. They can be activated upon injury and be involved in gliosis, characterised by proliferation or hypertrophy of several types of glial cells or, in its most extreme form, glial scar formation[38]. They then assume a reactive hypertrophic phenotype, characterised by the upregulation and rearrangement of the glia-specific cytoskeletal intermediate filament protein glial fibrillary acidic protein (GFAP)[39,40]. Hypertrophic activated astrocytes have been associated with BrMets as BrMets can induce the strong local activation of astrocytes[41]. In fact, accumulation of microglia and activated astrocytes around and within lesions has been shown, with astrocytes having direct contact with cancer cells[42,43].

As the most abundant cell type in the CNS, astrocytes account for the majority of the interactions that cancer cells will be exposed to during brain metastatic process, both in early and late stages, and are emerging as essential regulators of BrMets progression as they have a secretory nature and can function as oncogenic signals for the tumour cells[38,41,44]. However, it has been reported that they show different roles, depending on the stage of the disease[44]. Overall, initially astrocytes act as an innate host defense system preventing disease progression, while in late stages they can favour it, providing protection against tumour cell death[45,46].

1.2.3 Tumour-Infiltrating Lymphocytes (TILs)

Supporting evidence of the role played by immune infiltrating cells in brain tumours is increasing, although the CNS being allegedly considered an immune-privileged site. Despite brain's apparently limited capacity for inflammatory response, BrMets contain tumour-infiltrating lymphocytes (TILs), drivers of the 'selective pressure' on CNS tumours, in the tumour stroma and in an intratumoral location[47].

TILs, which include T and B cells at a density that varies according to tumour type and stage of the disease, can be a clinically significant prognostic biomarker in various types of tumours[48]. Their presence in tumours is generally a good prognostic sign and despite their role in BrMets being still not clear with conflicting data from different primary tumour sites, reviews of the literature concluded that TILs density in CNS metastases was strongly associated with improved overall survival (OS)[49,50,51].

BC has not been traditionally considered an immunogenic cancer type. Nevertheless, increasing evidences suggest that an effective immune response may greatly impact on the clinical behaviour of this malignancy. Also in BC, TILs are associated with favourable prognosis, especially in early TNBC and HER2+ BC phenotypes – the ones with more brain tropism –, and may positively influence the response to systemic therapies[52,53]. Studies with the same cutoff for TILs positivity suggested survival benefit in TNBC patients in their presence[54,55].

It is extremely difficult to characterise and quantify all TILs subpopulations, and the prognostic value of a specific subset may well not represent the total impact of TILs on survival, as TILs subsets have their own roles in the immune microenvironment and in metastatic BC. For example, CD4+ T cells include helper T (Th) and regulatory T (Treg) cells, whereas CD8+ lymphocytes are the main immune effector cells. The prognostic value of CD8+ T cells varies according to tumour type and, therefore, more prospective studies are warranted to confirm it. However, a meta-analysis with 25 studies concluded that high density of CD8+ T cells is an indicator of good prognosis in BC patients[56]. Furthermore, an association between higher amounts of CD8+ lymphocytes within the invasive margin and significantly longer disease-free survival (DFS) was reported in BCBrMets[57]. The study of the CD4+/CD8+ TILs ratio may be an important parameter in cancer patients. Although it varies between different types of cancer, an increase has been observed in patients with several cancers, such as BC[58,59].

1.2.4 Programmed Cell Death-Protein 1 (PD-1)/Programmed Cell Death-Ligand 1 (PD-L1) Axis

The effectiveness of immune-modulating agents within the CNS could be limited. However, there is growing evidence that immune therapies may be effective,

namely in patients with BrMets from melanoma, providing durable clinical responses[60]. In primary CNS tumours, a study showed promising preclinical data with immune-modulating antibodies[61]. Several lines of evidence suggest that T cells within the tumour microenvironment are the drivers of response to immune-modulation therapies[47,62].

Programmed cell death-protein 1 (PD-1), also known as CD279 (cluster of differentiation 279), helps regulating the autoimmune response by down-regulating the immune system and suppressing T-cell inflammatory activity. However, this can also suppress antitumour immunity. PD-1 is normally expressed on the surface of immune cells, but recent studies revealed a widespread tumour-intrinsic expression of PD-1 in cancer, such as melanoma and lung cancer[63,64].

PD-L1, also known as CD274 (cluster of differentiation 274) or B7-H1 (B7-Homolog 1), similar to programmed cell death-ligand 2 (PD-L2 or PDCD1LG2), is a PD-1 ligand and an immune regulatory transmembrane protein expressed by tumour cells[65]. Therefore, targeting the PD-1/PD-L1 axis, by blocking it, is associated with tumour regression in several malignancies (Figure 2).

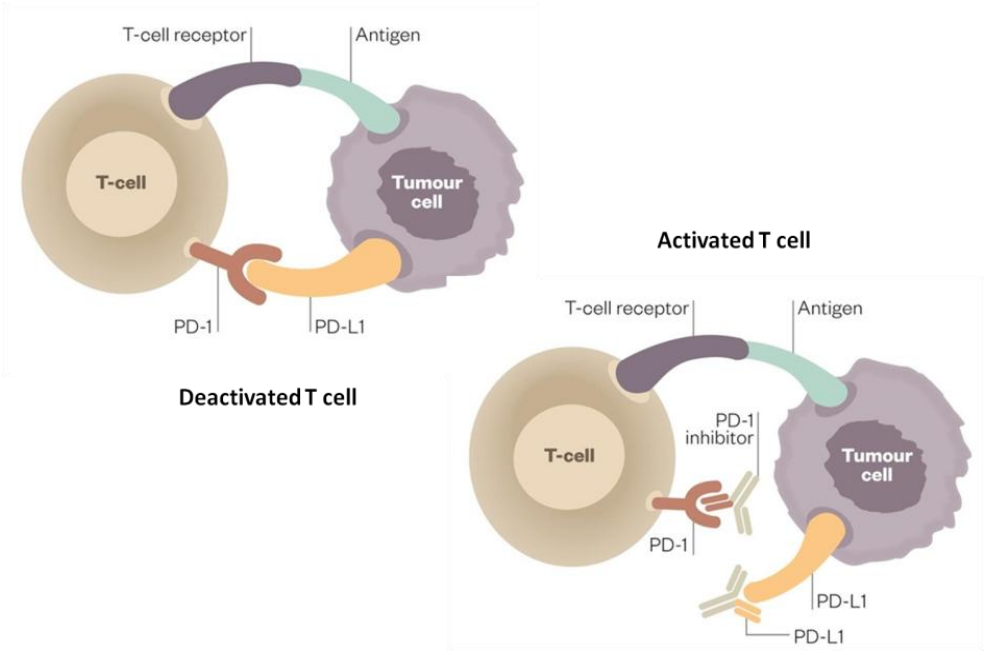


Figure 2 | Schematic representation of the PD-1/PD-L1 axis. T cells are deactivated upon PD-1 and PD-L1 interaction and activated when inhibitors block this interaction. Adapted from [103].

It has been described that a high level of PD-L1 expression in tumour cells correlates with poor prognosis in several cancers, including breast, lung and renal

cancers, and melanoma[66]. However, other studies have suggested a positive or inexistent correlation between PD-L1 expression and survival[67,68,69]. Specifically in BC, PD-L1 was shown to be most frequently expressed in basal-like tumours, though its expression was rare[70]. Thus, its prognostic value is controversial. Finally, a recent study defined PD-L1 and PD-L2 expression as a common occurrence in BCBrMets, irrespective of primary tumour or BCBrMets phenotypes[71]. The role played by PD-L2 is still not clear[66].

2 OBJECTIVES

The primary objective of this project was to retrospectively investigate if the immune compartment of BCBrMets, characterised by the presence of stromal TILs (total, CD4+ and CD8+), was associated with OS as endpoint, using a randomised retrospective cohort of patients with BrMets from other tumours as comparator.

As secondary objectives we proposed to: a) characterise the presence of activated astrocytes (using GFAP as a surrogate marker) and the expression of PD-L1 in BCBrMets, and to explore a possible association with the patients' outcome; b) assess the molecular alterations between primary breast tumours and paired BrMets, namely in ER, PR, HER2 and Ki67 expression.

3 MATERIALS AND METHODS

3.1 Clinical Samples

In this study we used a retrospective cohort of 56 formalin-fixed, paraffin-embedded (FFPE) samples from BrMets tissue. The use of tissue samples was approved by the institutional review board of *Hospital de Santa Maria – Centro Hospitalar Universitário Lisboa Norte* (HSM – CHULN) (approval number 556/14). All tissue samples used were initially obtained for pathological diagnosis and are part of the general sample archive of the Neuropathology Laboratory, HSM – CHULN. The requirement for written informed consent was waived due to the retrospective nature of the study. During the analysis, the observers were fully blinded for patients' personal data, as samples were identified by their registration number and, in a later phase, by their tissue microarrays (TMAs) positioning.

The cohort included 56 patients diagnosed with BrMets between 2009 and 2013 and followed at Department of Medical Oncology of HSM – CHULN; 34 (60.7%) women and 22 (39.3%) men, with a median age at BrMets diagnosis of 58.50 years (range 28-90 years), and with BrMets arising from BC (n=25, 44.7%), lung cancer (n=15, 26.8%), colon cancer (n=6, 10.7%), kidney cancer (n=6, 10.7%) and melanoma (n=4, 7.1%). The time between first recurrence and metastization to the brain varied between 0 – 5 BC patients had the brain as primary site of recurrence – and 24 months. The median survival after BrMets was 7.5 months. All of the patients received holocranial radiotherapy after surgical resection. Sample distribution per analysis is represented in Figure 3.

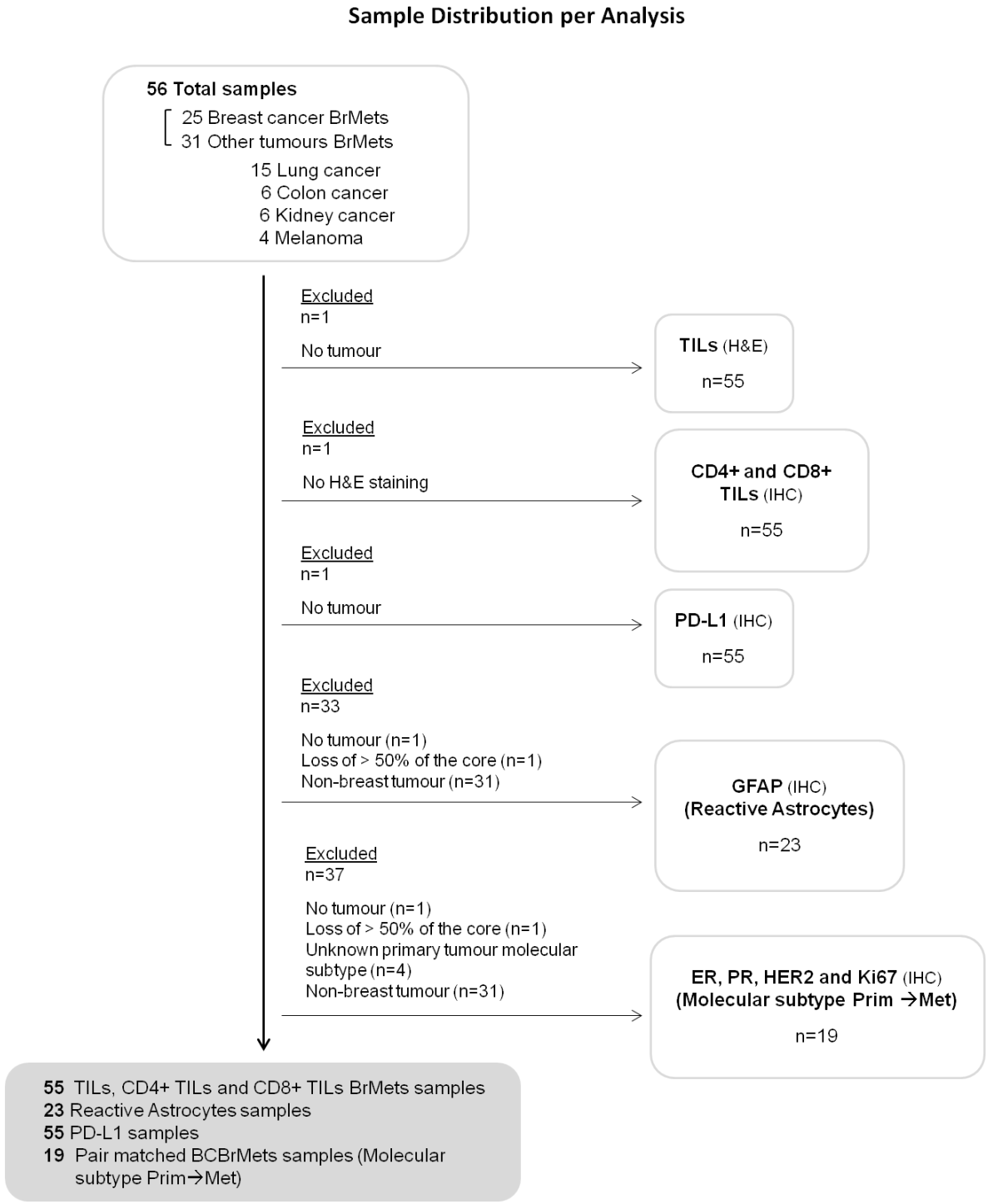


Figure 3 | Flowchart of sample distribution per analysis.

3.2 Haematoxylin and Eosin Staining (H&E)

For Haematoxylin and Eosin staining (H&E), 5 μm sections of TMAs were depparafinized in xylene, rehydrated in decreasing concentrations of ethanol (100%, 95%, 70%); each for 5 minutes (min) and placed in distilled water. Tissue sections

were stained with Harris Haematoxylin for 3 min, washed in running tap water for 5 min, and dipped in 70% ethanol. After staining with alcoholic eosin, tissue sections were dehydrated in increasing concentrations of ethanol, cleared in xylene for 10 min and mounted with a solvent-based mounting media, Quick-D (Klinipath).

3.3 Immunohistochemical Staining (IHC)

For immunohistochemistry (IHC), 5 μm sections of TMAs were processed using the PT Module Thermo Scientific for Tissue Specimens (Dako), with Antigen Retrieval pH 6.0 or pH 9.0 solution (Dako) at 94ºC for 20 min. Sections were washed with Phosphate Buffered Saline (Sigma) with 0.05% Tween 20 (VWR, PROLABO) (PBS-T) for 5 min at room temperature (RT). The activity of endogenous peroxidase was blocked with Blocked Endogenous Peroxidase Solution (Dako) for 10 min at RT, followed by three 5 min rinses in wash buffer. Total protein blockade was performed using Protein Block Solution (Dako) for 20 min at RT, followed by incubation with the following primary antibodies, diluted in Antibody Diluent (Dako): anti-CD4, anti-CD8, anti-GFAP, anti-HER2, anti-ERα, anti-PR and anti-Ki67 (for additional details and working conditions see Table 1). Tissue sections were rinsed in wash buffer two times, 10 min each, and incubated with the visualisation system Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako) for 1 hour (h) at RT. After incubation, slides were rinsed in wash buffer as described above, incubated with 3,3'-diaminobenzidine (DAB+ Chromogen, Dako REAL), and rinsed in PBS-T and Elix water, each for 5 min. Slides were counterstained with Harris Hematoxylin for 10 seconds (sec), washed in running water for 5 min, dehydrated, cleared and mounted as described for H&E staining.

PD-L1 staining was performed at Instituto de Patologia e Imunologia Molecular da Universidade do Porto (IPATIMUP), using the Ventana BenchMark XT Staining System, with a 36 min incubation time for the monoclonal mouse anti-human PD-L1 (1:70) (22C3, Dako) and the OptiView DAB IHC Detection Kit (Ventana Medical Systems). Membranous and cytoplasmic staining pattern was expected.

The absence of primary antibody was used as negative control for all the markers.

Table 1 | Antibodies and respective conditions used for the IHC staining in the present study.

Antibody	Manufacturer Clone	Expected Staining Pattern	Conditions
Monoclonal mouse anti-human CD4	Dako 4B12	Membranous	pH 6.0 1:100 1 h at RT 5 min DAB
Monoclonal mouse anti-human CD8	Dako C8/144B	Membranous and Cytoplasmic	pH 6.0 1:100 1 h at RT 5 min DAB
Monoclonal mouse anti-human GFAP	Dako 6F2	Cytoplasmic	pH 6.0 1:100 1 h at RT 1 min DAB
Monoclonal rabbit anti-human ERα	Dako EP1	Nuclear	pH 9.0 Ready-to-use 1 h at RT 5 min DAB
Monoclonal rabbit anti-human PR	Ventana 1E2	Nuclear and Membranous	pH 9.0 Ready-to-use 1 h at RT 5 min DAB
Polyclonal rabbit anti-human HER2/ErbB2	Cell Signaling Technology NA	Membranous	pH 9.0 1:50 ON at 4ºC 5 min DAB
Monoclonal mouse anti-human Ki67	Dako MIB-1	Nuclear	pH 9.0 1:100 1 h at RT 5 min DAB

h, Hour; *RT,* Room Temperature; *min,* Minute(s); *NA,* Not Applicable; *ON,* Overnight; *ºC,* Degree Celsius.

3.4 Histological Evaluation and IHC Scoring

All H&E and IHC slides were analysed by a Pathologist, with each case being represented by 3 cores. The evaluation and scoring of every biomarker resulted from the mean of the 3 corresponding cores per case. TILs density was presented as the percentage of total TILs in the tumour stromal compartment using visual assessment of H&E-stained TMAs. Samples were classified as: absent (0% TILs), slight (≤30% TILs), moderate (30%> TILs ≤60%), and marked (>60% TILs)[72]. CD4+ TILs and CD8+ TILs were classified as described for TILs after IHC staining and percentages were normalised to total stromal TILs.

PD-L1 expression was classified as positive (membranous and cytoplasmic staining in ≥1% of tumour cells or stromal TILs) or negative (membranous and cytoplasmic staining in <1% of tumour cells or stromal TILs)[73].

GFAP was used as a marker of reactive astrocytes. Astrocytes in tumour sections were classified qualitatively as not altered (normal GFAP cytoplasmic staining intensity) or altered (abnormal GFAP cytoplasmic staining intensity).

Hormone receptors (ER and PR), HER2, and Ki67 were classified according to the guidelines of the *Template for Reporting Results of Biomarker Testing of Specimens from Patients with Carcinoma of the Breast*, from the College of American Pathologists (2014). ER and PR were considered positive if ≥1% positive cells. For HER2, staining intensity was classified from 0 to 3: (0) absence of staining, (1) weak, (2) moderated and (3) strong staining, and samples were classified as negative (0 or 1+), equivocal (2+) or positive (3+). Ki67 was classified as low (<10% positive cells), borderline (20%-30% positive cells) or high (>30% positive cells).

3.5 Statistical Analysis

For statistical analysis, patients were dichotomised into *low* and *high*, according to TILs and CD8+ TILs percentage cutoff, selected using 12-months OS as endpoint and Cutoff Finder (http://molpath.charite.de/cutoff/)[74].

Demographic and clinicopathologic data of patients were described using frequencies for categorical variables, and central tendency and range for continuous variables. Univariate association of these characteristics and TILs and CD8+ TILs percentage was done through Kruskal-Wallis test, Fisher's exact test, $\chi 2$ test and Mann-Whitney test as applicable. OS was estimated using Kaplan-Meier curves and

differences were determined using the log-rank (Mantel-Cox) test. A significance level of *P*-value <0.05 was set for all statistical analyses.

Statistical analysis was carried out using the software GraphPad Prism 6 for Windows (GraphPad Software).

3.6 Cell Culture

All cell lines were cultured in supplemented Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) with 10% (v/v) fetal bovine serum (FBS) (Gibco) and 1% (v/v) Penicillin Streptomycin (Pen Strep, 10,000 Units/mL Penicillin, 10,000 µg/mL Streptomycin) (Gibco). Cells were kept at 37ºC with 5% CO_2 in a humidified atmosphere, and medium was changed every two or three days.

The BC brain tropic cell lines MDA-MB-231-BR HER2+ and MDA-MB-231-BR HER2- were kindly provided by Patricia S. Steeg and David Lyden Lab, at Cornell University. MCF-10A, MDA-MB-231, MDA-MB-435S, MCF7, ZR-75, SK-BR-3, MDA-MB-361 and PC-3 cell lines were purchased from ATCC. MDA-MB-231-BO2, T-47D and BT-474 cells were provided by Phyllippe Clézardin Lab, *Institut National de la Santé et de la Recherche Médicale* (INSERM).

3.7 RNA Extraction, cDNA Synthesis and Reverse Transcription - Quantitative Polymerase Chain Reaction (RT-qPCR)

Ribonucleic Acid (RNA) extraction (NZY Total RNA Isolation kit, NZYtech) was performed according to the manufacturer's protocol. Total RNA was quantified by spectrophotometry, using NanoDrop ND-1000 (Thermo Fisher Scientific) and 1 µg of total RNA was used to synthesize complementary DNA (cDNA), with Oligo(dT)18 primer and the NZY M-MuLV First-Strand cDNA Synthesis Kit (NZYTech), according to the manufacturer's instructions.

Genes of interest were amplified by semi-quantitative real time PCR in the ViiA 7 Real-Time PCR System (Applied Biosystems), using specific primers (Invitrogen, Table 2) in a 10 µL reaction volume with SYBR Green PCR Master Mix (Applied Biosystems).

Target gene expression was normalised against the housekeeping gene *GAPDH*, and data were analysed using the $2^{-\Delta\Delta Ct}$ method.

Table 2 | Specific primer sense and anti-sense sequences used for gene amplification.

Primer	Sense (5´-3´)	Anti-sense (5´-3´)
GAPDH	CAATGACCCCTTCATTGACC	TGGATTTCCATTGATGACA
PD-1	CGTGGCCTATCCACTCCTCA	ATCCCTTGTCCCAGCCACTC
PD-L1	AAATGGAACCTGGCGAAAGC	GATGAGCCCCTCAGGCATTT
PD-L2	GTCTTGGGAGCCAGGGTGAC	TGAAAAGTGCAAATGGCAAGC

4 RESULTS AND DISCUSSION

4.1 Prognostic Role of Stromal TILs and CD8+ TILs in BrMets

Histologic assessment of the immune context of tumours has been showing prognostic relevance[75]. Thus, the determination of the amount of immune infiltrate as well as its composition, in particular of prognostically relevant immune cell subtypes such as CD8+ T cells, for example, is becoming central.

As stated before, TILs and CD8+ TILs have been shown to have prognostic value across a broad range of tumour types and to be positively correlated with OS, suggesting that TILs might reduce metastatic spread[48,50,51,54]. Therefore, as a primary objective we aimed to quantify total, CD4+ and CD8+ stromal TILs in BCBrMets, and to assess their prognostic role in the context of brain metastatic disease.

Although we focused on BCBrMets, we used a retrospective cohort of FFPE TMAs, which included not only samples from BCBrMets but also from BrMets from other types of tumours. This allowed us to use these non-breast cancer samples as comparators in our analysis, enriching the results of this project. Demographic and clinicopathological characteristics of the 56 patients with BrMets, included in this study, 25 of which with BCBrMets, are presented in Tables 3 and 4, respectively. For the analysis of total TILs and CD8+ TILs on H&E and IHC tissues, respectively, we excluded 1 case due to absence of tumour in the TMAs cores used for H&E staining.

Table 3 | Demographic and clinicopathological characteristics of the 56 patients with BrMets originated from breast, lung, colon, kidney cancer and melanoma included in this study.

	Total (*N*=56)
Age at diagnosis of BrMets (years)	
Median	58.50
Range	28-90
IQR	15.75
Gender	
Female	34 (60.7%)
Male	22 (39.3%)
Primary tumour	
Breast cancer	25 (44.6%)
Lung cancer	15 (26.8%)
Colon cancer	6 (10.7%)
Kidney cancer	6 (10.7%)
Melanoma	4 (7.2%)
Time between BrMets and death (months)	
Median	7.5
Mean	11.07
Range	1-72
Survival status	
Deceased	54 (96.4%)
Alive	2 (3.6%)

BrMets, Brain Metastases; *IQR*, Interquartile Range.

Table 4 | Demographic and clinicopathological characteristics of the 25 patients with BCBrMets included in this study.

		Total (N=25)
Age at diagnosis of BrMets (years)		
Median		57
Range		28-86
IQR		17
Gender		
Female		25 (100%)
Male		0 (0%)
TNM stage		
IA		2 (8%)
IB		1 (4%)
IIA		7 (28%)
IIB		4 (16%)
IIIA		4 (16%)
IIIB		3 (12%)
IIIC		1 (4%)
IV		0 (0%)
Unknown		3 (12%)
Histology		
Ductal carcinoma		18 (72%)
Lobular carcinoma		1 (4%)
Unknown		6 (24%)
ER status‡		
Positive		7 (28%)
Negative		16 (64%)
NA		2 (8%)
PR status‡		
Positive		11 (44%)
Negative		12 (48%)
NA		2 (8%)
HER2 status‡		
Positive		10 (40%)
Negative		9 (36%)
Equivocal		4 (16%)
NA		2 (8%)
Ki67 status‡		
High		8 (32%)
Low		5 (20%)
Borderline		10 (40%)
NA		2 (8%)
Molecular subtype*	Primary tumour	Metastasis
Luminal	9 (36%)	7 (28%)
Luminal B HER2+	3 (12%)	5 (20%)
HER2+	7 (28%)	6 (24%)
TNBC	2 (8%)	5 (20%)
Unknown	4 (16%)	0 (0%)
NA	0 (0%)	2 (8%)
Molecular subtype discordance between primary tumour and metastasis		10/19 (52.6%)
Unknown		4/25 (16%)
NA		2/25 (8%)
Adjuvant therapy		25 (100%)

BrMets , Brain Metastases; *IQR* , Interquartile Range; *ER* , Oestrogen Receptor; *PR* , Progesterone Receptor; *HER2* , Human Epidermal Growth Factor Receptor type-2; *TNBC* , Triple Negative Breast Cancer; *NA* , Not Applicable, meaning absence of tumour or loss of > 50% of the cores.
‡*Satuses* refer to the metastasis; *Molecular subtype classification is according to the 2015 St Gallen Consensus Conference, recommended by the ESMO Clinical Practice Guidelines; Luminal comprises Luminal A and Luminal B HER2- subtypes.

TMAs H&E slides were used to observe tissue morphologic characteristics and also to quantify total TILs (Figure A1). Next, samples were dichotomised into low or high TILs, using the cutoffs 1%, 3% and 0.5% for BC cases, non-BC cases and all tumour types, respectively, as these were the best cutoff points found by Cutoff Finder after testing other cutoffs (median; positive (>0) vs negative (0)). These cutoffs were not close to the 50% cutoff point reported as the best for dichotomisation of lymphocyte-predominant BC, due to overall low TILs density in our TMAs samples. Moreover, 50% was chosen to analyse only lymphocyte-predominant BC, a subset of TNBC and we had only five TNBC samples in our fifty six tissue samples cohort[53].

CD4+ and CD8+ TILs were assessed by IHC and normalised to total TILs (Figures A2 and A3, respectively). Next, samples were dichotomised into low or high TILs, using the cutoffs 75%, 45% and 45% for BC cases, non-BC cases and all tumour types, respectively, like in TILs. Representative images of H&E and IHC staining are displayed in Figure 4.

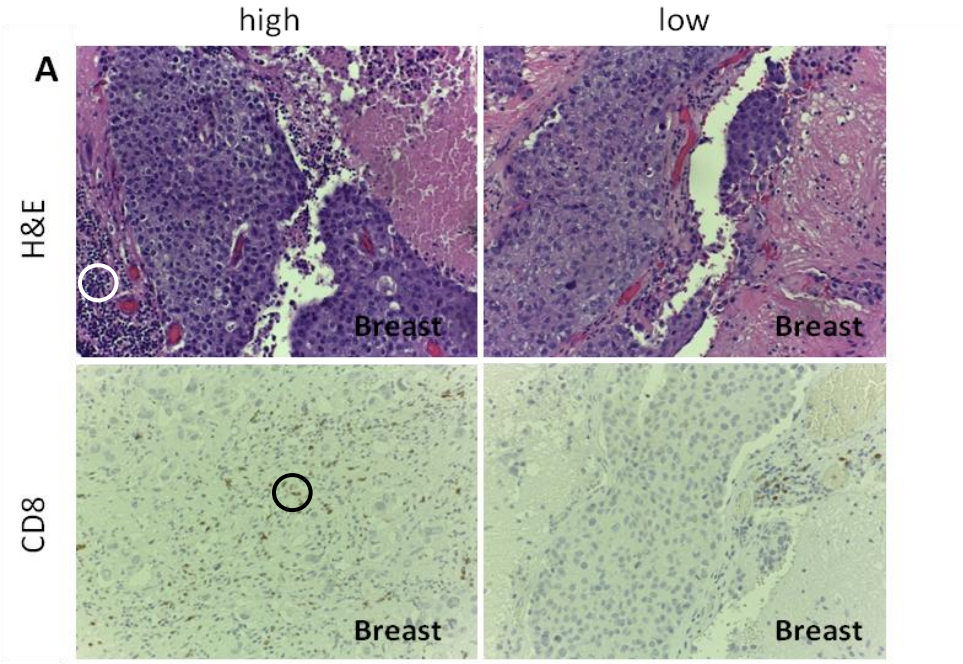


Figure 4 | Representative tissue sections with high or low total or CD8+ TILs in tissue samples from patients with BrMets from BC (A) and non-BC tumours (B). Example of TILs on H&E sections is indicated by a white circle; Example of CD8+ TILs in IHC sections is indicated by a black circle. Magnification X200.

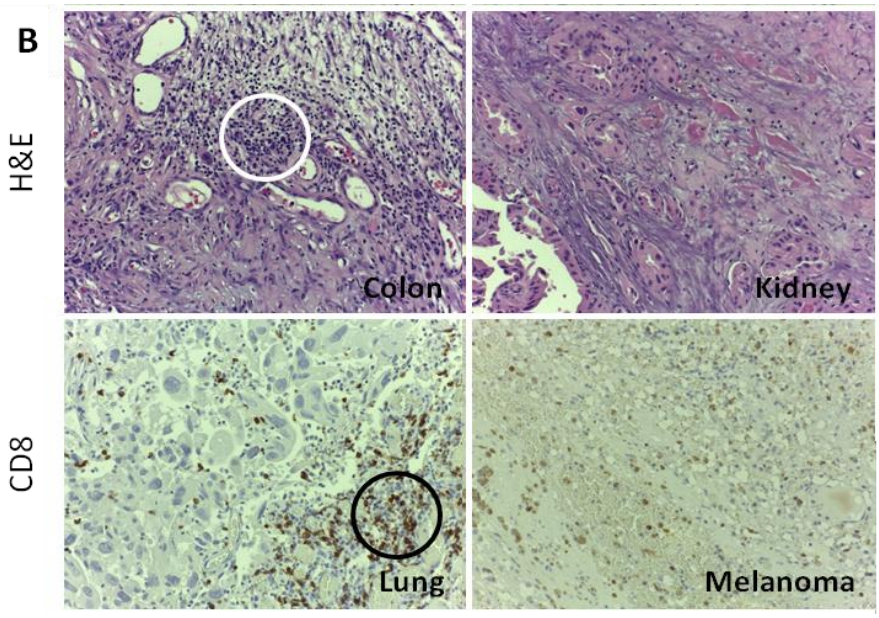


Figure 4 | Representative tissue sections with high or low total or CD8+ TILs in tissue samples from patients with BrMets from BC (A) and non-BC tumours (B). Low TILs and CD8+ TILs on the left, high TILs and CD8+ TILs on the right. Example of TILs on H&E sections is indicated by a white circle; Example of CD8+ TILs in IHC sections is indicated by a black circle. Magnification X200. (Continued)

Across all samples, total TILs ranged between 0% and 30%, corresponding to absent or slight TILs, respectively (Figure 5). Median TILs ranged between 1.33% and 8.33%, depending on tumour type.

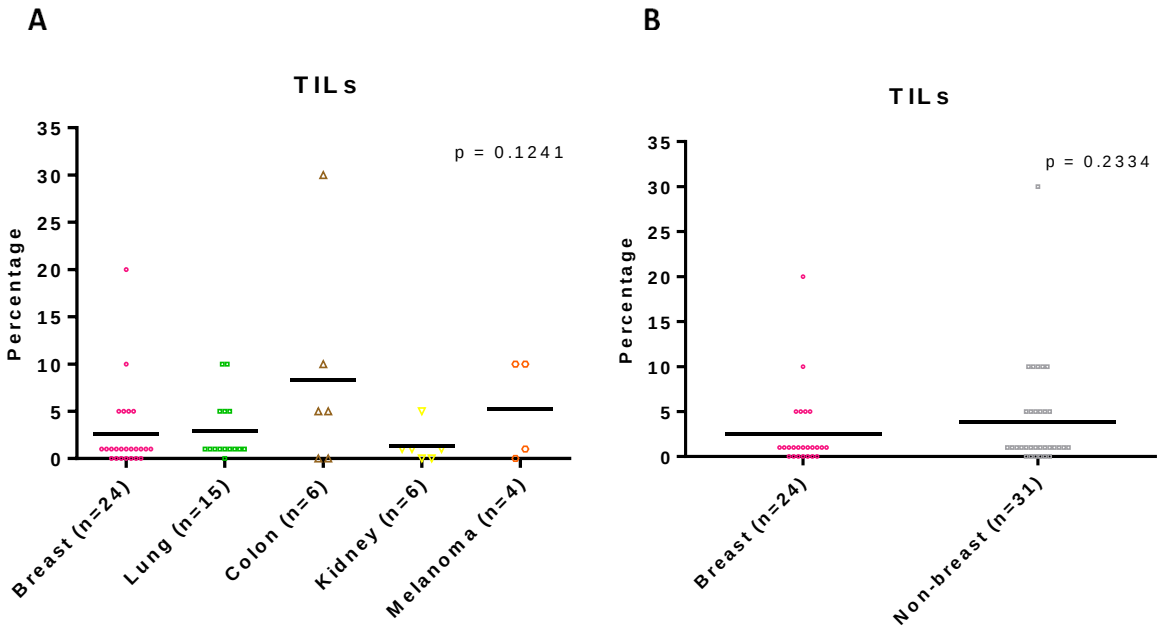


Figure 5 | Total TILs in BrMets according to the origin of the primary tumour (A) and comparing metastases from BC with metastases from non-breast tumours (B). Corresponding mean percentage, median percentage and range (%) are registered in the table (C). *P*-value was calculated with Kruskal-Wallis (A) or Mann-Whitney test (B), and significance was set as $p<0.05$.

C

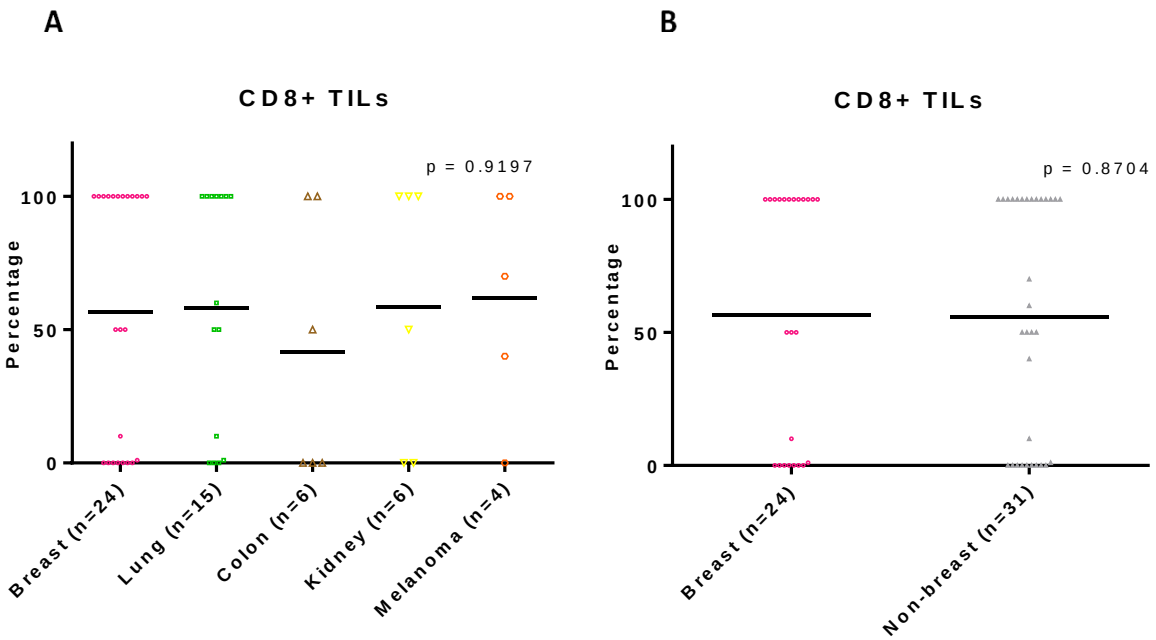
	Breast	Lung	Colon	Kidney	Melanoma	Non-breast
Mean percentage	2.54	2.93	8.33	1.33	5.25	3.88
Median percentage	1.00	1.00	5.00	1.00	5.50	1.00
Range	0-20	0-10	0-30	0-5	0-10	0-30

Figure 5 | Total TILs in BrMets according to the origin of the primary tumour (A) and comparing metastases from BC with metastases from non-breast tumours (B). Corresponding mean percentage, median percentage and range (%) are registered in the table (C). *P*-value was calculated with Kruskal-Wallis (A) or Mann-Whitney test (B), and significance was set as $p<0.05$. (Continued)

Regarding specific subsets of TILs, we observed the presence of both CD4+ and CD8+ TILs in BrMets, although CD4 staining was a rare event in this study, with only four positive cases. The scarce positive data prevented us from further investigating their relevance in this cohort. However, we must take into account that CD4+ TILs absence was consistently observed and that this may be of particular relevance and deserve further studies in larger cohorts. Moreover, the use of TMAs is a limitation of this study, inasmuch as TMAs surely limit the representativeness of the tissue heterogeneity.

Concerning CD8+ T cells, 23/55 cases (41.8%) scored 100%. Given the low percentages of total TILs, this implies that even in those cases of abundant CD8+ TILs, they correspond to a small part of the immune cell population. It would be important to quantify the other immune cell subpopulations in those BrMets system to further characterise the microenvironment of BrMets.

Distribution of CD8+ TILs across the different types of tumours is depicted in Figure 6. As in total TILs, we did not observe a difference between tumour types.

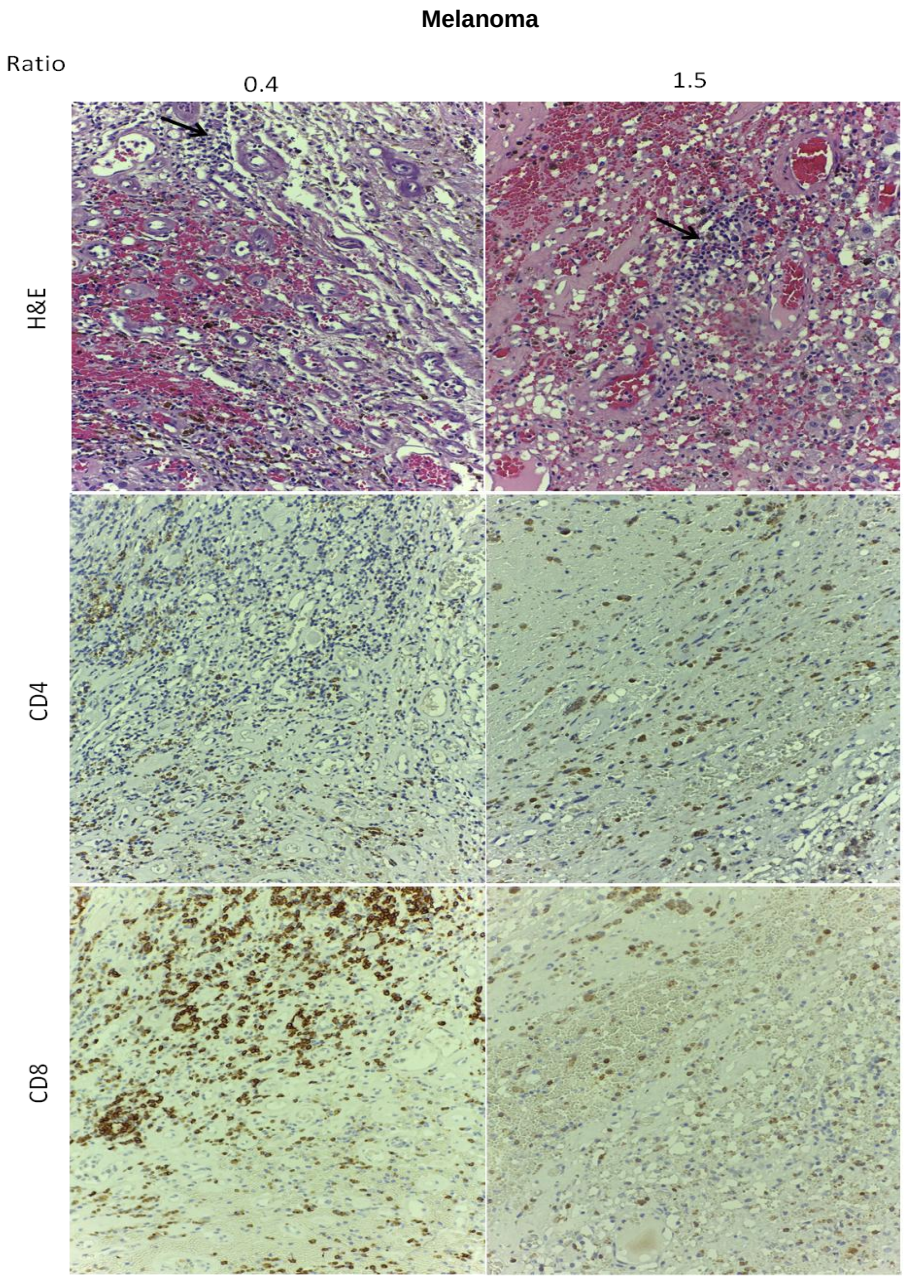


C

	Breast	Lung	Colon	Kidney	Melanoma	Non-breast
Mean percentage	56.71	58.07	41.67	58.33	62.00	55.60
Median percentage	75.00	60.00	25.00	75.00	70.00	1.000
Range	0-100	0-100	0-100	0-100	0-100	0-100

Figure 6 | CD8+ TILs in BrMets according to the origin of the primary tumour (A) and comparing metastases from BC with metastases from non-breast tumours (B). Corresponding mean percentage, median percentage and range (%) are registered in the table (C). *P*-value was calculated with Kruskal-Wallis (A) or Mann-Whitney test (B), and significance was set as $p<0.05$.

Since it has been suggested that the ratio CD4+/CD8+ TILs may impact the prognosis in melanoma, BC and squamous cell carcinoma of the cervix, we analysed the CD4+/CD8+ TILs ratio in the four cases with CD4+ TILs, two from melanoma, one from BC and one from lung cancer, even though it did not allow us any statistical inference (Figure 7)[58,59,76].



Primary Tumour	CD4+ (%)	CD8+ (%)	Ratio
Breast cancer	40	50	0.8
Melanoma	**60**	**40**	**1.5**
Lung cancer	40	60	0.7
Melanoma	**30**	**70**	**0.4**

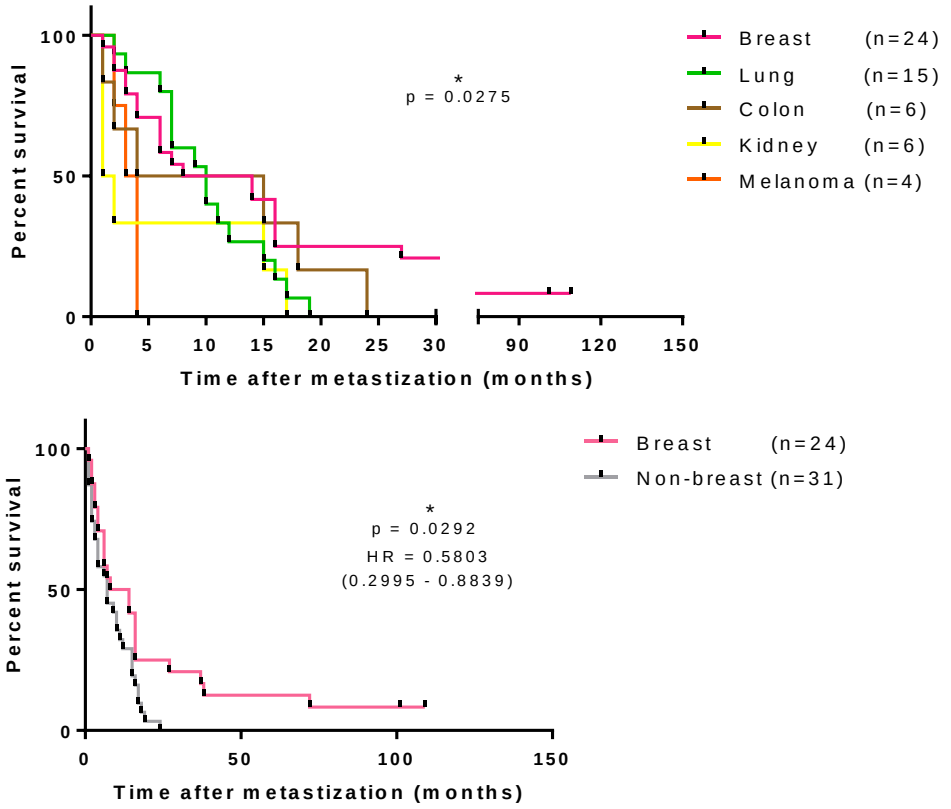
Figure 7 | Representative images of tissue sections with the lowest (left) and the highest (right) CD4+/CD8+ TILs ratios (bold in the table). TILs are pointed out by black arrows. Ratios of the cases with positive CD4 staining. Magnification X200.

TILs subsets have their own roles in BC progression and CD8+ TILs, the main effective cells in the immune response, have been related to better DFS[48,56,77].

So next we aimed to assess the prognostic role of total TILs and CD8+ TILs in BrMets, by quantifying its association with OS.

Samples were dichotomised into low and high TILs and CD8+ TILs according to best cutoff value when analysing OS as endpoint. The exception was the sub-analysis of TILs in BCBrMets, where we could not use Cutoff Finder, and selected the median as dichotomisation cutoff, since it was the method tested associated with the best *P-value*.

We started by assessing the contribution of the primary tumour type to survival rates and 12-months survival rates (Figures 8 and A4, respectively). The OS curves were different according to the tumour type (p=0.0275), and better for BC patients, in comparison to non-breast patients (p=0.0292, median survival 11 vs. 7 months).



	Breast	Lung	Colon	Kidney	Melanoma	Non-breast
Median survival time (months)	11	10	9.5	1.5	3.5	7

Figure 8 | Overall survival according to the origin of the primary tumour and comparing breast with non-breast metastatic disease (n=56). *P*-value was calculated using log-rank (Mantel-Cox) test, and significance was set as p<0.05.

Next we assessed the impact of total TILs and CD8+ TILs on OS (Figures 9 and 10, respectively).

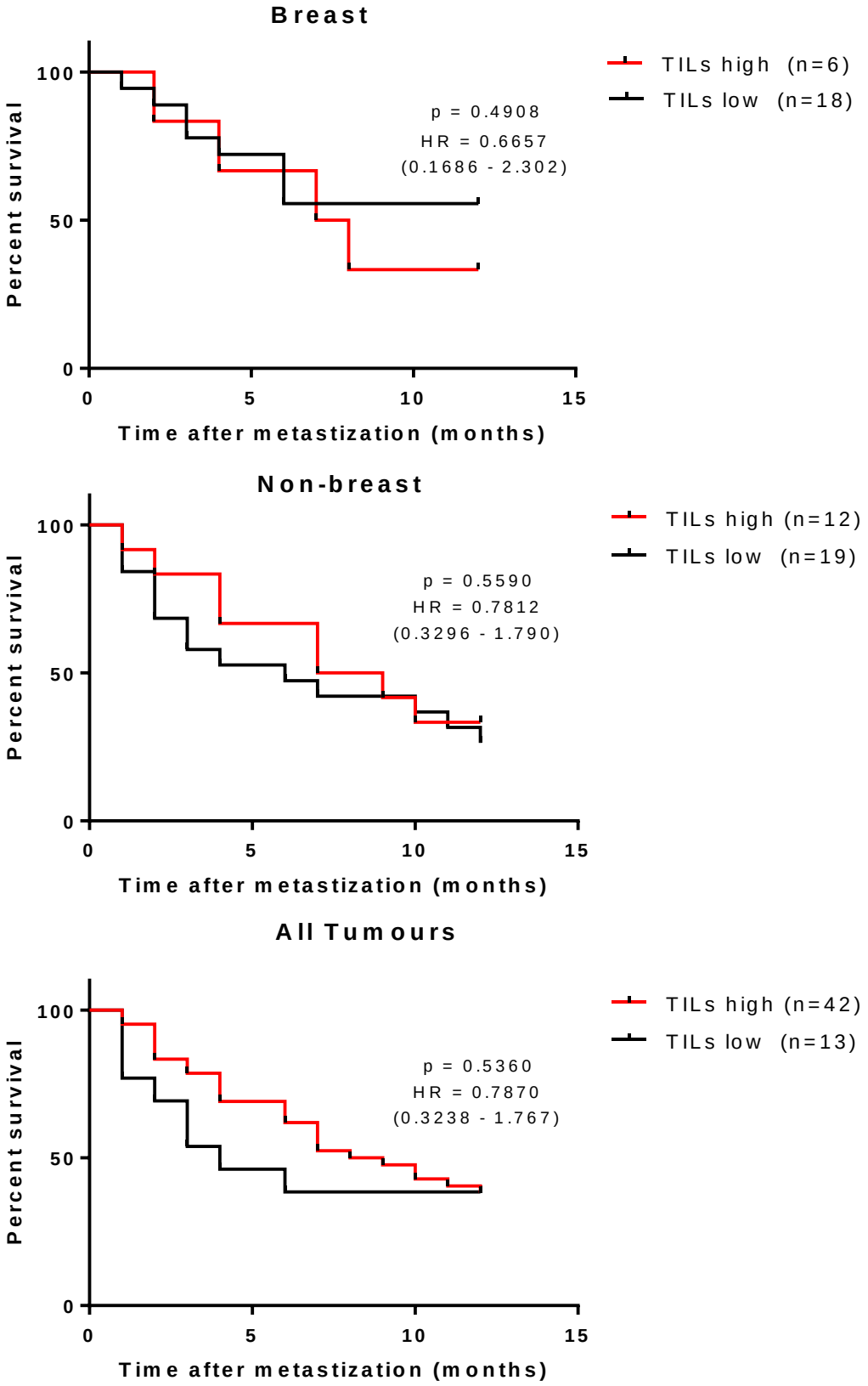


Figure 9 | 12-months-overall survival according to TILs percentage in BrMets from BC (n=24), all tumours (n=55), and non-BC (n=31). Cutoff values for dichotomisation (%) 1, 0.5, 3, respectively. *P*-value was calculated using log-rank (Mantel Cox) test, and significance was set as $p<0.05$.

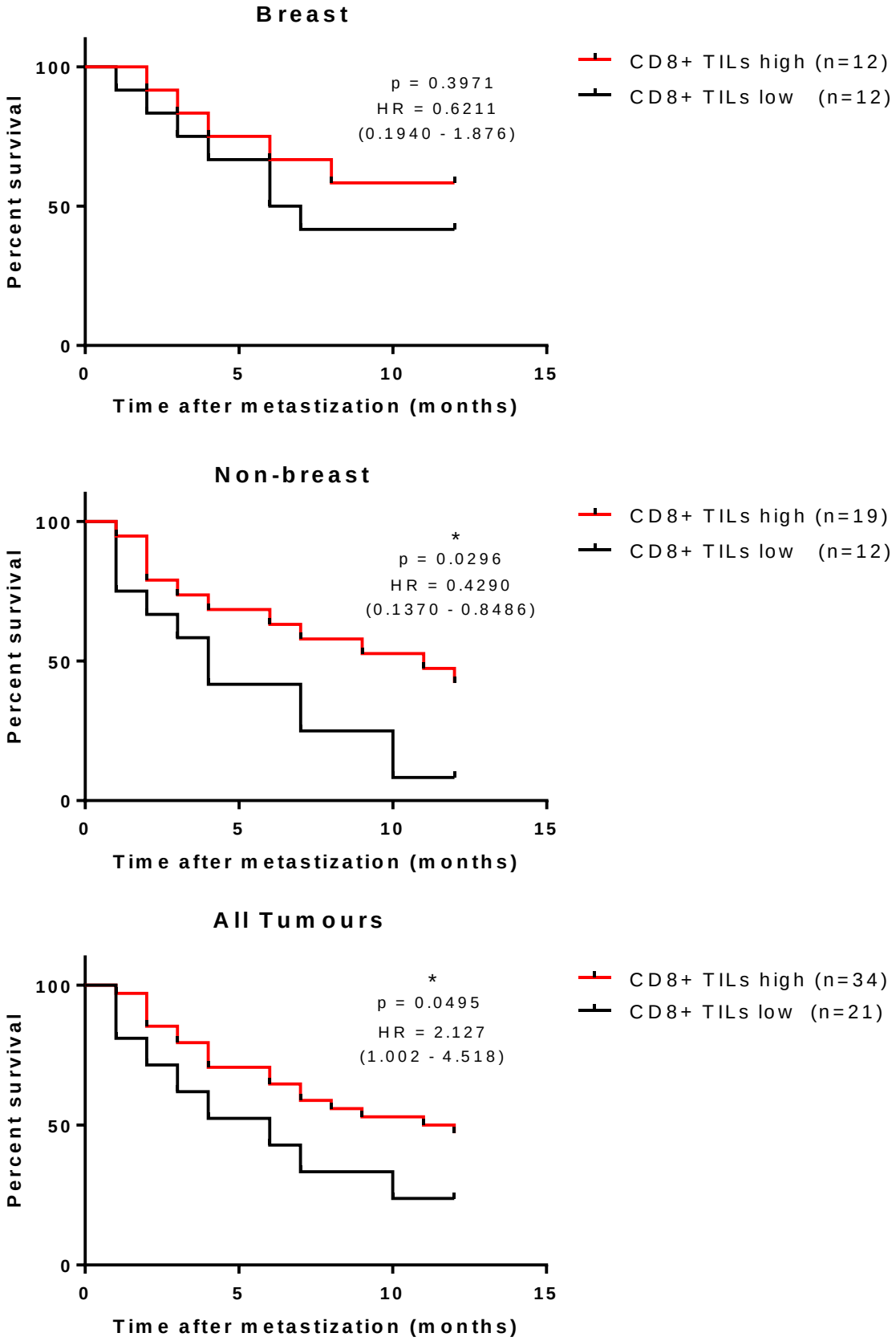


Figure 10 | 12 months-overall survival according to CD8+ TILs percentage in BrMets from BC (n=24), all tumours (n=55), and non-BC (n=31). Cutoff values for dichotomisation (%) 75, 45, 45, respectively. *P*-value was calculated using log-rank (Mantel Cox) test, and significance was set as $p<0.05$.

Our data showed that TILs do not have impact on OS in BCBrMets (p=0.4908, median survival undefined vs. 7.5 months), in BrMets from other tumours (p=0.5590, median survival 6 vs. 8 months), or in all tumours (breast plus non-breast, p=0.5360, median survival 4 vs. 8.5 months), although in this case there was a trend for an association between high TILs and better OS. However, CD8+ TILs (Figure 10), were significantly associated with better OS in non-BC (p=0.0296, median survival 4 vs. 11 months) and all tumors (p=0.0495, median survival 6 vs. 11.5 months). In BC, there was a trend for an association (p=0.3971, median survival 6.5 months vs. undefined), even though no statistical significance was reached. This analysis should be repeated in a larger group of patients with BCBrMets, since the immune contexture of BC has gained broad acceptance as an important clinical factor correlated with patient prognosis, and also therapy prediction in primary tumours[78].

Overall, our data support that TILs and in particular CD8+ TILs can be indicators of a less aggressive disease, since these were positively associated with survival. In fact, although the percentage of TILs was consistently low independently of tumour origin, the primary tumour was significantly associated with low or high TILs, upon dichotomisation (p=0.0014) (Table 5). There was also an association between gender and low (male) or high TILs (female) (p=0.0003). Tissues from BrMets from lung or skin cancers were predominantly low TILs, whereas in the case of breast, colon and kidney cancers the majority of the cases where classified has high TILs. It is important to take into account that apart from breast and lung cancer, the relative numbers of cases were very small. Moreover, there is a clear role of the immune system in lung cancer, and BC is not typically associated with an immune enrichment, therefore a cautious interpretation of these data and proper validation is required[79]. Nevertheless, future work should be done in order to study subtype immunogenicity of BC. The subcohort of BC should be enlarged to enable the assessment of TILs and CD8+ TILs as well as their association with patient outcome in the context of molecular subtypes of BC, since variable immunogenicity activity has been suggested before[80,81]. The presence of CD8+ T cells was associated with a reduction of hazard of BC-specific mortality, specifically in ER- tumours but not in ER+, in which no differences in survival were found concerning CD8+ infiltrate[82].

Table 5 | Association between clinicopathological characteristics of patients with BrMets originated from breast, lung, colon, kidney and melanoma and total and CD8+ stromal TILs.

	Stromal TILs			Stromal CD8+ TILs		
Clinicopathological characteristics (n=55)	**Low**	**High**	***p* -value**	**Low**	**High**	***p* -value**
Number of patients	28	27		21	34	
Age at diagnosis of BrMets (years)			0.3461 ¥			0.5674 ¥
Median	56	60		56	58.5	
Range	40-90	28-86		28-90	40-86	
IQR	13.75	19		16.5	17.5	
Gender			**0.0003 §**			0.7823 §
Female	10	23		12	21	
Male	18	4		9	13	
Primary tumour			**0.0014 †**			0.9377 †
Breast cancer	7 (25%)	17 (63%)		9 (37.5%)	15 (62.5%)	
Lung cancer	14 (50%)	1 (3.7%)		5 (33.3%)	10 (66.7%)	
Colon cancer	2 (7.1%)	4 (14.8%)		3 (50%)	3 (50%)	
Kidney cancer	2 (7.1%)	4 (14.8%)		2 (33.3%)	4 (66.7%)	
Melanoma	3 (10.7%)	1 (3.7%)		2 (50%)	2 (50%)	
Time between brain relapse and death (months)			0.8840 ¥			0.0705 ¥
Median	7	7		5	11	
Mean	10.04	12.27		8.25	12.88	
Range	1-38	1-72		1-38	1-72	
12-months OS			0.2518 ¥			**0.0441 ¥**
Median	4	8.5		6	11	

Bold indicates $p < 0.05$, meaning statistical significance.
¥ Mann Whitney test; † $\chi 2$ test; § Fisher's exact test
BrMets , Brain Metastases; *IQR* , Interquartile Range; *12-months OS* , Overall Survival with 12 months as endpoint.

Association between high CD8+ TILs and median OS was also significant by Mann-Whitney test analysis (p =0.0441) (Table 5).

Considering the literature, the relevance of TILs in BrMets is not totally unravelled[49]. The present study describes the percentage of TILs and CD8+ TILs in BrMets from different brain tropic primary tumours and data suggest that, widening the cohort, the percentages of TILs and CD8+ TILs in the stroma of the BrMets, breast and non-breast, in fact can become biomarkers of better prognosis, as in the literature. Overall, these results are supported by the literature, that has suggested TILs as prognostic biomarkers across different types of cancers, including BC, ovarian cancer, Non-Small Cell Lung Cancer (NSCLC) and melanoma[48,56,77]. It would be important to analyse TILs and CD8+ TILs in the respective primary tumours. A recent study published in the Annals of Oncology showed that some immune-oncology targets and macrophage and angiogenesis signatures have preserved expression and suggest therapeutic combinations for clinical testing[83]. In view of

these findings, to understand if and how TILs and CD8+ TILs vary during tumour progression and assess a possible relevance in terms of OS, would pave the way to new therapeutic options for BrMets in the future.

Furthermore, it is important to mention that we did not perform survival analysis adjusting for the different molecular subtypes of BC due to the small sample size (n=24). In our study, no association was found between clinicopathological features of BC patients and stromal TILs or stromal CD8+ TILs (Table 6). It would be interesting to further investigate it, inasmuch as TILs have suggested survival benefit in BC patients, namely in TNBC[50,56]. Moreover, the study of the genomes of the surviving cancers, and their comparison between different BC molecular subtypes could lead to more efficient therapeutic approaches for each. Finally, one cannot neglect the study of stromal TILs and stromal CD8+ TILs, along with other immune targets, in the primary lesions, in future studies. It would be strongly advisable to do so in order to clarify the role of immune surveillance in primary breast cancers regarding clonal heterogeneity and consequently the changes in BC to BC metastases immunogenicity.

Table 6 | Association between clinicopathological characteristics of patients with BCBrMets and total and CD8+ stromal TILs.

	Stromal TILs			Stromal CD8+ TILs		
Clinicopathological characteristics (n=24)	**Low**	**High**	***p* -value**	**Low**	**High**	***p* -value**
Number of patients	18	6		12	12	
Age at diagnosis of BrMets (years)			0.7331 ¥			0.2587 ¥
Median	56,5	57,5		55	60,5	
Range	28-72	40-86		28-86	46-72	
IQR	16	28		19,75	17,75	
TNM stage			1.0000 §			1.0000 §
I-III	17 (94.4%)	5 (83.3%)		11 (91.7%)	11 (91.7%)	
IV	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
Unknown	1 (5.6%)	1 (16.7%)		1 (8.3%)	1 (8.3%)	
ER status ǂ			0.6158 §			0.3615 §
Positive	6 (33.3%)	1 (16.7%)		2 (16.7%)	5 (41.7%)	
Negative	10 (55.6%)	5 (83.3%)		9 (75%)	6 (50%)	
NA	2 (11.1%)	0 (0%)		1 (8.3%)	1 (8.3%)	
PR status ǂ			0.6351 §			1.0000 §
Positive	9 (50%)	2 (33.3%)		5 (41.7%)	6 (50%)	
Negative	7 (38.9%)	4 (66.7%)		6 (50%)	5 (41.7%)	
NA	2 (11.1%)	0 (0%)		1 (8.3%)	1 (8.3%)	
HER2 status ǂ			1.0000 §			1.0000 §
Positive	8 (44.4%)	3 (50%)		6 (50%)	5 (41.7%)	
Negative	5 (27.8%)	3 (50%)		5 (41.7%)	3 (25%)	
Equivocal	3 (16.7%)	0 (0%)		0 (0%)	3 (25%)	
NA	2 (11.1%)	0 (0%)		1 (8.3%)	1 (8.3%)	
Ki67 status ǂ			1.0000 §			1.0000 §
High	4 (22.2%)	2 (33.3%)		3 (25%)	3 (25%)	
Low/Borderline	12 (66.7%)	4 (66.7%)		8 (66.7%)	8 (66.7%)	
NA	2 (11.1%)	0 (0%)		1 (8.3%)	1 (8.3%)	
Molecular subtype ǂ *			0.7288 †			0.7288 †
Luminal	4 (22.2%)	1 (16.7%)		4 (26.7%)	1 (11.1%)	
Luminal B HER2+	4 (22.2%)	1 (16.7%)		4 (26.7%)	1 (11.1%)	
HER2+	3 (16.7%)	1 (16.7%)		2 (13.2%)	2 (22.2%)	
TNBC	2 (11.1%)	2 (33.2%)		3 (20%)	1 (11.1%)	
Unknown	3 (16.7%)	1 (16.7%)		1 (6.7%)	3 (33.4%)	
NA	2 (11.1%)	0 (0%)		1 (6.7%)	1 (11.1%)	
Molecular subtype discordance between primary tumour and metastasis			1.0000 §			0.6372 §
Yes	6 (21.4%)	3 (50%)		6 (50%)	3 (25%)	
No	17 (60.7%)	2 (33.3%)		4 (33.4%)	5 (41.7%)	
Unknown	3 (10.7%)	1 (16.7%)		1 (8.3%)	3 (25%)	
NA	2 (7.2%)	0 (0%)		1 (8.3%)	1 (8.3%)	
Time between brain relapse and death (months)			0.8840 ¥			0.0705 ¥
Median	7	7		5	11	
Mean	10,04	12,27		8.25	12,88	
Range	1-38	1-72		1-38	1-72	
12-months OS			0.6993 ¥			0.4025 ¥
Median	12	7,5		6,5	12	

p <0.05 means statistical significance.

¥ Mann Whitney test; † χ2 test; § Fisher's exact test

BrMets , Brain Metastases; *IQR* , Interquartile Range; *ER* , Oestrogen Receptor; *PR* , Progesterone Receptor; HER2, Human Epidermal Growth Factor Receptor type-2; TNBC, Triple Negative Breast Cancer; NA, Not Applicable, meaning absence of tumour or loss of > 50% of the cores.

ǂ Statuses and Molecular subtype refer to the metastases; *Molecular subtype classification is according to the 2015 St Gallen Consensus Conference, recommended by the ESMO Clinical Practice Guidelines; Luminal comprises Luminal A and Luminal B HER2- subtypes.

NA , *Equivocal* and *Unknown* are not taken int account to the statistical test.

Finally, we only looked for stromal TILs as they are currently known to be the best parameter for characterisation of TILs[72]. Intratumoural TILs are described by several studies as more difficult to evaluate and do not provide additional predictive/prognostic value compared to stromal TILs.

4.2 Detection of Reactive Astrocytes in BrMets

As a secondary objective of this study, we intended to address the presence of reactive (or altered) glial cells, namely astrocytes, as they are the most abundant cells on the BrMets microenvironment, and might interact with cancer cells and other adjacent cell types[44]. To detect astrocytes we used the cytoskeletal intermediate filament protein GFAP as marker. GFAP is expressed in normal, reactive and neoplastic astrocytes[35]. All BCBrMets tissue sections (n=25) were observed and classified by a Pathologist, without GFAP staining intensity alterations (Figure 11). This suggests that astrocytes did not suffer phenotypic modifications in this particular group of patients.

Despite knowing from the literature that GFAP is a glia-specific protein and not astrocytes-specific[40,84], we expected to observe strong staining intensity, since astrocytes are usually characterised by the upregulation of GFAP in any brain injury[44]. Moreover, previous studies of BCBrMets have shown a profusion of activated astrocytes around and within the lesions[15,41].

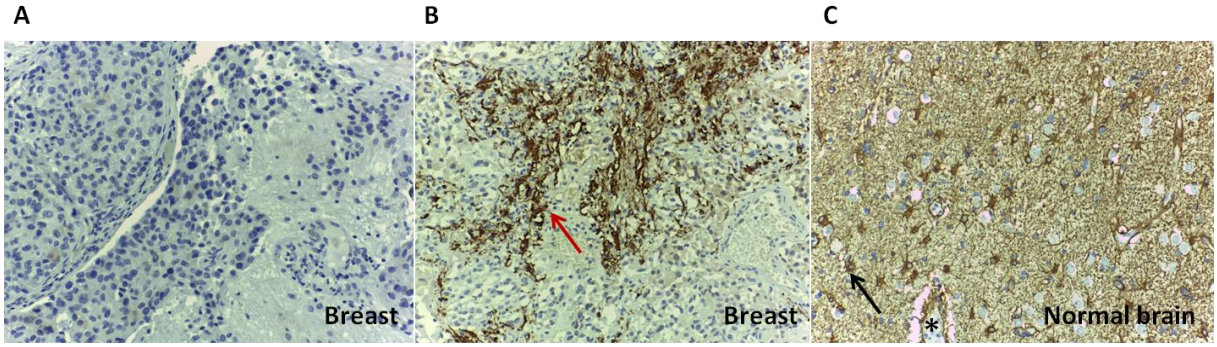


Figure 11 | Representative images of tissue sections of BC and normal brain stained for GFAP. Negative control (A); BCBrMet sample with glial elements (red arrow) (B); Normal brain parenchyma with stained astrocytes (black arrow) and other glial cells, and a blood vessel (*) (C). Magnification X200.

4.3 Characterisation of PD-L1 Expression in BrMets

Histologic assessment of tumours and their immune microenvironment is becoming really relevant in the clinical setting for patients with solid tumours[75]. Thus,

we aimed to determine the prevalence of PD-L1, an actionable immunotherapy target described to be expressed in some tumour cells[63], and to assess its correlation with survival outcomes.

PD-L1 expression was determined by IHC, and scored by a Pathologist according to intensity of staining and percentage of cells per intensity (Figure 12). In our cohort of BrMets (n=55), only four cases were positive for PD-L1 expression, with modified H-score of 10 for one BCBrMets and 20, 30 and 210 in three lung cancer BrMets.

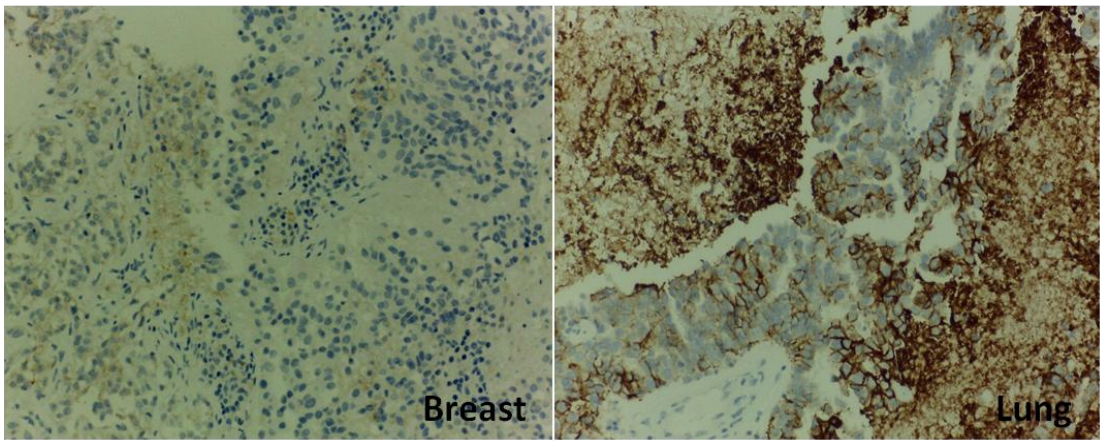


Figure 12 | Representative images of tissue sections immunostained for PD-L1. BC and lung BrMets with positive PD-L1 score, with staining intensities of 1+, and 3+, respectively. Magnification X200.

As mentioned in the Materials and Methods section, the modified H-score considers the frequency of positive cells (%) and staining intensity, and it has been described that elevated expression of PD-L1 is associated with worse prognosis and better response of BC patients to immune treatment[85]. However, and our data is in accordance to this, PD-L1 expression is rare in BC tissues, both in immune and tumour cells. Additionally, a significant enrichment of PD-L1 is registered in basal-like tumours, and our only positive BC case was in fact a TNBC (1/5 TNBC cases), according to the IHC analysis of the metastatic tissue (Table 3)[70]. The other four tumour types, lung cancer, colon cancer, kidney cancer, and melanoma, are known to be immunogenic, so we eventually envisaged more positive cases than we obtained[49,86,87]. However, all our tumour-specific subcohorts are small.

PD-L1 expression tends to decrease between primary tumours and BrMets and varies in BCBrMets according to the subtype of primary lesion[88]. A lower expression of 13/29 selected immune-oncology therapeutic targets in metastases in

which PD-L1 was included was found in a recent study[83]. Along with it, previously published data indicate an immune depleted state in metastatic lesions. Nevertheless, it is important to stress that the use of TMAs could have impaired results of heterogenic markers and underestimated true positivity.

Given the small number of positive results obtained, we were not able to proceed with further outcome analysis. However, to complement this study, we assessed the expression of PD-L1, and also PD-1 and PD-L2 in a panel of cancer cell lines, including two brain tropic clones of a TNBC cell line.

4.3.1 Expression of PD-1, PD-L1 and PD-L2 in Cancer Cell Lines

In order to quantify the gene expression of PD-1 (*PD-1*), PD-L1 (*PD-L1*) and PD-L2 (*PD-L2*) in cancer cell lines by RT-qPCR (Figure 13), we used two brain tropic clones from the MDA-MB-231 TNBC cell line, MDA-MB-231-BR HER2+ and MDA-231-BR HER2-, along with other nine BC cell lines (TNBC: MDA-MB-435S, MDA-MB-231, MDA-MB-231-BO2; HER2+: SK-BR-3; Luminal A: MCF7 and T-47D; Luminal B HER2-: BT-474, MDA-MB-361, ZR-75), and one prostate adenocarcinoma cell line (PC-3). The MCF-10A non-malignant mammary epithelial cell line was used to normalise the expression values and Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) was used as internal control for gene expression data normalisation.

In metastatic BC, there are few preclinical models to study the inherent heterogeneity of BrMets[89,90]. The MDA-MB-231 cell line has been the most broadly selected for brain tropism[90,91,92], as MDA-MB-231-BR resembles human craniotomy specimens in terms of proliferation, apoptosis, and a neuro-inflammatory response[43]. The proteome of this cell line has been compared with that of the parental cell line, MDA-MB-231. By means of protein quantitative analysis, a group of researchers has determined that 112/152 proteins were decreased and only 40/152 were increased, meaning that downregulation of specific proteins can be an important part of the underlying mechanism for BC cells to metastasize to the brain[93]. The Steeg P. laboratory has transfected cells with HER2 cDNA or a control vector after transducing them with enhanced green fluorescence protein (EGFP) in order to develop the MDA-MB-231-BR HER2+ cell line, a model system to study the relevance of HER2 overexpression in BrMets[89]. This laboratory has shown that the preclinical model mimics many characteristics of human BrMets.

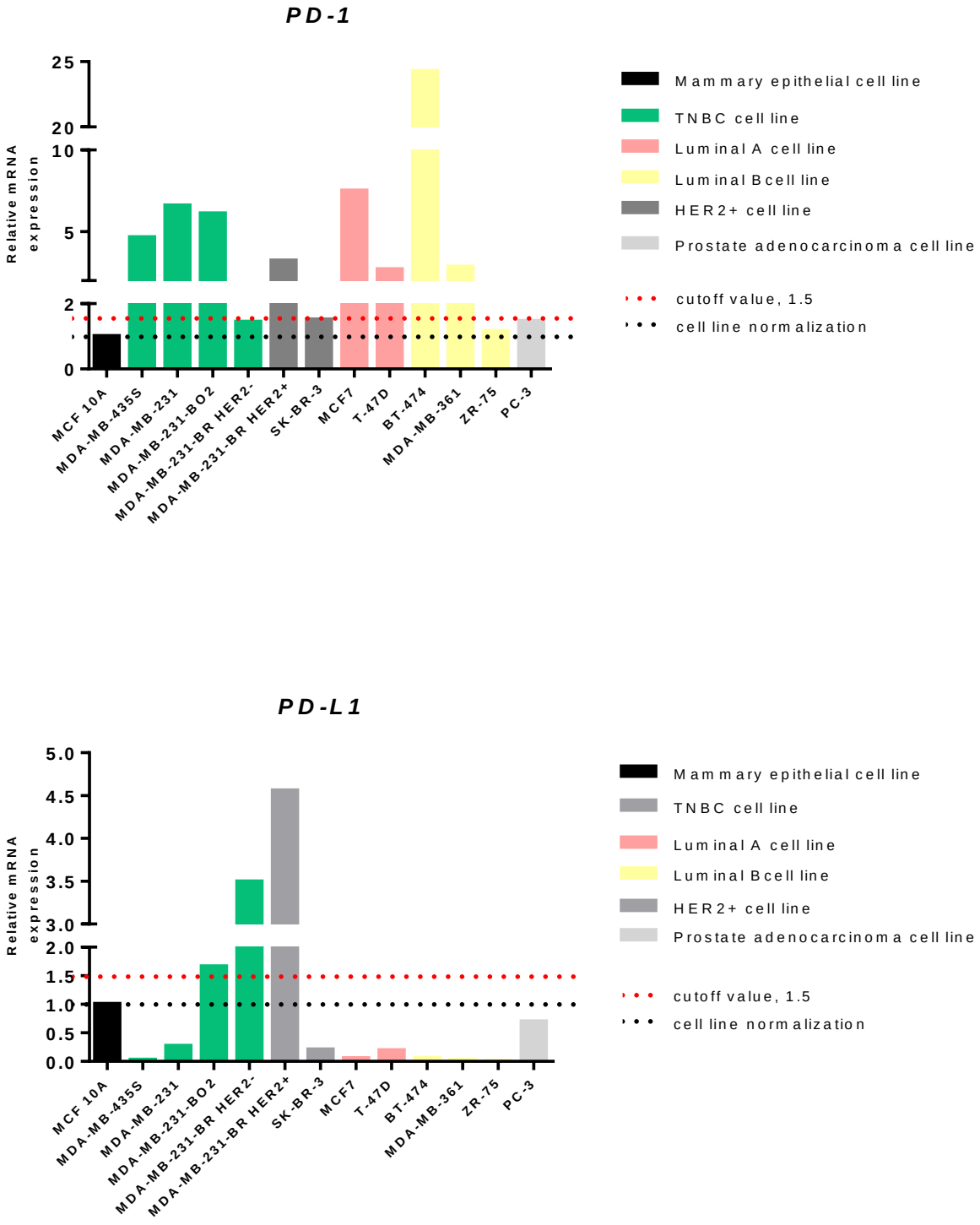


Figure 13 | *PD-1*, *PD-L1* and *PD-L2* expression in a panel of cancer cell lines. *PD-1* (A), *PD-L1* (B) and *PD-L2* (C) expression determination was performed by RT-qPCR in MDA-MB-435S, MDA-MB-231, MDA-MB-231-BO2, MDA-MB-231-BR HER2- (TNBC); MDA-MB-231-BR HER2+, SK-BR-3 (HER2+); MCF7, T-47D (Luminal A); BT-474, MDA-MB-361, ZR-75 (Luminal B HER2-) cell lines. Relative expression levels were calculated by comparative Ct method and values are presented as fold change ($2^{-\Delta\Delta Ct}$) relative to expression in the non-tumorigenic MCF10A cell line. *GAPDH* was used as housekeeping gene. Cutoff value for up-regulation: 1.5.

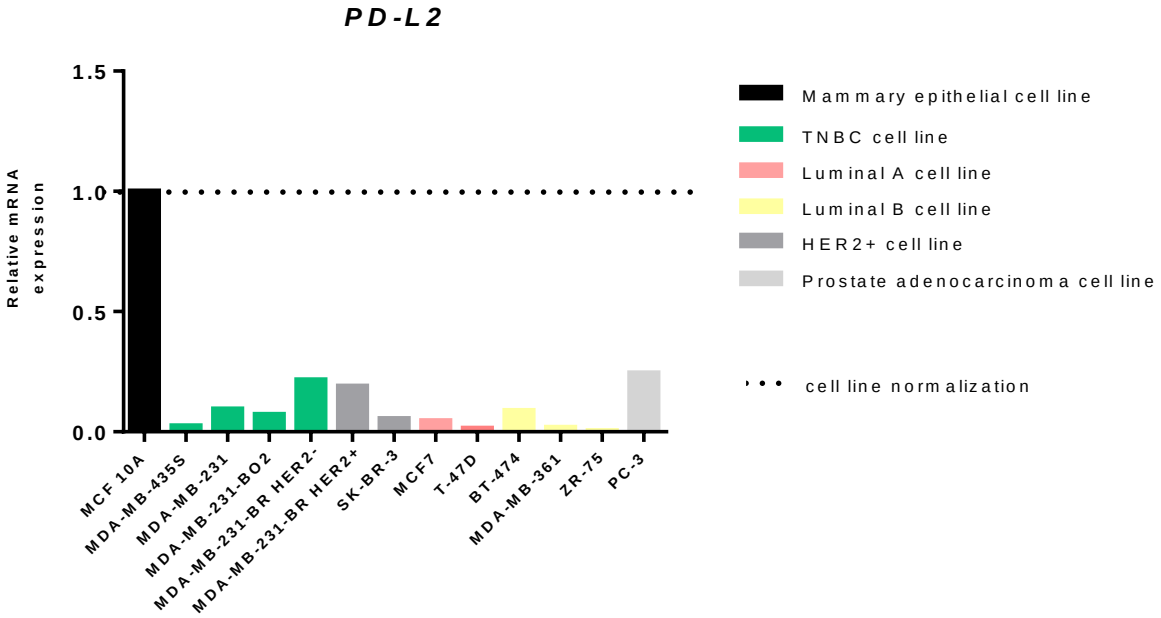


Figure 13 | *PD-1*, *PD-L1* and *PD-L2* expression in a panel of cancer cell lines. *PD-1* (A), *PD-L1* (B) and *PD-L2* (C) expression determination was performed by RT-qPCR in MDA-MB-435S, MDA-MB-231, MDA-MB-231-BO2, MDA-MB-231-BR HER2- (TNBC); MDA-MB-231-BR HER2+, SK-BR-3 (HER2+); MCF7, T-47D (Luminal A); BT-474, MDA-MB-361, ZR-75 (Luminal B HER2-) cell lines. Relative expression levels were calculated by comparative Ct method and values are presented as fold change ($2^{-\Delta\Delta Ct}$) relative to expression in the non-tumorigenic MCF10A cell line. *GAPDH* was used as housekeeping gene. Cutoff value for up-regulation: 1.5. (Continued)

PD-1 (Figure 13A) was upregulated in all tested cancer cell lines, with the exception of ZR-75, and the expression levels were decreased in the brain tropic clones when comparing to the parental TNBC cell line MDA-MB-231 and the bone tropic clone. PD-1 is described to be only expressed on the surface of immune cells, while its ligands, PD-L1 and PD-L2, are found expressed in tumour cells[63]. However, recent studies, which our data are along with, show intrinsic expression of the receptor in tumours, such as melanoma, liver cancer or NSCLC[63,64].

Regarding the ligands, PD-L2 (Figure 13C) was downregulated in all cancer cell lines, and PD-L1 (Figure 13B) was exclusively upregulated in the bone and brain tropic TNBC clones, particularly in the last ones. This suggests an association between the expression of PD-L1 in BrMets and the brain immune microenvironment, as we hypothesized at first and despite our results in the clinical cohort.

As far as we know, this is the first assessment of the expression of *PD-1*, *PD-L1* and *PD-L2* in the MDA-MB-231-BR HER2+ and MDA-MB-231-BR HER2- brain tropic clones, which makes it impossible for us to compare our results with reported values. The expression data of these genes was searched in databases, such as

Cancer Cell Line Encyclopedia (CCLE) and Oncomine but comparable results were not found.

4.4 ER, PR and HER2 Status in Primary Breast Tumours and Matched BrMets

It is known that during tumour progression molecular alterations occur due to clonal selection of the fittest cancer cells. In BC, metastases are not necessarily identical to primary tumours, concerning hormone receptor (HR) and HER2 expression, which impacts on treatment choice and efficacy. Therefore, whenever possible the status of HR and HER2 on metastatic tissue should be determined.

In our subcohort of 25 BCBrMets patients the HR, HER2, and Ki67 expression was unknown in clinical records, so we proposed to perform these determinations, and to verify if there was a molecular switch between primary tumour and metastasis (excluding Ki67 expression due to the lack of information on the primary tumour). Upon ER, PR, HER2, and Ki67 IHC, samples were evaluated by a Pathologist, and classified as Luminal, Luminal B HER2+, HER2+ or TNBC (Figure 14 and Table 4).

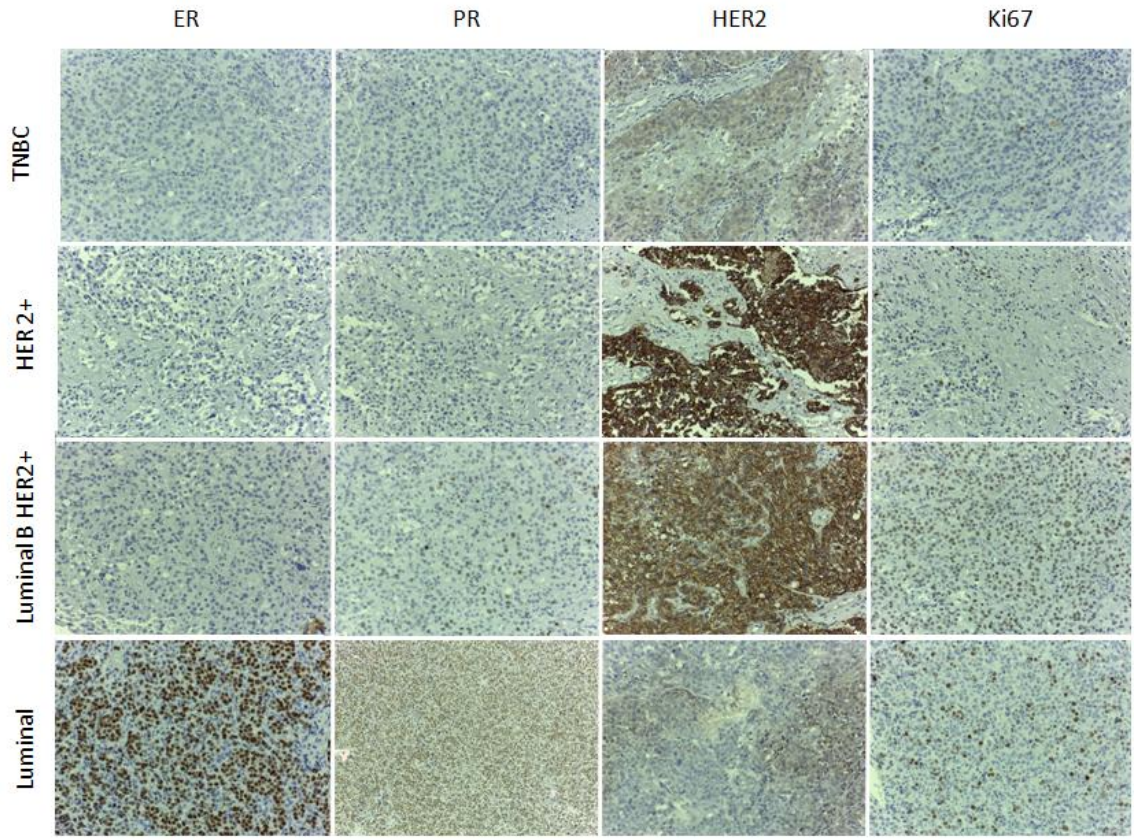


Figure 14 | Representative images of BrMets tissue sections immunostained for ER, PR, HER2 and Ki67, corresponding to different molecular subtypes of BC. TNBC: negative ER, PR, HER2 (1+), low Ki67; HER2+: negative ER, PR, positive HER2 (3+), low Ki67; Luminal B HER2+: negative ER, positive PR, positive HER2 (3+), low Ki67; Luminal: positive ER, PR, negative HER2 (1+), high Ki67. Magnification X200.

The median age of the patients at metastasis diagnosis was 57 years (range 28-86 years), and ductal BC was the predominant histologic subtype. Median brain metastases-free survival was 15.50 months (IQR=43.25), ranging between 1 and 119 months and median survival after diagnosis of brain metastasis was 8 months (IQR=12), ranging from 1 to 72 months. Survival after brain recurrence depends on factors that include, among others, the extent of extracranial disease, performance status, and local or systemic therapy administration. Nevertheless, great differences in survival after a diagnosis of brain metastases by receptor status have been shown in retrospective studies[11]. In this particular study, we did not analyse survival by subtype due the small size of the cohort.

In this study we had complete information about the primary tumour and metastasis subtype in 19 out of the 24 cases (79.2%). 10/19 cases (52.6%) had at least one alteration, comparing the breast tumour and brain metastasis, and all these ten cases changed the status of HR (Figure 15). In six cases there was loss of HR, and in four cases there was gain in HR expression.

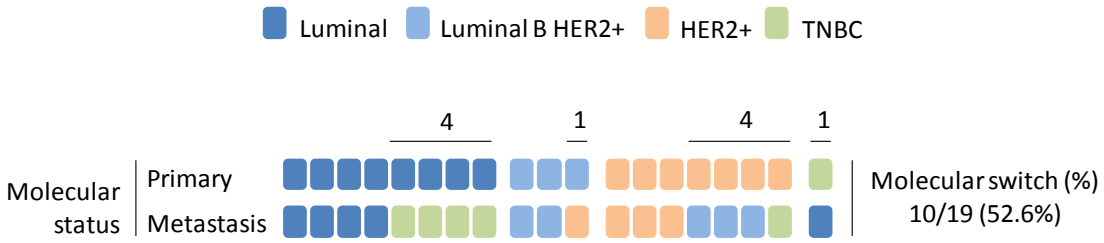


Figure 15 | Representative schema of the molecular status switch between BC primary tumours and paired BrMets. *HER2+,* Human Epidermal Growth Factor type 2-positive; *TNBC,* Triple Negative Breast Cancer.

Several studies have shown that expression of HR is not necessarily conserved during carcinogenesis and tumour progression. Loss of HR is the most common alteration and may be related to clonal selection of less differentiated cells during the metastatic process, for example, caused by adjuvant hormonal selective pressure[94,95]. However, conversion from negative to positive HR has also been described before[96].

A lower rate of discordance was found concerning HER2 expression. Only one case presented discordance, corresponding to loss of HER2. This is in accordance with the literature, since the HER2 status is more stable than that of HR[21]. Besides, from a tumour progression model perspective one would expect HER2 gain, result of gene amplification, due to the oncogenic role of this receptor[96].

The alterations described above led to a different classification of BrMets molecular subtype in comparison with primary tumors (Table 3). Thus, this pairwise analysis points out the need to evaluate HR and HER2 statuses in metastases as this may impact the treatment choice. For example, in patients whose switch is in the direction of HER2 and/or ER positivity (four cases in this subcohort). Moreover, compelling evidences suggest that primary and metastatic tumours are different. Cancer cells suffer from strong selective pressure and the vast majority is either eliminated or kept in a dormant state during metastatic colonisation. The ones that evade this selective pressure are able to manipulate the local microenvironment in order to create metastatic niches, which are tumour-supporting environments[97]. There are frequent genetic differences between primary tumours and metastases in BC from the same patient, suggesting distinct genetic profiles of primary tumours and corresponding metastases, the interactions of tumour cells and their microenvironment are regulated by different networks of cytokines and growth factors, and primary and metastatic brain tumours have been shown to be infiltrated by different populations of bone-marrow derived cells, which can be mobilised into the circulation and incorporate into tumour microenvironments, modulating them[30,98]. In a study on serous epithelial ovarian cancer, the genetic differences between primary tumours and metastases suggest that their major clone is not the same[99]. The differences mentioned above illustrate the relevance of studying the microenvironments of both tumours to compare their elements and unravel the alteration of tumour behavior from primary tumour to metastasis. The analysis of these parameters should elucidate possible crosstalks between elements of both tumours' microenvironments. It would be interesting to know which and how changes in the metastasis influenced HR and HER2 statuses.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

BrMets are a serious obstacle in cancer patient care and a significant indicator of dismal prognosis[41]. Although the CNS has been considered to be an immune-privileged site, increasing evidence supports the role of immune infiltrating cells and a strong association with TILs density and improved OS in CNS metastases[77]. This immune microenvironment may be targeted by immune-modulating therapies, such as immune checkpoint inhibitors[100]. Thus, the main objective of this project was to investigate the association between components of the immune compartment of BrMets (total TILs, and CD4+ TILs and CD8+ TILs) and their clinical relevance. Controversial results have been reported in published studies and little is known about whether TILs correlate with patient survival in different tumour types[101]. However, in the clinical setting, we obtained promising results that point stromal TILs and CD8+ TILs as biomarkers of better prognosis in patients with BrMets. Further studies are expected to confirm our findings in a broader cohort.

Our secondary objectives were more dependent on the size of the cohort, and our purpose to study events like the presence of reactive astrocytes and expression of PD-L1 will be pursued upon the expansion of this series of cases. The use of TMAs might have also been a limitation since immune infiltration may be heterogeneous and this heterogeneity is not captured by TMAs.

Furthermore, we also analysed a paired set of primary breast tumours and secondary lesions in the brain and found molecular alterations in a significant number of cases in our cohort. This stresses that determination of targetable proteins should be performed in metastases, independently of the findings in primary tissues, given that these patients may benefit from a different treatment strategy.

Overall, our results show that the relation between cancer cells and the host is constantly changing premises, so it must be mandatory for researchers and clinicians to work in close touch to unravel the mechanisms underlying the metastatic process in different organs. This could provide therapeutic alternatives in some specific windows of opportunity to patients with apparently no more, being the development of clinical trials regarding immunotherapies particularly desirable in the context of CNS pathologies.

6 REFERENCES

1. Nayak L, Lee EQ, Wen PY. Epidemiology of Brain Metastases. *Curr Oncol Rep.* 2012. 14(1):48–54.

2. Hall WA, Djalilian HR, Nussbaum ES *et al*. Long-term survival with metastatic cancer to the brain. *Medical Oncol.* 2000. 17:279–286.

3. Stelzer KJ. Epidemiology and prognosis of brain metastases. *Surg Neurol Int.* 2013. 4:S192–202.

4. Vernon AE, Bakewell SJ, Chodosh LA. Deciphering the molecular basis of breast cancer metastasis with mouse models. *Rev Endocr Metab Desord.* 2007. 8(3):199–213.

5. Soffietti R, Rudā R, Mutani R. Management of brain metastases. *J* Neurol. 2002. 249(10):1357–1369.

6. Cagney DN, Martin AM, Catalano PJ *et al.* Incidence and prognosis of patients with brain metastases at diagnosis of systemic malignancy: a population-based study. *Neuro Oncol*. 2017. 19(11):1511–1521.

7. Hoenerhoff MJ, Chu I, Barkan D *et al.* BMI1 Cooperates with H-RAS to Induce an Aggressive Breast Cancer Phenotype with Brain Metastases. *Oncogene.* 2010. 28(34):3022–3032.

8. Gavrilovic IT, Posner JB. Brain metastases: epidemiology and pathophysiology. *J Neurooncol.* 2005. 75(1):5–14.

9. Qian JM, Yu JB, Kluger HM *et al.* Timing and Type of Immune Checkpoint Therapy Affect the Early Radiographic Response of Melanoma Brain Metastases to Stereotactic Radiosurgery. *Cancer.* 2016. 1–8.

10. Kocher M, Soffietti R, Abacioglu U *et al.* Adjuvant Whole-Brain Radiotherapy Versus Observation After Radiosurgery or Surgical Resection of One to Three Cerebral Metastases : Results of the EORTC 22952-26001 Study. *J Clin Oncol.* 2017. 29:134–141.

11. McKee MJ, Keith K, Deal AM *et al.* A Multidisciplinary Breast Cancer Brain Metastases Clinic: The University of North Carolina Experience. *Oncologist.* 2016. 21(1):16–20.

12. Senkus E, Kyriakides S, Ohno S *et al.* Primary breast cancer : ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up clinical practice guidelines. *Ann Oncol.* 2015. 26(5):v8–v30.

13. Steeg PS, Camphausen KA, Smith QR. Brain metastases as preventive and therapeutic targets. *Nat Rev Cancer.* 2011. 11(5):352–363.

14. Bendell JC, Domcheck SM, Burstein HJ *et al.* Central Nervous System Metastases in Women who Receive Trastuzumab-Based Therapy for Metastatic Breast Carcinoma. *Cancer.* 2003. 2972–2977.

15. Witzel I, Oliveira-Ferrer L, Pantel K *et al*. Breast cancer brain metastases : biology and new clinical perspectives. *Breast Cancer Res.* 2016. 1–9.

16. Lin NU, Claus E, Sohl J. Sites of Distant Recurrence and Clinical Outcomes in Patients With Metastatic Triple-negative Breast Cancer. High Incidence of Central Nervous System Metastases. *Cancer*. 2008. 2638–2645.

17. Niwinska A, Murawska M, Pogoda K. Breast cancer brain metastases : differences in survival depending on biological subtype, RPA RTOG prognostic class and systemic treatment after whole-brain radiotherapy (WBRT). *Ann Oncol.* 2010. 942–948.

18. Zhang Q, Chen J, Yu X *et al*. Survival benefit of anti-HER2 therapy after whole-brain radiotherapy in HER2-positive breast cancer patients with brain metastasis. *Breast Cancer.* 2016. 23:732–739.

19. Weil RJ, Palmieri DC, Bronder JL *et al*. Breast Cancer Metastasis to the Central Nervous System. *Am. J. Pathol.* 2005. 167:913–920.

20. Dawood S, Broglio K, Esteva FJ *et al.* Survival among women with triple receptor-negative breast cancer and brain metastases. *Ann Oncol.* 2009. 20:621–627.

21. Broom RJ, Tang PA, Simmons C *et al.* Changes in Estrogen Receptor, Progesterone Receptor and Her-2/neu Status with Time : Discordance Rates Between Primary and Metastatic Breast Cancer. *Anticancer Res.* 2009. 29(5):1557–1562.

22. Gaedcke J, Traub F, Milde S *et al.* Predominance of the basal type and HER-2/neu type in brain metastasis from breast cancer. *Mod Pathol.* 2007. 864–870.

23. Niikura N, Liu J, Hayashi N *et al.* Loss of Human Epidermal Growth Factor Receptor 2 (HER2) Expression in Metastatic Sites of HER2-Overexpressing Primary Breast Tumors. *J Clin Oncol.* 2018. 593–599.

24. Nishimura R, Osako T, Okumura Y *et al.* Changes in the ER , PgR , HER2 , p53 and Ki-67 biological markers between primary and recurrent breast cancer : discordance rates and prognosis. *World J. Surg. Oncol.* 2011. 9:131.

25. Regitnig P, Schippinger W, Lindbauer M *et al.* Change of HER-2/neu status in a subset of distant metastases from breast carcinomas. *J Pathol.* 2004. 203:918–926.

26. Langley RR, Fidler IJ. The seed and soil hypothesis revisited — The role of tumor-stroma interactions in metastasis to different organs. *Int. J. Cancer.* 2011. 2527–2535.

27. Kienast Y, von Baumgarten L, Fuhrmann M *et al.* Real-time imaging reveals the single steps of brain metastasis formation. *Nat. Med.* 2009. 16:116–122.

28. Charles NA, Holland EC. The perivascular niche microenvironment in brain tumor progression. *Cell Cycle.* 2010. 9(15):3012–3021.

29. Carbonell WS, Ansorge O, Sibson N *et al*. The Vascular Basement Membrane as "' Soil "' in Brain Metastasis. *PLoS ONE.* 2009. 4(6):5857.

30. Lorger M. Tumor Microenvironment in the Brain. *Cancers.* 2012. 4(1):218–243.

31. Berghoff AS, Lassmann H, Preusser M *et al*. Characterization of the inflammatory response to solid cancer metastases in the human brain. *Clin Exp Metastasis*. 2013. 30:69–81.

32. Guirguis LM, Yang JC, White DE *et al.* Safety and Efficacy of High-Dose Interleukin-2 Therapy in Patients With Brain Metastases. *J Immunother.* 2008. 25:82–87.

33. Carson MJ, Doose JM, Melchior B *et al*. CNS immune privilege : hiding in plain sight. *Immunol Rev.* 2006. 213:48–65.

34. Patel KR, Lawson DH, Kudchadkar RR *et al.* Two heads better than one? Ipilimumab immunotherapy and radiation therapy for melanoma brain metastases. *Neuro Oncol.* 2015. 17(10):1312–1321.

35. Bos PD, Zhang XHF, Nadal C *et al.* Genes that mediate breast cancer metastasis to the brain. *Nature.* 2009. 459(7249):1005–1009.

36. Arshad F, Wang L, Sy C *et al.* Blood-Brain Barrier Integrity and Breast Cancer Metastasis to the Brain. *Pathology Research International.* 2011. 11:1–12.

37. Lin NU, Amiri-Kordestani L, Palmieri D *et al.* CNS Metastases in Breast Cancer : Old Challenge, New Frontiers. *Clin Cancer Res*. 2016. 19(23).

38. Termini J, Neman J, Jandial R. Role of the Neural Niche in Brain Metastatic Cancer. *Cancer Res.* 2014. 74(15):4011–4015.

39. Hol EM, Pekny M. Glial fibrillary acidic protein (GFAP) and the astrocyte intermediate filament system in diseases of the central nervous system. *Curr. Opin. Cell Biol.* 2015. 32:121–130.

40. Yoshimine T, Ushio Y, Hayakawa T *et al.* Immunohistochemical study of metastatic brain tumors with astroprotein (GFAP), a glia-specific protein. *J Neurosurg.* 1985. 62:414–418.

41. Ferraro GB, Kodack DP, Askoxylakis V *et al*. Closing the gap : astrocytes and brain metastasis. *Cell Res.* 2016. 26(9):973–974.

42. Zhang M, Olsson Y. Reactions of astrocytes and microglial cells around hematogenous metastases of the human brain. Expression of endothelin-like immunoreactivity in reactive astrocytes and activation of microglial cells. *J Neurol Sci.*1995. 26–32.

43. Fitzgerald DP, Palmieri D, Hua E *et al.* Reactive glia are recruited by highly proliferative brain metastases of breast cancer and promote tumor cell colonization. *Clin Exp Metastasis.* 2008. 25:799–810.

44. Wasileski D, Priego N, Fustero-Torre C *et al*. Reactive Astrocytes in Brain Metastasis. *Front. Oncol.* 2017. 7:298.

45. Kim SJ, Kim JS, Park ES *et al.* Astrocytes Upregulate Survival Genes in Tumor Cells and Induce Protection from Chemotherapy. *Neoplasia.* 2011. 13(3):286–298.

46. Valiente M, Obenauf AC, Xin J *et al.* Serpins Promote Cancer Cell Survival and Vascular Cooption in Brain Metastasis. *Cell.* 2015. 156(5):1002–1016.

47. Farber SH, Tsvankin V, Narloch JL *et al.* Embracing rejection : Immunologic trends in brain metastasis. *Oncoimmunology.* 2016. 5(7):1–11.

48. Yao W, He JC, Yang Y *et al.* The Prognostic Value of Tumor-infiltrating Lymphocytes in Hepatocellular Carcinoma: a Systematic Review and Meta-analysis. *Sci. Rep.* 2017. 1–11.

49. Berghoff AS, Fuchs E, Ricken G *et al.* Density of tumor-infiltrating lymphocytes correlates with extent of brain edema and overall survival time in patients with brain metastases. *Oncoimmunology.* 2016. 5(1):e1057388.

50. Berghoff AS, Preusser M. The inflammatory microenvironment in brain metastases : potential treatment target? *Chin Clin Oncol.* 2015. 4:1–9.

51. Barnes TA, Amir E. HYPE or HOPE: the prognostic value of infiltrating immune cells in cancer. *Br J Cancer.* 2017. 117:451–460.

52. Ogiya R, Niikura N, Kumaki N *et al.* Comparison of immune microenvironments

between primary tumors and brain metastases in patients with breast cancer. *Oncotarget.* 2017. 8(61):103671–103681.

53. Wein L, Savas P, Luen SJ *et al.* Clinical Validity and Utility of Tumor-infiltrating Lymphocytes in Routine Clinical Practice for Breast Cancer Patients: Current and Future Directions. *Front. Oncol.* 2017. 7:156.

54. Loi S, Sirtaine N, Piette F *et al.* Prognostic and Predictive Value of Tumor-Infiltrating Lymphocytes in a Phase III Randomized Adjuvant Breast Cancer Trial in Node-Positive Breast Cancer Comparing the Addition of Docetaxel to Doxorubicin With Doxorubicin-Based Chemotherapy : BIG 02-98. *J Clin Oncol.* 2018. 31:860–867.

55. Loi S, Michieles S, Salgado R *et al.* Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer : results from the FinHER trial. *Ann Oncol.* 2014. 1544–1550.

56. Mao Y, Qu Q, Chen X *et al.* The Prognostic Value of Tumor-Infiltrating Lymphocytes in Breast Cancer : A Systematic Review and Meta-Analysis. *PLoS ONE.* 2016. 1–13.

57. Sobottka B, Pestalozzi B, Fink D *et al.* Similar lymphocytic infiltration pattern in primary breast cancer and their corresponding distant metastases. *Oncoimmunology.* 2016. 5:1–10.

58. Shah W, Yan X, Jing L *et al.* A reversed CD4/CD8 ratio of tumor-infiltrating lymphocytes and a high percentage of CD4+FOXP3+ regulatory T cells are significantly associated with clinical outcome in squamous cell carcinoma of the cervix. *Cell Mol Immunol*. 2011. 8(1):59–66.

59. Yang X, Ren H, Sun Y *et al.* Prognostic significance of CD4/CD8 ratio in patients with breast cancer. *Int J Clin Exp Pathol.* 2017. 10:4787–4793.

60. Weber JS, Amin A, Minor D *et al.* Safety and clinical activity of ipilimumab in melanoma patients with brain metastases: retrospective analysis of data from a phase 2 trial. *Melanoma Res.* 2011. 530–534.

61. Cohen JV, Kluger HM. Systemic immunotherapy for the Treatment of Brain Metastases. *Front. Oncol.* 2016. 6:1–5.

62. Wilson EH, Weninger W, Hunter CA. Trafficking of immune cells in the central nervous system Find the latest version: Science in medicine Trafficking of immune cells in the central nervous system. *J Clin Invest.* 2010. 120:1368–1379.

63. Yao H, Wang H, Li C *et al.* Cancer Cell-intrinsic PD-1 and implications in Combinatorial immunotherapy. *Front. Immunol.* 2018. 9:1774.

64. Kleffel S, Posch C, Barthel SR *et al.* Melanoma Cell-Intrinsic PD-1 Receptor Functions Promote Tumor Growth. *Cell.* 2015. 162:1242–1256.

65. Shi L, Chen S, Yang L *et al*. The role of PD-1 and PD-L1 in T-cell immune suppression in patients with hematological malignancies. *J. Hematol. Oncol.* 2013. 6:74.

66. Sorensen SF, Zhou W, Dolled-Filhart M *et al.* PD-L1 Expression and Survival among Patients with Advanced Non–Small Cell Lung Cancer Treated with Chemotherapy. *Transl Oncol.* 2016. 9:64–69.

67. Wang X, Teng F, Kong Li *et al*. PD-L1 expression in human cancers and its association with clinical outcomes. *Onco Targets Ther.* 2016. 5023–5039.

68. Velcheti V, Schalper KA, Carvajal DE *et al.* Programmed death ligand-1 expression in

non-small cell lung cancer. *Lab Invest.* 2014. 94(1):107–116.

69. Yang CY, Lin MW, Chang Y *et al*. Programmed cell death-ligand 1 expression in surgically resected stage I pulmonary adenocarcinoma and its correlation with driver mutations and clinical outcomes. *Eur. J. Cancer*. 2014. 50:1361–1369.

70. Ali HR, Glont SE, Blows FM *et al.* PD-L1 protein expression in breast cancer is rare, enriched in basal-like tumours and associated with infiltrating lymphocytes. *Ann Oncol*. 2015. 1488–1493.

71. Duchnowska R, Peksa R, Radecka B *et al.* Immune response in breast cancer brain metastases and their microenvironment: the role of the PD-1/PD-L axis. *Breast Cancer Res.* 2016. 1–11.

72. Salgado R, Denkert C, Demaria S *et al.* The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILsWorking Group 2014. *Ann Oncol.* 2015. 259–271.

73. Polónia A, Pinto R, Cameselle-Teijeiro JF *et al*. Prognostic value of stromal tumour in fi ltrating lymphocytes and programmed cell death-ligand 1 expression in breast cancer. *J Clin Pathol.* 2017. 0:1–8.

74. Budczies J, Klauschen F, Sinn BV *et al*. Cutoff Finder: A Comprehensive and Straightforward Web Application Enabling Rapid Biomarker Cutoff Optimization. *PLoS ONE.* 2012. 7:1–7.

75. Ziai J, Gilbert HN, Foreman O *et al.* CD8 + T cell infiltration in breast and colon cancer: A histologic and statistical analysis. *PLoS ONE.* 2018. 1–18.

76. Hernberg MP, Muhonen T, Turunen JP *et al.* The CD4+/CD8+ ratio as a prognostic factor in patients with metastatic melanoma receiving chemoimmunotherapy. *J Clin Oncol.* 1996. 14(5):1690–6.

77. Harter PN, Bernatz S, Scholz A *et al.* Distribution and prognostic relevance of tumor-infiltrating lymphocytes (TILs) and PD-1/PD-L1 immune checkpoints in human brain metastases. *Oncotarget.* 2015. 6(38):40836–49.

78. Miller LD, Chou JA, Black MA *et al.* Immunogenic Subtypes of Breast Cancer Delineated by Gene Classifiers of Immune Responsiveness. *Cancer Immunol Res*. 2017. 4(7):600–610.

79. Carbone DP, Gandara DR, Antonia SJ *et al.* Non–Small Cell Lung Cancer: Role of the Immune System and Potential for Immunotherapy. *J Thorac Oncol.* 2016. 10(7):974–984.

80. Piura E, Piura B. Autoantibodies to Tumor-Associated Antigens in Breast Carcinoma. *J Oncol.* 2010.

81. Criscitiello C. Breast Care Tumor-Associated Antigens in Breast Cancer. *Breast Care.* 2012. 262–266.

82. Denkert C. The immunogenicity of breast cancer - molecular subtype matter. *Ann Oncol.* 2014. 1453–1455.

83. Szekely B, Bossuyt V, Li X *et al.* Immunological differences between primary and metastatic breast cancer. *Ann Oncol*. 2018. 29:2232–2239.

84. Sofroniew MV, Vinters HV. Astrocytes: biology and pathology. *Acta Neuropathol.* 2010. 7–35.

85. Muenst S, Schaerli AR, Gao F *et al.* Expression of programmed death ligand 1 (PD-L1) is associated with poor prognosis in human breast cancer. *Breast Cancer Res Treatment.* 2014. 146(1):15–24.

86. Wang HB, Yao H, Li CS *et al.* Rise of PD-L1 expression during metastasis of colorectal cancer: Implications for immunotherapy. *J Dig Dis.* 2017. 18(10):574–581.

87. Berghoff AS, Ricken G, Whilhelm D *et al.* Tumor infiltrating lymphocytes and PD-L1 expression in brain metastases of small cell lung cancer (SCLC). *J Neurooncol.* 2016. 130:19–29.

88. Tawfik O, Kimler BF, Karnik T *et al*. Clinicopathological Correlation of PD-L1 Expression in Primary and Metastatic Breast Cancer and Infiltrating Immune Cells. *Hum Pathol*. 2018. 80:170–178.

89. Palmieri D, Bronder JL, Herring JM *et al.* Her-2 Overexpression Increases the Metastatic Outgrowth of Breast Cancer Cells in the Brain. *Cancer Res.* 2007. 4190–4199.

90. Yoneda T, Williams PJ, Hiraga T *et al.* A Bone-Seeking Clone Exhibits Different Biological Properties from the MDA-MB-231 Parental Human Breast Cancer Cells and a Brain-Seeking Clone In Vivo and In Vitro. *J Bone and Miner Res.* 2001. 16(8):1486–95.

91. Heyn C, Ronald JA, Ramadan SS *et al.* In Vivo MRI of Cancer Cell Fate at the Single-Cell Level in a Mouse Model of Breast Cancer Metastasis to the Brain. *Magn Reson Medicine.* 2006. 56(5):1001–1010.

92. Perera M, Rebot EJ, Percy DB *et al.* In Vivo Magnetic Resonance Imaging for Investigating the Development and Distribution of Experimental Brain Metastases due to Breast Cancer. *Transl Oncol.* 2012. 5(3):217–225.

93. Dun MD, Chalkley RJ, Faulkner S *et al.* Proteotranscriptomic Profiling of 231-BR Breast Cancer Cells: Identification of Potential Biomarkers and Therapeutic Targets for Brain Metastasis. *Mol Cell Proteomics.* 2015. 14(9):2316–2330.

94. Simmons C, Miller N, Geddie W *et al.* Does confirmatory tumor biopsy alter the management of breast cancer patients with distant metastases? *Ann Oncol.* 2009. 1499–1504.

95. Cui X, Schiff R, Arpino G *et al*. Biology of Progesterone Receptor Loss in Breast Cancer and Its Implications for Endocrine Therapy. *J Clin Oncol.* 2018. 23:7721–7735.

96. Hoefnagel LDC, Vijver MJ, Slooten HJ *et al.* Receptor conversion in distant breast cancer metastases. *Breast Cancer Res.* 2010. 12(5):R75.

97. Pein M, Oskarsson T. Microenvironment in metastasis: roadblocks and supportive niches. *Am J Physiol Cell Physiol.* 2015. 309:C627–C638.

98. Popławski AB, Jankowski M, Erickson SW *et al.* Frequent genetic differences between matched primary and metastatic breast cancer provide an approach to identification of biomarkers for disease progression. *Eur J Hum Genet.* 2010. 18(5):560–568.

99. Brodsky AS, Fischer A, Miller DH *et al.* Expression Profiling of Primary and Metastatic Ovarian Tumors Reveals Differences Indicative of Aggressive Disease. *PLoS ONE.* 2014. 9(4):e94476.

100. Berghoff AS, Venur VA, Preusser M *et al*. Immune Checkpoint Inhibitors in Brain Metastases: From Biology to Treatment. *2016 ASCO Educational Book.* 2016.

101. Thomson AH, McGrane J, Mathew J *et al.* Changing molecular profile of brain metastases compared with matched breast primary cancers and impact on clinical outcomes. *Br J Cancer.* 2016. 114(7):793–800.

102. Rajendran L, Bali J, Barr MM *et al.* Emerging Roles of Extracellular Vesicles in the Nervous System. *J. Neurosci.* 2014. 34:15482–15489.

103. Guha M. The new era of immune checkpoint inhibitors: PD-1 and PD-L1 inhibitors induce a higher response rate across a wider range of tumours than other immunotherapies. *Pharm. J.* 2014.

7 APPENDICES

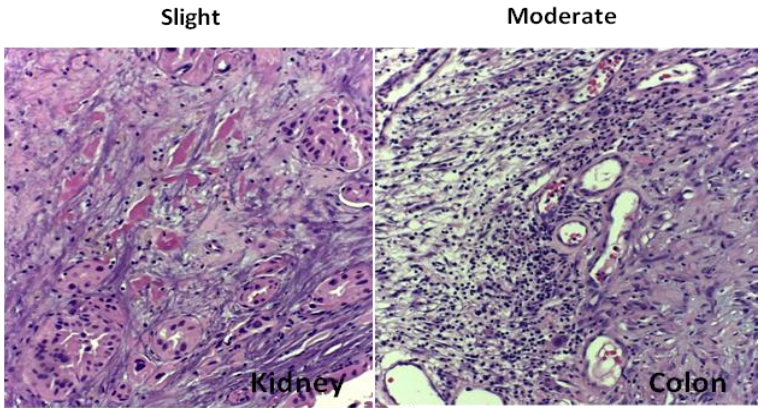


Figure A1 | Representative images of H&E tissue sections with scores found in this study. Magnification X200.

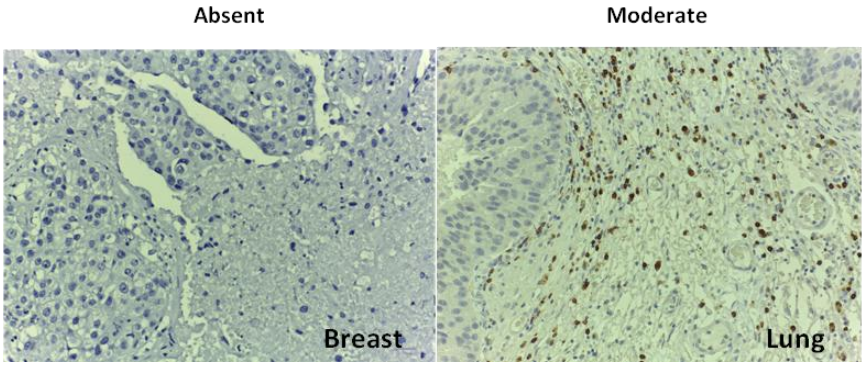


Figure A2 | Representative images of tissue sections immunostained for CD4 with scores found in this study. Magnification X200.

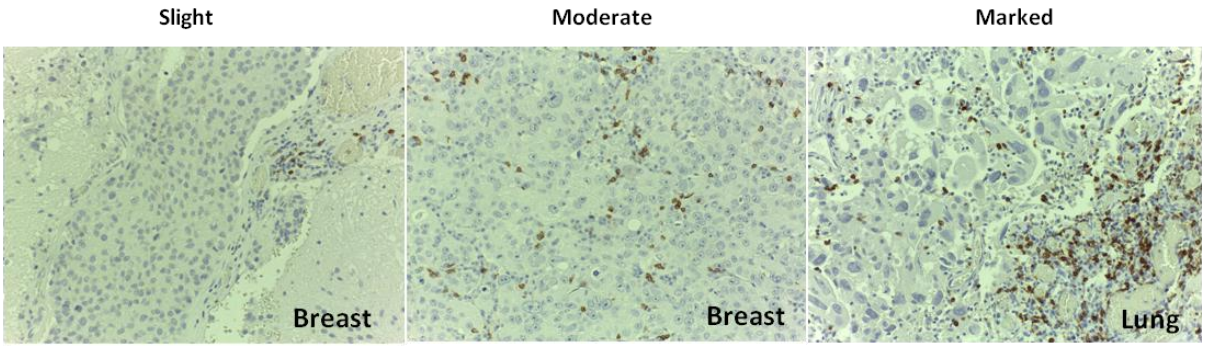


Figure A3 | Representative images of tissue sections immunostained for CD8 with scores found in this study. Magnification X200.

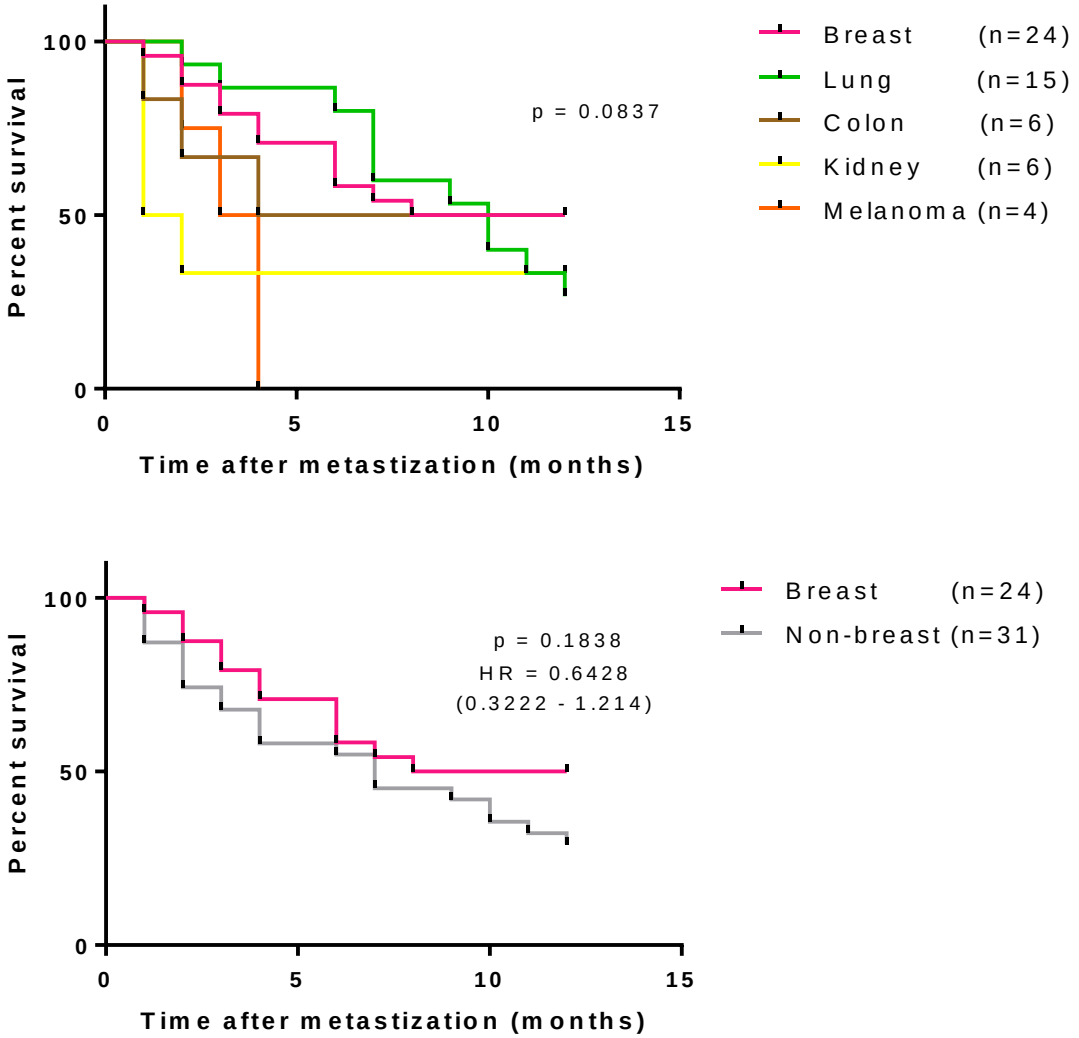


Figure A4 | 12 months-overall survival according to the origin of the primary tumour (top) and comparing breast with non-breast metastatic disease (bottom) (n=55). *P*-value was calculated using log-rank (Mantel-Cox) test, and significance was set as $p<0.05$.