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**Clinical impact and biological characterization of breast cancer-related
brain metastases: focus on gene expression profiling**

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Abstract

Background: Despite the clinical impact of breast cancer (BC) brain metastases (BMs), their biological complexity remains poorly understood. We here evaluate the genomic profile of BCBMs and compare it with paired primary BC samples to characterize biological changes during brain metastasization and their clinical impact in a retrospective real-world cohort.

Methods: Expression of 758 genes (BC360 Panel, nCounter), hormone receptor (HR) status and HER2 status were evaluated in BCBMs and matched primary BCs. Intrinsic subtyping was determined using PAM50 subtype predictor. Gene expression/PAM50 signature and OS correlations were analyzed using Cox-models. Median overall survival (OS) was calculated with Kaplan-Meier method. A False Discovery Rate corrected paired two-class SAM identified genes expression changes between paired BC and BMs samples.

Results: Seventy-five BCBM samples from 74 patients were analyzed: 30.7% HR+/HER2-, 41.3% HER2+, 28.0% HR-/HER2-. Intrinsic subtype was 36.0% Basal-like, 48.0% HER2-enriched, 14.7% Luminal B, 1.3% Normal-like; among HR+/HER2- BCs, 26.1% were Basal-like and 26.1% HER2-enriched. PAM50 basal-like signature was associated with worse OS overall ($p=0.014$) and within the HR+/HER2- subgroup ($p=0.024$). Among 21 primary BCs analyzed, 45% of Basal-like, 100% of Normal-like and 50% of Luminal-A shifted towards the HER2-enriched subtype in matched BMs. 388 genes were differentially expressed in BMs compared to primary BC, including genes involved in survival and migration (e.g. *FGFR4*), HER2-amplicon (e.g. *ERBB2*) and endocrine response (e.g. *ESR1*, *PGR*).

Conclusions: Non-luminal intrinsic subtypes are prevalent in BCBMs and basal-like genomic features are associated with worse survival. Recurrent gene expression modifications with potential therapeutic implications were observed in BCBMs.

1. Introduction

1.1 Breast cancer

1.1.1 Epidemiology and risk factors

Breast cancer (BC) is the most common malignancy in women, accounting for more than 2.2 million new cancer cases diagnosed worldwide per year, and the leading cause of cancer deaths in women worldwide.(1) BC is also the most common malignancy in women in Italy, with more than 55,000 new cases estimated in 2022.(2)

While the incidence of BC has increased in the last decades, BC mortality has consistently decreased. Thanks to the extensive use of screening and advances in systemic treatments, the 5-year overall survival is now around 88% and it is estimated that more than 830.000 women are living in Italy after a diagnosis of BC.(1,2)

Different risk factors, including genetic predisposition, family and personal history, lifestyle and environmental factors, participate in modulating the risk of developing BC. Age remains one of the most relevant risk factors, as shown by the fact that BC incidence increases with age, doubling on average every 10 years until menopause.(3) Among lifestyle and environmental factors, the most important are: reproductive history (such as menarche before age 12, late menopause after age 55, not having children or first birth after the age of 30); hormonal factors (e.g. hormone replacement therapy); lifestyle factors, such as physical inactivity, overweight, inappropriate alcohol use.(4) Approximately 5-10% of BC arise in the context of hereditary syndromes and although several genetic mutations are reported to be involved in the induction of breast carcinogenesis, *BRCA1* and *BRCA2* represent the two major genes related to hereditary BC, accounting for around one third of hereditary cases.(5)

1.1.2 Histopathological classification and clinical implications

BC represents a highly heterogeneous disease from both a biological and clinical standpoint. Clinically, BC is divided in three subgroups based on immunohistochemistry and in situ hybridization

(ISH): hormone receptor (HR) positive human epidermal growth factor receptor 2 (HER2) negative BC, HER2-positive BC and triple-negative (TN) BC. Each of these subtypes presents specific clinical characteristics and therapeutic possibilities.

In fact, recent advances in the understanding of the cellular and molecular biology of BC have led to the development of several targeted therapeutic agents. For patients with HR+/HER2- BC, in addition to several therapeutic options based on endocrine treatment, more recent combinations of endocrine treatment and targeted agents, such as cyclin-dependent kinase (CDK) inhibitors, have become available both in the advanced and in the early disease setting.(6–11)

For TNBC, classically defined by the lack of HR expression and the lack of HER2 overexpression, chemotherapy remains the cornerstone of systemic treatment, with the recent addition of immune checkpoint-inhibitors and antibody–drug conjugates (ADCs).(12,13)

Finally, the targeting of the HER2 driver oncogene has led to striking improvements in the outcome of women with HER2+ BC at all stages of disease. To date, several HER2-targeted treatments are available, including monoclonal antibodies (trastuzumab, pertuzumab), small tyrosine kinase inhibitors (lapatinib, neratinib, tucatinib), and ADCs (trastuzumab emtansine, trastuzumab deruxtecan).(14)

1.2 Brain metastases from breast cancer

1.2.1 Epidemiology

Among solid tumors, BC is the second most common source of brain metastases (BMs) and up to 30-50% of patients with metastatic BC will eventually develop central nervous system (CNS) involvement.(15,16) The incidence of BMs in metastatic BC patients is progressively increasing over time, mainly due to the use of more effective systemic treatment, better control of extracranial disease and longer survival of patients with metastatic BC, and to the widespread use of more sensitive imaging techniques.(17)

Several risk factors have been identified for the development of BMs, including younger age at diagnosis, the presence of lung metastases, a shorter disease-free survival interval, metastatic involvement of ≥ 4 axillary lymph nodes and high tumor grade.(18–21) Moreover, the biological subtype of the primary tumor plays a significant role in predisposing to CNS metastases, with BMs being particularly common in patients with HER2+ BC and TNBC.(16,22)

1.2.2 Prognosis

The diagnosis of BM in BC patients is generally considered as associated with worse prognosis compared to other metastatic sites and, despite improvements in therapeutical management, median overall survival (OS) range from 4 to 25 months.(23)

In this complex scenario, an adequate prediction of patient prognosis is needed to support the treatment decision making process. Efforts to prognosticate the outcome of patients with BCBM have led to the subsequent development of several different and eventually more precise scores.

Some of the first indexes, e.g. the Graded Prognostic Assessment (GPA), were originally developed based on data from cohorts including patients with different primary solid tumors.(24) Subsequently, acknowledging the relevant differences in clinical behavior and treatment options according to tumor histology, tumor-specific scores have been proposed. In 2012, Sperduto et al. identified, in a cohort of 400 BC patients with BM, Karnofsky Performance Status (KPS), BC subtype (as defined according to hormone receptors and HER2 status) and age as independent prognostic factors and these three variables were included in the breast-specific GPA index.(25) The accuracy of this score was then improved by the addition of number of BM (modified breast-GPA score) as proposed by Subbiah et al.(26) and further updated by the addition of presence/absence of extracranial metastases (updated breast-GPA).(27)

1.2.3 Locoregional treatments

Despite remaining an unmet medical need, over the last years treatment strategies for BCBMs have greatly evolved. Management of BCBMs therefore requires a multidisciplinary approach, integrating

local and systemic therapies tailored to patient-specific factors, including lesion number, size, location, symptoms, and overall performance status.

Surgical resection continues to play a pivotal role in BCBM management for both diagnostic and therapeutic purposes. Neurosurgery may be indicated when neuroimaging cannot conclusively differentiate BMs from other lesions, when no primary tumor is identified, or in patients with multiple primary tumors. Additionally, changes in molecular profiles compared to the primary tumor may impact treatment decisions, further supporting the need for tissue diagnosis in select cases. Therapeutically, surgery is most beneficial for patients with a single BM, controlled systemic disease, large symptomatic lesions (>3 cm), or tumors in critical brain regions such as the posterior fossa. Complete resection is strongly associated with improved local control and evidence indicates that en bloc resection is preferable to piecemeal removal, as it reduces both local recurrence and the risk of leptomeningeal dissemination. Techniques such as preoperative MRI, intraoperative neuronavigation, fluorescence-guided surgery, and cortical mapping enhance the likelihood of complete and safe resection. (28,29)

Stereotactic radiosurgery (SRS) has emerged as a cornerstone in BCBM management, particularly for patients with limited intracranial disease. Compared with whole-brain radiotherapy (WBRT), SRS offers superior preservation of cognitive function without compromising OS, as demonstrated in several randomized trials.(30,31) SRS delivers high-dose radiation with submillimeter precision, minimizing exposure to surrounding healthy brain tissue. Treatment doses are tailored according to lesion size and location, with lesions ≤ 20 mm typically receiving 24 Gy, while those 21–30 mm are treated with 18 Gy. Lesions >30 mm or located near critical structures are often treated with fractionated stereotactic radiotherapy to balance efficacy and minimize toxicity.(32,33) SRS has demonstrated effectiveness both as a primary treatment and in the postoperative setting. Postoperative SRS to the surgical cavity improves local control compared with observation and avoids the neurocognitive decline associated with WBRT.(34,35) Moreover, SRS can be repeated for new metastases without significantly increasing toxicity, making it particularly advantageous for patients with recurrent or progressive intracranial disease. Acute side effects are generally mild,

including headache, nausea, and transient cognitive changes, though radiation necrosis occurs in approximately 10% of patients.(29,36)

WBRT has historically been the standard for patients with multiple or unresectable BMs. Typical regimens include 30 Gy in 10 fractions or 20 Gy in 5 fractions, with consistent symptom relief and no significant OS benefit over localized therapies.(37,38) WBRT is now primarily indicated for patients with numerous large lesions. While WBRT improves intracranial control, it is associated with neurocognitive decline. Strategies such as hippocampal-avoidant WBRT combined with memantine have demonstrated reduced cognitive deterioration and better-preserved patient-reported quality of life, while maintaining local control.(39)

1.2.4 Systemic treatments

Over the past decades, several therapeutic agents have demonstrated clinically meaningful CNS activity, significantly improving patient outcomes.

In the HER2+ disease, the backbone of first-line therapy is a combination of chemotherapy with trastuzumab and pertuzumab.(40) Traditionally, the large size of monoclonal antibodies was thought to prevent them from crossing the intact blood brain barrier (BBB). Still, retrospective studies suggest some degree of intracranial activity. For instance, an analysis of 252 HER2+ patients with BMs showed that among 14 evaluable patients treated with dual blockade, 35.7% achieved complete intracranial remission and 57.1% partial remission.(41)

Due to their smaller size and enhanced BBB penetration, HER2-targeted tyrosine kinase inhibitors (TKIs) have generated substantial interest. Lapatinib, a dual HER2/EGFR TKI, combined with capecitabine demonstrated intracranial response rates of 20–38% in patients pretreated with radiotherapy.(41–43) Moreover, in the phase II LANDSCAPE trial involving untreated HER2+ BCBMs, the combination achieved an objective CNS response rate of 65.9% and a median time to CNS progression of 5.5 months.(44) These findings provided proof-of-concept that HER2-targeted TKIs can induce clinically meaningful CNS responses.

Recent therapeutic innovations have markedly altered the management of HER2+ BCBMs. The phase II/III HER2CLIMB trial evaluated tucatinib, a highly selective HER2 TKI, in combination with

trastuzumab and capecitabine in patients pretreated with trastuzumab–pertuzumab and T-DM1. The study included patients with both active and stable BMs, making it the first randomized trial to assess the survival benefit of systemic therapy in active BCBMs. Among 291 patients with BMs, tucatinib significantly improved overall survival (21.6 vs 12.5 months) and yielded higher intracranial response rates, with an objective response rate of 47.3% in patients with active BMs.(45) Similarly, trastuzumab deruxtecan (T-DXd), a next-generation HER2-targeted ADC, has demonstrated unprecedented intracranial activity. Indeed, several small single-arm trials reported relevant intracranial responses rates among patients with HER2+ BCBMs (including patients with active BMs), ranging from 44.4% to 73.3%.(46–48) Moreover, a pooled analysis of the DESTINY-Breast01/02/03 trials confirmed robust CNS efficacy, with intracranial response rates of ~45% and CNS progression-free survival of 12–18 months. More recently, the phase 3b/4 DESTINY-Breast12 study, enrolling 263 patients with HER2+ BCBMs, reported a 12-month CNS-PFS of approximately 58% in both stable and active disease, with an intracranial response rate of 71.7%. Collectively, these results establish T-DXd as a highly effective therapeutic option for HER2+ BCBMs.(49)

While the management of HER2+ BCBMs has advanced substantially, the therapeutic landscape for HER2- remains less developed. Nevertheless, up to 70% of patients diagnosed with BMs have HER2- BC, and the incidence of BMs in HR+/HER2- metastatic BC appears to be rising, potentially due to improved extracranial disease control and prolonged survival.(50)

In HR+/HER2- BC, BMs typically develop later in the disease course, and evidence regarding the intracranial activity of endocrine therapy is scarce. Preclinical models suggested that CDK4/6 inhibitors might cross the BBB and delay BM progression. However, pivotal clinical trials largely excluded patients with CNS disease. In a dedicated phase II trial, abemaciclib achieved an intracranial clinical benefit rate of 24% in heavily pretreated patients, supporting a potential role for CDK4/6 inhibitors in this setting.(51)

ADCs are among the most significant therapeutic advances in HER2- metastatic BC, though data on their intracranial efficacy are limited. In HER2-low disease, T-DXd yielded an intracranial response rate of 41.7% and a median duration of intracranial response of 7.2 months in the DEBBRAH trial, albeit in only 12 patients.(52) Sacituzumab govitecan, an anti-TROP2 ADC, has been approved for

both HR+/HER2- and TNBC. In the ASCENT trial, among 61 TNBC patients with stable BMs, sacituzumab govitecan provided a modest median PFS benefit (2.8 vs 1.6 months), consistent with real-world findings showing a PFS of ~3 months and disease control rates of ~42%.^(53–55)

1.3 Gene expression profiles of breast cancer and brain metastases

1.3.1 PAM50 (Prediction Analysis of Microarray 50)

Over the past 25 years, advances in molecular biology and high-throughput technologies have enabled the simultaneous analysis of thousands of genes, significantly enhancing our understanding of the biological heterogeneity and complexity of BC. Based on gene expression profiling, BCs have been stratified into five intrinsic molecular subtypes:

- Luminal A
- Luminal B
- HER2-enriched
- Basal-like
- Normal-like.⁽⁵⁶⁾

Although the initial definition of intrinsic subtypes relied on the assessment of global gene expression (mRNA) across large gene sets, subsequent studies demonstrated that the evaluation of a more limited 50-gene panel (known as Prediction Analysis of Microarray 50, PAM50) was sufficient to accurately identify the main molecular subtypes. The PAM50 classifier thus represents the minimal gene set required to maintain diagnostic accuracy while improving feasibility in clinical practice.⁽⁵⁷⁾ Importantly, this molecular classification goes beyond the conventional immunohistochemical or in situ hybridization (IHC/ISH) assessment of HR and HER2 status. By simultaneously analyzing 50 distinct genes, the PAM50 assay provides a more refined stratification of BC, offering additional prognostic and predictive information beyond that provided by pathological stage, histological grade, and standard clinical biomarkers.⁽⁵⁷⁾

This molecular framework has revolutionized the conceptualization of BC, underscoring that it is not a single disease entity but rather a spectrum of biologically and clinically distinct subtypes. Such insights have laid the groundwork for personalized therapeutic strategies, facilitating both prognostic assessment and the identification of patients who may benefit from targeted or tailored treatment approaches.

1.3.2 Evolution of breast cancer biology between primary tumors and brain metastases

In BC, it is well established that the biological characteristics of the disease (i.e., ER, PR, and HER2) may frequently vary between the primary tumor and metastatic sites. For this reason, current guidelines for the treatment of metastatic BC recommend, whenever clinically feasible, reassessment of tumor biology by repeating a diagnostic biopsy of distant metastases.(58) However, given the potential risks associated with brain biopsy and the fact that surgical resection is indicated only for a minority of patients with BCBMs, biological recharacterization of BMs is possible only in a limited subset of patients.

Previous studies conducted on small cohorts of paired samples of primary BC and corresponding BMs have reported that BCBMs may acquire specific alterations during the metastatic process, both at the immunohistochemical level and in the expression of clinically relevant genes.

Using immunohistochemical expression of HR and HER2, a recent multicenter analysis published in 2020, including 193 patients with BCBMs, demonstrated a discrepancy in the expression of at least one marker among ER, PR, or HER2 between primary tumors and their paired BMs in 36.3% of cases, with a consequent subtype switch in 22.8% of cases. More specifically, the frequency of this discordance appeared to vary considerably depending on the receptor status of the primary tumor: 37.5% of HR+/HER2– tumors (21/56) underwent subtype conversion, while only 10.2% of HR–/HER2+ primaries (4/39) exhibited subtype modification in BMs.(59) These findings have been corroborated by other studies, such as those conducted by Duchnowska et al.(60) and Thomson et al.,(61) who reported conversion from HER2- to HER2+ status in BMs (according to clinical definitions based on IHC and ISH) in 16% (10/61) and 18% (5/28) of cases, respectively. Similarly, a meta-analysis including data from 1,373 patients revealed discordance in at least one of the three

investigated markers (ER, PR, HER2) in 40% of cases. In this cohort, the most frequently observed changes in BMs were loss of ER expression (11%), loss of PR expression (15%), and acquisition of HER2 expression (9%).(62)

The explanation for this phenomenon remains controversial. One hypothesis suggests a role of intratumoral heterogeneity: in a primary tumor harboring diverse cellular populations with variable receptor expression, HR- or HER2+ subpopulations might exhibit a stronger neurotropic propensity compared to other clones, and could thus be positively selected in BMs.(59) Additionally, the use of endocrine therapy, either adjuvant or in the metastatic setting, might exert selective pressure, favoring the expansion of metastatic clones lacking these receptors.(62)

Only a limited number of studies, based on small sample sizes, have investigated differences in gene expression between primary BC and corresponding BMs. A study on 20 paired samples reported the acquisition of a considerable number of recurrent, potentially targetable alterations in BMs. Beyond a significant increase in ERBB2 (HER2) mRNA levels in BMs, notable upregulation of FGFR4 (observed in 30% of primary/metastasis pairs), FLT2 (20% of cases), AURKA (10% of cases), and EGFR (10% of cases) was also documented. Furthermore, the same study reported a significant reduction in ESR1 mRNA and in cytokeratin gene expression in BMs compared to primary tumors.(63) Another study analyzing gene expression profiles of 61 BC metastases (across nine different metastatic sites) found that BMs were characterized by low expression of genes associated with immune system activation, both relative to primary tumors and to other metastatic sites.(64)

Collectively, these limited findings suggest that BC may acquire specific biological alterations during metastatic colonization of the CNS. A deeper understanding of these mechanisms could provide a valuable basis for identifying new targets for novel therapeutic strategies as well as putative prognostic and predictive biomarkers. In the current study, we aimed to evaluate the transcriptomic profile of BCBMs and assess its prognostic implications. In addition, to characterize biological changes acquired by BC during brain metastasization we evaluated the transcriptomic profile of matched primary BC samples and compared it with that of their paired BMs.

2. Methods

2.1 Patients and samples

BC patients undergoing neurosurgery at three institutions between 2003 and 2019 were retrospectively identified. Eligibility criteria included: age ≥ 18 years, a diagnosis of metastatic BC, neurosurgery for BC-related BM performed as part of clinical care, and availability of archival histological material obtained from the neurosurgical procedure. Formalin-fixed paraffin-embedded (FFPE) BM samples and, when available, FFPE paired primary BC samples were centralized for analysis at the University of Padova. Among these, this work has benefited from 450 white slides and the expertise of Professor Valérie Rigau responsible for the collections “Neurology” and “CEREMET-LR” of the Biological Resource Center of Montpellier University Hospital – <http://www.chu-montpellier.fr> (BB-0033-00031).(65)

Clinical/anatomopathological data were retrospectively collected from medical records using a standardized electronic form. HR and HER2 status was evaluated on primary tumor and on BCBM according to current clinical guidelines. Other core clinical variables collected included: number of BMs, Karnofsky Performance Status, treatments received before and after BM diagnosis, survival outcomes. Prior to analysis, the dataset was cleaned to remove duplicate entries and harmonized across institutions to ensure uniform variable definitions. Modified breast-GPA score was evaluated according to published criteria.(26)

This study was approved by involved Institutional Review Boards and Ethics Committees and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from participants when needed according to the involved site legislation.

2.2 Gene expression analysis

FFPE samples were reviewed for tumor tissue quality and quantity (all samples presented $>40\%$ of tumor cells). RNA was extracted from five 5- μm sections using the Reliaprep TM RNA cell miniprep system (Promega) according to the manufacturer’s protocol. Concentration and quality of samples were assessed using the Qubit RNA HS Assay Kit on Qubit 4 Fluorometer (Thermo Fisher Scientific)

and the High Sensitivity RNA kit on TapeStation 4200 (Agilent Technologies), to ensure they had concentration ≥ 20 ng/ μ L and to evaluate RIN and DV200.

The starting material was 100 to 500 ng of RNA depending on the percentage of fragments with dimensions greater than 200 bp (DV200 value). Samples were hybridized with panel probes for 19 hours at 65°C at both institutions and then complexes were processed on the nCounter Analysis System. Cartridges were scanned at 555 FOVs. Profiling was carried out using the commercially available NanoString™ (Seattle, WA) Breast Cancer 360 Expression Assay according to manufacturer's instructions. The Breast Cancer 360 panel includes 758 target probe pairs, 18 housekeeping genes used for normalization, 6 exogenous positive control RNA targets that range linearly from 128 fM to 0.125 fM, and 8 exogenous negative control sequences.

Raw data were quality control assessed and normalized using the NanoString™ nSolver™ analysis software following the manufacturer's recommendations.

2.3 Statistical analysis

Intrinsic molecular subtyping was determined using the previously reported PAM50 subtype predictor.(66)

Correlations between expression of each gene/PAM50 signature, BC subtype and OS were studied using uni/multivariate Cox-models. All tests were two-sided ($p \leq 0.05$).

For survival analysis, patients for which multiple BM samples were analyzed were only counted once and the first BM sample collected was used for analyses. Median overall survival (OS) from first BM diagnosis was calculated using the Kaplan-Meier method. Given the exploratory intent and limited sample size, no multiplicity correction was applied for analyses assessing the association between single-gene expression and OS. A False Discovery Rate (FDR) corrected paired two-class SAM was used to identify changes in single genes expression between paired BC and BMs samples

Analyses were performed using R software 4.0.5.

3. Results

3.1 Patient and tumor characteristics

Seventy-five evaluable BCBMs samples from 74 BC patients (all female) diagnosed with BM who underwent surgical resection between 2003 and 2019 at one of the participating institutions were included in this study: Centre Hospitalier Universitaire (CHU), Montpellier France (N = 22); Istituto Oncologico Veneto IRCCS, Padova Italy (N = 34); Montefiore Medical Center, Bronx NY, USA (N = 19) (**Figure 1**).

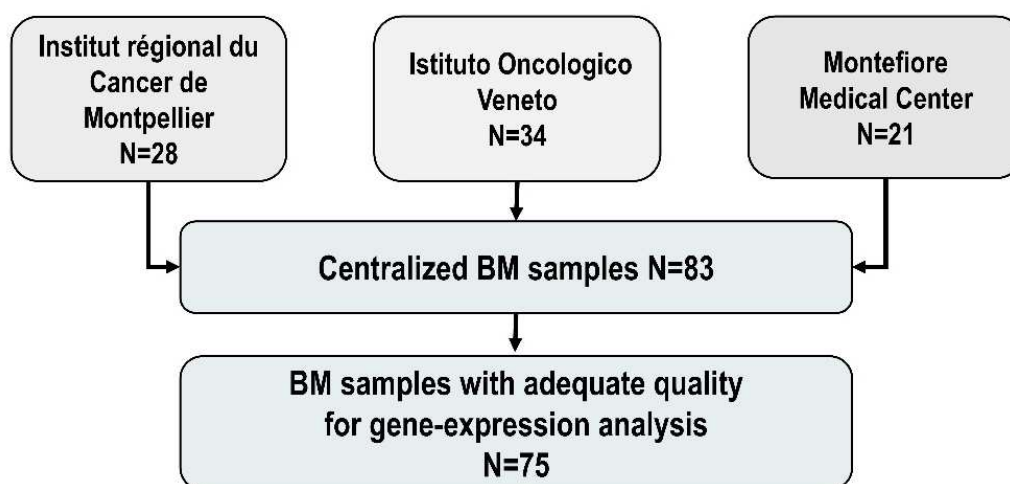


Figure 1. REMARK flowchart for breast cancer brain metastases samples.

Patients' characteristics are reported in **Table 1**.

Table 1. Patient characteristics (Total N=74, except for breast cancer subtype on brain metastases)

	n	%
Breast cancer subtype on brain metastases (N=75)		
HR+/HER2-	23	30.7
HR+/HER2+	11	14.7
HR-/HER2+	20	26.7
TNBC	21	28.9
Number of brain metastases on imaging		
1	60	81.1
2	6	8.1
3	3	4.1
≥4	5	6.7
Karnofsky performance status at first BM diagnosis		
90-100	18	24.3
70-80	33	44.6
≤60	7	9.5
NA	16	21.6
Modified breast-GPA		
0-1	2	2.7
1.5-2	18	24.3
2.5-3	29	39.2
3.5-4	9	12.2
NA	16	21.6
Brain metastases at first diagnosis of stage IV BC		
Yes	49	66.2
No	23	31.1
NA	2	2.7
Systemic therapy before neurosurgery		
Yes	27	36.5
No	46	62.2
NA	1	1.3
Presence of extra-CNS disease at BM diagnosis		
Yes	37	50.0
No	28	37.8
NA	9	12.2
Systemic therapy after neurosurgery		
Yes	54	73.0
No	14	18.9
NA	6	8.1
Radiotherapy after neurosurgery		
Yes	55	74.3
No	12	16.2
NA	7	9.5

Abbreviations: CNS, central nervous system; HER2, human epidermal growth factor receptor type 2; HR, hormone receptor; TNBC, triple-negative breast cancer; GPA Graded Prognostic Assessment

Median age at time of primary BC diagnosis and BM diagnosis was 44 years (range 30-77) and 50 years (range 33-77), respectively. BC subtyping on the BM was available for all 75 samples and was TNBC for 21 samples (28.0%), HR+/HER2– for 23 samples (30.7%), and HER2+ for 31 samples (41.3%).

3.2 Overall survival from first brain metastases diagnosis and clinical characteristics

At a median follow-up from BM diagnosis of 46.9 months, 48 patients (64.9%) had died. Median OS from BM diagnosis was 25.4 months (95% CI 18.4-32.4).

The only baseline clinical variable associated with OS following first BM diagnosis in this study cohort was BC subtype (median OS: 9.4 months for TNBC; 29.6 months for HR+/HER2–; 30.5 months for HER2+ BC; log-rank $p=0.020$; **Figure 2**), while the number of BM, performance status, the presence/absence of extra-CNS disease at time of BM diagnosis, and modified breast-GPA score were not significantly associated with survival outcome, likely due to particular clinical features of this highly selected patient cohort (univariate OS Cox models for these and other variables reported in **Table 2**). Therefore, also taking into account the potential association between BC subtype and the expression of a significant number of genes, BC subtype evaluated on the BM was used as a correction factor in subsequent analyses testing the association between gene-expression features and OS.

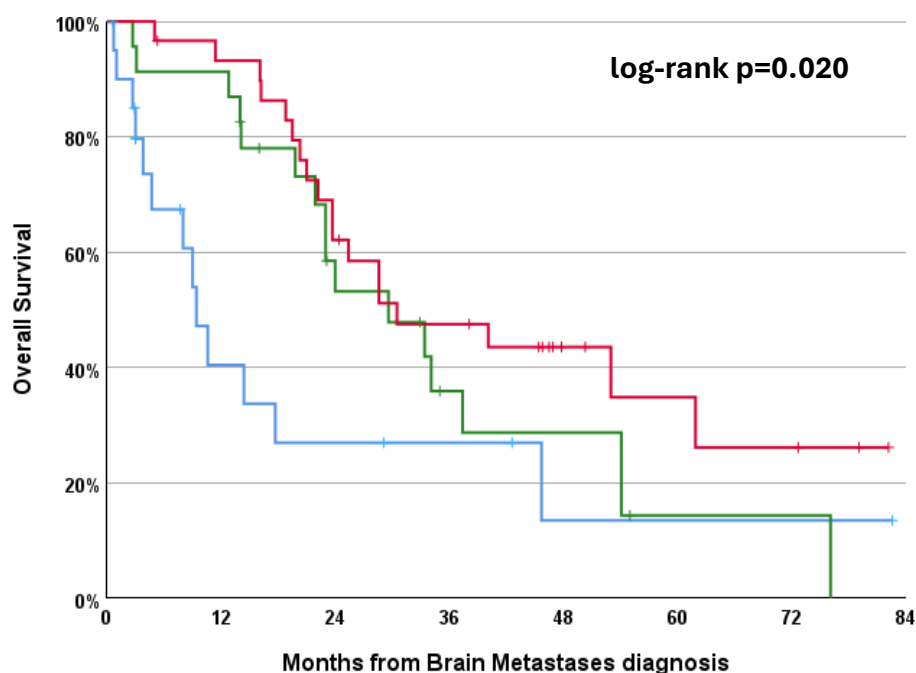


Figure 2. Kaplan-Meier Curves for overall survival from brain metastases diagnosis according to breast cancer subtype evaluated on brain metastasis sample (triple-negative=blue; HR+/HER2-=green; HER2+= red)

Table 2. Univariate Cox models for overall survival from brain metastases diagnosis

	Hazard Ratio (95%CI)	p value
Number of brain metastases		
1	Ref	Ref
2	1.37 (0.58-3.28)	0.474
3	1.44 (0.34-6.09)	0.619
≥4	2.14 (0.83-5.54)	0.116
Karnofsky performance status		
90-100	Ref	Ref
70-80	1.10 (0.53-2.29)	0.790
≤60	1.78 (0.61-5.19)	0.293
Modified breast-GPA		
0-1	3.13 (0.64-15.3)	0.159
1.5-2	1.01 (0.38-2.67)	0.979
2.5-3	0.71 (0.29-1.74)	0.458
3.5-4	Ref	Ref
Brain metastases at first diagnosis of stage IV BC		
Yes	0.79 (0.43-1.45)	0.448
No	Ref	Ref
Presence of extra-CNS disease at BM diagnosis		
Yes	1.72 (0.89-3.33)	0.109
No	Ref	Ref

Abbreviations: GPA, Graded Prognostic Assessment; CNS, central nervous system; BCBM, breast cancer brain metastases

3.3 PAM50 Intrinsic subtype of breast cancer brain metastases

PAM50 non-luminal subtypes were extensively represented in BCBMs, both overall and in each BC subtype (**Figure 3; Table 3**).

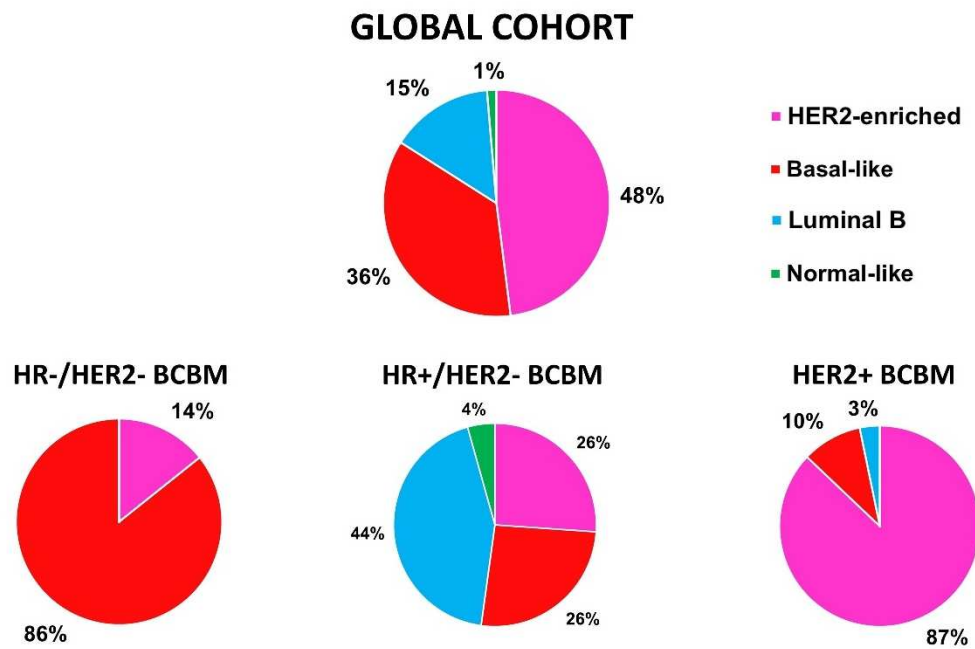


Figure 3. Distribution of PAM50 intrinsic subtype within the overall study cohort of breast cancer brain metastases and within each IHC-based subgroup (IHC assessed on the brain metastasis).

Table 3. Distribution of PAM50 intrinsic subtypes according to IHC-based breast cancer subtype (as assessed on the brain metastasis sample)

PAM50 Intrinsic subtype	HR+/HER2- BCBM	HER2+ BCBM	HR-/HER2- BCBM
	N (%)	N (%)	N (%)
HER2-enriched	6 (26.1%)	27 (87.1%)	3 (14.3%)
Basal-like	6 (26.1%)	3 (9.7%)	18 (85.7%)
Luminal B	10 (43.5%)	1 (3.2%)	0
Normal-like	1 (4.3%)	0	0
Luminal A	0	0	0

Indeed, HER2-enriched (48.0%) and Basal-like (36.0%) were the most represented subtypes in the overall study cohort, with only a limited number of BM classified as Luminal-B (14.7%) or Normal-like (1.3%). As expected, the HER2-enriched subtype represented the large majority of HER2+ BMs (87.1%), while the Basal-like subtype was the most represented subtype in HR-/HER2- BMs (85.7%). However, PAM50 non-luminal subtypes were well represented even among HR+/HER2- BMs, constituting more than half of these samples (Basal-like 26.1%, HER2-enriched 26.1%), with the remaining half categorized as Luminal-B (43.5%), and merely one sample classified as Normal-like (4.3%).

3.4 Association between PAM50 intrinsic subtyping and signatures and overall survival

We first assessed the prognostic impact of PAM50 intrinsic subtypes (evaluated on the BM) on survival from first BMs diagnosis. As expected, basal-like intrinsic subtype was associated with a worse OS as compared to other intrinsic subtypes ($p=0.021$; median OS for basal-like tumors 14.4 months 95% CI 2.5-26.3, for HER2-enriched tumors 29.6 months 95%CI 22.7-36.5, for Luminal B tumors 37.4 95% CI 29.8-45.0; Normal-like not reported as only one case was assessed, **Figure 4**). Due to the limited number of patients assessable in the present study, it was not possible to address if this could also be observed in each BC subtype separately, although a non-significant numerically worse median OS for patients with basal-like BM was observed in both HR+/HER2- BMs (24 vs 34.1 months) and in HER2+ BMs (21 vs 30.5 months). Moreover, among patients with HR+/HER2- BCBMs, patients with luminal PAM50 subtype presented a numerically better median OS than

patients with other subtypes (median OS for luminal subtypes 37.4 months 95% CI 22.6-52.2, for other subtypes 23.0 months 95%CI 21.2-24.8, $p=0.090$).

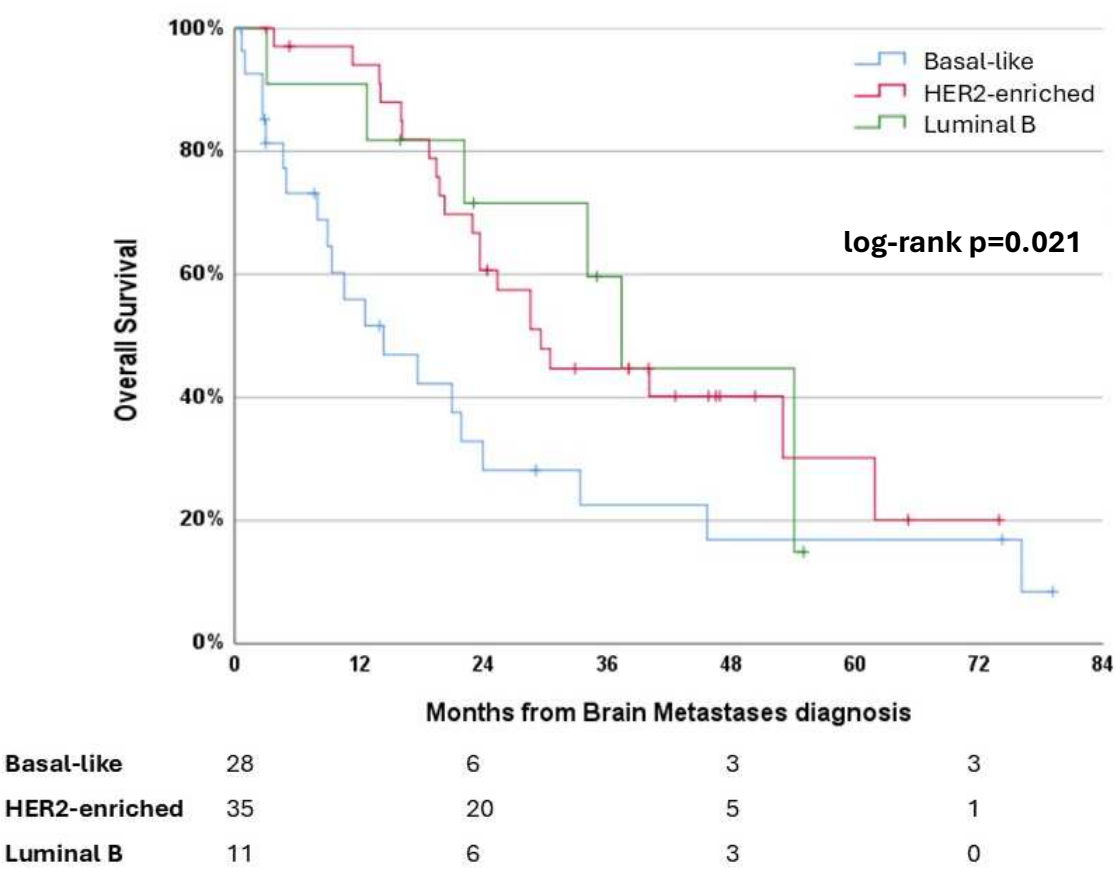


Figure 4. Kaplan-Meier Curves for overall survival from brain metastases diagnosis according to PAM50 intrinsic subtype evaluated on brain metastasis sample

To more precisely assess the association between tumor biology, as assessed by the PAM50 algorithm, and survival from BMs diagnosis, we then assessed the prognostic role of the PAM50 gene signatures, overall and in each BC subtype separately (**Table 4**).

Table 4. Association between each PAM50 signature (as a continuous variable) and overall survival from brain metastasis diagnosis (univariate Cox models) in the overall study cohort (N=74) and in each breast cancer subtype (evaluated on the brain metastasis samples)

PAM50 Signatures (continuous)	Overall		HR+/HER2-		HER2+		TNBC	
	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
Basal like	3.38 (1.28-8.92)	0.014	6.70 (1.28-35.03)	0.024	1.21 (0.08-17.75)	0.890	0.77 (0.05-11.03)	0.848
HER2-enriched	0.46 (0.13-1.60)	0.223	3.47 (0.17-71.05)	0.420	0.81 (0.05-12.88)	0.882	1.54 (0.14-17.67)	0.727
Luminal A	0.22 (0.05-1.03)	0.054	0.06 (0.01-0.75)	0.029	1.08 (0.03-36.54)	0.965	12.08 (0.01-10831.36)	0.473
Luminal B	0.24 (0.06-0.99)	0.049	0.19 (0.02-1.79)	0.147	0.43 (0.02-11.58)	0.619	0.86 (0.05-16.51)	0.919
Normal-like	5.07 (0.86-29.80)	0.073	6.40 (0.29-140.91)	0.239	2.18 (0.05-92.66)	0.685	0.82 (0.04-16.83)	0.895

Abbreviations: HR, hazard ratio; CI confidence interval; HR, hormone receptor; HER2, human epidermal growth factor receptor type 2; TNBC, triple-negative breast cancer

The PAM50 Basal-like signature as a continuous variable was significantly associated with a worse OS, both overall (p=0.014) and within the HR+/HER2- BCBMs subgroup (p=0.024), while this was not observed in the HER2+ and HR-/HER2- BCBMs subgroups. The PAM50 Luminal-A signature as a continuous variable was significantly associated with a better OS within the HR+/HER2- BCBMs subgroup (p=0.029) and, although not statistically significant, overall (p=0.054), while this was not observed in the HER2+ and HR-/HER2- BCBMs subgroups. In the overall study population, the PAM50 Luminal-B signature as continuous variables was also significantly associated with better OS (p=0.049), but this was not observed in any of the specific BC subgroups, although this might be partially explained by the limited number of patients analyzed.

3.5 Association between single gene expression and overall survival

To more extensively evaluate the impact of tumor biology on survival from BM diagnosis, we then assessed the association between the expression of single genes in BM tissue and OS from BM diagnosis using a univariate Cox Model.

The expression of 43 genes was significantly associated with OS ($p < 0.05$; Table 3); in particular, for 24 genes higher expression was associated with a longer OS from BM diagnosis, while for 19 genes higher expression was associated with a shorter OS from BM diagnosis. This association was confirmed as statistically significant independently from BC subtype (multivariate Cox Model corrected by BC subtype) for 8 and 7 genes, respectively (**Table 5**).

Table 5. Univariate and Multivariate Cox Models (corrected by breast cancer subtype) assessing the association of single gene mRNA levels in the BM tissue and overall survival from brain metastasis diagnosis for the 43 genes significantly associated with overall survival at univariate analysis. Genes with significant p-values after correcting by breast cancer subtype at multivariate analysis showed in bold.

Gene	Details	Univariate Cox Model		Multivariate Cox Model (corrected by subtype)	
		HR (95% CI)	p-value	HR (95% CI)	p-value
ANXA9	annexin A9	0.88 (0.77-0.99)	0.040	0.9 (0.79-1.01)	0.081
BCL11A	BCL11 transcription factor A	1.16 (1.01-1.35)	0.039	1.11 (0.96-1.29)	0.144
BCL2L1	BCL2 like 1	0.59 (0.4-0.87)	0.008	1.07 (0.86-1.33)	0.538
BLVRA	Biliverdin Reductase A	0.75 (0.58-0.98)	0.035	0.82 (0.63-1.06)	0.127
CCND1	cyclin D1	0.84 (0.71-0.99)	0.037	0.86 (0.73-1.01)	0.073
CD276	CD276 molecule (B7-H3)	0.41 (0.24-0.7)	0.001	0.41 (0.25-0.67)	0.001
CDC7	cell division cycle 7	1.54 (1.1-2.15)	0.011	1.36 (0.97-1.92)	0.078
CDH3	cadherin 3	1.23 (1.03-1.47)	0.025	1.21 (1.01-1.44)	0.041
CDK6	cyclin dependent kinase 6	1.29 (1.05-1.58)	0.015	1.29 (1.04-1.6)	0.019
CEACAM5	CEA cell adhesion molecule 5	0.91 (0.84-0.99)	0.038	0.95 (0.87-1.04)	0.283
CKB	creatine kinase B	1.25 (1.06-1.47)	0.007	1.33 (1.13-1.56)	0.001
CPA3	carboxypeptidase A3	0.81 (0.68-0.97)	0.020	0.83 (0.69-0.99)	0.040
ENO1	enolase 1	1.47 (1.02-2.1)	0.037	1.48 (1.03-2.13)	0.033
ERBB2	erb-b2 receptor tyrosine kinase 2	0.88 (0.78-0.99)	0.029	1.01 (0.81-1.24)	0.959
FAM214A	family with sequence similarity 214. A	0.75 (0.58-0.97)	0.026	0.84 (0.63-1.11)	0.217
FOXC1	forkhead box C1	1.24 (1.01-1.52)	0.041	1.11 (0.89-1.39)	0.334
GABRP	gamma-aminobutyric acid type A receptor subunit pi	1.12 (1.02-1.22)	0.020	1.08 (0.98-1.19)	0.139
GDF15	growth differentiation factor 15	0.81 (0.7-0.94)	0.004	0.84 (0.73-0.97)	0.018
GZMA	granzyme A	0.76 (0.58-1)	0.047	0.78 (0.59-1.01)	0.060
HOXB3	homeobox B3	0.88 (0.78-0.99)	0.028	0.86 (0.77-0.97)	0.016
IL13RA1	interleukin 13 receptor subunit alpha 1	0.59 (0.42-0.84)	0.003	0.65 (0.46-0.92)	0.015
IL4R	interleukin 4 receptor	0.69 (0.49-0.98)	0.036	0.76 (0.53-1.08)	0.126
KRT17	keratin 17	1.12 (1.01-1.25)	0.030	1.08 (0.98-1.2)	0.137
KRT5	keratin 5	1.12 (1.02-1.23)	0.020	1.08 (0.98-1.19)	0.100
KRT6B	keratin 6B	1.13 (1.01-1.26)	0.027	1.11 (1-1.23)	0.048
KRT7	keratin 7	1.19 (1.03-1.38)	0.020	1.27 (1.09-1.48)	0.003
LAMB3	laminin subunit beta 3	1.22 (1.02-1.45)	0.030	1.19 (0.99-1.43)	0.066
LFNG	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	0.83 (0.69-0.98)	0.033	0.89 (0.73-1.08)	0.225
LINC02381	Long Intergenic Non-Protein Coding RNA 2381	0.72 (0.58-0.9)	0.003	0.7 (0.55-0.87)	0.002
MLPH	melanophilin	0.82 (0.73-0.93)	0.002	0.87 (0.76-1)	0.043
MYC	MYC proto-oncogene	1.23 (1.03-1.46)	0.019	1.19 (0.99-1.42)	0.057
PCNA	proliferating cell nuclear antigen	1.47 (1.01-2.13)	0.042	1.24 (0.83-1.86)	0.299
PLAT	plasminogen activator tissue type	0.71 (0.55-0.93)	0.013	0.76 (0.57-1)	0.046
PUM1	pumilio RNA binding family member 1	2.13 (1.1-4.14)	0.026	1.73 (0.87-3.46)	0.120

RB1	RB transcriptional corepressor 1	0.66 (0.47-0.93)	0.016	0.79 (0.54-1.15)	0.219
SERBP1	SERPINE1 mRNA binding protein 1	2.07 (1.18-3.62)	0.011	0.79 (0.54-1.15)	0.219
SHC2	SHC adaptor protein 2	0.8 (0.65-0.97)	0.025	0.8 (0.66-0.98)	0.035
SIGIRR	single Ig and TIR domain containing	0.71 (0.5-1)	0.047	0.75 (0.54-1.04)	0.089
SLC44A4	solute carrier family 44 member 4	0.92 (0.85-0.99)	0.026	0.95 (0.88-1.03)	0.235
SPDEF	SAM Pointed Domain Containing ETS Transcription Factor	0.88 (0.77-1)	0.048	0.98 (0.83-1.14)	0.757
TFDP1	transcription factor Dp-1	1.39 (1.07-1.81)	0.015	1.2 (0.89-1.63)	0.235
TFF3	trefoil factor 3	0.92 (0.86-1)	0.041	0.95 (0.88-1.03)	0.206
TTYH1	tweety family member 1	1.15 (1.01-1.3)	0.032	1.16 (1.03-1.31)	0.014

3.6 Changes in PAM50 intrinsic subtyping between paired breast cancer and primary tumor samples

To evaluate whether biological features observed in BCBMs were already present in the primary BC or were specifically acquired by BC during metastasization to the brain, we compared the genomic profile of 21 paired primary BC matched to BM samples (**Figure 5**).

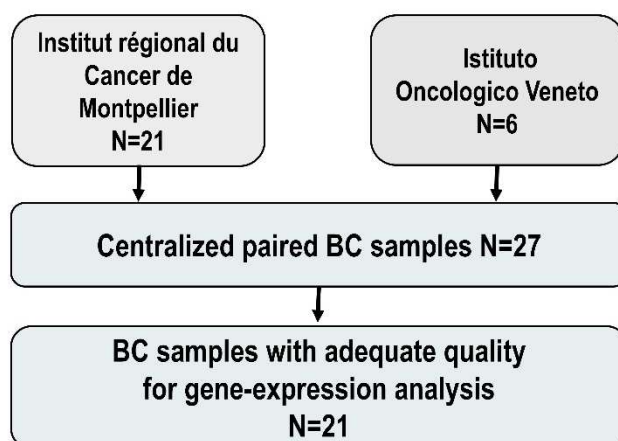


Figure 5. REMARK flowchart for paired primary breast cancer samples

The BC phenotype of primary BCs and matched BMs as assessed by IHC and ISH by clinical practice is reported in **Table 6**.

Table 6. Changes in IHC-based breast cancer subtype in primary breast cancer samples and paired brain metastases (N=22).

		Brain metastases subtype			
		HR+/HER2-	HR-/HER2+	HR+/HER2+	TNBC
Primary breast cancer subtype	HR+/HER2-	4	0	0	1
	HR-/HER2+	0	4	2	0
	HR+/HER2+	0	3	2	0
	TNBC	1	1	0	4

Abbreviations: HR, hormone receptor; HER2, human epidermal growth factor receptor type 2; TNBC, triple-negative breast cancer

Discordance in HR status evaluated on the primary BC and matched BM was observed in 33% (N=7) of patients: 4 (19.0%) with loss of previous HR positivity and 3 (14.3%) with acquisition of HR positivity. HER2 status appeared to be more stable with only one patient (4.8%) showing acquisition of HER2 positivity on the BM.

However, when PAM50 intrinsic subtyping was evaluated in the primary BC and paired BM, a shift towards HER2-enriched PAM50 subtype in the brain metastatic site was observed (**Figure 6**).

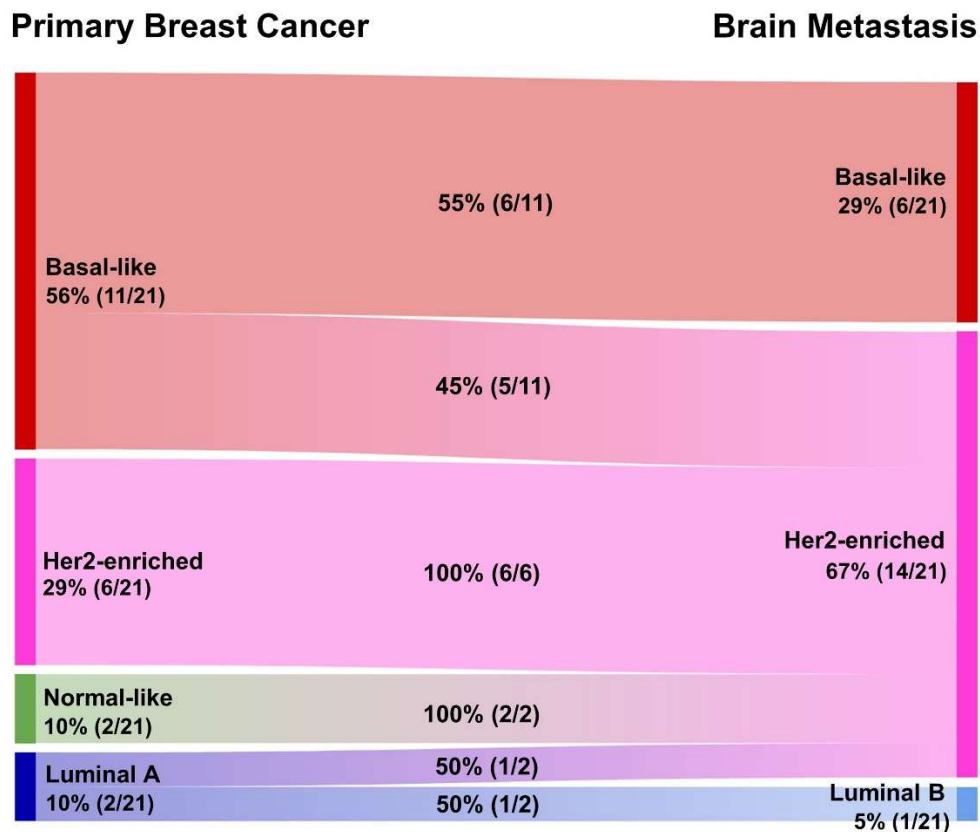


Figure 6. Sankey plot showing shifts in PAM50 breast cancer intrinsic subtype from primary breast cancer and matched brain metastasis (N=21).

In particular, 45% of cases classified as Basal-like on the primary tumor were re-classified as HER2-enriched on the BM sample, as were 100% of tumors classified as Normal-like and 50% of tumors classified as Luminal-A on the primary sample. On the contrary, all cases classified as Her2-enriched on the primary sample were reconfirmed as HER2-enriched in the BM sample.

Interestingly, the 8 patients for which a switch towards a HER2-enriched on the BM sample was observed constituted a quite heterogeneous patient subgroup. Only 4 patients presented a HER2+ BM according to clinical classification; for 3 of them the primary BC was also HER2+ (1 Luminal-A to HER2-enriched; 1 Basal-like to HER2-enriched; 1 Normal-like to HER2-enriched), while in 1 case acquisition of HER2 positivity on the BM was observed (clinically HR-/HER2- to HR-/HER2+; PAM50 intrinsic subtype Normal-like to HER2-enriched). The remaining 4 patients for which a switch towards a HER2-enriched on the BM sample was observed presented a BM classified as HER2- according to clinical classification: 1 case presented a HR-/HER2- BM (clinically: HR-/HER2- to HR-/HER2-; PAM50 intrinsic subtype Basal-like to HER2-enriched), while 3 cases presented HR+/HER2- BMs.

Indeed, of the 11 HR+/HER2- BM samples which were characterized as non-luminal subtype (HER2-enriched or Basal-like) according to the PAM50 algorithm, 4 had an available matched primary BC (all clinically classified as HR+/HER2-). On the matched primary BC, 3 were classified as Basal-like (two subsequently switching to HER2-enriched on the BM) and 1 as Normal-like (subsequently switching to HER2-enriched on the BM).

Consistently with changes observed in PAM50 subtyping, we also observed a significant increase in expression of the HER2-enriched (FDR-corrected Wilcoxon paired signed rank test $p < 0.001$) and Luminal-B ($p = 0.001$) and a significant decrease in Luminal-A ($p < 0.001$) and Normal-like ($p = 0.001$) PAM50 signatures in BMs as compared to their paired primary tumors (**Figure 7**). On the other side, the expression of the PAM50 Basal-like signature did not show significant changes in BMs as compared to their primary tumors.

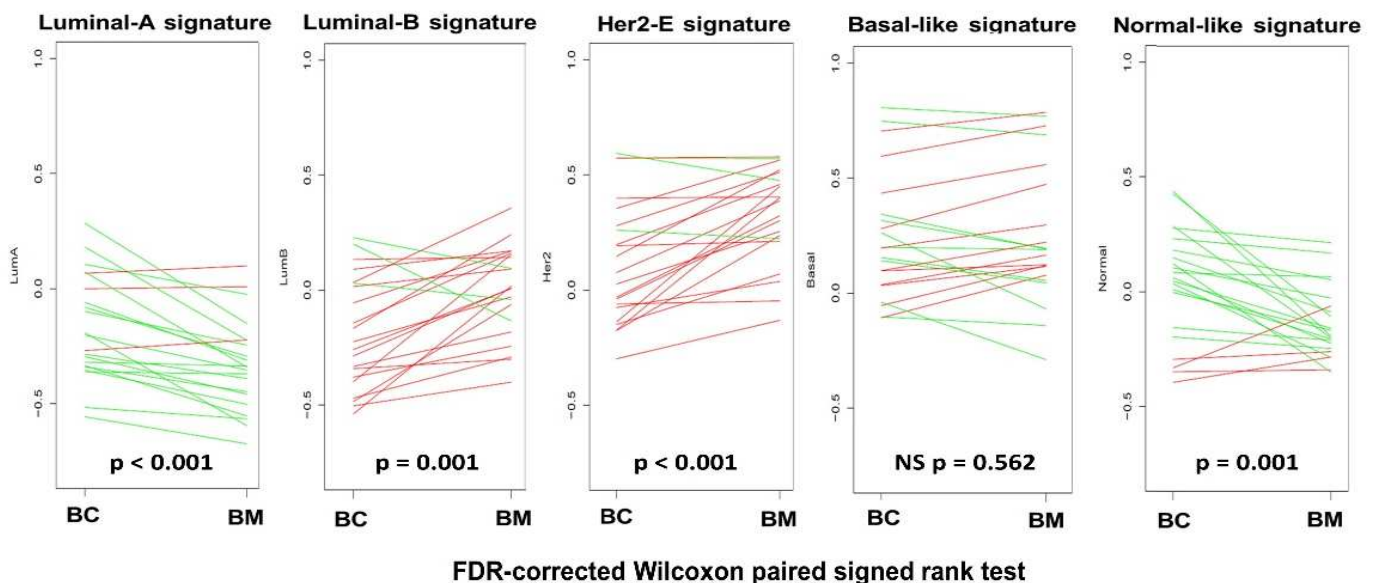


Figure 7. Changes in expression of each PAM50 subtype gene signature between primary breast cancer (BC) and matched brain metastasis (BM). Lines are colored according to gene expression dynamics: increase (red), decrease (green).

3.7 Changes in single-gene expression between paired breast cancer brain metastases and primary tumor samples

To more extensively evaluate modifications in tumor biology acquired during metastasization to the brain, we then assessed changes in the expression of single genes expression between paired BC and BMs samples using a False Discovery Rate (FDR) corrected paired two-class SAM analysis. Among the 758 evaluated genes, 68 and 320 genes, respectively, were significantly up- and downregulated in BMs as compared to primary BC (FDR<5%; **Table 7** and **Table 8**).

Table 7. Genes up-regulated in brain metastases as compared to paired primary breast cancer samples at paired SAM analysis (pairs N=21) with False Discovery Rate <5%

Gene ID	Score(d)	Fold Change	q-value(%)
BNIP3	4,993239	3,024056	0
AGT	4,113785	3,229681	0
HBB	4,063223	5,578653	0
GRB7	3,827858	2,283492	0
CRYAB	3,726344	2,792016	0
SOX2	3,715879	3,499829	0
GDF15	3,708871	2,87091	0
FGFR4	3,4243	2,937386	0
TOP2A	3,269882	2,01109	0
RRM2	3,236376	1,876504	0
HDAC2	3,161606	2,519038	0
NRCAM	3,134905	2,723198	0
ZIC2	3,10993	3,34255	0
TYMS	2,998716	1,684664	0
BIRC5	2,958634	2,084372	0
ANLN	2,866786	1,7055	0
SPP1	2,805701	2,792301	0,197244
PALB2	2,804567	1,878813	0,197244
MLPH	2,791584	1,936119	0,197244
PSMC4	2,761813	1,531243	0,197244
CDC25B	2,734915	1,747762	0,197244
PHGDH	2,684937	1,883259	0,197244
TTK	2,635277	1,971484	0,197244
WNT7B	2,630778	2,06212	0,197244
HMGA1	2,62429	1,756393	0,197244
PTTG1	2,536117	1,91998	0,309278
FOXM1	2,526853	1,591318	0,309278
CDKN3	2,5158	1,859372	0,309278
GRB2	2,505826	1,623047	0,309278
CKMT1A	2,502953	2,066187	0,309278
ADM	2,494643	2,644635	0,309278
ELF3	2,492552	1,844468	0,309278
CXADR	2,435559	1,738542	0,309278
PMS2	2,420328	1,638605	0,309278

Gene ID	Score(d)	Fold Change	q-value(%)
CDK1	2,389456	1,710117	0,705471
BMP7	2,377256	2,423412	0,705471
ALDOA	2,344923	1,631867	0,705471
MRPL19	2,325172	1,422391	0,705471
ORC6L	2,309294	1,693607	0,705471
POLQ	2,211996	1,723799	1,081393
CDCA5	2,169236	1,491256	1,081393
CDH2	2,153571	2,044387	1,081393
TMPRSS2	2,139935	1,739592	1,081393
G6PD	2,137833	1,557831	1,081393
E2F5	2,137496	1,504665	1,081393
GRIN1	2,121166	2,84394	1,081393
NCAM1	2,111329	2,182349	1,081393
CEP55	2,1072	1,619982	1,081393
CNTFR	2,093852	1,926751	2,009836
MAP3K12	2,085248	1,85069	2,009836
CHI3L1	2,066582	1,974698	2,009836
CAMK2B	2,064574	2,203012	2,009836
FAM83D	2,029479	1,542342	2,009836
COL2A1	1,978123	1,605217	2,009836
MCM2	1,920251	1,461819	3,276254
CDH1	1,871497	1,391428	3,276254
IL20RB	1,809861	1,607204	4,703264
SHMT2	1,805471	1,439705	4,703264
KIFC1	1,788206	1,549866	4,703264
ERBB2	1,769101	1,421117	4,703264
CCNE1	1,748352	1,492187	4,703264
CCNB1	1,741996	1,414653	4,703264
MAPK10	1,720135	1,768986	4,703264
FOXC2	1,716087	1,749334	4,703264
FOXA1	1,713562	1,615921	4,703264
UBB	1,702924	1,363127	4,703264
MIS18A	1,696582	1,345362	4,703264
SKP1	1,684775	1,407796	4,703264

Table 8. Genes down-regulated in brain metastases as compared to paired primary breast cancer samples at paired SAM analysis (pairs N=21) with False Discovery Rate <5%

Gene ID	Score(d)	Fold Change	q-value(%)
SFRP2	-7,65108	0,097177	0
COL6A3	-7,40047	0,145822	0
TPSAB1	-7,3711	0,095205	0
MMP11	-7,32608	0,077828	0
GREM1	-7,26134	0,133488	0
VCAN	-6,79142	0,177147	0
DPT	-6,60156	0,122881	0
ASPN	-6,12214	0,130308	0
CPA3	-5,85433	0,124872	0
MMP3	-5,74811	0,176354	0
FBN1	-5,59949	0,212979	0
COMP	-5,56751	0,122462	0
INHBA	-5,51416	0,249428	0
FAP	-5,41996	0,199417	0
SNAI2	-5,36964	0,29921	0
PARP4	-5,31879	0,340336	0
JAK3	-5,31501	0,25964	0
CXCL12	-5,23281	0,314289	0
THBS4	-5,16961	0,138903	0
IL3RA	-5,13705	0,285857	0
CFD	-5,045	0,236629	0
LTB	-4,94046	0,199036	0
CCL21	-4,83808	0,141215	0
GAS1	-4,82644	0,207627	0
IL2RB	-4,81389	0,229868	0
SRPX	-4,72941	0,250076	0
MMP14	-4,68717	0,272805	0
CD27	-4,67716	0,292461	0
IL10RA	-4,51034	0,310793	0
BCL2	-4,49318	0,32501	0
CDH5	-4,40277	0,283702	0
KRT14	-4,3169	0,14406	0
PDCD1LG2	-4,30019	0,369555	0
TIGIT	-4,27246	0,302564	0
TNFAIP6	-4,25643	0,365647	0
PTGER3	-4,2546	0,244637	0
PDGFRB	-4,25052	0,432485	0
THBS2	-4,15519	0,322594	0
GNLY	-4,06982	0,317019	0
TWIST2	-4,06392	0,300463	0
PDGFRA	-3,99976	0,363491	0
GZMA	-3,99527	0,315257	0
DCN	-3,94696	0,269934	0

Gene ID	Score(d)	Fold Change	q-value(%)
CLEC14A	-2,19394	0,575508	0
AR	-2,17972	0,562972	0
FANCF	-2,1626	0,683879	0
FZD10	-2,13781	0,517241	0
TNKS2	-2,12807	0,644674	0
CLEC5A	-2,09481	0,578586	0
LAMA3	-2,06731	0,533146	0
ZFYVE9	-2,06196	0,685138	0,197244
COLEC12	-2,05025	0,55533	0,197244
TNF	-2,0395	0,606132	0,197244
HLA.DRA	-2,03027	0,521795	0,197244
ESR1	-2,01539	0,469525	0,197244
PSMB10	-2,01131	0,679533	0,197244
EMCN	-2,00508	0,516105	0,197244
BAX	-1,99669	0,734566	0,197244
DLL1	-1,97131	0,602042	0,197244
CD8B	-1,96432	0,5315	0,197244
SOX17	-1,96344	0,526971	0,197244
CXXC5	-1,95981	0,699953	0,197244
FSTL1	-1,94788	0,617043	0,197244
JAK2	-1,9441	0,658506	0,197244
IL4R	-1,94168	0,675124	0,197244
ADD1	-1,94053	0,716882	0,197244
PYCARD	-1,94009	0,666059	0,197244
CXCR6	-1,9194	0,563803	0,197244
ITPR1	-1,90076	0,632325	0,197244
MLH1	-1,89652	0,7524	0,197244
AREG	-1,89105	0,450417	0,197244
KIAA0040	-1,8856	0,655739	0,197244
ITGB3	-1,86783	0,603007	0,309278
CD84	-1,86667	0,593646	0,309278
GZMB	-1,86387	0,585722	0,309278
DTX1	-1,86256	0,564337	0,309278
NEIL2	-1,86036	0,680264	0,309278
CACNA1H	-1,85734	0,584103	0,309278
CD36	-1,85416	0,520774	0,309278
GADD45G	-1,852	0,555737	0,309278
ALDH1A1	-1,85069	0,568596	0,309278
IL2RA	-1,84316	0,585965	0,309278
JAG1	-1,84196	0,677296	0,309278
PDCD1	-1,8347	0,560393	0,309278
BAG1	-1,82739	0,743121	0,309278
JAM2	-1,82734	0,591173	0,309278

HLA.DOB	-3,93928	0,321163	0
MME	-3,92295	0,305863	0
RAC2	-3,83557	0,302353	0
WNT2	-3,82916	0,290866	0
FST	-3,82553	0,312012	0
KLRK1	-3,78514	0,347043	0
FAM198B	-3,77981	0,359627	0
IGF1R	-3,72671	0,408088	0
IGF1	-3,72396	0,343017	0
CAV1	-3,71563	0,430027	0
MS4A2	-3,70796	0,316903	0
THY1	-3,68122	0,391781	0
TCF4	-3,6347	0,399628	0
MAF	-3,62528	0,439308	0
NKG7	-3,58562	0,347508	0
ELK3	-3,56768	0,475191	0
HLA.DRB1	-3,54906	0,398688	0
BMP8A	-3,52953	0,276833	0
OLFML2B	-3,51529	0,427353	0
CD8A	-3,50399	0,330581	0
IL7R	-3,48062	0,290605	0
PSMB9	-3,47107	0,402068	0
TGFB1	-3,44456	0,479478	0
KRT5	-3,39067	0,231802	0
WNT10A	-3,3837	0,395067	0
ISM1	-3,38269	0,371255	0
MMP9	-3,36455	0,286037	0
SFRP1	-3,31739	0,316504	0
PIK3CD	-3,31232	0,382964	0
CCL5	-3,30981	0,362247	0
FGF7	-3,23694	0,318836	0
CXCL13	-3,18877	0,190588	0
COL11A1	-3,09227	0,325386	0
TEK	-3,08795	0,430667	0
ADAM12	-3,04569	0,346815	0
NPR1	-3,04503	0,433184	0
PIK3CG	-3,04035	0,481908	0
CYBB	-3,03089	0,467931	0
IKZF3	-3,01682	0,394755	0
MFNG	-3,01634	0,455775	0
CXorf36	-3,01366	0,45632	0
HLA.DMB	-3,0096	0,532229	0
CCND2	-2,9947	0,455725	0
THBS1	-2,95384	0,425037	0
IDO1	-2,93238	0,315275	0
JCAD	-2,92866	0,470749	0
TGFBR2	-2,92002	0,440517	0

LRRC32	-1,82561	0,671556	0,309278
BCL6B	-1,82485	0,595042	0,309278
GPX3	-1,81928	0,513108	0,309278
CDKN2D	-1,80118	0,660972	0,309278
TAP1	-1,79913	0,595928	0,309278
TLR4	-1,79774	0,665637	0,309278
ITGB6	-1,79437	0,579006	0,309278
HOXA5	-1,79224	0,523833	0,309278
CCND1	-1,79192	0,612351	0,309278
PLA2G2A	-1,79123	0,538242	0,309278
KDR	-1,78626	0,659795	0,309278
GABRP	-1,77687	0,481387	0,309278
BCL2A1	-1,76938	0,720404	0,309278
ERCC1	-1,76834	0,67745	0,309278
PPP2CB	-1,7536	0,717364	0,309278
OAZ1	-1,74969	0,710633	0,309278
HOXA9	-1,72555	0,54751	0,309278
CD68	-1,7223	0,726807	0,309278
HEMK1	-1,70856	0,705159	0,309278
FGL2	-1,67831	0,547043	0,705471
CACNG4	-1,67634	0,545031	0,705471
SMAD3	-1,6751	0,691398	0,705471
MED1	-1,67169	0,7658	0,705471
FHL1	-1,65999	0,590256	0,705471
ARID1A	-1,65688	0,767359	0,705471
GLI3	-1,65125	0,652805	0,705471
MRE11	-1,64869	0,712887	0,705471
BCL11A	-1,6359	0,576179	0,705471
NFATC1	-1,62704	0,616852	0,705471
ACTR3B	-1,61014	0,725425	0,705471
LFNG	-1,60741	0,67518	0,705471
PDK4	-1,6064	0,578688	0,705471
HAS1	-1,59329	0,617393	0,705471
SYTL4	-1,5646	0,697843	0,705471
PRKCB	-1,5637	0,571995	0,705471
ACTB	-1,54508	0,798888	1,081393
GADD45B	-1,53933	0,697928	1,081393
CLDN1	-1,51915	0,62066	1,081393
SOCS2	-1,51697	0,66018	1,081393
ADCY9	-1,50972	0,707037	1,081393
TCF7L1	-1,50695	0,680285	1,081393
CCR1	-1,50504	0,726759	1,081393
GSK3B	-1,4632	0,797577	1,081393
RELN	-1,4622	0,558018	1,081393
CACNA2D1	-1,45606	0,65815	1,081393
VIM	-1,45571	0,672384	1,081393
MLLT3	-1,45138	0,735583	1,081393

WRN	-2,91529	0,521572	0
HLA.DQA1	-2,90489	0,359001	0
CXCL9	-2,90344	0,201249	0
HLA.DPB1	-2,89801	0,391007	0
PIM1	-2,89756	0,49082	0
HLA.DQB1	-2,88716	0,433162	0
FZD7	-2,88567	0,453839	0
SPN	-2,88235	0,419304	0
SFRP4	-2,84886	0,341623	0
CD34	-2,82389	0,46289	0
FOXP3	-2,81837	0,443871	0
AXIN2	-2,7612	0,531938	0
HLA.DPA1	-2,75425	0,427354	0
SCARA5	-2,75092	0,38814	0
RUNX3	-2,74361	0,496988	0
RASGRP1	-2,73779	0,426526	0
TAP2	-2,72503	0,522131	0
MEOX2	-2,70621	0,360646	0
POLR2A	-2,70487	0,575344	0
FLI1	-2,70123	0,528543	0
HLA.A	-2,68032	0,470717	0
DDR2	-2,67163	0,577569	0
ITGAV	-2,67032	0,581936	0
TGFB3	-2,65512	0,537711	0
CTSW	-2,64483	0,461327	0
LAMC2	-2,64276	0,424912	0
TWIST1	-2,63381	0,398578	0
TIE1	-2,58577	0,510279	0
AKT3	-2,56791	0,472846	0
SP1	-2,56751	0,650123	0
SERPINH1	-2,52893	0,658588	0
SF3A1	-2,52064	0,647082	0
PLAT	-2,51482	0,498813	0
HDAC6	-2,51068	0,637004	0
ICAM1	-2,50187	0,549702	0
TBX1	-2,50173	0,5313	0
LAMB3	-2,49657	0,47043	0
ACVRL1	-2,48768	0,501872	0
PDGFB	-2,47835	0,590647	0
WNT5B	-2,47462	0,501062	0
HLA.B	-2,45672	0,529884	0
SHE	-2,45378	0,534385	0
CD19	-2,44698	0,448709	0
OGN	-2,42964	0,471952	0
GZMM	-2,4198	0,493516	0
GJB2	-2,41807	0,386943	0
RORA	-2,40728	0,524848	0

MIA	-1,42879	0,659435	1,081393
NFKBIZ	-1,42019	0,724148	1,081393
EIF4E2	-1,41282	0,767496	1,081393
RORB	-1,40924	0,622571	1,081393
CDKN2A	-1,40836	0,681709	1,081393
RBL2	-1,40532	0,784118	1,081393
PPP2R1A	-1,40229	0,792081	2,009836
FLT3	-1,40052	0,64485	2,009836
TFF1	-1,39607	0,581273	2,009836
LPL	-1,38662	0,71171	2,009836
CXCL10	-1,37878	0,53491	2,009836
SERPINB5	-1,37623	0,670355	2,009836
SOCS1	-1,37391	0,70928	2,009836
IL12RB2	-1,36536	0,652326	2,009836
PRKX	-1,35984	0,683535	2,009836
NCAPH2	-1,35181	0,782913	2,009836
ITGB1	-1,34025	0,742735	2,009836
POLD1	-1,3367	0,758735	2,009836
CD276	-1,32694	0,777954	2,009836
MMP7	-1,3259	0,570196	2,009836
IL6R	-1,3197	0,739029	2,009836
MUS81	-1,31441	0,779278	2,009836
PTEN	-1,30825	0,72446	2,009836
JAK1	-1,30262	0,810424	2,009836
IL11RA	-1,29245	0,721511	2,009836
BBC3	-1,28169	0,781482	2,009836
IL1B	-1,27861	0,705736	2,009836
RASGRF2	-1,27607	0,627613	2,009836
EDNRB	-1,24803	0,709208	2,009836
KRT17	-1,24499	0,604247	2,009836
NSD1	-1,2404	0,795419	2,009836
RBL1	-1,23905	0,808441	2,009836
CDK6	-1,23201	0,738313	2,009836
TBC1D9	-1,22918	0,737166	2,009836
NOD2	-1,21305	0,7834	3,276254
HDAC1	-1,21172	0,783295	3,276254
PAX5	-1,20535	0,641012	3,276254
KRT6B	-1,19488	0,623282	3,276254
HES1	-1,18321	0,796992	3,276254
CACNG1	-1,18288	0,691845	3,276254
SMURF2	-1,18153	0,835364	3,276254
CDKN1C	-1,17518	0,737168	3,276254
ETV7	-1,1729	0,726841	3,276254
DTX3	-1,16826	0,746475	3,276254
SMC1B	-1,16287	0,694273	3,276254
CXCL8	-1,15752	0,678418	3,276254
CDC14A	-1,15249	0,716401	3,276254

BMPR2	-2,40334	0,663616	0
ECM2	-2,39061	0,544996	0
AGTR1	-2,38742	0,450337	0
CCR2	-2,38717	0,498007	0
HGF	-2,38667	0,523031	0
ATM	-2,38592	0,563541	0
IRX1	-2,3443	0,390515	0
ENPP2	-2,34343	0,534702	0
MYCT1	-2,33142	0,566369	0
PGR	-2,32757	0,378504	0
LEF1	-2,32108	0,53401	0
IL6	-2,31899	0,530799	0
ZEB2	-2,31576	0,535844	0
SCUBE2	-2,30505	0,456461	0
FGF18	-2,29213	0,503401	0
HDC	-2,27895	0,492713	0
MMRN2	-2,26766	0,557575	0
HEG1	-2,23644	0,512413	0
RAD52	-2,23153	0,653022	0
TYMP	-2,22295	0,580055	0
HLA.E	-2,21993	0,57759	0
PIK3R5	-2,21225	0,569405	0
BORCS7	-2,2009	0,645638	0

PFDN2	-1,15218	0,82825	3,276254
TLE3	-1,12785	0,803626	3,276254
LINC02381	-1,12558	0,791278	3,276254
CACNG6	-1,12476	0,66073	3,276254
BLVRA	-1,12117	0,81207	3,276254
BNC2	-1,1171	0,689512	3,276254
WNT11	-1,10077	0,706511	3,276254
DDB2	-1,09838	0,743328	3,276254
KIT	-1,09138	0,710403	4,703264
TNFSF10	-1,07803	0,728278	4,703264
PRF1	-1,07325	0,762744	4,703264
MAML2	-1,05687	0,765025	4,703264
BCAS1	-1,05608	0,74148	4,703264
WNT6	-1,05268	0,72712	4,703264
EP300	-1,04752	0,850592	4,703264
POPDC3	-1,04005	0,7146	4,703264
ROCK2	-1,03275	0,842914	4,703264
EFNA3	-1,0101	0,789007	4,703264
ROBO4	-1,0082	0,764641	4,703264
HNF1A	-0,99843	0,701562	4,703264
KAT2B	-0,99241	0,770183	4,703264
RPS6KA5	-0,98315	0,835451	4,703264
LEMD1	-0,98097	0,781875	4,703264

Upregulated genes were enriched in genes involved in survival (e.g. FGFR4), proliferation and cell cycle regulation (e.g. CCNB1, CCNE1, CDK1, TOP2A), and receptor tyrosine kinase signaling and signal transduction (e.g. ERBB2, GRB7, GRB2) (**Figure 8A**). Downregulated genes were enriched in genes involved in endocrine response (e.g. ESR1, PGR), inflammation and immune response (e.g. CD8A, CCL5, PDCD1LG2 [PD-L2], TNF), and angiogenesis (e.g. PDGFRB, TIE1) (**Figure 8B**). Expression of several cytokeratins (e.g. KRT5, KRT14, KRT17) and of CAV1 (gene codifying for caveolin-1) was also significantly downregulated in BMs as compared to matched primary tumors.

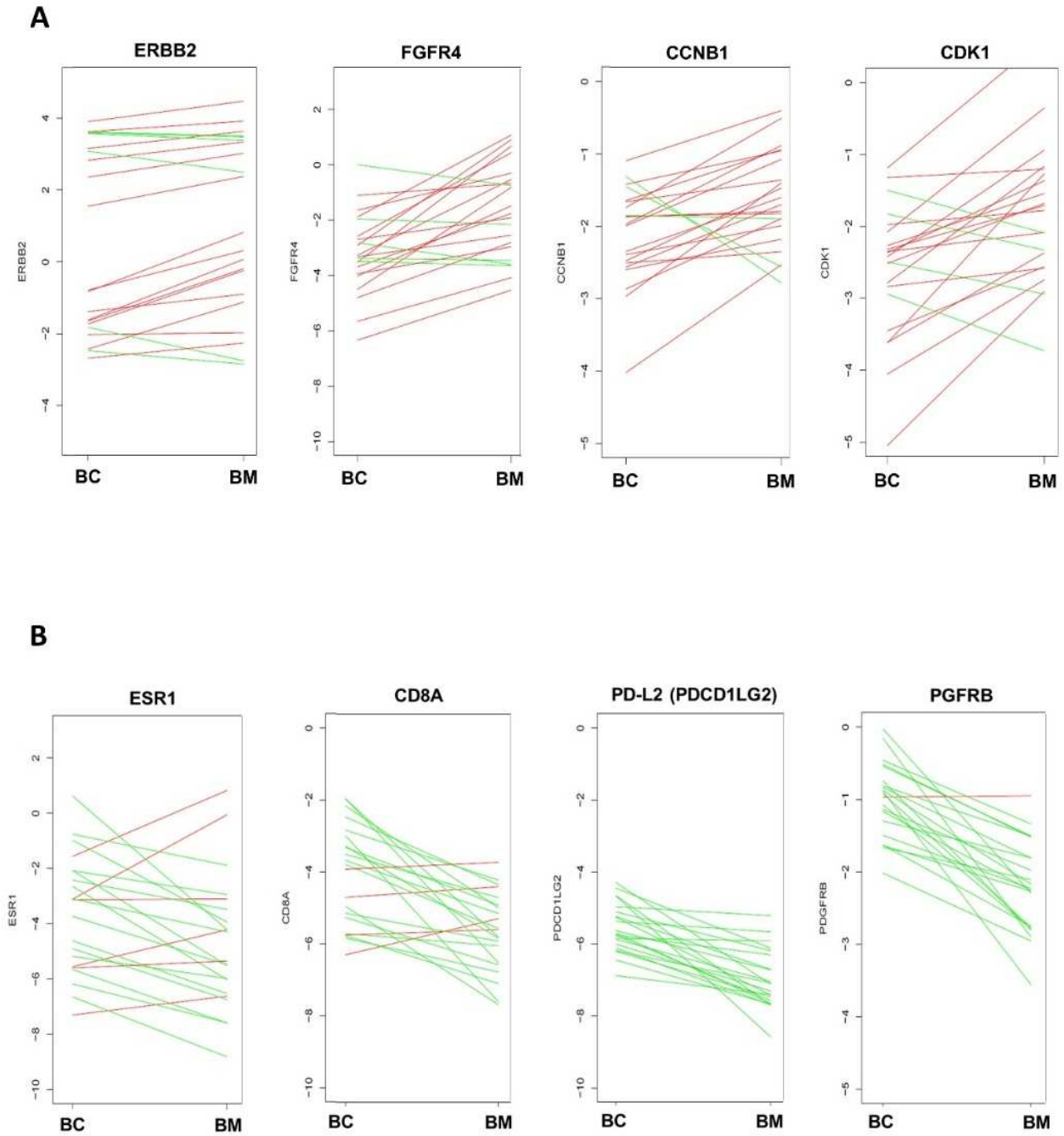


Figure 8. Changes in single-gene expression between primary breast cancers and matched brain metastasis. Examples of changes in expression of relevant genes up-regulated (A) and down-regulated (B) in matched brain metastasis (BM) as compared to primary breast cancer (BC). Lines are colored according to gene expression dynamics: increase (red), decrease (green).

4. Discussion and conclusions

Over the last two decades, gene expression profiling has had a considerable impact on our understanding of BC biology. We here report the transcriptomic profile of a large cohort of BCBMs, assess its association with patient prognosis and compare it with the transcriptomic profile of matched primary BC samples to shed light on the biological processes relevant for BC metastasization to the brain niche.

4.1 PAM50 subtype and brain metastasization

Through this approach we identified a very high prevalence of non-luminal intrinsic subtypes (HER2-enriched and basal-like) in resected BCBMs. While the high prevalence of Basal-like tumors within HR-/HER2- BCBMs is in line with what generally observed in primary BC, HER2-enriched tumors appeared to be more represented within the HER2+ subgroup than expected. Indeed, while the large majority of HR-/HER2+ primary BCs are usually classified as HER2-enriched, generally only around half of HR+/HER2+ primary BCs are described as HER2-enriched, with the other half classified as Luminal (A or B) subtype.(67,68) Our observation might potentially be explained by a higher propensity of HER2-enriched HER2+ BCs to metastasize to the brain or by the acquisition of a HER2-enriched subtype during brain metastasization. Indeed, in a previous study assessing 677 HER2+ stage I-III BC patients, we reported that, even within HER2+ BC, HER2-enriched tumors were more prone to develop BMs as compared to other PAM50 subtypes (10-year incidence 3.8% vs 0.6%, $p=0.005$). (69) On the other hand, when we compared paired primary BC and BM samples, we observed a significant discordance in PAM50 subtyping with a consistent shift towards HER2-enriched subtype, and this was observed even in paired samples which consistently remained HER2-positive both on the primary BC and BM sample. This observation is consistent with what observed in another small study on 17 paired primary BC and BMs, where a PAM50 molecular subtype conversion was observed in 8/17 (47.1%) matched pairs, with half (3/6) of luminal A primary BCs converting to HER2-enriched subtype on the BM.(70) Indeed, this might represent the effect of a positive selection of tumor clones presenting a more HER2-enriched phenotype during brain

metastasization, in line with what we previously observed in early HER2+ BC. Moreover, this appears to differ from what generally observed during metastatic recurrence, as previous studies have reported that intrinsic molecular subtype is largely maintained between primary BCs and paired metastatic non-CNS samples, except for luminal A disease which more frequently converts to a luminal B subtype and only rarely (around 15% of cases) converts to HER2-enriched phenotype.(71) Therefore, the switch towards a HER2-enriched subtype observed might represent a biological feature specific to the brain metastatic niche, with potential therapeutic implications.

We also observed an unexpected high frequency of PAM50 non-luminal subtypes (Basal-like, HER2-enriched) among HR+/HER2- BMs, constituting half of these samples. This represents a very high proportion when compared to the relative rarity of non-luminal intrinsic subtypes in this context, which generally represent less than 10% in early HR+/HER2- BC and around 20% in metastatic HR+/HER2- BC.(72,73) Gene-expression data from matched primary BC samples was available only for a very limited number of patients with non-luminal HR+/HER2- BCBM, showing that in 3 out of 4 cases the primary BC was already classified as non-luminal.

Although limited by the small sample size and the specific clinical characteristics of our cohort, this observation suggests that non-luminal disease within HR+/HER2- BC might potentially present a specific metastatic tropism, thus adding to the increasing evidence that non-luminal disease within HR+/HER2- BC represents distinct biological and clinical features. In addition, we also observed that within the subgroup of patients with HR+/HER2- BCBMs, tumor biology as assessed by the gene signatures of PAM50 algorithm was significantly associated with patient's prognosis (with a higher expression of the PAM50 basal-like signature emerging as a predictor of worse OS, and a higher expression of the PAM50 Luminal-A signature associated with more favorable outcomes). These observations might carry a therapeutic significance as retrospective analyses of prospective trials have reported distinct therapeutic effects of different therapeutic agents in non-luminal compared to luminal HR+/HER2-metastatic BCs and some clinical trials are currently underway to try to test this hypothesis.(73)

4.2 Single gene expression: changes and prognostic impact

Moreover, gene expression profiling of BMs and matched primary BCs highlighted recurrent gene-expression modifications in metastatic samples. Consistently with a previous report on 20 patients with paired samples,(74) we observed in 15 BMs out of 21 (71%) an increase in ERBB2 (HER2) expression and FGFR4 expression as compared to the primary BC, while a decrease in ESR1 expression levels was observed in 15 BMs out of 21 (71%). On one side, this might potentially represent a therapeutic target as high ERBB2 mRNA levels have been previously reported to be associated with higher response rate to T-DM1,(75,76) and its role in predicting response to other HER2-targeted ADCs is currently being assessed. In addition, the decrease in CAV1 in BM might also underlie a higher sensitivity to HER2-targeted antibodies. In fact, caveolin-1 is well-known to be involved in HER2 cell membrane dynamics and its depletion has been previously associated with an increase in HER2 half-life and availability at the cell membrane resulting in improved trastuzumab binding.(77) On the other side, the decrease in expression of genes involved in endocrine response (e.g. ESR1, PGR) might underpin a general decrease in endocrine sensitivity in this context. Moreover, we also observed a consistent increase in CCNE1 mRNA expression levels in BMs as compared to respective primary tumors, which in the context of HR+/HER2- metastatic BC has been reported to be associated with a relative resistance to Cdk4/6 inhibitors.(78,79) In addition, the activation of FGFR4 signaling pathway has also been described as an important factor in luminal disease progression and resistance to endocrine therapy in the metastatic setting; however, it might be disrupted using small molecules inhibiting FGFR4, therefore also representing an attractive therapeutic target to be explored.(80,81) Of note, in addition to changes in gene expression, immunohistochemical analyses of HR and HER2 status also revealed the emergence of clinically targetable entities in BMs (i.e. three HR- cases shifted to HR+, and one HER2- case shifted to HER2+). These findings highlight the clinical relevance of reassessing biomarker status in BMs, as changes in HR and HER2 expression may open therapeutic opportunities, and underscore the interest for the exploration of non-invasive approaches, such as radiomics, to reassess tumor biology on BCBMs.

We also observed a consistent decrease in BMs, as compared to primary tumors, in the expression of genes related to inflammation and immune response (e.g. CD8A, CCL5, PDCD1LG2 [PD-L2], TNF). This trend was observed both for genes involved in promoting immune activation (e.g. CD8A) and for some genes codifying for immune checkpoint molecules and typically associated with immunosuppression (e.g. PDCD1LG2 [PD-L2]). This might recapitulate a pattern frequently observed in BC (especially in triple-negative and HER2+ BC) in which presence of immune infiltrate is generally associated with an activation of the immune system, characterised by an increase in both cytotoxic and pro-tumoral components of the immune system (e.g. CD8+ and FOXP3+ cells).(82,83) Overall, the observation of a decreased expression of these genes in BMs suggests that the brain niche might be characterized by a more immunologically "cold" microenvironment than the primary tumor. This is consistent with previous studies reporting that lower levels of immune infiltrate are generally observed in BMs as compared to their primary tumors and that lower levels of expression of immune-related genes are generally observed in brain lesions as compared to other metastatic sites in BC patients.(64,84) It is also in line with previous reports showing that immune infiltrate in BCBMs is predominantly composed of microglia/macrophages, while the lymphocytic compartment is scarcely represented.(85)

Interestingly, when we assessed association between single gene expression and survival only a limited number of inflammation and immune related genes showed a significant prognostic impact, namely CD276 (an immune checkpoint molecule), Granzyme A (a cytotoxic T lymphocyte enzyme), IL4R and IL13RA1 (subunits of the type II IL4 receptor, typically low in nonlymphoid cells but up-regulated in tumors). In line with what previously discussed, higher expression of these genes was associated with better OS from BM diagnosis. The association between single gene expression and survival observed in this study should be considered an exploratory analysis and requires further confirmation in independent studies. Nevertheless, the highlighted genes might represent interesting potential therapeutic targets. For instance, CD276 (B7-H3) is currently being investigated as a target for novel immunotherapy agents and CAR-T cell therapies across various solid tumors.(86) Additionally, high LINC02381 expression has been linked to BC and other solid tumors, such as gliomas, to activation of the PI3K/AKT signaling pathway via FN1 and IGF1R.(87,88) Our results

might therefore suggest that PI3K/AKT signaling may play a significant role in BCBMs beyond specific mutational alterations, which is also in line with similar studies assessing the genomic profile of BMs.(89)

Overall, this study presents some relevant limitations as it included a retrospective cohort of BCBM patients, therefore warranting future validation in independent cohorts of BCBMs. Due to the hypothesis-generating nature of the analyses and the limited sample size, multiplicity correction was not adopted for analyses assessing the association between single gene expression and OS, thus potentially increasing the risk of false-positive results. Moreover, differences in terms of tumor cellularity between BMs and primary tumors may have influenced the change observed in some pairs from normal-like subtype on the primary tumor (potentially due to contamination by normal breast tissue) to HER2-enriched subtype on the BM. However, the same trend towards switching to HER2-enriched subtype was observed in tumors originally classified as Basal-like on the primary tumor sample, thus supporting the hypothesis that the shift towards HER2-enriched subtype is driven by a genuine biological effect. Furthermore, the inclusion of patients who underwent neurosurgery may have introduced selection bias, as these patients likely represent a subgroup with more favorable prognosis. The predominance of single BM cases may limit external validity, as patients with multiple metastases often differ clinically and therapeutically. Additionally, the wide time interval covered by the study and the recent evolution of therapeutic options especially in the HER2+ subgroup may limit the transferability of survival results to a contemporary cohort. Finally, this study did not use a whole transcriptome approach, therefore potentially relevant genes not included in the specific panel might have been missed.

Nevertheless, the study also presents several strengths: this BCBM cohort represents one of the largest to date to study matched primary and BM samples using a comprehensive genomic assessment, including expression of more than 700 genes, and one of the few to evaluate PAM50 intrinsic subtype profiling of primary BC and BCBMs, thus allowing for the identification of relevant biological modifications acquired during brain metastasization and potentially overlooked by other techniques.

Indeed, the integration of PAM50 intrinsic subtyping and comprehensive genomic profiling offered the opportunity to unveil the biological heterogeneity of BCBMs and the association between biological features of BC and clinical outcomes, even in the context of BMs. Moreover, it highlighted the dynamic nature of tumor biology during the metastatic process. Indeed, the identification of recurrent genomic modifications acquired by BC during metastasization to the brain might carry potential therapeutic implications, especially in the context of the limited systemic treatment options available for HER2- BC patients with BMs, and therefore warrants further exploration in translational and clinical studies.

5. Bibliography

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