UNIVERSITAT DE BARCELONA

Uncovering the molecular and cellular mechanisms of metastatic dormancy in luminal breast cancer

Juan Miguel Cejalvo Andújar

Programa de Doctorado en Biomedicina
Universitat de Barcelona

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PhD Thesis presented by
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Thesis Directors:
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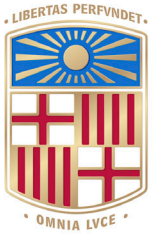
Thesis Tutor:
Dr. Manuel Palacín

September, 2019



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FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

Programa de Doctorat en Biomedicina

Uncovering the molecular and cellular mechanisms of metastatic dormancy in luminal breast cancer

Institute for Research in Biomedicine, IRB Barcelona
Institut d'Investigacions Biomèdiques August Pi i Sunyer, IDIBAPS

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CERTIFICAN:

Que la presente tesis doctoral, titulada "UNCOVERING THE MOLECULAR AND CELULLAR MECHANISM OF METASTATIC DORMANCY IN LUMINAL BREAST CANCER", que presenta Don Juan Miguel Cejalvo Andújar para optar al Grado de Doctor por la Universidad de Barcelona, ha sido realizada bajo su dirección dentro del programa de Biomedicina, y que se encuentra finalizada y lista para su presentación a fin de que pueda ser juzgada por el tribunal correspondiente.

Y para que así conste, firman la presente en Barcelona, a 22 de octubre de 2019.

Fdo.: Dr. Aleix Prat

Fdo: Dr. Roger Gomis

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INTRODUCTION

INTRODUCTION

1. Introduction

Breast cancer is the most common cancer in women and represents the most common cause of cancer-related death in women worldwide[1]. Advances in early detection, prevention, risk stratification, and therapeutic strategies as well as supportive care for patients have resulted in important improvements in mortality and reduction of cancer relapse. However, around 20% of the patients will present metastatic disease. Advanced breast cancer is still an incurable disease with a median overall survival (OS) of ~3 years, while the 5-year survival of only ~25%[2]. The median overall survivals of patients with triple-negative (TNBC), hormone receptor (HR)-positive/HER2-negative and HER2-positive diseases are approximately 12, 20, and 56 months, respectively[3 4 5 6].

The need of a deeper understanding of cancer biology, inter- and intra-tumor heterogeneity, and as well as, the biology behind the progression of tumor cells toward metastasis is urgent. To date, evidence suggests that both intrinsic properties of breast cancer cells and host organ microenvironment participate actively to this matter[7].

In general, detectable distant breast cancer metastases occur years, or even decades, after primary tumor diagnosis. These secondary lesions are supposed to originate from disseminated tumor cells that underwent a period of dormancy[8] which is the result of equal rated of cell proliferation and cell death[9]. However, the molecular factors that promote the formation of detectable metastasis from disseminated tumor cells are largely unknown. To try to explore this phenomenon, several studies were conducted to identify the molecular differences between primary tumor and their matched metastatic lesions[10]. At the DNA level, although significant differences have been observed, the majority (80-85%) of molecular alterations are preserved. For example, the discordance of HER2 gene amplification by FISH in primary

versus metastatic tissue is 3-10%[11]. Similarly, at the protein level, estrogen and progesterone receptors by immunohistochemistry (IHC) are discordant in 13-28% of cases[12]. Despite it, these results suggest that minor but important molecular changes occur during metastatic progression such as *ESR1* mutations[13].

The genomic revolution has transformed the landscape of clinical research in cancer, increasing the understanding of cancer biology, the identification of driver mutations as potential targets, and the mechanisms of sensitivity and resistance to conventional and newer targeted therapies. In terms of global gene expression, four main molecular subtypes [Luminal A, Luminal B, HER2-enriched (HER2-E) and Basal-like], and a normal breast-like group, have been identified and intensively studied for the last 15 years in early breast cancer[14 15 16 17]. Known as the "intrinsic subtypes of breast cancer", these groups of tumors have revealed critical differences in tumor development[18 19], survival[20 21 22], and response to treatment[23 24]. Importantly, the information provided by the intrinsic subtypes complements and expands the information derived by classical clinical parameters (e.g., age, node status, tumor size, histologic grade) and pathologic markers [estrogen receptor (ER), progesterone receptor (PR), and HER2][25 26], all of which are routinely used today in the clinic to stratify patients for prognostic predictions and to select treatments.

The greatest obstacles for improving the breast cancer outcomes include (1) the limited proportion of breast tumors found to have actionable mutations, (2) the need to integrative DNA- and RNA-based approaches, (3) mixed results in terms of patient outcomes and molecular targeted, and (4) biologic heterogeneity across population and intrapatient tumor.

2. Intrinsic Molecular Subtypes of Breast Cancer

Breast cancer is a clinically and biologically heterogeneous disease. According to the expression of single biological biomarkers such as ER, PR, and HER2, breast cancer can be classified today into the following 4 main pathological-based groups: ER-positive and/or PR-positive and HER2-negative (HR+/HER2-negative), ER-positive and/or PR-positive and HER2-positive (HR+/HER2+), ER-negative and PR-negative and HER2-positive (HR-negative/HER2+) and TNBC. This pathology-based classification is routinely used in the clinic to stratify patients for prognostic predictions and to select treatments.

In the last decade, gene expression profiling has had a considerable impact on our understanding of breast cancer biology. Nowadays, we have extensively characterized 4 main intrinsic molecular subtypes of breast cancer (Luminal A, Luminal B, HER2-E, Basal-like) and a normal breast-like group. These entities have shown significant differences in terms of incidence, risk factors, prognosis and treatment sensitivity. More importantly, these molecular entities have shown to provide additional prognostic and predictive information beyond pathological-based calissification[27 28 29].

At the RNA and protein level, Luminal A and B subtypes are largely different according to the expression of two main biological process: proliferation/cell cycle-related and luminal/hormone-regulated pathways. Compared to Luminal A, Luminal B tumors have higher expression of proliferation/cell cycle-related genes or proteins (e.g. MKI67 and AURKA) and lower expression of several luminal-related genes or proteins such as the PR and FOXA1, but not the ER, which is found similarly expressed between the two luminal subtypes and can only help in distinguish luminal from non-luminal disease. At the DNA level, Luminal A tumors show lower number of mutations across the genome, lower number of chromosomal copy-number changes (e.g. lower rates of CCND1 amplification), less TP53 mutations (12% vs. 29%), similar GATA3 mutations (14% vs 15%) and more PIK3CA (45%

vs 29%) and MAP3K1 mutations (13% vs 5%) compared to Luminal B tumors. Interestingly, a subgroup of Luminal B tumors is found hypermethylated, and a subgroup of Luminal A (6.3–7.8%) and Luminal B (16.4–20.8%) tumors show HER2-amplification/overexpression[30].

The HER2-E subtype is characterized at the RNA and protein level by the high expression of HER2-related and proliferation-related genes and proteins (e.g. ERBB2 and GRB7), intermediate expression of luminal-related genes and proteins (e.g. ESR1 and PGR) and low expression of basal-related genes and proteins (e.g. keratin 5 and FOXC1). At the DNA level, these tumors show the highest number of mutations across the genome, and 72% and 39% of HER2-E tumors are TP53 and PIK3CA mutated, respectively. Although the majority (68%) of HER2-E have HER2 overexpression/amplification, we should expect to identify the HER2-E subtype within HER2-negative disease. Interestingly, the HER2-E subtype has been found uniquely enriched for tumors with high frequency of APOBEC3B-associated mutations. APOBEC3B is a cytidine deaminase, which converts cytosine to uracil during RNA editing and retrovirus or retrotransposon restriction, and may induce mutation clusters in human tumors[31]. Several studies have now linked APOBEC genetic signatures with the HER2-E subtype in breast cancer[32]. Intriguingly, both the APOBEC signatures and the HER2-E profile have been associated with high mutational burden and high expression of immune genes and immune infiltration.

The Basal-like subtype is characterized at the RNA and protein level by the high expression of proliferation-related genes (e.g. MKI67) and keratins typically expressed by the basal layer of the skin (e.g. keratins 5, 14 and 17), intermediate expression of HER2-related genes, and very low expression of luminal-related genes. At the DNA level, these tumors show the second highest number of mutations across the genome, mostly are hypomethylated, and 80% and 9% of Basal-like tumors are TP53 and PIK3CA mutated, respectively. BRCA1-mutated breast cancer is associated with Basal-like disease. Finally, HER2 overexpression/amplification is found in 2.1–17.4% of tumors with a Basal-like profile.

a. Subtype distribution within pathology-based groups

In 2009, Parker and colleagues introduced a clinically applicable gene expression-based test, known as PAM50, which identifies the main breast cancer intrinsic molecular subtypes in formalin-fixed paraffin embedded tumor tissues by using 50 genes[33]. Since then, this assay has allowed the identification of these molecular entities across a large number of studies, including tumor samples from various phase III clinical trials. Among them, we have finally selected 31 trials in which PAM50 and pathological markers were assessed in primary breast cancer. The characteristics used to define clinicopathological subtype were IHC evaluation for hormone receptors and IHC and/or FISH results for HER2.

In order to assess the concordance between these two classifications and to understand the biologic heterogeneity of breast cancer, we have combined the data across these independent cohorts for a total of 21,113 samples. We defined clinicopathological subtype category as Luminal (HR+/HER2-), Luminal/HER2+ (HR+/HER2+), HER2+ (HR-/HER2+) and TNBC (ER-/HER2-). All tumors were assigned to an intrinsic molecular subtype Luminal A (LumA), Luminal B (LumB), HER2-E, Basal-like, and Normal-like group. The majority studies have performed a standardized version of the PAM50 assay (RT-qPCR-based or nCounter-based), while others have performed the microarray-based version of the PAM50 assay.

HR and HER2 statuses, and PAM50 data, were available in 16,286 tumors. In the other 22.86% (4,827 out of 21,113) we do not have complete information about ER, PR or HER2 expression. The distribution of the pathology-based groups in this combined dataset was as follow: 46.26% (n=9,768) HR+/HER2-negative, 9.80% (n=2,069) HER2+/HR+, 7.30% (n=1,542) HER2+/HR-negative, 13.77% (n=2,907) TNBC, 8.83% (n=1,865) HER2+, and 14.03% (n=2,962) HR+. The distribution of the intrinsic subtypes in this combined dataset was as follows: 38.60% (n=8,151) Luminal A, 24.85% (n=5,248) Luminal B, 18.97% (n=4,004) HER2-enriched, 14.33% (n=3,025) Basal-like and 3.25% (n=685) Normal-like (Fig. 1).

Within each pathology-based group, all the intrinsic molecular subtypes were identified, albeit with different proportions. In HR+/HER2-negative disease, 5.64% and 2.24% of tumors were identified as HER2-enriched and Basal-like, respectively (Fig 1B). In TNBC, 10.59%, 3.53% and 2.06% were identified as HER2-E, Luminal A and Luminal B, respectively (Fig. 1C). Finally, 45.35% of HER2+ tumors were not HER2-E (Fig. 1D). Subtype distribution within HER2+ disease was not entirely explained by HR status. Within HER2+/HR+ disease, 31.64% and 2.46% of the tumors were HER2-E and Basal-like, respectively (Fig. 1E). Within HER2+/HR- negative disease, 13.82%, 6.16% and 2.08% were identified as Basal-like, Luminal A and Luminal B, respectively (Fig. 1F).

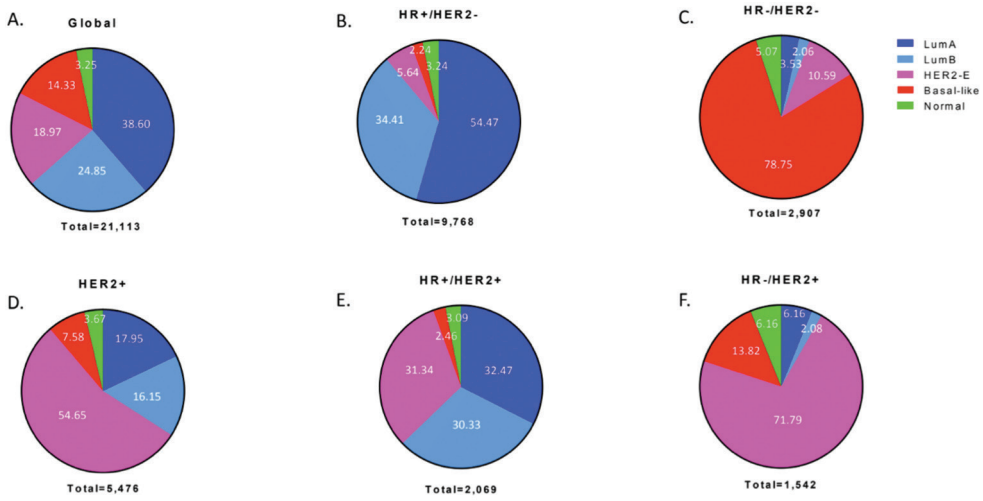

Fig. 1. Molecular heterogeneity of early breast cancer. Intrinsic subtype distribution (A) in global cohort; (B) in HR+/HER2-; (B) in HR+/HER2-; (C) in HR-/HER2-; (D) in HER2+; (E) HER2+/HER2+; and (F) HR-/HER2+

This pooled analysis finally demonstrated two relevant situations: 1) The clinicopathological classification should not be considered synonymous of intrinsic subtype profile, suggesting that the two methods currently used to define breast cancer characteristics should not be considered the same; 2) breast cancer is a heterogeneous disease and all the intrinsic molecular subtypes is represented in each classical subgroup.

b. Clinical implications between molecular subtypes and pathological classification:

HR+/HER2-negative versus non-Luminals

Luminal breast cancer represents about 70% of all new diagnosis[34]. These patients have the best prognosis but, despite the benefit of endocrine therapy, recurrence appears in approximately 10-15%[35]. PAM50 classification suggests that it could be useful to predict both prognostic and predictive benefit deriving from the use of hormonotherapy in adjuvant setting, offering the possibility to personalize treatment for luminal breast cancer patients.

In neoadjuvant setting Ki67 expression has been used as a surrogate indicative of the effect of endocrine treatment outcome. ACOSOG Z1031 trial[36] assessed the preoperative aromatase inhibitor treatment. Basal-like and HER2-E patients presented high pre- and post-treatment Ki67 levels consistent with endocrine resistance. And both Luminal A and Luminal B tumors were highly endocrine-therapy responsive, however Luminal B had significantly higher post-treatment Ki67 levels, according with the worse prognosis associated with this subtype. In the same line, Dunbier et al.[37] also showed that Luminal A and B obtained similar benefit from neoadjuvant anastrozole treatment, despite Luminal B initially having higher levels of Ki67, while Basal-like and HER2-E presented poor reductions in Ki67 upon treatment, so poorer responses.

Adjuvant endocrine therapy is the backbone of systemic treatment in hormone receptor positive breast cancer. In order to assess for both prognostic and predictive value of PAM50, it was identified in a cohort of premonopausal women with primary breast cancer (NCIC CTG MA.12)[38] where they were randomized of tamoxifeno vs placebo. The intrinsic subtypes analysis were prognostic for both DFS and OS, luminal subtypes presented a statistically significant benefit from adjuvant tamoxifen

(HR: 0.52, p=0.009). In particular, patients diagnosed with HER2-E subtype were shown to have the lowest benefit while the Luminal A the highest benefit deriving from the use of tamoxifen.

A similar analysis to evaluate response to multi-agent neoadjuvant chemotherapy was also performed in the same setting of patients. Data demonstrated that non-Luminal (Basal-like and HER2-E) showed higher pCR rates than luminals (Luminal A and B) (30% vs 8-9%)[39].

According to the data presented, PAM50 analysis seems to be able to screen patients who will really benefit from hormonotherapy rather than chemotherapy. So, in patients with HR+/HER2-negative PAM50 risk of recurrence score associated with other clinicopathological parameters could provide a relevant tool to take decisions about adjuvant systemic treatment[40]. To further support this hypothesis, PAM50 assay was performed in a cohort of postmenopausal luminal patients who participated in the ABCSG-8 trial (5 years of adjuvant tramoxifen vs tamoxifen 2 years followed by anastrozole for 3 years without chemotherapy). DFS analysis showed a significantly difference between Luminal A and B (HR: 2.49, p<0.001). The 15-year late DFS estimated were 92.8% for Luminal A and 86.2% for the luminal B, this magnitude of prognostic differences was greater between ROR low- and high-risk groups (absolute risk distant recurrence of 2.4% in low ROR and 17.5% in the high ROR group)[41]. So both, ROR groups and breast intrinsic subtype demonstrated clinically meaningful differences with respect to early and late risk of distant recurrence. Therefore, with this evidence there is a significant and clinically relevant discrimination between these two groups. This finding could help to avoid unwarranted overtreatment. The identification of a toll able to discern whom patient will benefit from only hormonal therapy from whom will not, is fundamental to personalize treatment, avoiding chemotherapy in those patients who will not be sensitive to this treatment and who will only benefit from an endocrine approach.

In advanced disease, endocrine therapy is the preferred option for luminal breast cancer. Nowadays, in the presence of visceral disease, unless there is proven visceral crisis or endocrine resistance. In 2016 the first study reporting the prognostic and predictive value of intrinsic subtype in first-line HR+ metastatic breast cancer was published. Interestingly, it was possible to observe that the identification of intrinsic subtypes could drive treatment. In particular, it was observed that those patients diagnosed with HR+/HER2- disease but with a HER2-E profile according to PAM50, may finally benefit from the combination of endocrine therapy with Lapatinib (median progression free survival, 6.49 vs 2.60 months, p= 0.02)[42]. This event could justify the clinical relevance of the identification of the intrinsic subtype in advanced disease to plan personalized treatment.

HER2-E versus HER2+

HER2 overexpression or amplification is known to be an independent poor prognostic factor but it also recognized to be a predictive factor of response to anti-HER2 treatment. Classically randomized clinical trials have demonstrated the clinical efficacy of trastuzumab plus chemotherapy in treatment of HER2+ breast cancer disease. When a HER2 positive breast cancer is newly diagnosed and treated with trastuzumab plus chemotherapy, the DFS and OS rates expected are 84% and 92% at five years[43]. However, heterogeneity of this subset of breast cancer has an impact in responses and clinical outcomes.

Nevertheless, the prognostic value of HER2 status disappeared when subtypes were taken into account in the absence of HER2 targeting, each intrinsic subtypes showed similar survival regardless of their HER2 status[44 45]. Several trials in neoadjuvant and adjuvant setting have test the implication of molecular subtype in predicting treatment responses:

1. Neoadjuvant Treatment

Neoadjuvant setting allows direct and early observation of the response to treatment. This approach provides a potential surrogate marker as pCR. Some trials have been used to assess the capability of intrinsic molecular subtype to predict tumor response.

A retrospective analysis of NOAH trial showed that HER2-E disease presents a high response rate with the addition of trastuzumab at the chemotherapy regimen. The combination benefit was observed also in terms of DFS in the HER2-E subtype (HR= 0.43) compared with non-HER2-E tumors (HR= 0.87)[46].

Combined administration of two different HER2-targeted agents has been tested. In PAMELA trial, a phase II single group, was assessed the efficacy of dual HER2 blockade (trastuzumab plus lapatinib) without chemotherapy. HER2-E subtype was associated with pCR compared with patients who had non-HER2-E profile (41% vs 10%), this finding was independent of hormone receptor status[47]. The use of antiHER2 blockades in neoadjuvant setting was also evaluated in the NeoALTTO, a phase III randomized clinical trial, compareing trastuzumab, lapatinib, or the combination followed by the addition of paclitaxel. The expression of ERBB2 was the most significant predictor of pCR, followed by HER2-E subtype. A third clinical trial, CherLOB phase II study[48], evaluated the impact of tumor-related and immune-related diversity of HER2+ disease on the response to neoadjuvant chemotherapy plus trastuzumab, lapatinb or their combination. In this case, the highest rate of pCR was observed for the HER2-E (50%), followed by Basal-like, Luminal B and Luminal A (p= 0.026). In agreement with these results, HER2-E achieved the greater pCR (70%), compared with luminal A (34%) and B (36%) in CALGB40601 trial. This is a randomized phase III trial[49], where was examined the impact of trastuzumab plus lapatinib added to paclitaxel.

In conclusion the molecular diversity of HER2+ breast cancer disease was reflected in PAM50 subtype, showing different response to neoadjuvant treatment.

2. Adjuvant Treatment

In the N9831 trial[50], HER2+ patients were analyzed to assess the association between intrinsic subtype and clinical outcome. The PAM50 subtypes were statistically significantly associated (irrespective of therapy) with DFS. Patients with Basal-like tumors showed worse DFS compared with the others subtypes. In the arm with chemotherapy alone, there was no statistically significant association between subtypes and DFS. However, a statistically significant association was observed between survival outcome and intrinsic subtype among patients who received trastuzumab and Basal-like group showed less benefit than the others subtypes (HR= 1.06, p=0.87). A possible answer to these results could be that in NSABP B-31[51] (a randomized phase III clinical trial to compare chemotherapy alone or chemotherapy plus trastuzumab) all PAM50 intrinsic subtypes received benefit from trastuzumab treatment in terms of DFS. Both the HER2-E subtype and the non-HER2-E subtype improved the outcomes, the HR for trastuzumab arm in the HER2-E subtype was 0.44, and in the non-HER2-E was 0.47, $p< 0.001$. However, both studies were limited in power by the relative small number of Basal-like tumors. Further investigations are needed.

TNBC versus Basal-like

Triple negative breast cancer is pathologically defined by the absence of HR and HER2 expression. This subgroup benefits only from chemotherapy, there is no predictive factors of response to use targetable drugs for these group of patients. Several clinical trials with multi-agent chemotherapy have evaluated the ability of intrinsic subtype to predict response and/or survival in early disease. In GEICAM2006-03 and MDACC neoadjuvant trials[52] were assessed the pCR, none of the intrinsic subtype evaluated was found significantly associated with pCR. However, among patients with basal-like phenotype, low expression of the luminal

A signature and high expression of proliferation score were statistically significant associated with pCR. In general, among the different pathological subtypes, the Basal-like shows consistently greater pCR rates.

To predict DFS in adjuvant treatment, PAM50 were analyzed in TN breast cancer patients from GEICAM/9906. Within TN breast cancer of the basal-like subtype, these two signatures (the low expression of luminal A and high expression of proliferation score), were confirmed a statistical association with DFS.

In metastatic setting, the TNT clinical trial compared treatment with carboplatin and docetaxel, both standard chemotherapy. PAM50 analysis showed a better objective response rate in non-basal subtypes with docetaxel (73.7%) than with carboplatin treated group (16.7%)[53]. And there was no difference between Basal-like group. Thereby, sub-classification of TN breast cancer into intrinsic subtype can help to identify prognostic and predictive biomarkers.

As conclusion, basing on these data, it is possible to suggest that multigene assays could provide prognostic and predictive information beyond pathological parameters and may support more-informed treatment decision[54]. The molecular subtypes of breast cancer significantly extend our knowledge about behavior of the disease. This makes necessary that in future clinical trials should consider stratifying patients by intrinsic molecular subtype.

3. Tumor and microenvironment alteration during metastasis

Metastasis frequently develops years after the removal of a primary tumor, from a minority of disseminated tumor cells (DTCs) that survived as latent entities through unknown mechanism. Before diagnosis and treatment, primary tumors may release large numbers of cancer cells into the circulation. Although a majority of the DTCs perish in the blood-stream or soon after infiltrating distant organs, a minority may survive as latent seeds in host tissues. Thereby, patients who are clinically considered

disease-free after cancer treatment may carry thousands DTC in the bone marrow and other organs as liver, lung or brain.

Latent metastasis is a major concern in the clinic, up to know, very little is known about the nature of dormant DTCs and the mechanisms that allow these cells to remain quiescent, evade immunity, retain tumor-initiating capacity, and evolve into aggressive metastasis.

One hypothesis is that DTCs are tumor-initiating cells that enter in quiescence by the action of growth inhibitory signals from the host tissue stroma. However, organs that host DTCs, such as the bone marrow, liver, and lungs, support cell proliferation as part of their normal tissue homeostasis and regenerative processes, raising questions as to whether stromal growth inhibitory signals are persistent enough to enforce long-term metastatic latency[55].

Another important point, is the role of immunity in latent metastasis. The interplay between cancer cells and the immune system plays a crucial role in tumor progression. Metastatic latency may therefore require DTCs to be in equilibrium with the immune system.

a. The immune landscape of breast cancer

A fundamental role of the immune system is maintenance of tissue homeostasis by continuous immunosurveillance and initiation of inflammatory reactions that involve the activation of innate and adaptive immune cells. Neoplastic transformation alters the orderly structure of tissues and induces immune responses that can eliminate incipient tumors. However, immunocompetent individuals also develop cancers in spite of the immunosurvillance. In these situations malignant cells escape immune control and a tumor develops[56]. Three different parameters are taken in account to evaluate immune response in solid tumors, such as tumor infiltrating lymphocyte, mutation burden and PD-L1 expression.

The host immune response

Tumor cells suffer several genomic alterations, generating neoantigens that can be identified by the immune system. The interaction between the immune system and tumor cells, also called immunoediting, goes through three phases: elimination, equilibrium, and escape. In the elimination process, the innate and adaptive arms of the immune system recognize incipient cancer cells by this neoantigens presented on their surface in association with MHC-I or by the distress signals usually expressed by transformed cells that have undergone chromosomal changes (aneuploidy or hyperploidy) and eliminate them.

Equilibrium is reached when the immune system fails to eliminate the transformed cells but stops them from progressing. This can be conceived as the dormancy phase of cancer. This phase is mediated by equilibrium between cells and cytokines that promote elimination such as CD8+ cytotoxic T cells, NK cells, T-helper 1 (Th1), or DC while other cells promote tumor progression (Th2, and Foxp3+ regulatory T [Terg]). In addition, the tumor microenvironment sends concomitant dangerous signals to the host immune system (as inflammatory, hypoxic, and often necrotic microenvironment). B cells and plasma cells can also adopt a positive or negative antitumor associations depending on contextual factors.

Therefore, infiltrating immune cells can function to control tumor growth, but can also help to create an immunosuppressive environment in which the tumor can progress (Fig 2).

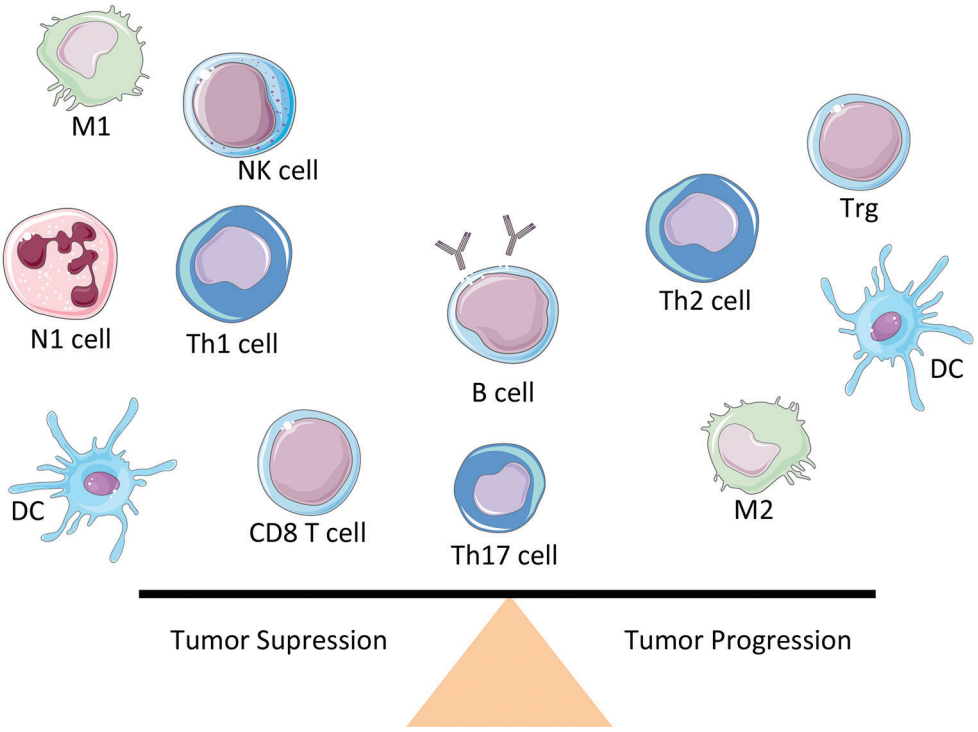

Fig. 2. The cellular cross-talk between different leukocyte subsets and their predominant contribution to either pro- or antitumor activities, including myeloid lineage leukocytes, tumor-associated macrophages with either protumorigenic (M2) or antitumorigenic (M1) properties, helper T-cell subsets, cytotoxic T cells, regulatory T cells, B cells, dendritic cells and myeloid-derived suppressor cells are shown. These cells play central roles in shaping the microenvironment via the factors they produce thereby driving either an immune-mediated anti- or protumor activities in the microenvironment.

Some cancers present hundreds or even thousand mutations, representing a large repertoire of antigens that could be recognized by the immune system. But despite expression of abundant antigens, most cancers progress and evade immune system. Many immune escape mechanisms have been identified, including local immune suppression, induction of tolerance, dysfunction in T-cell signaling, and evasion of immune destruction by expression of endogenous “immune checkpoint” that normally terminate immune responses after antigen activation[57].

Although patients are most frequently diagnosed in the escape phase, even at these advanced disease stages, immune parameters have been recognized as directly or indirectly influencing patient survival. The exact composition of the immune infiltrate can vary widely within and between tumors and modulate the effectiveness of the antitumor response. It seems that adaptive immunity mediated by T and B lymphocytes provides the critical foundation for effective and sustained antitumor responses. Therefore, defining these immune phenotypes may aid in predictive biomarker development for classes of immunotherapy.

In breast cancer, tumor infiltration by cytotoxic CD8+ T cells was strongly associated with patient survival, and response to therapy. The presence of CD4+ regulatory T cells (Treg) has been associated with both good and bad prognosis. Among the other CD4+ T-cell subpopulations, Th1 cells (the principal cellular source of interferon-γ) have been associated with favorable clinical outcomes, whereas Th2 cells have been reported to be associated with dampening of the antitumor response. Th17 cells, producers of the proinflammatory IL-17, appear to have variable effects depending on the surrounding cytokine milieu, which may in part be linked with the organ site and tumor type. The precise role of tumor-infiltrating B cells is currently not well defined and remains controversial.

Despite the heterogeneity of intratumor lymphocytes it is interesting that the degree of tumor-infiltrating lymphocytes (TILs) has shown to have prognostic and predictive value in HER2+ and TNBC in spite of a lack of information on the immune subpopulations.

Thus, despite the inability of the immune system to reject a clinically detectable tumor, an organized immune response at the tumor site may signal the generation of immunological memory with the potential to effectively control residual disease.

Different methods to evaluate the host immune response

Flow cytometry is a common approach to immune cell profiling and it is able to characterize immune cell subsets by multiple markers, quantitative data acquisition, wide availability, and possess also the ability to examine small subpopulations of interest. However, fresh tissue is required and no information is provided on the organization of the immune infiltrate or relationship to other microenvironmental structures. Nevertheless, recent study of TILs in invasive breast carcinoma found a significant positive correlation between fresh tumor tissue analyzed by flow cytometry and IHC-stained sections. Nowadays, a semiquantitative H&E-based TILS assessment provides clinically relevant information. This technique is affordable and accessible.

Another way to evaluate the immune context of cancer is studying mRNA profiling of tumor tissue. By doing that, it is possible to detect "immune signatures", using the level of expression of immune-related genes to describe the composition and functional status of the immune infiltrate[58]. No information is provided on the distribution of the infiltrate with this method furthermore this technology is currently restricted to a research setting. This method is more complex and more difficult to be implemented in clinical practice.

Immunotherapy in Breast Cancer

Recently, new therapies that reactivate anticancer immune responses to cancer have been introduced in clinical practice improving clinical outcomes and several studies are on-going to evaluate new drugs and combinations. Clinical trials have demonstrated durable responses in different solid tumors, nevertheless, the benefit within tumor types vary widely.

One of the most studied mechanisms leading to tumor immune evasion is the expression of immune checkpoint molecules such as cytotoxic T lymphocyte antigen-4 (CTLA4) and programmed death ligand-1 (PD-L1), both on tumor cells

and on infiltrating immune cells. By blocking these signaling pathways, immune checkpoint inhibitors can reactivate the host immune system to recognize and control the tumor cells.

A particular challenge in immunotherapy is the identification of predictive biomarkers to discern responders from non-responders and to guide disease-management decision. For this reason, there is a strong need in identifying consistent, largely applicable, and clinically validated biomarkers.

Emerging data suggest that patients whose tumors overexpress PD-L1 by IHC have improved clinical outcomes with anti-PD1-directed therapy tumors. Nevertheless, the role of PD-L1 expression and its relation with response to checkpoint inhibitors is actually controversial.

Interestingly, in breast cancer, TILs were shown to correlate with pathological complete response (pCR) after neo-adjuvant chemotherapy in all breast cancers. However, a significant correlation between TILs at diagnosis and overall survival was only observed in TNBC and HER2.

Immune genes expression

The development of gene expression profiling of tumors has enabled to identify prognostic gene expression signatures and patient selection for targeted therapies. Recently several studies have evaluated the association of immune-related gene expression in patients with different solid tumors treated with immunotherapy. Among them, the IFN-inflammatory immune gene expression signature was associated with both enhanced overall response (OR) rates and PFS in patients with melanoma treated with an anti PD-1 monoclonal antibody, pembrolizumab[59]. Another examples of signature predicting response to immunotherapy, includes an eight-gene signature reflecting preexisting immunity, the T-effector/IFNγ signature, explored in a phase II trial of previously treated non–small cell lung carcinoma[60].

If validated, the implementation of these or others signatures will require the use of robust and reproducible genomic-based platforms to select treatment in clinical practice.

According to all these considerations, we have to assume, that we are at the beginning of a new paradigm in breast cancer therapy. However, at present, the biologic diversity of breast cancer remains elusive. Futhermore, the burden of mutations is lower in breast cancer than in classically immunologically tractable cancers, such as melanoma or lung cancer, and breaking immune tolerance appears to be more difficult.

b. Chromosomal instability

Chromosomal instability (CIN) results in alterations in chromosome number or structure, developing intercellular genetic heterogeneity and therefore intratumor heterogeneity. Numerical CIN is characterized by gain or loss of whole chromosomes (aneuploidy), while structural CIN is characterized by gain or loss of fractions of chromosomes.

This alteration is a hallmark of solid tumors, and it has been implicated in tumor evolution, increased invasiveness, poor prognosis and, therapy resistance. Furthermore, it has been reported that CIN is highest in the most aggressive and metastatic cancer types

Breast cancer has different patterns of chromosomal alterations, as there are some DNA amplifications associates with the Luminal B and HER2-E subtypes. One of the most notable and classic examples is amplification of *HER2*, but there are other amplified sites that include *FGFR1*, *MYC*, *CCND1*, *MDM2*, and *ZNF217*[61].

Finally, CIN in breast cancer has been correlated with higher tumor grade, poorer survival and shorter times to recurrence. These observations suggest that CIN have important clinical implications.

Nevertheless, modest level of genetic diversity provides an advantage in tumors, excessively high rates of CIN cause tumor death, as inviable progeny. These considerations suggest that cancer cell to survive needs a balance and an optimal range of CIN (Fig. 3).

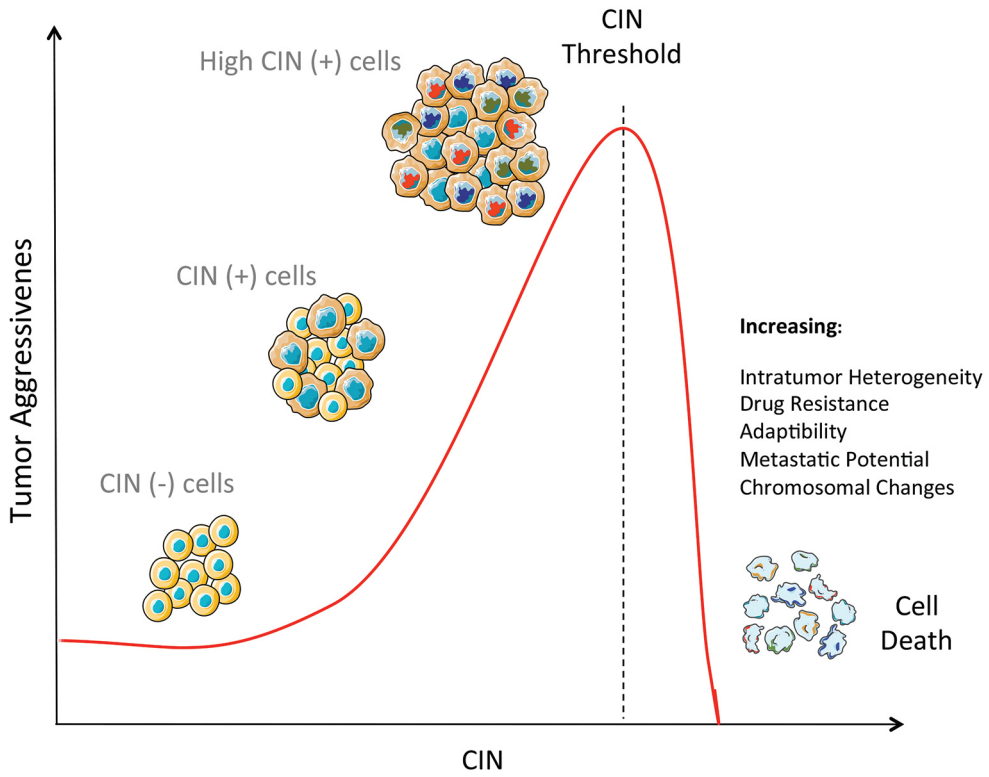

Fig. 3. High levels of CIN cause excessive chromosome loss and cell death, whereas modest levels of CIN provide sufficient genetic diversity for cancer.

A deeper understanding of the characteristics associated with CIN and the development of clinically applicable biomarkers are needed both for patient stratification and to leverage new therapeutic opportunities.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Most biological changes occur during metastatic progression of breast cancer. Thus, we hypothesized that there is a different biology behavior between primary and metastatic breast cancer. For this reason, we proposed a transcriptomic approach in paired primary and metastatic tissues from a cohort of metastatic breast cancer patients.

OBJECTIVES

Aim 1. To characterize metastatic disease and analyze the PAM50 subtypes between PT and MT

Aim 2. To analyzed the gene expression changes between PT and MT

Aim 3. To explore the role of chromosomal instability in the metastasis process

Aim 4. To explore the implication and changes of immune-system gene expression between both setting

MATERIAL AND METHODS

1. Study population

This retrospective study included non-consecutive female patients over the age of 18 years with a histologic diagnosis of metastatic breast cancer detected at the time of diagnosis, at first relapse or after successive disease progressions. Tissues were collected from five independent sources: GEICAM/2009-03 ConvertHER trial[62], Hospital Clínico Universitario de Valencia, Vall d'Hebrón Institute of Oncology, University-AO Papardo and Hospital Clinic of Barcelona. To be included, samples were required to have a formalin-fixed paraffin-embedded (FFPE) tissue sample from primary and metastatic tumor. Biopsies were performed by core biopsy or surgical process, according to the routine clinical practice of the hospitals. For each sample, receptor status (ER, PR, and HER2) were analyzed at the local laboratory.

2. Gene expression analysis and gene list

All primary and metastatic tissues were analyzed using the same methodology. A section of FFPE breast tissue was first examined with a hematoxylin and eosin staining to confirm the diagnosis and determine the tumour surface area. For RNA purification, three 10-μm FFPE slides were cut for each tumor, and macro-dissection was performed, when needed, to avoid normal breast contamination. A minimum of approximately 100 ng of total RNA was used to measure the expression of 105 breast cancer-related genes and 5 housekeeping genes (ACTB, MRPL19, PSMC4, RPLP0, and SF3A1) using the nCounter platform (Nanostring Technologies)[63]. Data was log base2–transformed and normalized using the housekeeping genes. Raw data have been deposited in the Gene Expression Omnibus under the accession number GSE92977.

The list of 105 breast cancer–related genes includes genes from the following three signatures: PAM50 intrinsic subtype predictor (n = 50)[64], claudin-low subtype predictor (n = 43)[65], VEGF/Hypoxia signature (n = 13)[66]. In 8 addition, we included individual genes that have been found to play an important role in breast cancer [i.e., CD24[67], CRYAB[68], ERBB4[69], PIK3CA[70], PTEN, RAD17[71], RAD50, and RB1]. In the following table, there is the complete list of genes:

PAM50 signature	ACTR3B, ANLN, BAG1, BCL2, BIRC5, BLVRA, CCNB1, CCNE1, CDC20, CDC6, CDCA1, CDH3, CENPF, CEP55, CXXC5, EGFR, ERBB2, ESR1, EXO1, FGFR4, FOXA1, FOXC1, GPR160, GRB7, KIF2C, KNTC2, KRT14, KRT17, KRT5, MAPT, MDM2, MELK, MIA, MKI67, MLPH, MMP11, MYBL2, MYC, NAT1, ORC6L, PGR, PHGDH, PTTG1, RRM2, SFRP1, SLC39A6, TMEM45B, TYMS, UBE2C, UBE2T
Claudin-low signature	CCNB1, CDC20, CDH3, ERBB2, FOXA1, GRB7, PTTG1, ADM, AXL, CAV1, CLDN3, CLDN4, CLDN7, DDR1, DSP, EMP3, EPCAM, ERBB3, ESRP1, EVI2A, F11R, FBN1, GATA3, GNG11, GRHL2, JUP, KRT19, KRT8, LEPRE1, LHFP, MET, MPP1, NT5E, PLOD1, PVRL3, RAB25, SH2B3, SPINT1, SPINT2, TMEM158, VAMP8, VIM, ZEB1
VEGFR/Hypoxia	RRAGD,FABP5, UCHL1, GAL, PLOD1, DDIT4, VEGFA, ADM, ANGPTL4, NDRG1, PNP, SLC16A3, FLVCR2
Others	CD24, CRYAB, ERBB4, PIK3CA, PTEN, RAD17, RAD50, RB1

Table 1. List of 105 breast cancer-related genes.

We analyzed the RNA sequencing (RNAseq) in 102 patients of this corhot. RNAseq had been used to identify biomarkers related with immune cells and chromosomal instability (CIN70[72]). We assessed 16 immune-related signatures[73 74] [i.e., CD8+ T, Th2 cells, innate immune, macrophages, NK cells, neutrophils, dendritic cells, mastocytes and B and T cells] and CIN (Table 2). In addition, we evaluated the following 6 immune-related genes including: *CD4*, *CD274*, *CTLA4*, *CD8A*, *CD45*, and *PDCD1*.

Mastocytes	PRG2, CTSG, TPSAB1, MS4A2, SLC18A2, CPA3, TPSB2, GATA2, HDC, LOH11CR2A, SIGLEC6, ELA2, CMA1, PGDS, MLPH, ADCYAP1, SLC24A3, CALB2, KIT, TAL1, ABCC4, PPM1H, MAOB, HPGD, SCG2, PTGS1, CEACAM8, MPO, NR0B1, LOC339524
iDC	CD1B, VASH1, F13A1, CD1E, MMP12, FABP4, CLEC10A, SYT17, MS4A6A, CTNS, GUCA1A, CARD9, CD1E, ABCG2, CD1A, PPARG, RAP1GAP, SLC7A8, GSTT1, PDXK, FZD2, CSF1R, HS3ST2, CH25H, LMAN2L, SLC26A6, BLVRB, NUDT9, PREP, TM7SF4, TACSTD2, CD1C
DC cells	CD209, CCL17, HSD11B1, CCL13, CCL22, PPFIBP2, NPR1
Macrophages	CXCL5, SCG5, SULT1C2, MSR1, CTSK, PTGDS, COLEC12, GPCA, MSR1, PCOLCE2, CHIT1, PTGDS, KAL1, CLEC5A, ME1, DNASE2B, CCL7, FN1, CD163, GM2A, SCARB2, BCAT1, RAI14, MSR1, COL8A2, APOE, CHI3L1, ATG7, CD84, FDX1, MS4A4A, SGNS1, EMP1, CYBB, CD68
Neutrophils	CSF3R, LILRB2

NK cells	LOC643313, GAGE2, ZNF747, XCL1, XCL2, AF107846, SLC30A5, NM_014114, MCM3AP, TBXA2R, CDC5L , LOC730096, FUT5, FGF18, MRC2, RP5-886K2.1, PN, PSMD4, PRX, FZR1, ZNF205, AL080130, ZNF528, MAPRE3, BCL2, NM_017616, ARL6IP2, SPN, PDLIM4, NM_014274, LDB3, ADARB1, SMEK1, TCTN2, TINAGL1, IGFBP5, ALDH1B1, NCR1
Innate immune	TIRAP, ECSIT, TLR8
Th2	PMCH, AHI1, PTGIS, CXCR6, EVI5, IL26, MB, NEIL3, GSTA4, PHEX, SMAD2, CENPF, ANK1, ADCY1, AI582773, LAIR2, SNRPD1, CXCR6, MICAL2, DHFR, WDHD1, BIRC5, SLC39A14, HELLS, LIMA1, CDC25C, CDC7, GATA3
CD8+T	PRF1, CD8A, GZMM, CD8B, FLT3LG
Cytotoxic cells	KLRD1, KLRF1, GNLY, CTSW, KLRB1, SIGIRR, ZBTB16, RUNX3, APOL3, RORA, APBA2, WHDC1L1, DUSP2, GZMA
CD83	TNFSF5, IKBKAP, RELA, NFKBIA, NFKB1, TNFRSF5, DUSP1, MAP3K14, IKBKG, MAP3K1, IKBKB, TNFAIP3, CHUK, TRAF6, TRAF3
B cells	MS4A1, TCL1A, HLA-DOB, PNOC, KIAA0125, CD19, CR2, BLK, IGHG1, COCH, OSBPL10, IGHA1, TNFRSF17, ABCB4, BLNK, GLDC, MEF2C, IGHM, FAM30A, SPIB, BCL11A, GNG7, IGKC, CD72, MICAL3, BCL11A, BACH2, CCR9, QRSL1, DTNB, HLA-DQA1, SCN3A, QRSL1, SLC15A6
T cells	PRKCQ, CD3D, CD3G, CD28, LCK, TRAT1, PRKCQ, BCL11B, CD2, LCK, TRBC1, ITM2A, SH2D1A, CD6, CD96, NCALD, GIMAP5, CD3E, SKAP1
Macrophages + Th1	PTPN22, PLCB2, CLEC4A, HLA-DOA, FMNL1, TAGAP, SRGN, CSF1R, CD33, C1QB, C1QC, C1QA, CTSS, CD300LF, IRF8, SCIMP, MNDA, AOAH, WDFY4, CD84, TFEC, CYBB, PIK3CG, SLCO2B1, HLA-DPA1, HLA-DPB1, HLA-DRA, HLA-DMB, HLA-DMA, CD74, SAMSN1, MPEG1, TNFRSF1B, ARHGAP25, ARHGAP30, CARD11, MS4A6A, PARVG, GPR65, CXorf21, IL12RB1, FAM78A, FGD2, SLEPLG, CD37, HCLS1, WAS, NCF4, ITGB2, FERMT3, SPI1, MYO1F, CYTH4, TNFAIP8L2, AIF1, LST1, SNX20, BTK, LCP2, PTPRC, EVI2B, NCKAP1L, CD53, PLEK, CD4, SASH3, IL10RA, DOCK2, FYB, ILZF1, CCR5, SPN, ARHGAP9, LILRB1, LILRB4, FCER1G, SLC7A7, LAIR1, HAVCR2, LAPTM5, CD86, PIK3R5, GAB3, RASAL3, TMC8, KLHL6, APBB1IP, SLA. INPP5D, LRRC25, SIGLEC9, SIGLEC7, CD68, ITGAX, ADAP2, C3AR1, EVI2A, WIPF1, FGL2, IGSF6, LILRB2, BIN2, SIGLEC10, TLR8, RNASE6
Tgd	TARP, C1orf61, TRGV9, CD160, FEZ1
Th1	IFNG, LTA, APBB2, DOK5, IL12RB2, APOD, ZBTB32, CD38, CSF2, CTLA4, CD70, DPP4, EGFL6, BST2, DUSP5, LRP8, IL22, DGKI, CCL4, GGT1, LRRN3, SYNGR3, ATP9A, BTG3, CMAH, HBEGF, SGCB
CIN70	ACTL6A, CDAN3, CDK1, CDC6, CDC20, CKS2, CTPS1, DHCR7, DKC1, ECT2, ELAVL1, EZH2, FEN1, FOXM1, GPI, MSH6, H2AFX, H2AFZ, HDGF, MAD2L1, MCM2, MCM7, NDUFAB1, NEK2, PCNA, RAD21, RFC4, RRM1, RRM2, SRSF2, SP1, AURKA, TACC1, TOP2A, TTK, UNG, PRDM2, CDC45, PRC1, CCNB2, AURKB, PTTG1, TRIP13, MELK, NCAPD2, KIF20A, RNASEH2A, RAD51AP1, CELF1, UBE2C, ZWINT, OIP5, CCT5, TPX2, NUP205, NEMP1, NCAPH, KIF4A, LSM4, ATAD2, NXT1, ZWILCH, CDCA8, CEP55, MCM10, ASF1B, PBK, CMAS, C20orf24, TGIF2, CDCA3, PDCD2L

Table 2. List of gene signatures.

3. Intrinsic subtype and Cluadin-low intrinsic subtype

All tumors were assigned to an intrinsic molecular subtype of breast cancer (luminal A, luminal B, HER2-E, and basal-like) and the normal-like group using the previously reported PAM50 subtype predictor[75]. Also, we applied the previously reported 9-Cell line claudin-low predictor[76]. Only a sample was identified as claudin-low independently of the PAM50 subtype call.

4. Gene signatures

The expression of 10 independent signatures was evaluated as a continuous variable. The PAM50 predictor calculates, for each sample, the correlation coefficient to each of the 5 PAM50 centroids (luminal A, luminal B, basal-like, HER2-enriched, and normal-like). Each centroid was considered a single signature. In addition, the PAM50 predictor outputs a risk of recurrence (ROR) score at 10 years. The ROR score based on subtype (ROR-S) and subtype and proliferation (ROR-P) were developed in a micro-array-based cohort of node-negative, untreated early breast cancer[77]. In addition, we evaluated the following three signatures: proliferation score, which is the mean expression of 11 proliferation-related genes[78], VEGF/Hypoxia signature[79], which is the mean expression of 13 hypoxia-related genes, and claudin-low signature (as a continuous variable)[80].

5. Statistical analysis

$\chi 2$ tests were performed to determine the differences in the distribution of variables. To identify genes whose expression was significantly different between paired primary and metastatic samples, we used a paired two-class significance of microarrays (SAM) with a false discovery rate (FDR) <5% (35). Biologic analysis of gene lists was performed with DAVID annotation tool (http://david.abcc.ncifcrf.gov/; ref. 36). Time to tumor recurrence (TTR) was defined as the period of time from surgery to the date of the first distant relapse. Overall survival from metastatic disease (OSmet) was defined as the period of time of metastatic disease to death or last follow-up. Estimates of survival were from the Kaplan–Meier curves and

tests of differences by the log-rank test. Univariate Cox models were used to test the independent prognostic significance of each variable. All statistical computations were carried out in R v2.15.1 (http://cran.r-project.org). All statistical tests were two sided, and the statistical significance level was set to less than 0.05.

RESULTS

1. Intrinsic Subtypes and gene expression profiles in primary and metastatic breast cancer

a. Clinical–pathologic characteristics

A total of 123 patients were included (Table 3). The median age at breast cancer diagnosis was 52.5 years (range, 28–90). In primary disease, the immunohistochemical analyses showed 73.17% (n = 90) of patients had HR-positive (HR+), 15.45% (n = 19) HER2-positive (HER2+), and 9.76% (n = 12) triple-negative disease. In metastatic disease, 69.92% (n = 86) of patients had HR+, 19.51% (n = 24) HER2+, and 9.76% (n = 12) triple-negative disease. No significant differences (P > 0.502) were observed in the distribution of the three IHC groups in primary versus metastatic disease. Fourteen patients (11.38%) presented with de novo metastatic disease. Median follow-up and OSmet were 76.5 and 84 months, respectively (Fig. 4)

	n (%)	
n	123 (100)	
Age, median (range)	52.5 (28 - 90)	
	PT	**MT**
HER2 status		
Positive	19 (15.4)	24 (19.5)
Negative	100 (81.3)	97 (78.9)
Unknown	4 (3.3)	2 (1.6)
ER status		
Positive	99 (80.5)	101 (82.1)
Negative	22 (17.9)	20 (16.3)
Unknown	2 (1.6)	2 (1.6)
PR status		
Positive	92 (74.8)	74 (60.2)
Negative	29 (23.6)	48 (39.0)
Unknown	2 (1.6)	1 (0.8)

Table 3. Clinical characteristics of the cohort.

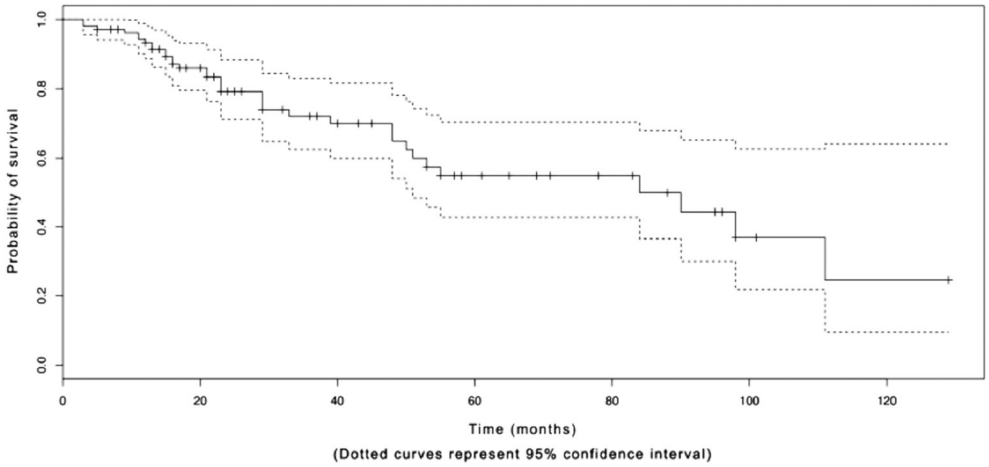

Fig. 4. Overall survival from recurrence to death or last follow-up of the entire cohort.

b. Type of metastatic tissues

The organs of origin of the metastatic biopsies analyzed in this study were skin (n = 35; 28.4%), lymph nodes (n = 24; 19.5%), liver (n = 20; 16.3%), bone (n = 16; 13%), lung (n = 7; 5.7%), ovarian and peritoneum (n = 7; n = 5.7%), pleural (n = 6; 4.9%) and others (n = 8; 6.5%), including brain, pericardial fluid, and colon metastases (Fig. 5).

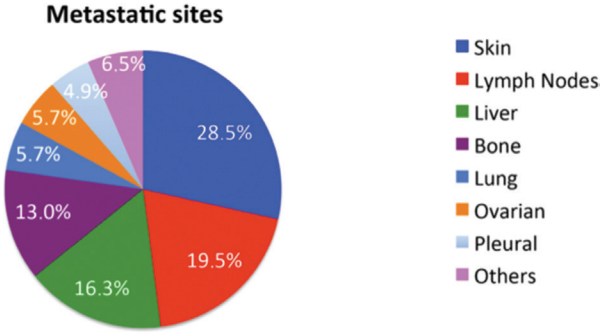

Fig. 5. Organs of origin of the metastatic biopsies.

c. Subtype distribution

The distribution of the PAM50 intrinsic subtype classification in primary tumor versus metastatic disease was 39% versus 26% for luminal A ($P = 0.029$), 26% versus 35.8% for luminal B ($P = 0.097$), 11.4% versus 22% for HER2-E ($P = 0.026$) and 9.8% versus 12.2% for basal-like tumors ($P = 0.540$) (Fig. 6).

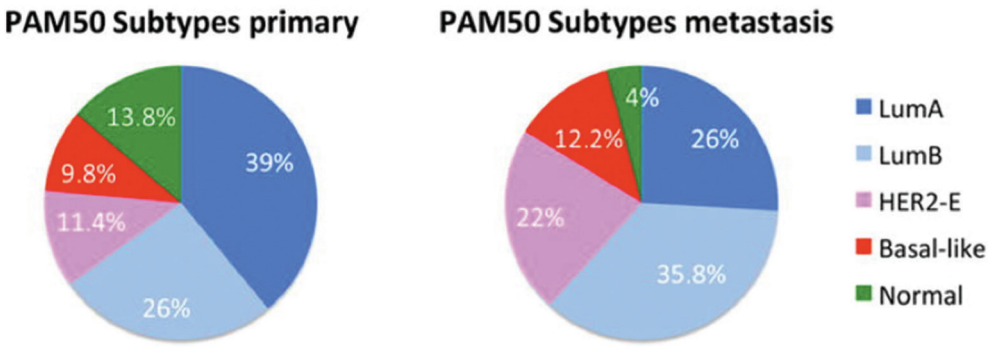

Fig. 6. Distribution of intrinsic subtype in primary versus metastatic disease.

Individually, subtype concordance was high for basal-like (100%), HER2-E (76.9%), and luminal B (70.0%) tumors. Regarding luminal A primary tumors, 44.7% remained luminal A in the metastasis, switching to luminal B in 40.4% and HER2-E in 14.9% of the cases. Overall, primary luminal tumors (A and B combined) changed to a HER2-E in 14.28%, despite 81% of them being clinically HER2 negative. Cohen kappa coefficient was 0.38 [95% confidence interval (CI), 0.27–0.5, $P < 0.001$] (Table 4).

	Metastatic disease				
Primary disease	***Basal-like***	***HER2-E***	***Luminal A***	***Luminal B***	***Genes differentially expressed (FDR<5%)***
Basal-like	12 (100%)	0	0	0	0
HER2-E	2 (15.38%)	10 (76.92%)	1 (7.7%)	0	7
Luminal A	0	7 (14.9%)	21 (44.68%)	19 (40.42%)	25
Luminal B	0	4 (13.33%)	5 (16.67%)	21 (70%)	8

Table 4. Subtype concordance between primary and metastatic disease. *, FDR, false discovery rate.

Indeed, we observed that the 8 patients, whose tumors changed from luminal A/B in primary disease to HER2-E in metastatic disease showed an increase in *FGFR4* expression but not *ERBB2* expression (Fig. 7).

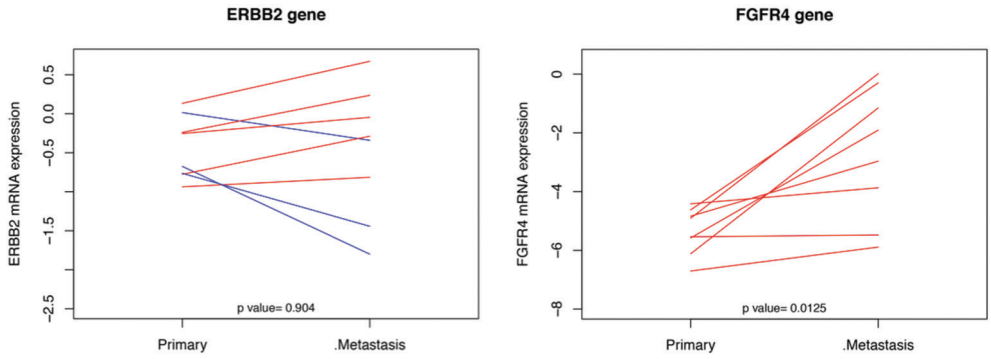

Fig. 7. HER2 and FGFR4 mRNA expression changes between HER2-negative/Luminal A-B primary and HER2-negative/HER2-E metastasis breast cancer.

These results did not changed when the claudin-low classification was investigated as no claudin-low tumor was identified in this series. Finally, we observed that liver and lung metastases showed the highest and lowest subtype conversion rate (75% and 14%), respectively. However, these results by site of metastasis need further validation, due to the small sample sizes (Table 5).

Metastatic Site		n	%
Lung, n=7	Conversion	1	14.3
	No Conversion	6	85.7
Pleura, n= 6	Conversion	4	66. 7
	No Conversion	2	33.3
Skin, n= 35	Conversion	13	37.1
	No Conversion	22	62.9
Nodes, n= 24	Conversion	12	50.0
	No Conversion	12	50.0
Bone, n= 16	Conversion	6	37.5
	No Conversion	10	62.5
Liver, n= 20	Conversion	15	75.0
	No Conversion	5	25.0
Ovarian and peritoneum, n= 7	Conversion	3	42.9
	No Conversion	4	57.1
Others, n= 8	Conversion	4	50.0
	No Conversion	4	50.0

Table 5. Different Intrinsic subtype conversion across the different organs of metastasis.

d. Expression changes of individual signatures

We evaluated the expression changes of each individual signature between primary tumor and their paired metastatic samples. Luminal A and normal-like signatures were found significantly less expressed in metastatic tumors than in primary tumor. In contrast, luminal B, HER2-E, and proliferation signatures were found more expressed in metastatic tumors than in primary tumors. Finally, the expression of basal-like, VEGF/hypoxia and claudin-low signatures was similar between primary and metastatic disease (Fig. 8).

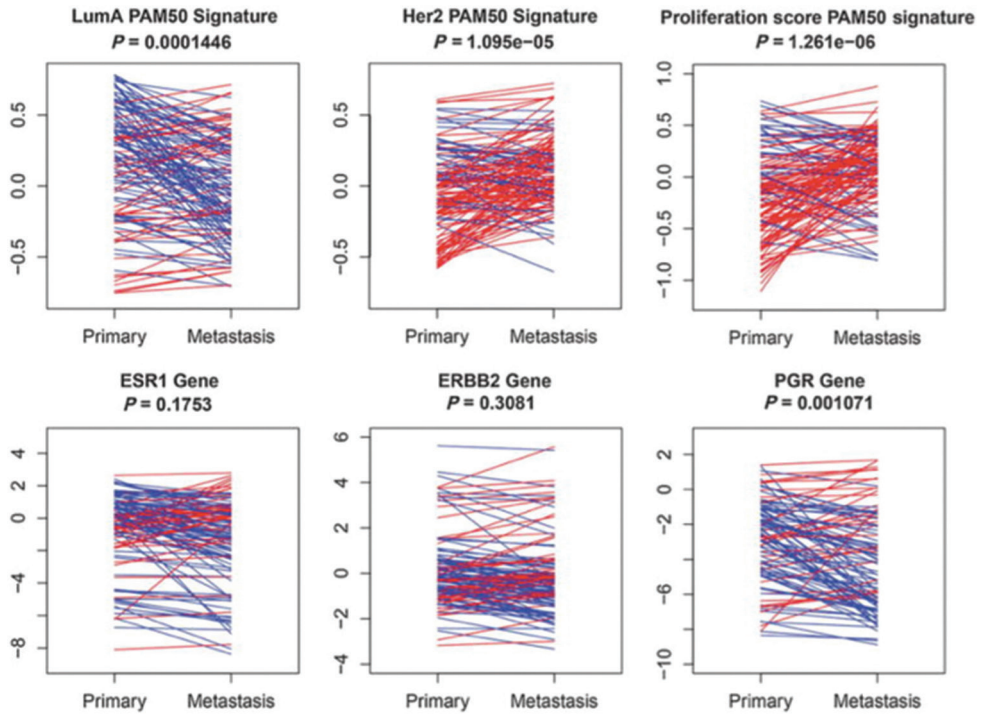

Fig. 8. Gene and signature expression changes between primary and metastasis. P value was obtained after performing a paired t test.

e. Expression changes of individual genes

Among 105 breast cancer–related genes, 16 and 31 genes were found up- and downregulated in metastatic tissues compared to primary tissues (FDR < 5%) (Table 6). The upregulated gene list was enriched for genes involved in survival and

migration (e.g., *FGFR4*), cell cycle (e.g., *CDC6* and *CCNB1*), and DNA repair (e.g., *TYMS*). The downregulated gene list was enriched for genes involved in response to hormone stimulus (e.g., *BCL2* and *PGR*; Fig. 7), differentiation (e.g., *GATA3*) and chromatin regulation (e.g., *CXXC5*).

Gene name	Gene symbol	Score (d)	Fold change	FDR (%)
Fibroblast growth factor receptor 4	FGFR4	3.38	1.74	0
Cell division cycle 6	CDC6	2.14	1.29	1.90
Maternal embryonic leucine zipper kinase	MELK	1.99	1.27	1.90
Pituitary tumor-transforming	PTTG1	1.80	1.21	1.90
Cell division cycle 20	CDC20	1.79	1.23	1.90
Thymidylates synthetase	TYMS	1.75	1.22	1.90
Centrosomal protein	CEP55	1.73	1.24	1.90
Cyclin B1	CCNB1	1.71	1.19	1.90
Phosphoglycerate dehydrogenase	PHGDH	1.69	1.23	1.90
Keratin 8, Type II	KRT8	1.68	1.17	1.90
Ribonucleotide reductase M2	RRM2	1.67	1.22	1.90
Epithelial cell adhesion molecule	EPCAM	1.50	1.15	3.60
BCL2-Associated athanogene	BAG1	1.42	1.14	3.60
Ubiquitin-conjugating enzyme E2T	UBE2T	1.38	1.17	4.82
V-Myb myeloblastosis viral oncogene homolog-like 2	MYBL2	1.36	1.21	4.82
Serine Peptidase Inhibitor, Kunitz Type, 2	SPINT2	1.33	1.13	4.82
Transmembrane protein 158	TMEM158	−6.29	−1.34	0
Keratin 17, Type I	KRT17	−5.55	−1.22	0
Matrix metallopeptidase 11	MMP11	−5.39	−1.32	0
Secreted frizzled-related protein 1	SFRP1	−4.26	−1.39	0
Zinc finger E-box binding homeobox 1	ZEB1	−4.19	−1.57	0
Progesterone receptor	PGR	−3.72	−1.40	0
Crystallin, alpha B	CRYAB	−2.90	−1.59	0
Melanoma inhibitor activity	MIA	−2.82	−1.55	0
V-Myc myelocytomatosis viral oncogene homolog	MYC	−2.69	−1.67	0
N-Acetyltransferase 1	NAT1	−2.31	−1.62	0
Microtubule-associated protein tau	MAPT	−2.25	−1.64	0
Estrogen receptor	ESR1	−2.07	−1.65	0
Retinoid-inducible nuclear factor	CXXC5	−2.07	−1.80	0
Fibrilin 1	FBN1	−1.99	−1.72	0
AXL receptor tyrosine kinase	AXL	−1.88	−1.78	0
Forkhead box A1	FOXA1	−1.87	−1.72	0

Solute carrier family 16	SLC16A3	−1.82	−1.78	0
Lipoma HMGIC fusion partner	LHFP	−1.74	−1.78	0
Solute carrier family 39 member 6	SLC39A6	−1.73	−1.75	0
Caveolin 1	CAV1	−1.59	−1.78	0
GATA binding protein 3	GATA3	−1.54	−1.79	0
Erb-B2 receptor tyrosine kinase 2	ERBB2	−1.52	−1.82	0
B-Cell CLL/Lymphoma 2	BCL2	−1.41	−1.80	1.35
DNA damage inducible transcript 4	DDIT4	−1.17	−1.85	3.60
Claudin 7	CLDN7	−1.10	−1.87	3.60
Cadherin 3	CDH3	−1.10	−1.82	3.60
Adrenomedullin	ADM	−1.07	−1.83	4.82
Phosphatase and tensin homolog	PTEN	−1.06	−1.91	4.82
Forkhead Box C1	FOXC1	−1.03	−1.82	4.82
MET proto-oncogene, receptor tyrosine kinase	MET	−1.02	−1.83	4.82
Vimentin	VIM	−0.99	−1.88	4.82

Table 6. List of up- downregulated genes differentially expressed between metastatic vs. primary disease across all samples (FDR<5%).

A similar analysis was performed within each of the subtypes identified in primary disease. Concordant with the subtype changes, 25, 8, 7, and 0 genes were found differentially expressed in luminal A, luminal B, HER2-E, and basal-like primary disease, respectively, compared with metastatic disease (Table 7).

Luminal A			Luminal B			HER2-E		
Gene Symbol	Score (d)	FDR (%)	Gene Symbol	Score (d)	FDR (%)	Gene Symbol	Score (d)	FDR (%)
FGFR4	3.54	0	MMP11	-3,73	0	TMEM158	3,05	0
MMP11	-4.18	0	TMEM158	-3,14	0	MMP11	2,46	0
TMEM158	-4.16	0	NAT1	-2,79	0	CD24	1,87	0
KRT17	-3.74	0	PGR	-2,45	0	KRT17	1,75	0
ZEB1	-3.59	0	KRT17	-2,27	0	KRT19	1,70	0
PGR	-3.14	0	ESR1	-2,13	0	NDRG1	1,69	0
GATA3	-3.11	0	MAPT	-2,05	0	SLC16A3	1,53	0
SFRP1	-3.08	0	ZEB1	-1,79	0			
FOXA1	-2.72	0						
ESR1	-2.63	0						
MIA	-2.21	0						
NAT1	-2.07	0						
MAPT	-2.00	0						
MYC	-1.94	0						
CRYAB	-1.92	0						
LHFP	-1.85	0						
BCL2	-1.82	0						
SLC16A3	-1.80	0						
SLC39A6	-1.77	0						
ERBB2	-1.64	0						
AXL	-1.43	2.27						
CAV1	-1.40	2.27						
CXXC5	-1.36	2.27						
FBN1	-1.30	4						
CDH3	-1.29	4						

Table 7. List of up- and downregulated genes differentially expressed between metastatic vs. primary disease across all samples by intrinsic subtype.

f. Association with overall survival

We also evaluated the ability of the 10 signatures to predict OSmet in primary (Fig. 9A) versus metastatic (Fig. 9B) disease. Interestingly, no signature consistently predicted OSmet in both primary and metastatic disease. In primary disease, basal-like signature was found associated with worse outcome (HR = 1.50, P = 0.007), while the VEGF/Hypoxia signature was associated with a better outcome (HR = 0.65, P = 0.016). In metastatic disease, proliferation was found associated with worse outcome (HR = 1.40, P = 0.047).

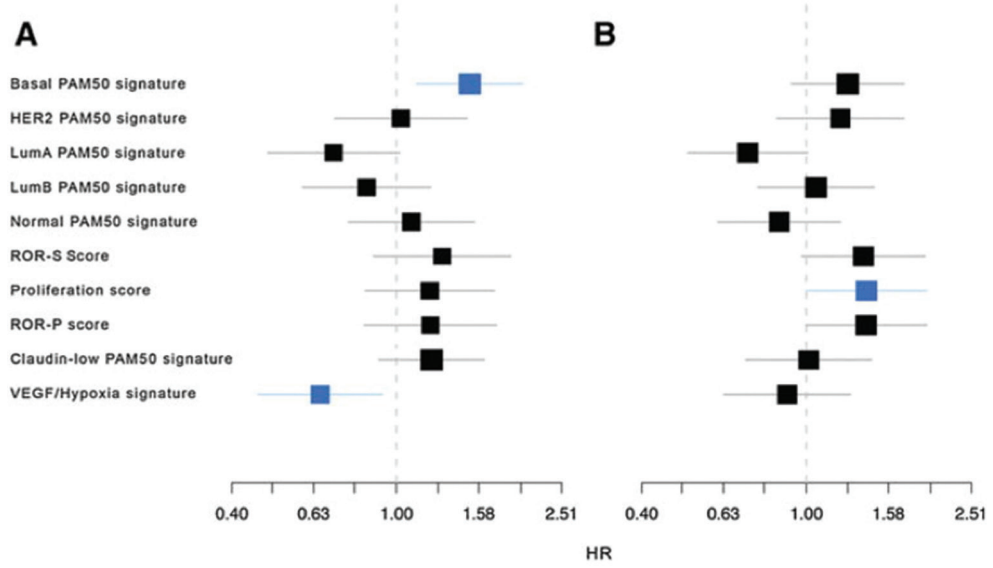

Fig. 9. Association of 10 signatures with OSmet when evaluated in primary (A) and metastatic (B) disease. Each signature was evaluated as a continuous variable and was standardized to have a mean of 0 and a SD of 1. The size of the square is inversely proportional to the SE; horizontal bars represent the 95% Cls of HRs. Statistically significant variables are shown in blue. Each gene signature was evaluated in a univariate analysis.

These results suggested that OSmet might be better predicted by measuring either the primary tumor or the metastatic tumor depending on the biological process (e.g., proliferation) being evaluated. To further explore the role of this signature, we evaluated the ability of each individual gene to predict OSmet in primary versus metastatic disease. Among 105 genes, 14 and 10 genes were finally found associated with OSmet in primary and metastatic disease, respectively. Interestingly, only one gene (*GATA3*) consistently predicted favorable outcome in both settings (Fig. 10). In primary disease, high expression of 13 of the 14 genes was found associated with better outcome. These 13 genes (e.g., *PGR*, *ESR1*, and *FOXA1*) were mostly tracking luminal-related biological processes. On the contrary, high expression of 8 of the 10 genes in metastatic disease was found associated with worse outcome. These 8 genes (e.g. *MYC*, *CCNE1*, and *CCNB1*) were mostly tracking cell cycle/proliferation-related biological processes.

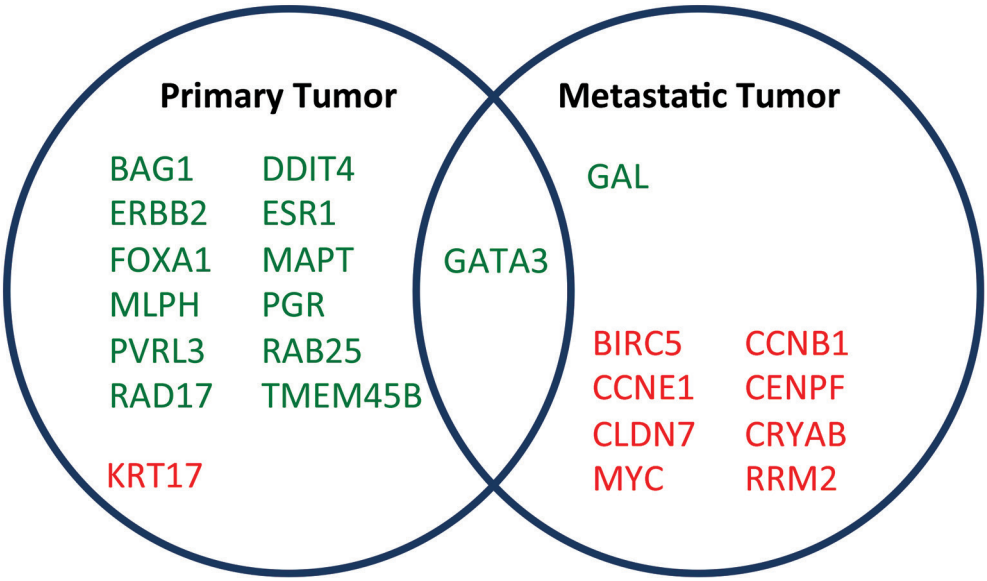

Fig. 10. Venn diagram of genes that predict overall survival from the data of recurrence when analyzed in primary versus metastatic disease. Green, genes associated with good prognosis; red, genes associated with poor prognosis.

Finally, we explored the ability of each gene signature to predict OSmet in patients with tumors with no subtype conversion (n = 59) versus patients with tumors without subtype conversion (n = 49). The results revealed that in patients with no subtype conversion, the association profile of signatures with OSmet were very similar when the primary or the metastatic tumors were evaluated. However, in patients with subtype conversion, the associations of signatures with OSmet were generally different when the primary or the metastatic tumors were evaluated. Among them, the HER2-E signature was found significantly associated with poor outcome (HR = 1.86, P = 0.046) when evaluated in metastatic tumors but not when evaluated in primary disease (Fig. 11).

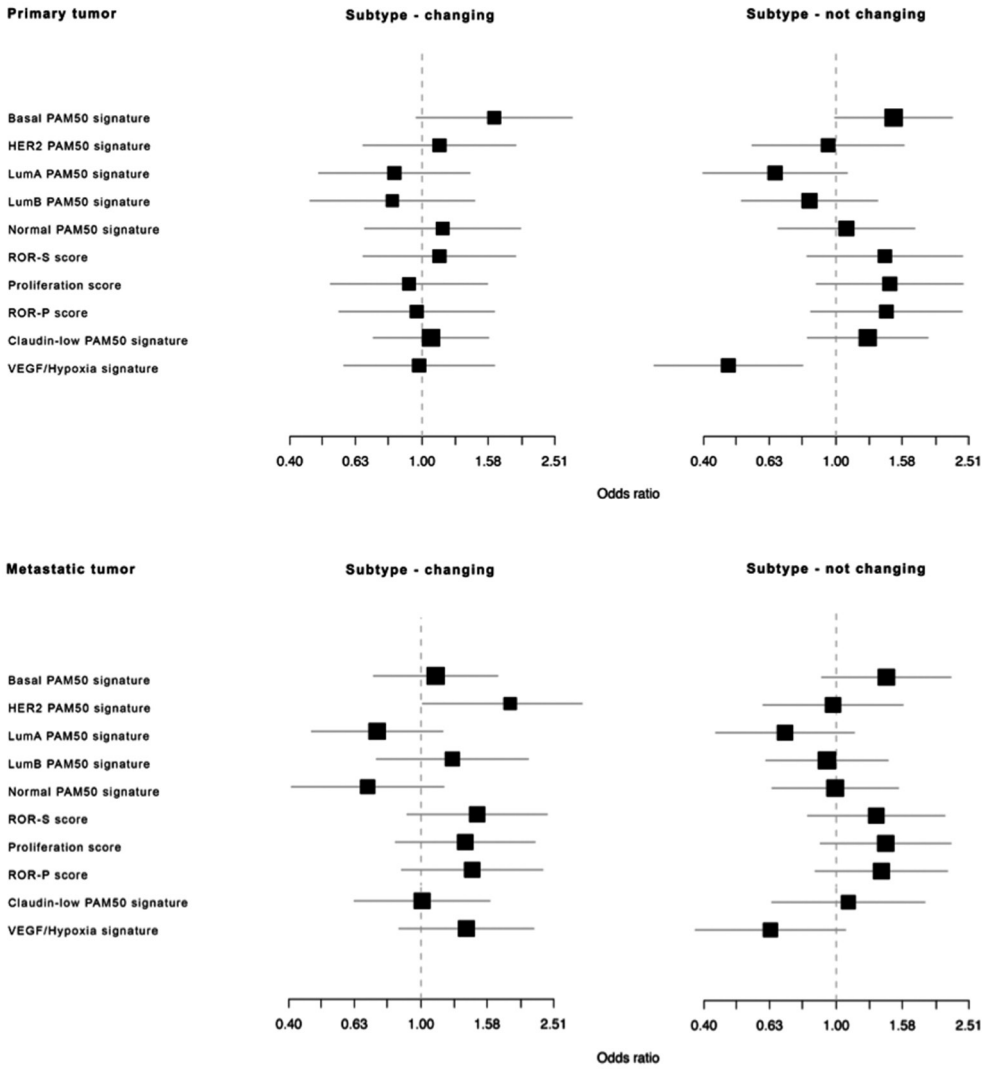

Fig. 11. Association of 10 signatures with OSmet when evaluated in primary metastatic disease with subtype conversion vs. without subtype conversion.

g. Magnitude of gene expression changes versus TTR

To evaluate if the gene expression changes observed in metastatic tissues are a reflection of tumor evolution over time, we plotted the magnitude of change of the expression of each signature versus TTR (Fig. 12). The results revealed a positive correlation between TTR and HER2-E (corr = 0.324, $P < 0.001$), luminal B (corr = 0.27, $P = 0.004$), Proliferation score (corr = 0.291, $P = 0.002$), and ROR-P (corr = 0.295,$P = 0.001$). In contrast, normal-like and luminal A signatures showed a negative correlation with TTR (corr = −0.285, $P = 0.002$; corr = −0.219, $P = 0.019$, respectively).

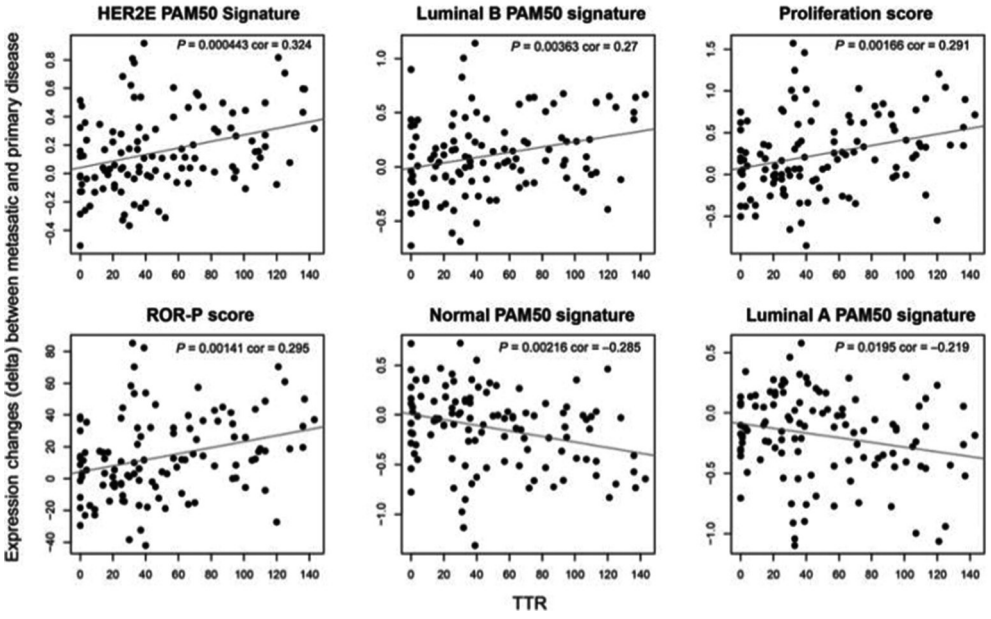

Fig. 12. Correlation between time to tumor recurrence (TTR) and the magnitude of gene/signature expression changes between primary and metastatic disease.

Gene-by-gene analysis revealed a positive correlation between TTR and the magnitude of change of genes implicated in cell proliferation (*CEP55*: corr = 0.244, $P = 0.024$), mitogenesis, and differentiation biological process (*FGFR4*: corr = 0.211, $P = 0.044$). In contrast, a negative correlation was observed with genes that

participate in cell-to-cell adhesion (*CLDN4*: corr = −0.207, $P = 0.027$; *F11R*: corr = −0.237, $P = 0.01$), regulation of DNA damage repair (*RAD17*: corr: −0.226, $P = 0.017$), tumor suppression (*GRHL2*: corr = −0.186, $P = 0.05$), mammary gland development (*PGR*: corr = −0.203, $P = 0.045$), and that attenuate cell migration (*ESRP1*: corr = −0.252, $P = 0.006$).

h. Conclusions

Here, we explored RNA-based expression differences between paired primary and metastatic breast tumors and made the following observations: (I) intrinsic molecular subtype is largely maintained during metastatic recurrence, except for luminal A disease, which converted to luminal B and HER2-enriched in 55% of the cases; (II) metastatic tissues show higher expression of proliferative and lower expression of luminal-related genes compared to primary tumors, except for basal-like disease, which seems to be very stable from a RNA-based perspective; (III) different biological processes can predict overall survival from recurrence when evaluated in primary versus metastatic disease; (IV) an intriguing relationship seems to exist between the time taken to develop detectable metastases and the aggressiveness of the tumor, indicating that a tumor might evolve towards a more aggressive phenotype as time evolves.

Previous studies have evaluated the rates of change of the three classical pathologic biomarkers (i.e., ER, PR, and HER2) between primary and metastatic tumors[81 82]. Overall, the rates of ER, PR, and HER2 conversion were 13%, 28%, and 3%–10%, respectively[83]. Among the three genes, we also observed *PGR* as the top downregulated gene in metastatic compared with primary tissues. Nonetheless, the three classical biomarkers are largely maintained in the metastatic setting, which is concordant with our findings using the basal-like, HER2-enriched, and luminal A/B intrinsic subtype classification. At the same time, prior gene expression-based studies with smaller number of patients are concordant with our findings[84 85 86]. However, Lee and colleagues evaluated the PAM50 intrinsic subtypes in 17 paired

samples of primary and brain metastasis, and subtype conversion was observed in 47.1% of the cases, which is higher than the 30.9% conversion rate observed in our study. However, similar to our study, a large proportion of luminal A primary tumors (1/6) changed to non-luminal A disease, and all basal-like primary tumors (n = 6) remaining basal-like at recurrence[87].

Other studies have evaluated changes in somatic mutations and gene copy-number aberrations (CNA) between primary and metastasis. For example, Meric-Bernstam and colleagues[88] performed targeted DNA sequencing of 3,320 exons of 182 cancer-related genes plus 37 introns from 14 genes in 74 tumors. In 33 matched primary and recurrent tumors, 97 of 112 (86.6%) somatic mutations were concordant. Of identified CNAs, 136 of 159 (85.5%) were concordant. There was an increased frequency of *CDK4/MDM2*amplifications in recurrences, as well as gains and losses of other actionable alterations. The authors concluded that analysis of recurrent tumors before treatment may provide additional insights, as both gains and losses of targets are observed. In another study, Ding and colleagues described the genomic analyses of four DNA samples from an Africo-American patient with basal-like breast cancer: peripheral blood, the primary tumor, a brain metastasis, and a xenograft derived from the primary tumor. Of the 50 validated point mutations and small indels, 48 were detectable in all three tumors. Overall, while additional somatic mutations, copy number alterations, and structural variations occurred during the clinical course of the disease, most of the original mutations and structural variants present in the primary tumor were propagated.

Similar to prior studies looking at DNA alterations, we did not identify large absolute expression changes at the RNA level between primary and metastatic disease. Nevertheless, 47 genes were found differentially expressed, mostly within luminal A/B disease. Among them, *FGFR4* was detected as the top upregulated gene in metastatic disease. Interestingly, this gene is found in the PAM50 gene list and its overexpression is characteristic of the HER2-E intrinsic subtype. Fibroblast

growth factor receptors are involved in development, differentiation, cell survival, migration, angiogenesis and carcinogenesis[89]. Dimerization of the receptor leads to intracellular phosphorylation of receptor kinase domains and intracellular signal transduction, including *RAS/RAF/MEK* and *PI3K/AKT* pathways[90]. These evidences suggest that *FGFR4* could drive the HER2-E phenotype in metastatic lesions with a HER2-negative/HER2-E profile. Indeed, the patients whose tumors changed from luminal A/B in primary disease to HER2-E in metastasic disease showed an increase in *FGFR4* expression but not *ERBB2* expression. Of note, HER2-E subtype has been associated with estrogen-independent growth and poor outcome in patients with HR+/HER2-negative breast cancer treated with anti-estrogens[91 92]. Further mechanistic studies are needed to elucidate the role of *FGFR4* in metastatic disease.

Currently, large-phase III clinical trials, especially within HR+/HER2-negative disease, are not taking into account this biological heterogeneity, such as proliferation, which is not well captured by HR and HER2 statuses. For example, patients with a luminal A profile following endocrine therapy might be treated with second-line endocrine therapy while those that change to a HER2-negative/HER2-E or luminal B profile might be treated with chemotherapy or other novel combinatory strategies such as endocrine therapy and*CDK4/6* inhibition. Overall, this result suggests that, although there is some stability of the intrinsic subtype, approximately 40% of the tumors will change subtype, highlighting the need to biopsy metastatic disease to better understand the clinical and biological evolution of a tumor.

Another interesting observation was the significant correlation between the magnitude of gene expression changes of various signatures between primary and metastasis disease and the time from diagnosis to tumor recurrence. Specifically, we observed that the longer was the time to recurrence, the more aggressive the tumors become based on proliferation and expression of luminal genes. This suggests that there is an intrinsic evolution of tumor cells towards a more aggressive phenotype as time elapses. However, the correlation coefficients were weak and thus the magnitude

of gene expression changes might also be explained by other variables such as the treatments received in (neo)adjuvant setting.

Despite the interesting founding, this study has several limitations worth noting. First, this is a retrospective study using tumor samples from different hospitals and a selection bias is plausible. Second, patients received different adjuvant and/or metastatic systemic treatments and thus we could not evaluate treatment effects on tumor biology or survival. However, subtype conversion of the 14 patients with de novo metastatic disease was found to be 57.1%, suggesting that subtype conversion is independent of treatment effects. For all these reasons, more studies are needed to address this particular question. Third, metastatic tumor biopsies were not always collected at the time of the diagnosis of recurrent disease. Fourth, we did not analyze DNA mutations such as *ESR1* whose incidence is known to increase during tumor progression. Further studies will be able to evaluate if the gene expression changes observed during progression of luminal breast cancer are related to the appearance of *ESR1* mutations.

To conclude, most biological changes occurring during metastatic progression of breast cancer are largely unknown today. Here, we compared intrinsic molecular subtype and expression of individual genes in paired primary and metastatic tissues. Our results suggest that although intrinsic subtype is largely maintained during metastatic progression, luminal/HER2-negative tumors acquire a luminal B or HER2-E profile during metastatic progression, likely reflecting tumor evolution and/or acquisition of estrogen-independency.

2. Immune-related genes expression profiles

The aim of this analysis is to assess the prognostic value and changes of immune-related genes expression profiles across intrinsic molecular breast cancer subtypes. Furthermore, we explored how immune signatures evolve during disease progression.

a. Clinical-pathologic characteristics and subtypes distribution

A total of 102 patients were included in this analysis. The median age at breast cancer diagnosis was 52.5 years (range, 28–90). In primary disease, the immunohistochemical analyses showed 74.5% (n = 76) of patients were luminals, 15.7% (n = 16) HER2-positive (HER2+), and 7.8% (n= 8) triple-negative disease. In metastatic disease, 70.6% (n = 72) of patients were luminals, 18.6% (n= 19) HER2+, and 8.8% (n= 9) triple-negative disease. Median OS was 85.5 months (Table 8).

	n (%)	
n	102 (100)	
Age, median (range)	52.5 (28 - 90)	
	PT	**MT**
HER2 status		
Positive	16 (15.7)	19 (18.6)
Negative	83 (81.4)	82 (80.4)
Unknown	4 (3.9)	1 (1.0)
ER status		
Positive	83 (81.4)	83 (81.4)
Negative	17 (16.7)	17 (16.7)
Unknown	2 (2.0)	2 (2.0)
PR status		
Positive	75 (73.5)	62 (60.8)
Negative	25 (24.5)	39 (38.2)
Unknown	2 (2.0)	1 (1.0)
Subtype-IHC		
Luminal	76 (74.5)	72 (70.6)
Luminal/HER2	8 (7.8)	12 (11.8)
HER2	8 (7.8)	7 (6.9)
TNBC	8 (7.8)	9 (8.8)
Unknow	2 (2.0)	2 (2.0)

Table 8. Clinical characteristics of the cohort with RNA-seq analysis.

The distribution of the PAM50 intrinsic subtype classification in primary tumor versus metastatic disease was 39.22% versus 26.47% for luminal A, 28.43% versus 34.31% for luminal B, 11.76% versus 23.53% for HER2-E and 7.84% versus 10.78% for basal-like tumors (Fig. 13).

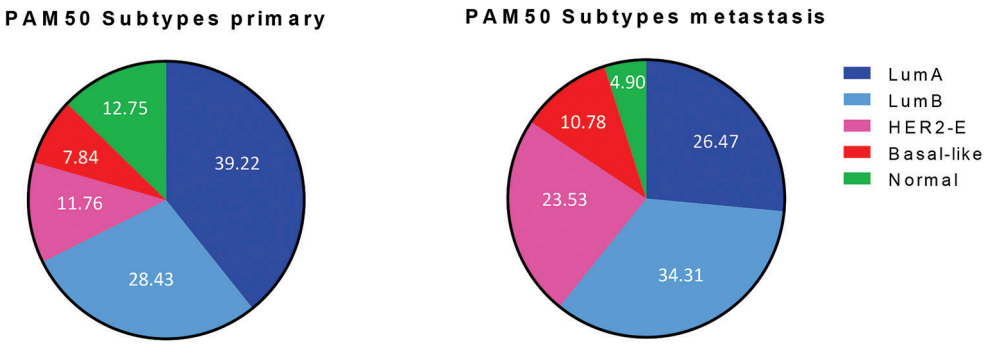

Fig. 13. Distribution of intrinsic subtypes in primary versus metastatic disease.

Individually, subtype concordance was high for basal-like (100%), HER2-E (66.67%), and luminal B (62.07%) tumors. Regarding luminal A primary tumors, 45% remained luminal A in the metastasis, switching to luminal B in 37.5% and HER2-E in 15% of the cases. Overall, primary luminal tumors (A and B combined) changed to a HER2-E in 14.49%, despite 70% of them being clinically HER2 negative. (Table 9).

(%)	Metastatic disease				
Primary disease	*Luminal A*	*Luminal B*	*HER2-E*	*Basal-like*	*Normal –like*
Luminal A	18 (45)	15 (37.5)	6 (15)	–	1 (2.5)
Luminal B	5 (17.24)	18 (62.07)	4 (13.79)	–	2 (6.90)
HER2-E	1 (8.33)	–	8 (66.67)	2 (16.67)	1 (8.33)
Basal-like	–	–	–	100 (100)	–
Normal-like	3 (23.08)	2 (15.38)	6 (46.15)	1 (7.69)	1 (7.69)

Table 9. Subtype concordance between primary and metastatic disease.

b. Immune-related genes expression across intrinsic molecular breast cancer subtypes

We performed an analysis of 6 immune-related genes (*CD4*, *CD8A*, *CD45*, *CTLA4*, *PDCD1* and *CD274*) across intrinsic molecular breast cancer subtypes in primary and metastatic samples.

Very similar patterns of immune-related gene expression were observed between both settings. Where Basal-like group showed the highest expression followed by HER2-E. While Luminal subtypes presented less gene expression in both early and advanced disease (Fig. 14).

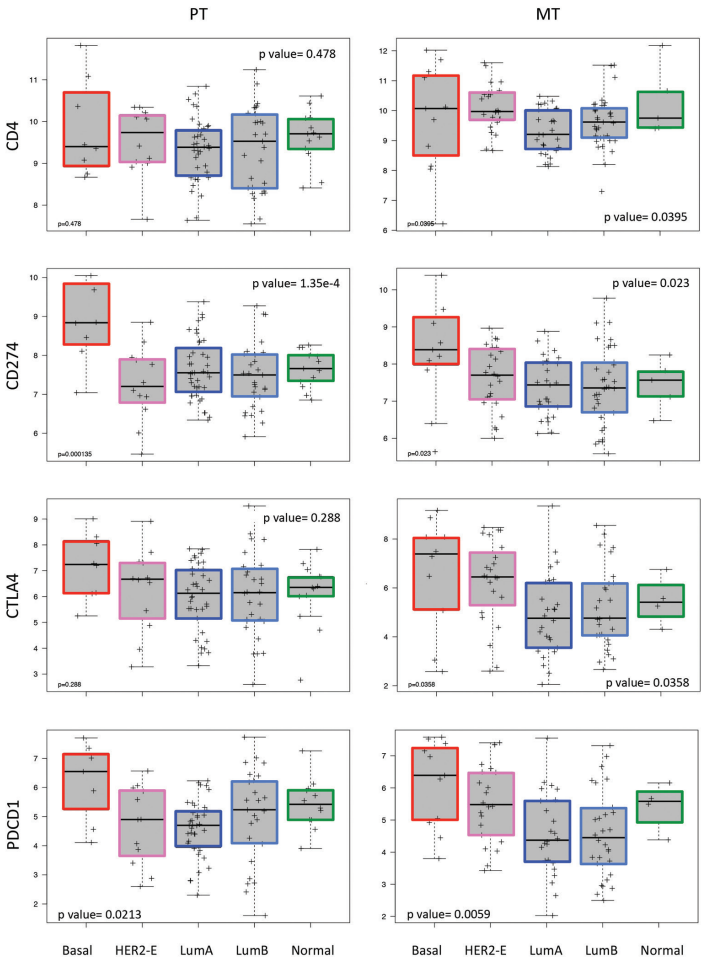

Fig. 14. Expression of selected genes across the molecular intrinsic subtypes of breast cancer in primary (left) and metastatic (right) tumor.

We performed an analysis of several immune signatures using public available data from transcriptional profile of immune cell subpopulations. When we analyzed the expression of these immune signatures across the intrinsic subtypes, most of them, including T and B cells, and macrophages, they were found to be upregulated in Basal-like in both primary and metastatic tumor (Fig. 15).

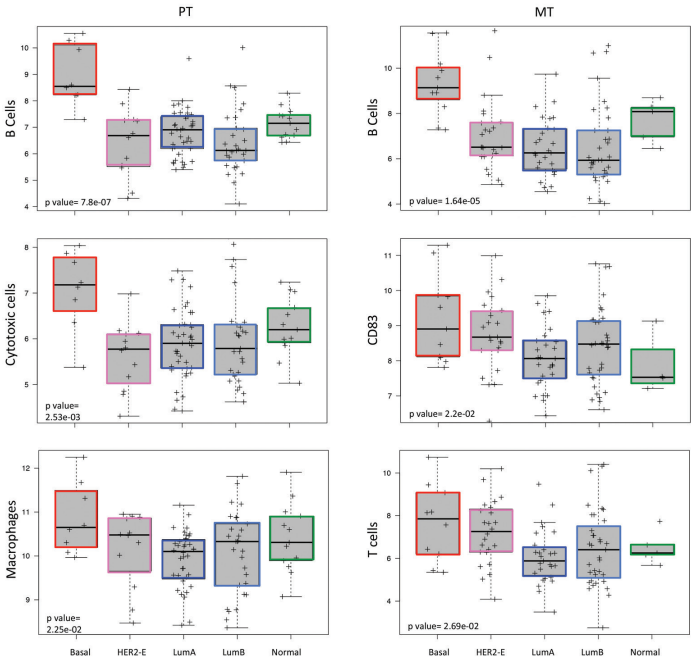

Fig. 15. Expression of immune signatures across the molecular intrinsic subtypes of breast cancer in primary (left) and metastatic (right) tumor.

To better understand the evolution of the immune response, we evaluated the expression changes of each gene between primary and metastatic disease. In our analysis, *CD4* was more expressed in metastatic tumor (p= 0.016), and especially in Luminal A (p= 0.025) and HER2-E subtypes (p= 0.004). Interestingly, *CTLA4* and *PDCD1*, both immune checkpoint pathway inhibitors, were downregulated in the metastatic tissues. In this case, *CTLA4* was found significantly less expressed in metastatic tumor (p= 0.009), especially in Luminal B (p= 0.011). Meanwhile, *PDCD1* presented a significant downregulation only in Luminal B (p= 0.044) (Fig. 16).

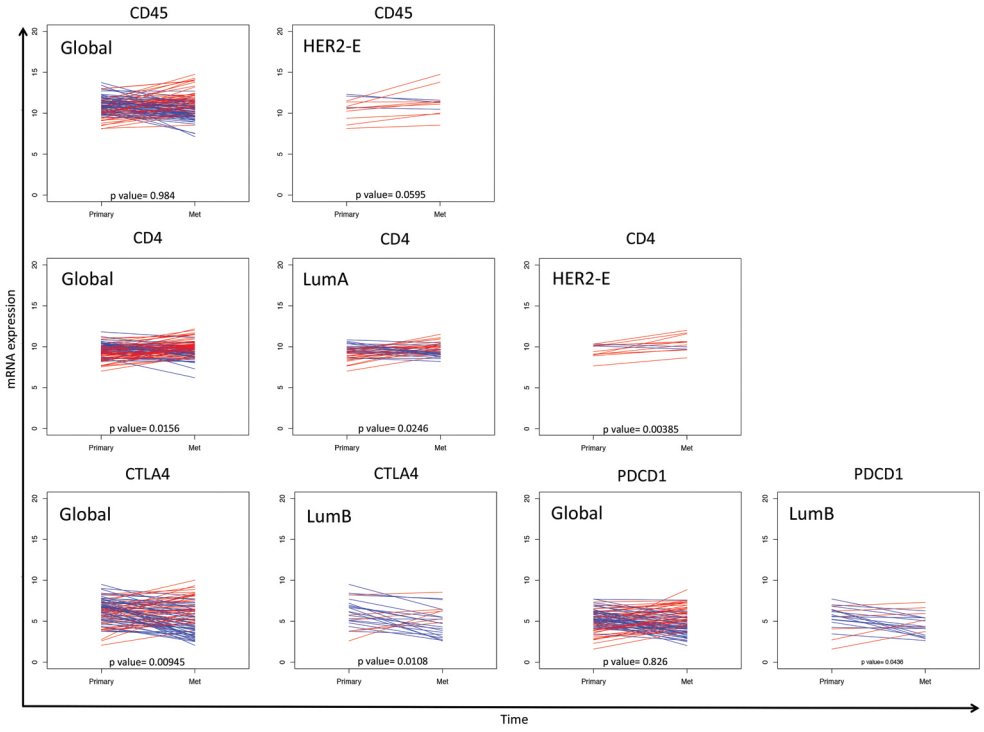

Fig. 16. Immune- gene expression changes between primary and metastasis disease.

Finally, we explored the association between the PAM50 signatures and the 6 immune-related genes. The results showed a positive correlation between all of them and Basal-like signature. In contrast, a negative correlation was found with Luminal A signature (Fig. 17). Luminal B signature showed a negative correlation with PDCD1, meanwhile HER2-E subtype presented a positive correlation with CD4 (Fig. 17).

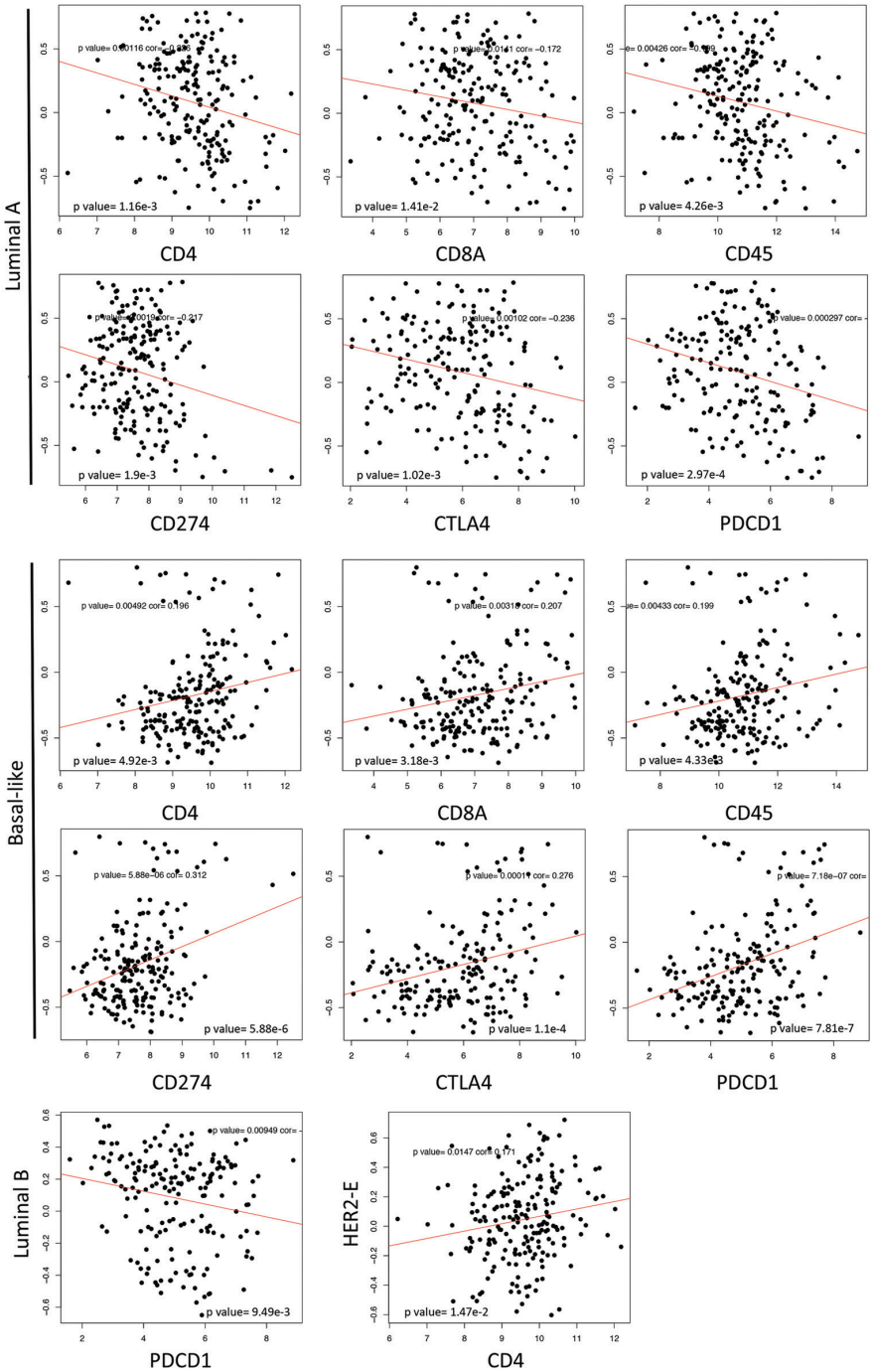

Fig. 17. Correlation between 6 immune-related genes and PAM50 signatures.

Similar data were observed when we analyses the relation between PAM50 and immune signatures. Basal-like and HER2-E showed a positive correlation, in contrast, Luminal A and B presented a negative correlation with immune signatures (Fig. 18).

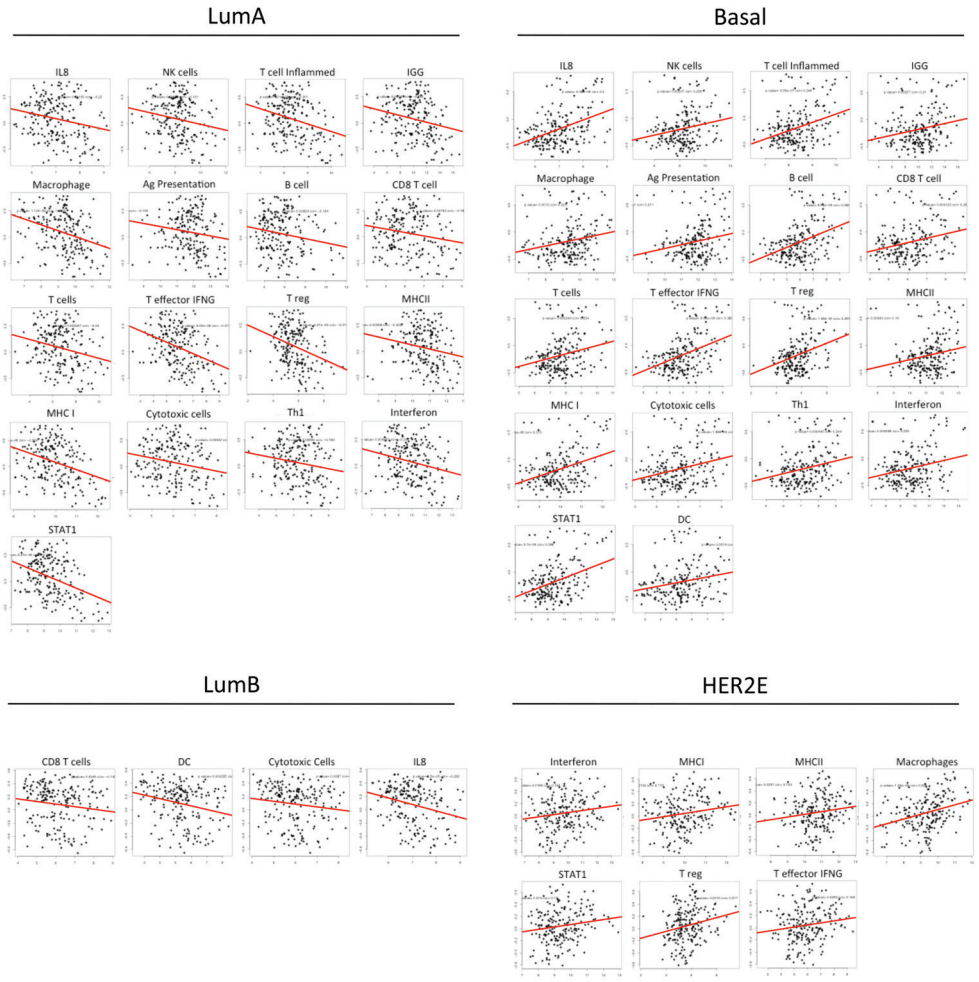

Fig. 18. Correlation between immune signatures and PAM50 signatures.

To gain deeper insights the complex spatiotemporal dynamics of the tumor-immune interaction during tumor progression, we investigated both innate immune cell (dendritic cells, mast cells, macrophages, NK cells, and neutrophils) and adaptive immune cells (B, T helper 1, T helper 2, T γ δ).

Innate immune signature was significantly higher expressed in metastatic tissue vs primary samples in the global cohort ($p= 6.1e^{-1}$). We found some subpopulation cells upregulated such as dendritic cells ($p= 9.68e^{-6}$), Th2 (p= 0.006), and Th1 (p=0.024).

When we assessed the signatures across PAM50 subtypes, we found a significant upregulation of innate immune system in Luminal A ($p= 1.19e^{-4}$) and HER2-E (0.002) tumors in the metastatic disease. When we focused in immune subpopulations cells, we found dendritic cells (p= 0.005), macrophages (p= 0.005), and Th2 (p= 0.004) upregultaed in Luminal A. And also, dendritic cells (p= 0.011), macrophages and Th1 (p= 0.018), Tgd (p= 0.039), cytotoxic cells (p= 0.038), antigen presentation (p= 0.013), and macrophages (p= 0.002) signatures were upregulated in HER2-E (Fig. 19).

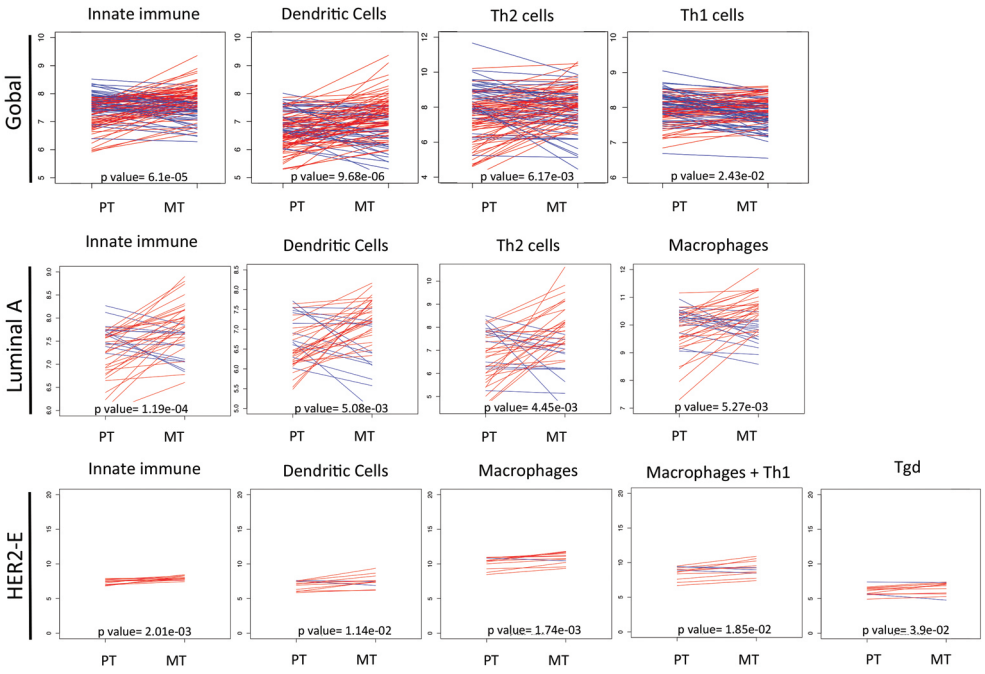

Fig. 19. Innate immune signatures expression change between primary and metastasis. *P* value was obtained after performing a paired t test

c. Immune-related genes expression associated with prognostic value

Additionally, we tested the ability of the 6 immune-related genes to predict prognosis in primary versus metastatic setting. Of note, no single gene consistently predicted prognosis in both primary and metastatic disease. Furthermore, no single gene predicted DFS by measured at primary tumor.

In metastatic disease, *CD274*, *CD8A* and *CD4* were found to be associated with better outcomes in terms of OS (HR= 0.51, p= 0.003; HR= 0.63, p= 0.026; HR= 0.72, p= 0.039; respectively). However, in primary tumor none of these genes were related with survival (Fig. 20).

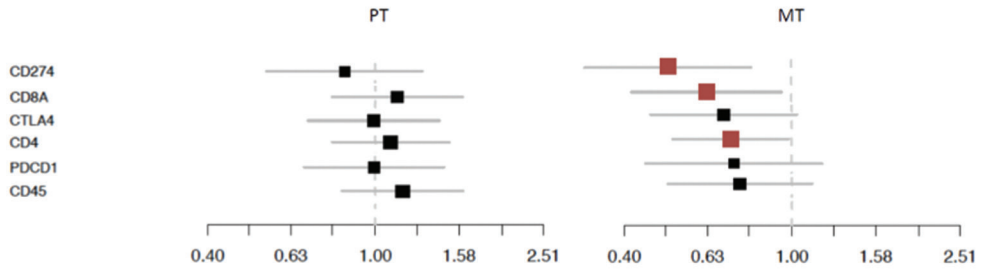

Fig. 20. Association of 6 immune-related genes with OS evaluated in primary (A) and metastatic (B) disease.

All innate immune signatures were associated with good prognosis in terms of OS in the metastatic group (HR<1, p< 0.05). Adaptive immunity mediated by T and B lymphocytes are crucial for effective and sustained antitumor responses. In this case only Th2 was related with worse outcome in primary tumor (OS, HR: 1.56, p= 0.048) (Fig. 21).

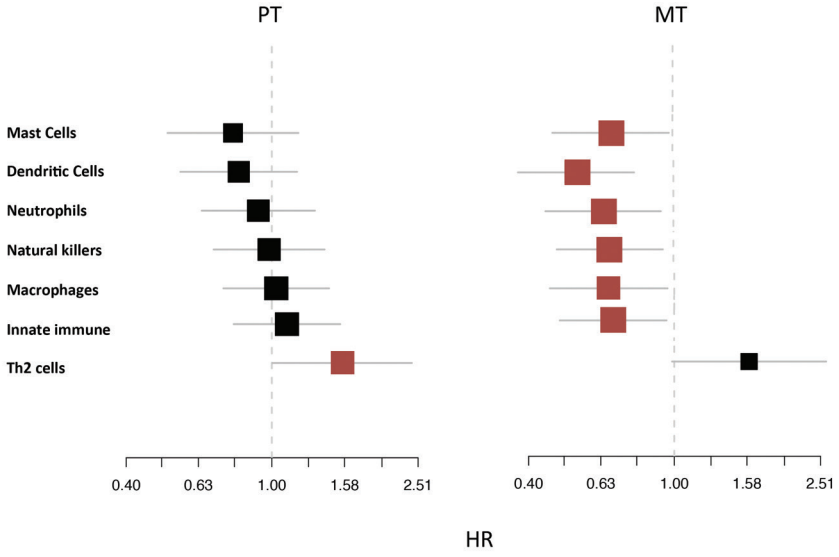

Fig. 21. Association of immune signatures with OS when we evaluated in primary and in metastatic disease.

Furthermore, CD8+ T cell was associated with favorable overall survival in metastatic disease when we measured in metastatic tissue (Osmet, HR: 0.66, p= 0.04).

d. Conclusions

The immune microenvironment of breast cancer differs according to tumor biology. In summary, our data propose that the expression of immune genes varied significantly between different intrinsic subtypes. These observations were based on assessment of immune-related genes expression.

We showed an inverse correlation between luminal tumors and immune genes. Therefore, Basal-like followed by HER2-E were more immunogenic than luminal breast cancer subtypes. This suggests that luminal breast cancer is more immunologically "cold" than non-luminal, and thus, luminal tumors would be immunologically silent.

Interestingly, controversial data regarding the literature was found, because metastatic tumors presented more immunoreactivity than primary tumors.

Our results also add the utility of RNA-based immune biomarkers for predict prognosis. There was a robust association between immune genes expression measured at metastatic tumors and favorable patient outcomes, in contrast, this effect was not found in primary tumor. Only Th2 cells were related with bad prognosis, these cells has been widely described as promoting metastasis and it was related to worse overall survival.

3. Chromosomal instability

One of the most consistent characteristics of human tumors is CIN. Despite the important role in cancer biology, the molecular mechanisms underlying CIN are poorly understood, and it is unfrequently evaluated in the clinic due to the technical

difficulty and a lack of clear therapeutic application. To evaluate the role of CIN in breast cancer, we used a gene expression signature of chromosomal instability published in Nature Genetics (Carter SL, et al. Nature Genetics. 2006).

a. CIN signature expression between paired primary and metastatic samples

We evaluated the expression changes of chromosomal instability signature between primary and metastatic samples. We found a global significantly enrichment in the metastatic setting (p= 2.11 e^{-3}), this increased expression of CIN70 signature was especially due to Luminal A subtype (p= 6.74 e^{-4}) (Fig. 22 A and B).

When we focused the analysis in Luminal A patients in the primary tumor, we observed that the increase expression was associated with patients whose tumors switched the subtype in the metastasis to Luminal B or HER2-E (p= 2.18 e^{-8}) (Fig. 22 C).

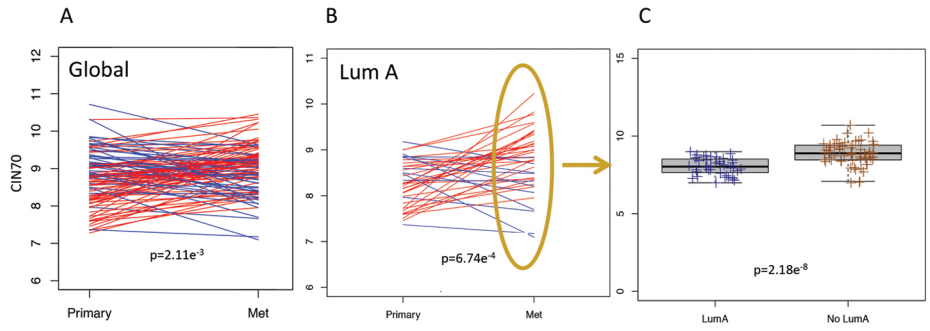

Fig. 22. CIN70 signature expression changes between primary and metastasis (A). CIN70 signature expression changes in Luminal A patients at primary tumor (B). CIN70 expression in metastatic biopsies derived from primary luminal A patients: differences between luminal A vs non-luminal A (C).

Chromosomal instability was related with tumor phenotype. The most aggressive subtypes as Basal-like and HER2E showed the highest expression of CIN followed by Luminal B, while Luminal A presented less chromosomal instability expression in both primary and metastatic disease (p= 1.57 e^{-12} and p= 3.63 e^{-13}, respectively)

(Fig. 23 A). Furthermore, CIN signature presented a positive correlation with RORP (corr = 0.82, p= 6.7 e^{-51}) and Proliferation signature (corr = 0.812, p= 3.41 e^{-49}). Interestingly, a negative correlation was observed with CESP signature (corr = -0.36, p= 1.79 e^{-24}) (Fig. 23 B).

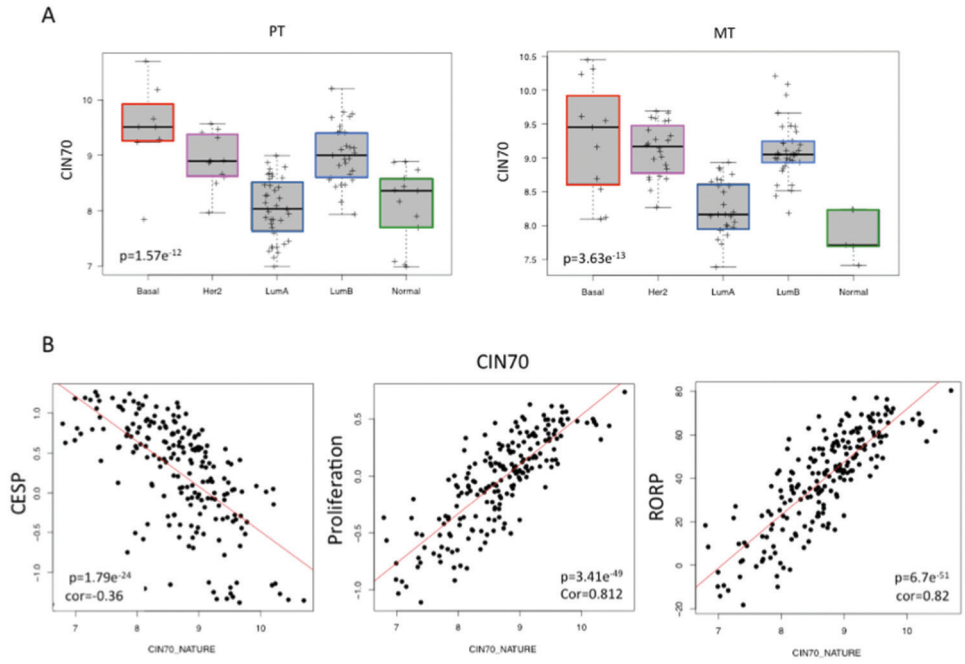

Fig. 23. Expression of CIN70 signature across PAM50 subtypes of breast cancer in primary (left) and metastatic (right) tumor (A). Correlation between CIN70 signature and some signatures as CESP, Proliferation and RORP (B).

According with these data, the highest expression of CIN70 signature was associated with shorter disease-free survival (p= 8.79 e^{-4}). Furthermore, this signature was related with worse overall survival, but our results were not no statistically significant (p= 0.524) (Fig 24).

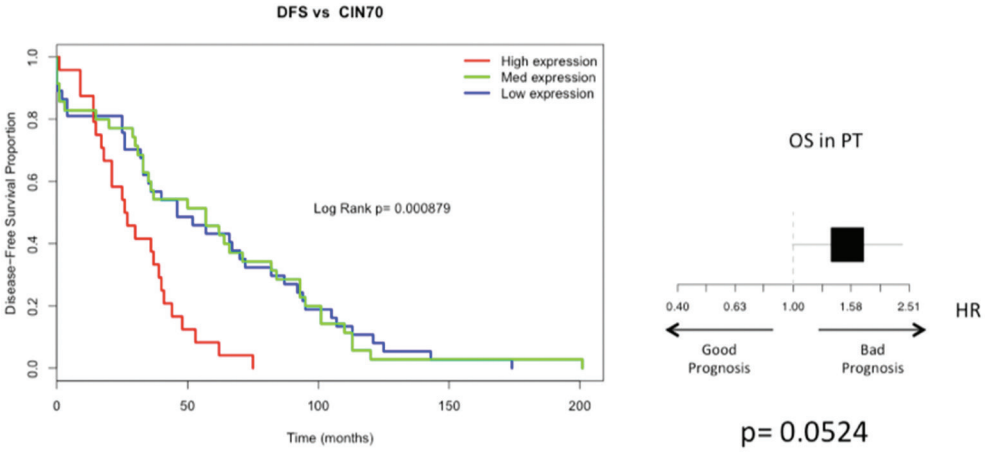

Fig. 24. Kaplan-Meier disease-free survival by CIN70 signature in breast cancer patients (A). Forest plot showing hazard ratio for overall survival related with CIN70 at primary tumor.

b. Conclusions

CIN provides cells plasticity, due to this plasticity the cells could acquire new properties to develop metastasis during dormancy status and induce the change in breast cancer subtypes.

Chromosomal instability could be related with phenotypic adaptation and produce tumor evolution, in fact, patients diagnosed with Luminal A breast cancer which tumor switched to luminal B or HER2E in the metastasis presented an increased in CIN expression.

Furthermore, CIN70 was related with more aggressive cancer subtypes and represented a poor prognosis signature in terms of DFS.

DISCUSSION

DISCUSSION

Metastasis is the main cause of death for patients with breast cancer. Many studies have characterized the genomic landscape of breast cancer during its early stages. By performing such molecular analyses, it was possible to detect that several genomic alterations are acquired during the evolution of cancers from their early to late stages. Among several genes, recently, *ERBB2* and *ESR1* mutations have been identified as acquired alteration over the course of disease[93]. These mutations were also related to acquired resistance to treatment in particular for luminal BC.

Here, to try to further explore molecular differences, we compared a transcriptomic analysis performed in paired primary and metastatic tissues from a cohort of metastatic breast cancer patients. To do so, we explored RNA-based expression differences between paired primary and metastatic breast tumors.

Our results showed different biology behavior between primary and metastatic breast cancer. Although intrinsic subtype was largely maintained during metastatic progression, it was possible to observe that luminal/HER2-negative tumors acquired a luminal B or HER2-E profile during metastatic progression. This switch suggests the tumor evolution and the acquisition of new properties of metastases.

Most cancers have an abnormal chromosomal content characterized by clonal changes in chromosomal structure and number. It is also know that more than 90% of solid tumors show some degree of genomic disbalances and are aneuploid[94]. The heterogeneity of tumors is a marker of ongoing chromosomal instability in cancer. CIN contributes to the transformation into a more malignant phenotype by altering the equilibrium between oncogenes and tumor suppressor genes, leading to the acquisition of new malignant properties. Gain and loss of chromosomal material in neoplastic cell populations provides cells plasticity that leads to the survival of the fittest clones. The evolution of cancer cells from benign tumor to invasive metastasis appears to correlate with increased aneuploidy and karyotypic complexity.

Therefore, CIN could be related with phenotypic adaptation and produce tumor evolution during metastasis disease. In our cohort of patients, those who were diagnosed with Luminal A breast cancer which tumor switched to luminal B or HER2E in the metastasis presented an increased in CIN expression signature.

Moreover, CIN has been widely associated with poor patients prognosis, metastasis, and resistance to chemotherapies. Indeed in our cohort, CIN was related with more aggressive cancer subtypes and represented a poor prognosis signature in terms of DFS.

Thus, understanding the mechanisms that cause chromosomal instability as well as mechanisms that allow genetic changes that promote acquire malignant features, offers an attractive possibility to interfere with tumor aggressiveness, and enhance the efficiency of cancer therapy.

The last aspect that we explored was the potential difference in tumor microenvironment and immune system between primary tumors and metastases. The interaction between tumor cells and stromal cells is of increasing interest and it was demonstrated to be crucial for both tumor progression and suppression. Cancers evolve through continuous interaction with immune cells in the tumor microenvironment. In fact, tumor-induced immunological changes causing the progression to metastatic disease, even before disseminated cancer cells have reached a secondary organ. Systemic immune tolerance and changes in the character of circulating myeloid cells can predispose a tumor for success in seeding a metastatic site. As tumor cells metastasize to distant tissue sites, they are swarmed by distinct sets of immune populations that can both aid in and inhibit metastasis formation[95].

Tumor-infiltrating immune cells was one of the most studied part of this phenomenon. TILs are supposed to play a central role in tumor control and response to therapy. Greater tumor infiltrating lymphocyte count and increased immune-

related gene expression are associated with better survival in early stage triple negative, HER2-positive and high risk luminal breast cancer, even in the absence of any systemic adjuvant therapy. Which genomic features drive high or low immune infiltration remains unclear.

The past decade has seen a revolution in cancer treatment with the development of immunotherapy towards the use of antibodies that modulate immune responses against tumors. The immune-checkpoint blockade (ICB) works by blocking the receptor and/or ligand interactions of molecules, such as CTLA-4 and PD-1 or PD-L1. ICB therapies have shown significant clinical benefit for several solid tumors. Unfortunately, there is still an unmet clinical need for identify which patients could benefit for this target therapy .

Detection of PD-L1 by IHC measurement has been evaluated as a predictor of response to anti-PD(L)-1 treatment and has been demonstrated to be a valid biomarker in some solid tumors. Several antibodies and pathological scores have been developed and used to try to identify those patients who will benefit from the use of an ICB. However, PD-L1 quantitation for immunotherapy response prediction is no longer precise and there is a need for improved biomarkers of response.

The presence of TILs might confer a prognostic and a predictive impact. But also, the immune gene expression signatures represent an emerging predictive biomarker. In our analysis, there was a robust association between immune genes expression measured at metastatic setting and favorable patient outcomes, meanwhile, this effect was not found in primary tumor. To try to complete the evaluation of those factors related to immune activation in solid tumors, another parameter has been evaluated, the tumor mutation burden (TMB). A minority of somatic mutations in tumor DNA can give rise to neoantigens, which are recognized and targeted by immune system. The more somatic mutations a tumor has, the more neoatingens it is also likely to form. Importantly, this is one of the most relevant factors that

influence the ability of T cells to recognize and kill tumor cells. It could be logic to hypothesize that the tumors with the highest TMB are more likely to respond to immune checkpoint blockade therapy. For example, tumor mutation and neoantigen load predict improved PFS and OS for melanoma patients who were treated with adoptive T cell transfer therapy[96].

We detected differences in the immune microenvironment between primary and metastatic lesions and across different molecular subtypes. Our results suggest that immune system has different implications in primary and metastatic tumors with different prognostic value.

Overall, immune signatures expression was higher in metastases compared with primary tumors. Basal-like followed by HER2-E were the two molecular subtypes most immunologically active. In contrast, luminal breast cancer which presents less degree of difference between cancer cell and normal cell, presented less immune-gene related expression, suggesting that luminal tumors would be immunologically silent. The role of immunosuppressive cells in the induction or maintenance of this immune suppressed state in luminals tumors warrants further investigations.

The interaction between the tumor microenvironment and tumors is an important area for exploration. This knowledge could lead to different strategies for the choice of immunotherapy of breast cancer subtypes. Deeper analysis of interaction between tumor-cells and immune-cells is likely reveal advanced biomarkers that will prove fruitful in identifying patients populations responsive to current immunotherapy and will benefit the search for novel targets for therapeutic modulation.

Finally, our study highlights the importance of molecular characterization of metastatic disease. mRNA expression profiles provide a new tool to explore the distinct nature of breast cancer subtypes.

CONCLUSIONS

CONCLUSIONS

• Intrinsic molecular subtype is largely maintained during metastatic recurrence, except for luminal A disease, which converted to luminal B and HER2-enriched in 55% of the cases.

• Metastatic tissues show higher expression of proliferative and lower expression of luminal-related genes compared to primary tumors, except for basal-like disease, which seems to be very stable from a RNA-based perspective.

• Different biological processes can predict overall survival from recurrence when evaluated in primary versus metastatic disease.

• An intriguing relationship seems to exist between the time taken to develop detectable metastases and the aggressiveness of the tumor, indicating that a tumor might evolve towards a more aggressive phenotype as time evolves.

• Metastatic tumors presented more immunoreactivity than primary tumors. Where, Basal-like followed by HER2-E were more immunogenic than luminal breast cancer subtypes. This suggests that luminal tumors would be immunologically silent.

• There was a robust association between immune genes expression measured at metastatic setting and favorable patient outcomes, meanwhile, this effect was not found in primary tumor. Only Th2 cells were related with bad prognosis.

• Chromosomal instability could be related with phenotypic adaptation and produce tumor evolution. In fact, patients diagnosed with Luminal A breast cancer which tumor switched to luminal B or HER2E in the metastasis presented an increased in CIN70 expression.

• CIN70 was related with more aggressive cancer subtypes and represented a poor prognosis signature in terms of DFS.

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