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ORIGINAL ARTICLE

# Gene expression profiling in a retrospective real-world cohort of breast cancer brain metastases and paired primary tumors identifies biological changes with potential therapeutic implications

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

**Materials and methods:** Expression of 758 genes (BC360 Panel, nCounter), hormone receptor (HR) status, and human epidermal growth factor receptor type 2 (HER2) status were evaluated in BCBMs and matched primary BCs. Intrinsic subtyping was determined using the PAM50 subtype predictor. Gene expression/PAM50 signature and overall survival (OS) correlations were analyzed using Cox models. Median OS was calculated using the Kaplan—Meier method. A false discovery rate-corrected paired two-class SAM identified gene expression changes between paired BC and BM samples.

**Results:** Seventy-five BCBM samples from 74 patients were analyzed: 30.7% HR-positive/HER2-negative, 41.3% HER2-positive, and 28.0% HR-negative/HER2-negative. Intrinsic subtype was 36.0% basal-like, 48.0% HER2-enriched, 14.7% luminal B, and 1.3% normal-like; among HR-positive/HER2-negative 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-positive/HER2-negative subgroup ($P = 0.024$). Among 21 primary BCs analyzed, 45% of basal-like, 100% of normal-like, and 50% of luminal A shifted toward the HER2-enriched subtype in matched BMs. Three hundred and eighty-eight genes were differentially expressed in BMs compared with 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.

**Key words:** breast cancer, brain metastases, gene expression, intrinsic subtype

## INTRODUCTION

Brain metastases (BMs) represent a major clinical challenge in patients with solid tumors, affecting ∼20% of all patients with metastatic cancer. Breast cancer (BC) is one of the solid tumors more frequently involved in the development of BMs and higher incidences occur in triple-negative BC (TNBC) or human epidermal growth factor

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receptor type 2 (HER2)-positive BC, where 30%-50% of patients with metastatic BC will eventually develop a central nervous system (CNS) involvement.[1-3]

Despite progress in BC treatments, treatment options for BMs remain limited and are still mostly based on local treatment with radiotherapy and, if applicable, surgical resection of the tumor mass, with only limited activity of most systemic therapies in the CNS. Therefore, patients with BM still face a poorer prognosis, as compared with patients without BMs, and a higher risk of neurological impairment which negatively affects quality of life.[1] Optimization of therapeutic options is therefore warranted to improve the prognosis of BC patients diagnosed with BMs.

In this context, the characterization of the molecular mechanisms underlying metastasization of BC to the CNS might allow for the identification of new therapeutic strategies and putative prognostic and predictive biomarkers.[4] Indeed, previous analyses on a limited number of paired primary BC and BM samples reported the acquisition in the BM of recurrent expression changes in clinically actionable genes, which might be potentially used to inform targeted therapy selection.[5] However, despite the clinical impact of BCBMs and the potential therapeutic relevance of a molecular characterization, their biological complexity still remains poorly understood.

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.

## MATERIALS AND METHODS

### *Patients and samples*

BC patients undergoing neurosurgery at three institutions between 2003 and 2019 were retrospectively identified. Eligibility criteria included: age $\geq$18 years, a diagnosis of metastatic BC, neurosurgery for BC-related BM carried out 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 Prof. 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).[6]

Clinical/anatomopathological data were retrospectively collected from medical records using a standardized electronic form. Hormone receptor (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, and survival outcomes. Before analysis, the dataset was cleaned to remove duplicate entries and harmonized across institutions to ensure uniform variable definitions. The modified breast Graded Prognostic Assessment (GPA) score was evaluated according to published criteria.[7]

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. ESMO-Guidance for Reporting Oncology Real-World Evidence checklist and flowchart were applied (Supplementary Methods, available at https://doi.org/10.1016/j.esmoop.2025.105507).

### *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, Madison, WI) 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, Waltham, MA) and the High Sensitivity RNA kit on TapeStation 4200 (Agilent Technologies, Santa Clara, CA), to ensure they had a concentration of $\geq$20 ng/μl and to evaluate RIN and DV200.

The starting material was 100-500 ng of RNA depending on the percentage of fragments with dimensions >200 bp (DV200 value). Samples were hybridized with panel probes for 19 h at 65°C at both institutions and then complexes were processed on the nCounter Analysis System. Cartridges were scanned at 555 fields of view. 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.

### *Statistical analysis*

Intrinsic molecular subtyping was determined using the previously reported PAM50 subtype predictor.[8]

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 whom 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-gene expression between paired BC and BM samples.

Analyses were carried out using R software 4.0.5.

## RESULTS

### Patient and tumor characteristics

Seventy-five evaluable BCBM 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 ($N = 34$); and Montefiore Medical Center, Bronx NY, USA ($N = 19$) (REMARK flowchart, Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2025.105507).

Patients' characteristics are reported in Table 1. Median age at the time of primary tumor and BM diagnosis was 44 years (range 30-77 years) and 50 years (range 33-77 years), respectively.

BC subtyping on the BM was available for all 75 samples and was TNBC for 21 samples (28.0%), HR-positive/HER2-negative for 23 samples (30.7%), and HER2-positive for 31 samples (41.3%).

**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 |

BC, breast cancer; BM, brain metastasis; CNS, central nervous system; GPA, Graded Prognostic Assessment; HER2, human epidermal growth factor receptor type 2; HR, hormone receptor; NA, not available; TNBC, triple-negative breast cancer.

### 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% confidence interval (CI) 18.4-32.4 months].

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-positive/HER2-negative; 30.5 months for HER2-postive BC; log-rank $P = 0.020$; Supplementary Figure S2, available at https://doi.org/10.1016/j.esmoop.2025.105507), while the number of BM, performance status, the presence/absence of extra-CNS disease at the 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 Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2025.105507). Therefore, also taking into account its potential association with the expression of several 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.

The association between time-dependent treatment-related variables (having received systemic treatment and having received radiotherapy after neurosurgery) and OS was assessed (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2025.105507). BC subtype and having received radiotherapy after neurosurgery were the only independent factors associated with OS at multivariate Cox models. Therefore, to evaluate the potential confounding effect of radiotherapy, exploratory analyses using both these variables as correction factors were conducted.

### 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 1; detailed data reported in Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2025.105507). 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 BMs classified as luminal B (14.7%) or normal-like (1.3%). As expected, the HER2-enriched subtype represented the large majority of HER2-positive BMs (87.1%), while the basal-like subtype was the most represented subtype in HR-negative/HER2-negative BMs (85.7%). However, PAM50 non-luminal subtypes were well represented even among HR-positive/HER2-negative 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 identified as normal-like (4.3%).

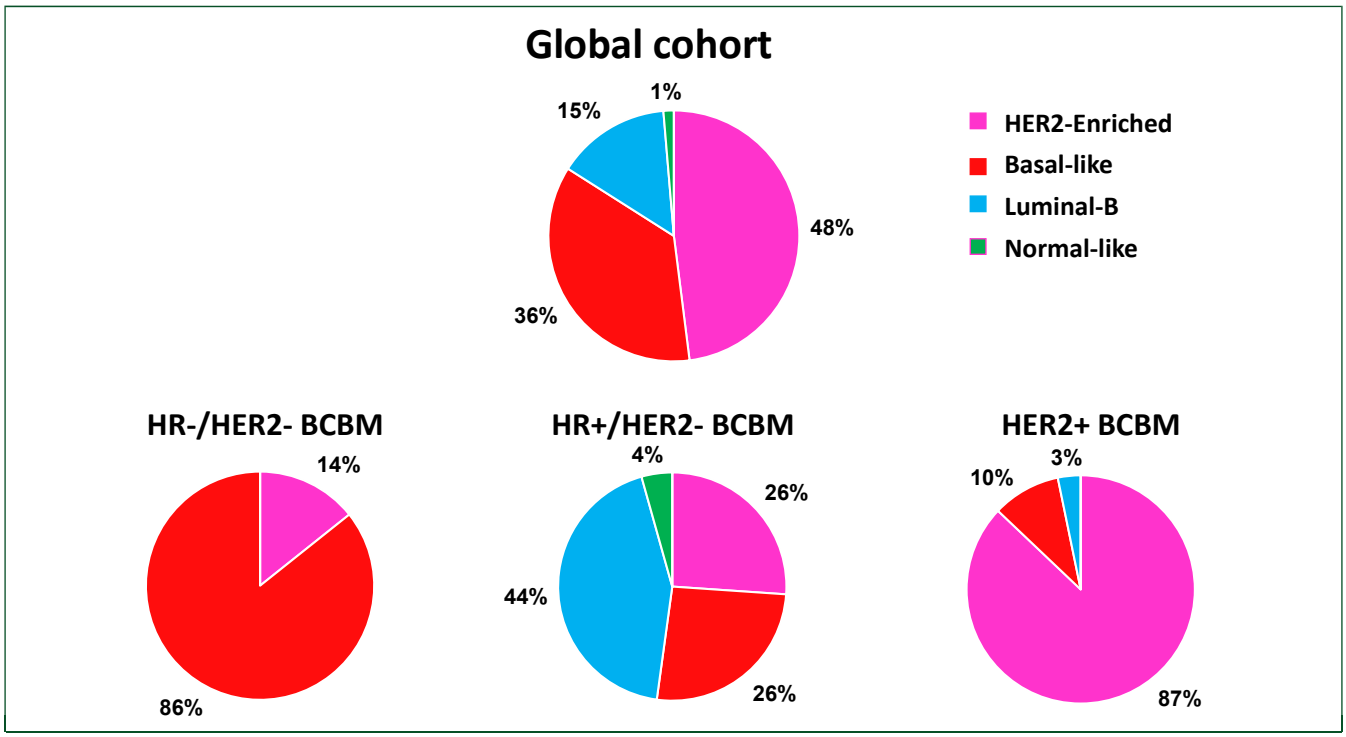


**Figure 1. Distribution of PAM50 intrinsic subtype within the overall study cohort of breast cancer brain metastases and within each IHC-based subgroup (IHC assessed on the brain metastasis).**
BCBM, breast cancer brain metastasis; HER2, human epidermal growth factor receptor type 2; HR, hormone receptor; IHC, immunohistochemistry.

### *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 BM diagnosis. As expected, basal-like intrinsic subtype was associated with a worse OS as compared with other intrinsic subtypes ($P$ =0.021; median OS for basal-like tumors 14.4 months, 95% CI 2.5-26.3 months; for HER2-enriched tumors 29.6 months, 95% CI 22.7-36.5 months; for luminal B tumors 37.4 months, 95% CI 29.8-45.0 months; normal-like not reported as only one case was assessed, Supplementary Figure S3, available at https://doi.org/10.1016/j.esmoop.2025.105507). 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-positive/HER2-negative BMs (24 versus 34.1 months) and in HER2-positive BMs (21 versus 30.5 months). Moreover, among patients with HR-positive/HER2-negative BCBMs, patients with the 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 months; for other subtypes 23.0 months, 95% CI 21.2-24.8 months, $P = 0.090$).

To more precisely assess the association between tumor biology, as assessed by the PAM50 algorithm, and survival from BM diagnosis, we then assessed the prognostic role of the PAM50 gene signatures, overall and in each BC subtype separately (Table 2).

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-positive/HER2-negative BCBM subgroup ($P = 0.024$), while this was not observed in the HER2-positive and HR-negative/HER2-negative BCBM subgroups. The PAM50 luminal A signature as a continuous variable was significantly associated with a better OS within the HR-positive/HER2-negative BCBM subgroup ($P = 0.029$) and, although not statistically significant, overall ($P = 0.054$), while this was not observed in the HER2-positive and HR-negative/HER2-negative BCBM 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.

Similar results were obtained at multivariate analyses after correction for having received radiotherapy after neurosurgery (Supplementary Table S3, available at https://doi.org/10.1016/j.esmoop.2025.105507).

### *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

**Table 2. 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 | |
|---|---|---|---|---|---|---|---|---|
| | Hazard ratio (95% CI) | *P* value | Hazard ratio (95% CI) | *P* value | Hazard ratio (95% CI) | *P* value | Hazard ratio (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 (0.01-10 831.36) | 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 |

Statistically significant values are indicated in bold.
CI, confidence interval; HR, hormone receptor; HER2, human epidermal growth factor receptor type 2; TNBC, triple-negative breast cancer.

subtype) for eight and seven genes, respectively (Table 3). The majority of these associations (13 out of 15 genes) were confirmed at multivariate analysis also corrected for having received radiotherapy (Supplementary Table S4, available at https://doi.org/10.1016/j.esmoop.2025.105507).

**Table 3. 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**

| Gene | Details | Univariate Cox model | | Multivariate Cox model (corrected by BC subtype) | |
|---|---|---|---|---|---|
| | | Hazard ratio (95% CI) | *P* value | Hazard ratio (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** |

Genes with significant *P* values after correcting by breast cancer subtype at multivariate analysis are shown in bold.
CI, confidence interval.

### *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 (REMARK flowchart, Supplementary Figure S4, available at https://doi.org/10.1016/j.esmoop.2025.105507).

The BC phenotype of primary BCs and matched BMs as assessed by immunohistochemistry (IHC) and in situ hybridization by clinical practice is reported in Figure 2A. Discordance in HR status evaluated on the primary BC and matched BM was observed in 33% ($n = 7$) of patients: four (19.0%) with loss of previous HR positivity and three (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 toward HER2-enriched PAM50 subtype in the brain metastatic site was observed (Figure 2B). 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 eight patients for whom a switch toward a HER2-enriched on the BM sample was observed constituted a quite heterogeneous patient subgroup. Only four patients presented a HER2-positive BM according to clinical classification; for three of them the primary BC was also HER2-positive (one luminal A to HER2-enriched; one basal-like to HER2-enriched; one normal-like to HER2-enriched), while in one case acquisition of HER2 positivity on the BM was observed (clinically HR-negative/HER2-negative to HR-negative/HER2-positive; PAM50 intrinsic subtype normal-like to HER2-enriched). The remaining four patients for whom a switch toward a HER2-enriched on the BM sample was observed presented a BM classified as HER2-negative according to clinical classification: one case presented a HR-negative/HER2-negative BM (clinically: HR-negative/HER2-negative to HR-negative/HER2-negative; PAM50 intrinsic subtype basal-like to HER2-enriched), while three cases presented HR-positive/HER2-negative BMs. Indeed, of the 11 HR-positive/HER2-negative 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-positive/HER2-negative). On the matched primary BC, three were classified as basal-like (two subsequently switching to HER2-enriched on the BM) and one 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 with their paired primary tumors (Figure 2C). On the other side, the expression of the PAM50 basal-like signature did not show significant changes in BMs as compared with their primary tumors.

### *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-gene expression between paired BC and BM samples using an FDR-corrected paired two-class SAM analysis.

Among the 758 evaluated genes, 68 and 320 genes, respectively, were significantly up- and down-regulated in BMs as compared with primary BC (FDR <5%; Supplementary Tables S5 and S6, available at https://doi.org/10.1016/j.esmoop.2025.105507). Up-regulated 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*) (Supplementary Figure S5A, available at https://doi.org/10.1016/j.esmoop.2025.105507). Down-regulated 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*) (Supplementary Figure S5B, available at https://doi.org/10.1016/j.esmoop.2025.105507). Expression of several cytokeratins (e.g. *KRT5*, *KRT14*, *KRT17*) and of *CAV1* (gene codifying for caveolin-1) was also significantly down-regulated in BMs as compared with matched primary tumors.

## DISCUSSION

Over the last two decades, gene expression profiling has had a considerable impact on our understanding of BC biology. We reported the transcriptomic profile of a large cohort of BCBMs, assessed its association with patient prognosis, and compared 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.

### *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-negative/HER2-negative BCBMs is in line with what is generally observed in primary BC, HER2-enriched tumors appeared to be more represented within the HER2-positive subgroup than expected. Indeed, while the large majority of HR-negative/HER2-positive primary BCs are usually classified as HER2-enriched, generally only around half of HR-positive/HER2-positive primary BCs are described as HER2-enriched, with the other half classified as luminal (A or B) subtype.[9,10] Our observation might potentially be explained by a higher propensity of HER2-enriched HER2-positive BCs to metastasize to the brain or by the acquisition of a

A

| | | 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 |

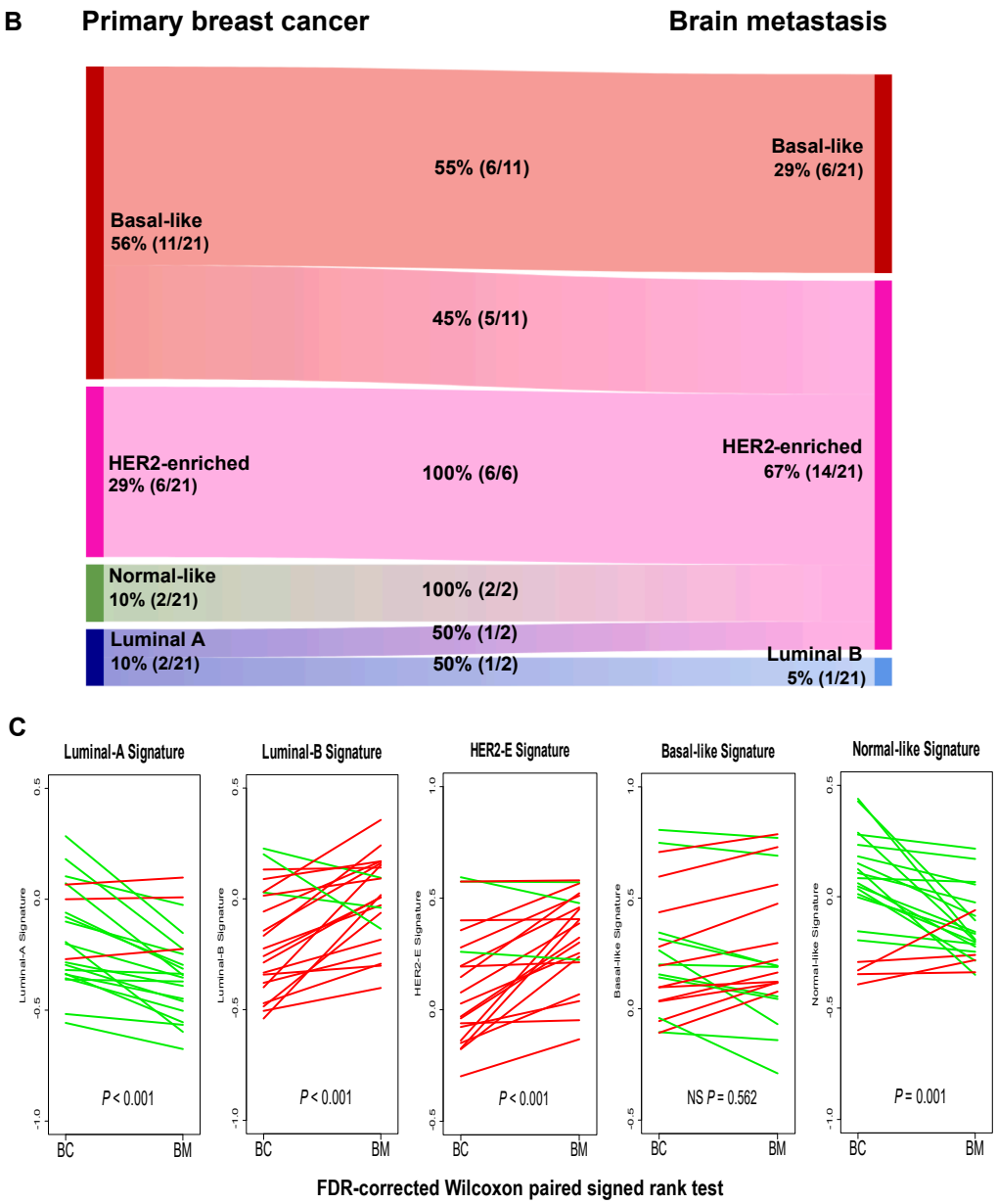


**Figure 2. Changes in subtyping between primary breast cancers and matched brain metastasis.** (A) Changes in IHC-based breast cancer subtype in primary breast cancer samples and paired brain metastases ($N = 22$). (B) Sankey plot showing shifts in PAM50 breast cancer intrinsic subtype from primary breast cancer and matched brain metastasis ($N = 21$). (C) 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).
FDR, false discovery rate; HER2, human epidermal growth factor receptor type 2; HR, hormone receptor; IHC, immunohistochemistry; NS, not significant; TNBC, triple-negative breast cancer.

HER2-enriched subtype during brain metastasization. Indeed, in a previous study assessing 677 HER2-positive stage I-III BC patients, we reported that, even within HER2-positive BC, HER2-enriched tumors were more prone to develop BMs as compared with other PAM50 subtypes (10-year incidence 3.8% versus 0.6%, $P = 0.005$).[11] 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 toward the HER2-enriched subtype, and this was observed even in paired samples which consistently remained


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HER2-positive both on the primary BC and BM sample. This observation is consistent with what was 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.[12] 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-positive BC. Moreover, this appears to differ from what was generally observed during metastatic recurrence, as previous studies have reported that the 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 the HER2-enriched phenotype.[13] Therefore, the switch toward 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-positive/HER2-negative BMs, constituting half of these samples. This represents a very high proportion when compared with the relative rarity of non-luminal intrinsic subtypes in this context, which generally represent <10% in early HR-positive/HER2-negative BC and around 20% in metastatic HR-positive/HER2-negative BC.[14,15] Gene expression data from matched primary BC samples were available only for a very limited number of patients with non-luminal HR-positive/HER2-negative BCBM, showing that in three out of four 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-positive/HER2-negative BC might potentially present a specific metastatic tropism, thus adding to the increasing evidence that non-luminal disease within HR-positive/HER2-negative BC represents distinct biological and clinical features. In addition, we also observed that within the subgroup of patients with HR-positive/HER2-negative BCBMs, tumor biology as assessed by the gene signatures of PAM50 algorithm was significantly associated with patients' 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 with luminal HR-positive/HER2-negative metastatic BCs and some clinical trials are currently under way to try to test this hypothesis.[15]

### *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,[5] we observed in 15 BMs out of 21 (71%) an increase in ERBB2 (HER2) expression and FGFR4 expression as compared with 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 trastuzumab emtansine,[16,17] and its role in predicting response to other HER2-targeted antibody—drug conjugates 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.[18] On the other hand, 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 with respective primary tumors, which in the context of HR-positive/HER2-negative metastatic BC has been reported to be associated with a relative resistance to cyclin-dependent kinase 4/6 inhibitors.[19,20] In addition, the activation of the 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.[21,22] 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-negative cases shifted to HR-positive, and one HER2-negative case shifted to HER2-positive). 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 with 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-positive BC) in which presence of immune infiltrate is generally associated with an activation of the immune system, characterized by an increase in both cytotoxic and pro-tumoral components of the immune system (e.g. CD8+ and FOXP3+ cells).[23,24] Overall, the observation of a decrease in 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 with their primary tumors and that lower levels of expression of immune-related genes are generally observed in brain lesions as compared with other metastatic sites in BC patients.[25,26] 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.[24]

Interestingly, when we assessed the 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 was previously discussed, higher expression of these genes was associated with better OS from BM diagnosis. In this study, we also assessed the association between single-gene expression and survival. This exploratory analysis requires further confirmation in independent studies; nevertheless, the highlighted genes deserve further exploration as potential therapeutic targets. For instance, CD276 (B7-H3) is currently being investigated as a target for novel immunotherapy agents and chimeric antigen receptor T-cell therapies across various solid tumors.[27] 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.[28,29] 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.[30]

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 the HER2-enriched subtype on the BM. However, the same trend toward switching to the HER2-enriched subtype was observed in tumors originally classified as basal-like on the primary tumor sample, thus supporting the hypothesis that the shift toward the 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-positive 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 >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-negative BC patients with BMs, and therefore warrants further exploration in translational and clinical studies.

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## DISCLOSURE

GG reports fees for advisory role from Gilead, Seagen, and Menarini; personal fees as an invited speaker from Eli Lilly, Novartis, and MSD; travel support from Gilead, Eli Lilly, Pfizer, Novartis, Daiichi Sankyo, AMGEN, and AstraZeneca. MVD reports personal fees for consultancy/advisory role from: Eli Lilly, Pfizer, Novartis, Seagen, Gilead, MSD, Exact Sciences, AstraZeneca, Roche, Daiichi Sankyo, and Roche. MB reports travel support from Eli Lilly. LB reports advisory board for Novocure and Servier. FM reports personal fees from Roche, Novartis, Pfizer, Seagen, Menarini, MSD, Gilead, and AstraZeneca. WJ reports grants, personal fees, and non-financial support from AstraZeneca; personal fees and non-financial support from Eisai; personal fees and non-financial support from Novartis; personal fees and non-financial support from Roche; personal fees and non-financial support from Pfizer; personal fees and non-financial support from Eli Lilly; personal fees from MSD; personal fees from BMS; personal fees and non-financial

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## DATA SHARING

Data can be made available through request to the corresponding author after fulfilment of legal/ethical requirements. We warmly encourage investigators interested to contact the corresponding author.

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