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DESCIFRANDO EL PERFIL TRANSCRIPTÓMICO DEL CÁNCER DE MAMA EN MUJERES JÓVENES: NUEVAS PERSPECTIVAS

TESIS DOCTORAL

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ESCUELA DOCTORAL

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La vida cobra sentido cuando se hace de ella una aspiración a no renunciar a nada.
Sorprenderse, extrañarse, es comenzar a entender.
Ciencia es todo aquello sobre lo cual siempre cabe discusión.

Sólo cabe progresar cuando se piensa en grande,
solo es posible avanzar cuando se mira lejos.

-José Ortega y Gasset-

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"Només desitge que tingues sort, que siga llarg el viatge, que la vida et done temps, que t'acaricien els ocells de l'aire, que t'atrape cada dia la mort, de morir-se de riure. Que el cor et recorde a cada cop que cal omplir el camí d'aventura, que t'abracen altres llunes mar enllà, que el camí no et domestique, que fer-se el dur no t'adultere, que cada final siga un principi."

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Perquè crec que vos sentiríeu orgullosos de mi.

Porque soy como el árbol, talado que retoño, aún tengo la vida – Miguel Hernández

ABSTRACT

Breast cancer (BC) in very young women (VYBC), defined as those aged under 35 years, presents biological and clinical differences compared to older patients with breast cancer (OBC). Previous studies have shown discordance in tumor characteristics and treatment responses between younger and older patients, notably within luminal subtypes. This study aims to elucidate the transcriptomic landscape of BC in VYBC compared to OBC, focusing on identifying age-associated molecular differences that could decipher personalized treatment strategies for these patients.

This study included 49 BC patients treated at Hospital Clínico Universitario de Valencia. RNA sequencing was performed on formalin-fixed samples from 22 VYBC (age ≤35 years) and 27 OBC (≥50 years) patients. Differential expression analysis was managed, and gene set enrichment analysis (GSEA) was performed to identify differentially expressed genes. Deconvolution analysis using MCP-counter and quanTIseq algorithms were realized to evaluate tumor cellular composition. Histological examination described tumor infiltrating lymphocytes and tertiary lymphoid structures. Statistical analyses were assessed using R (version 4.0.2).

Within the VYBC cohort, 12 patients (55%) had luminal tumors, and 10 (45%) had non-luminal subtypes, compared to 17 (63%) luminal and 10 (37%) non-luminal tumors in the OBC group. Transcriptomic analysis revealed significant age-associated differences particularly in the luminal subtype (133 and 106 genes down- and up-regulated [fold change ±2] adjusted p value < 0.05). Luminal VYBC tumors exhibited greater proliferation signatures (p= 5.17e-05) and ki67 (IHC, p= 0.021), increased chromosomal instability (CIN70, p= 1e-06), and higher expression of immune-related gene signatures compared to OBC tumors. Interestingly, despite there were no differences in estrogen receptors (ER) and progesterone receptors (PR) by IHC, lower expression was found at RNA level in luminal VYBC patients (ESR1: p= 0.0027, PGR: p= 0.0476). Deconvolution analysis revealed increased immune cells infiltration in luminal VYBC tumors, identifying them as immunoreactive tumors, characterized by high infiltration of cytotoxic T lymphocytes (CTLs) and overexpression of immune checkpoint molecules such as PD-1 and its ligand PD-L1. These findings were corroborated by histological evidence of higher TILs and TLS in VYBC tumors. Finally, PAM50-based chemoendocrine score (CES) was also determined for luminal samples showing higher

levels of CES, which are predictive of better response to chemotherapy in luminal VYBC samples.

In conclusion, this study provides a comprehensive understanding of molecular landscape of luminal BC in very young women. We found age-related gene expression changes not only in cancer cells but also in the tumor microenvironment. These data suggest that luminal VYBC could be more endocrine-resistant and immunoreactive than OBC. Accordingly, personalized therapeutic approaches in this underrepresented and high-risk subgroup are needed.

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ABBREVIATIONS AND ACRONYMS

ASIR	Age standardize incidence rate
BC	Breast cancer
CC	Correlation coefficients
CES	Chemoendocrine score
CES-C	Chemotherapy-sensitive
CES-E	Endocrine-sensitive
CES-U	Chemotherapy-uncertain
CIN	Chromosomal instability
CTLS	Cytotoxic T lymphocytes
DEG	Differentially expressed genes
ER	Estrogen receptors
ESR1	Estrogen receptor
FDR	False discovery rate
FFPE	Formalin-fixed paraffin-embedded
GSEA	Gene set enrichment analysis
GO	Gene Ontology
HER2+	HER2-positive
HR+/HER2-	Hormone receptor positive/HER2-negative
ICI	Immune checkpoints inhibitors
IHC	Immunohistochemistry
LMR	Lymphocyte-monocyte ratio
LNR	Lymphocyte-neutrophil ratio
OBC	Older breast cancer patients
PCA	Principal Component Analysis
PR	Progesterone receptor
TGF-β	Transforming growth factor beta
TILS	Tumor infiltrating lymphocytes
TME	Tumor microenvironment
TN	Triple negative
TLS	Tertiary lymphoid structures
VYBC	Very young breast cancer patients

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INTRODUCTION

INTRODUCTION

1. Breast cancer: concept and magnitude of the problem

1.1. Epidemiology

Breast cancer (BC) is the most common cancer in women and represents the second leading cause of cancer-related deaths worldwide (1).

Global incidence of breast cancer was estimated approximately 2.3 million new cases in 2020, resulting in around 685,000 deaths **(Figure 1)** (2). Over the last 30 years the global incidence of breast cancer has increased by 57,8% with a rate increase of 0.5% per year. It is estimated that by 2050, the age-standardized incident rate (ASIR) will be 59.63/100,000 and it will increase of 32.13% compared with 2019. (**Figure 2a,c**) Moreover, Xu et al estimated that by 2050 age-standardized death rate (ASDR) in female will be 16.42/100,000, an increase of 4.69% compared with 2019 **(Figure 2d)** These results highlight the subgroup of very young breast cancer patients and emphasize the magnitude of the actual situation (3).

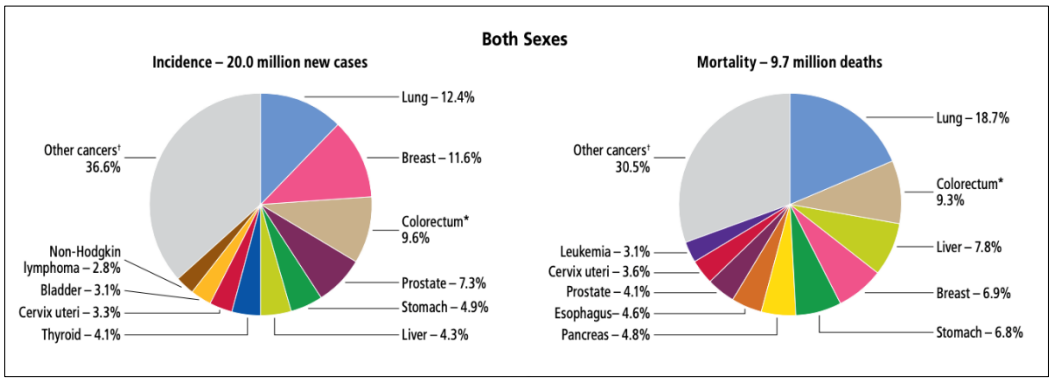

Figure 1. *Pie charts with the distribution of cases and deaths for the top five cancers in 2022 for both sexes. (GLOBOCAN 2022, World Health Organization).*

There are significant geographic disparities in breast cancer incidence rates, with less than 40/100,000 females in some African and Asian countries compared with another regions as Australia/New Zealand, North America and parts of Europe that exceeded 80/100,000 (**Figure 3**). Due to growth and aging, by 2040, the global burden of breast cancer is expected to rise over 3 million new cases and 1 million deaths per year (4).

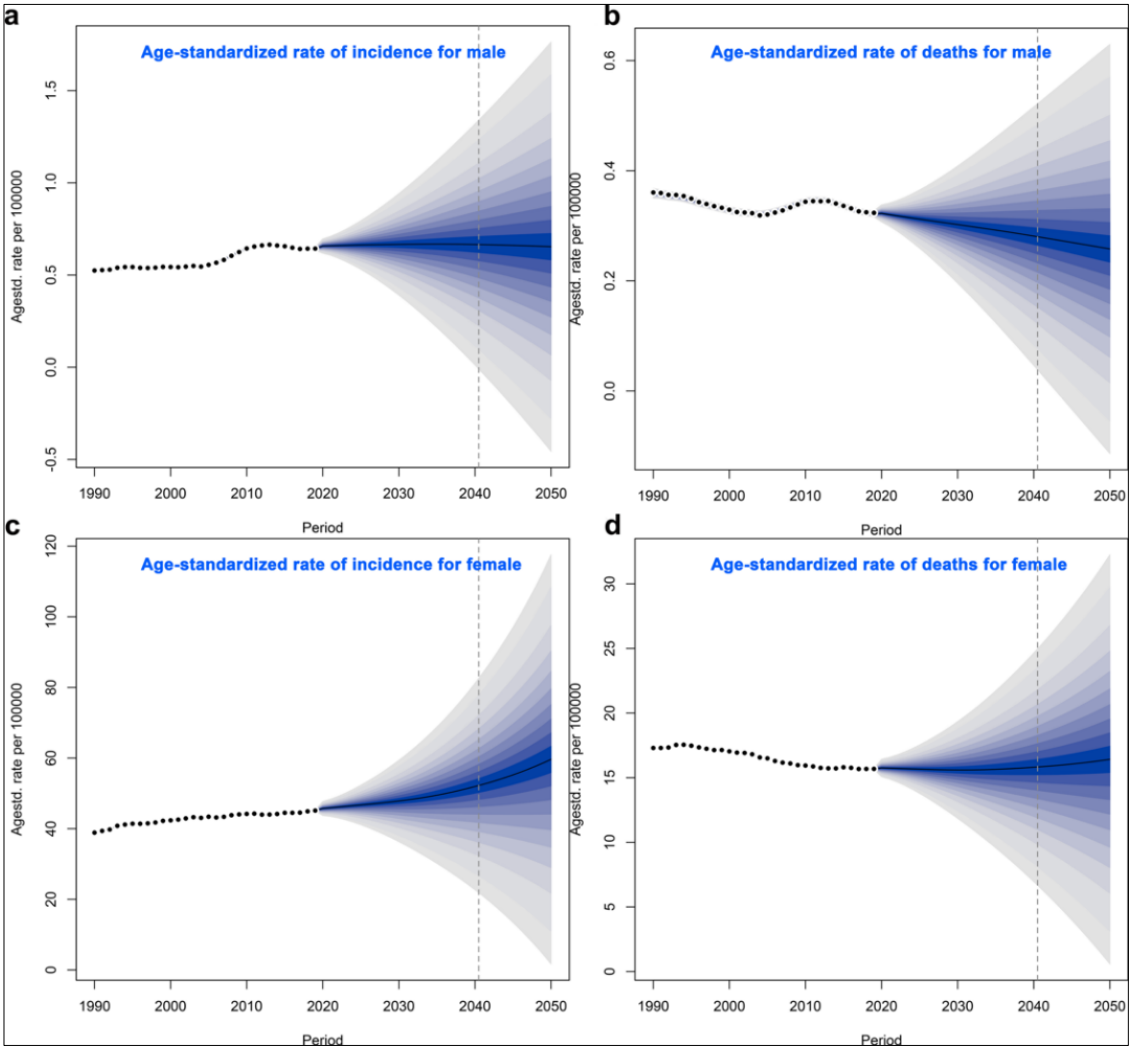

Figure 2. *Future forecast of global burden disease in breast cancer.* ***(a)*** *The ASIR per 100,000 for male;* ***(b)*** *The ASDR per 100,000 for male;* ***(c)*** *The ASIR per 100,000 for female;* ***(d)*** *The ASDR per 100,000 for female (3).*

As opposed to incidence, mortality rates are lower in developed countries (12.7 per 100,000) than in developing countries (19.8 per 100,000), due to the limited access to screening programs, the diagnosis at late stages, and poor access to treatments (**Figure 3**)

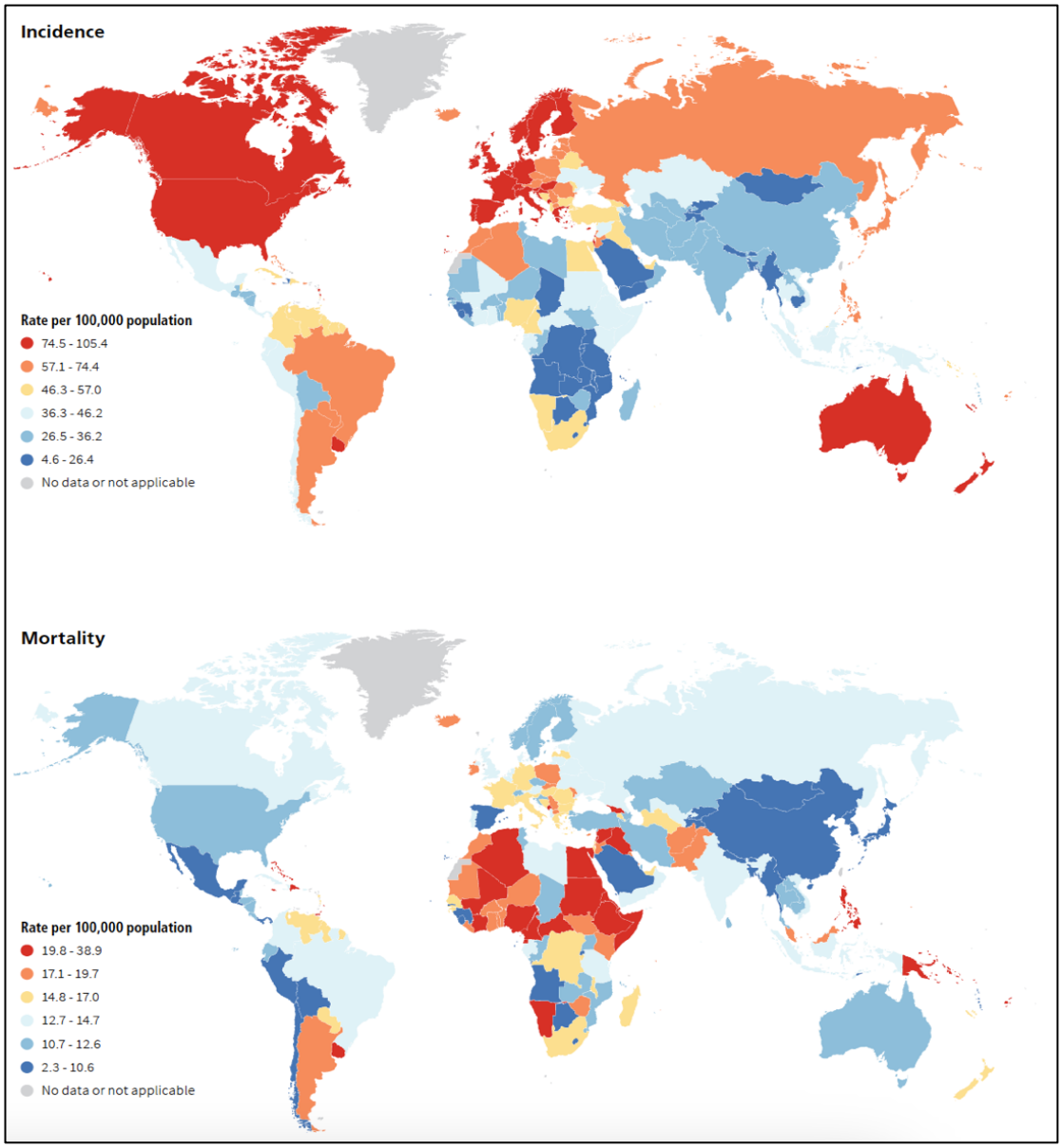

Figure 3. *International variation in female breast cancer incidence and mortality rates, 2022 (GLOBOCAN 2022, World Health Organization).*

1.2. Risk factors

Breast cancer risk factors have been widely studied; recent studies focus on the interplay between various factors. Harmful and early alcohol use initiation have been associated with increased in breast cancer risk (5). Pregnancy and obesity (concretely body mass index) were associated to higher Nottingham Prognostic Index scores, however family history and age showed negative correlation (6).

Family history of breast cancer is a known risk factor, although most patients don't have a documented family history, the absence, not necessary imply a lower risk. High

penetrance gene mutations as BRCA1, BRCA 2, PALB2 are associated with high risk of breast cancer and risk reduction strategies may consider in these patients (prophylactic mastectomy, chemoprevention and genetic counselling among others) (7). Approximately half of breast cancers appear in women who have no identifiable breast cancer risk factors another than gender and age. Various risk assessment models have been developed to incorporate genetic factors and estimate breast cancer risk (8).

Chen et al through mendelian analyses have demonstrated the potential causal relationship between breast cancer and 23 known and suspected risk factors such as menarche, body mass index, smoking and some blood biomarkers (9).

Another interesting data is related on microbial alterations, these alterations are link with obesity, aging and a periodontal disease suggests that a microbial dysbiosis could be a BC factor risk. All these microbial dysbiosis are related with risk factors mentioned before and confirmed the interconnection between them. These data underscore the importance of consider multiple factors in breast cancer risk assessment and prevention strategies (10).

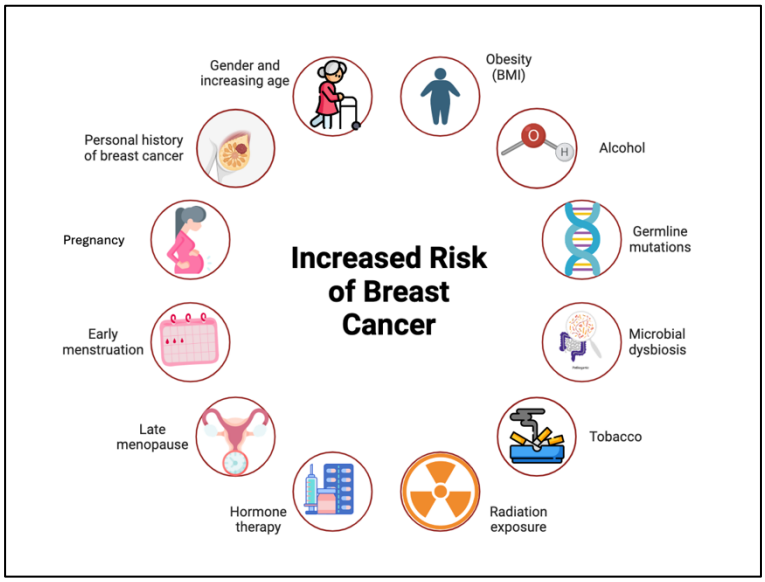

Figure 4. *Breast cancer risk factors.*

In recent decades, the risk factors associated with cancer development in younger populations have gained importance in medical research. While evidence points to an etiological role of exposures to risk factors during early life and young adulthood, the

specific effects of individual exposures remain largely unknown. The early-life exposome (diet, lifestyle, obesity, environmental exposures, and the microbiome) has undergone substantial changes since the mid-20th century, showing variable patterns globally. The early-onset cancer epidemic may represent one manifestation of increasing trends in chronic diseases among younger and future generations.

Prospective cohort studies incorporating electronic health records and early-life biospecimen collection could enable information of these factors and their associations with numerous future health outcomes, including cancer. Therefore, raising awareness of the early-onset cancer epidemic and improving early-life environmental conditions, should be immediate goals to reduce the burden of both early and later-onset cancers (11).

2. Breast cancer in very young women

BC is a complex and heterogeneous disease that affects women across various age groups. Although it is usually recognized as a disease predominantly detected in older women, the incidence of BC among very young women (defined as those diagnosed at 35 years of age or younger) is increasing and presents unique clinical and biological challenges. This subset of patients often exhibits a more aggressive phenotype and less favourable outcomes compared to older women, regardless of stage at diagnosis (12).

Several factors contribute to these poor outcomes, including diagnostic delays, unfavourable pathologic features, and inadequate treatment compliance (13). Notably, there is a higher proportion of HER2-positive (HER2+), and triple-negative (TN) BC among younger women, which are the BC subtypes associated with poorer prognosis (14–16). However, hormone receptor-positive/HER2-negative (HR+/HER2-) is still the most frequent subtype. Although HR+ BC generally has a more favourable prognosis, it remains a clinical challenge in young women.

Indeed, women diagnosed with luminal BC under 40 years of age have a statistically significant increased risk of BC death than postmenopausal patients. Interestingly, this increase in mortality rate was not observed among women with either triple-negative or HER2-positive subtypes. These observations highlight the need for a deeper understanding of the disease in very young patients, which could impact clinical management strategies for this subgroup. Moreover, this subset of patients is underrepresented in clinical trials.

In recent years, the advent of high-throughput technologies has revolutionized cancer research, enabling a comprehensive analysis of the genomic and molecular landscape of tumors. Currently, Oncotype DX is considered a valuable tool for tailoring adjuvant treatment for early-stage HR+ BC patients. Nevertheless, results from the RxPONDER clinical trial suggest that Oncotype DX is less predictive of chemotherapy benefits in premenopausal patients with node-positive BC compared to postmenopausal women. Transcriptomic profiling has emerged as a powerful tool to unravel molecular alterations and key biological processes, as well as to identify new molecular subtypes and potential targeted therapies.

1.3. Biological characteristics in Very Young Breast Cancer patients

VYBC patients presents more aggressive tumor biology and poorer outcomes compared to older patients as we commented before (17,18). Higher proliferation rates, more aggressive phenotypes, and distinct genomic profiles are distinguished features of these tumors (19). Waks et al revealed mutational differences in PIK3CA, GATA3, and ARID1A alterations between young and older patients with luminal A breast cancer (20).

VYBC patients have intermediate- to high-risk genomic signatures (21) and mutations in structural protein-coding genes (22). Transcriptional patterns reflecting increased proliferation and oncogenic signalling were identified in this subgroup, independently of molecular subtypes. A novel 6-gene signature pointing in aggressive tumor features, tumor proliferation, and reduced survival was associated with more aggressive clinical and pathological features of breast cancer and had independent prognostic value for predicting outcomes (23).

Across multiple subtypes these patients present lower mutation burdens but are enriched for driver mutations and copy-number alterations and presents hallmarks of premature senescence. Probably they also exhibit unique immune microenvironment characteristics, that may affect immunotherapy responses (19,24).

These findings underline the importance of biological age-specific considerations in clinical management to improve outcomes for young breast cancer patients. Further research is needed to develop age-specific biomarkers to define treatment strategies in these patients.

1.4. Transcriptomic analysis in breast cancer

Multi-omics approaches have demonstrated a better understanding of BC heterogeneity. Single-cell have revealed complex cellular ecosystems, identifying new immune cell populations and stromal-immune clusters associated with clinical outcomes (25). Advancing in omics techniques, such as, liquid biopsy, immune-omics, interact-omics, holds significant potential for early and non-invasive BC diagnosis (26). Moreover, genomic and transcriptomic analyses of paired primary tumors and metastases have described enriched genomic alterations and subtype switching (27).

Integration of genomics, transcriptomics, and proteomics have provided deeper insights into molecular subtypes, gene expression patterns, and protein regulation (28). In summary, new research focus on the importance of integrating multiple omics strategies are needed to decipher BC heterogeneity and develop personalized treatment strategies.

3. The immune landscape in breast cancer

BC is significantly influence by the immune microenvironment. Immune system plays a fundamental role in maintaining tissue homeostasis through monitoring and triggering inflammatory responses of innate and adaptative immune cells.

Recent studies have identified different immune subtypes among breast tumors, characterized by biological functions, immune cell signatures, and clinical outcomes (29). Immune checkpoint inhibitors (ICI) have shown promise in treating advanced triple-negative breast cancer (30). However, the immunosuppressive TME remains a significant barrier for treatment effectiveness (31). Some strategies are needed to overcome this resistance, including combination therapies and targeting specific immune cell populations (32). Understanding the relation between systemic and local immune responses is crucial for developing new approaches in BC treatment (33).

1.5. The host immune response

The cancer-immune set point, determined by tumor host and environmental factors, influences the capacity and timing of anticancer responses (34). Host-intrinsic factors as genetics, epigenetics, and immunity with the exposome, impact ICI response and toxicity (35).

TME contains different immune cell populations, including tumor-infiltrating lymphocytes (TILs), which serve as prognostic biomarkers in HER2+ and TNBC (34). TILs are immune cells that have migrated to the tumor tissue and TME. CD8+ cytotoxic T lymphocytes are the predominant TIL cell population and correlate with survival. CD4+ T helper cells can have dual activity as anti-tumor (Th1) and pro-tumor (Th2) effects. Regulatory T cells (Tregs) generally promote tumor growth by suppressing anti-tumor immunity (**Figure 5**).

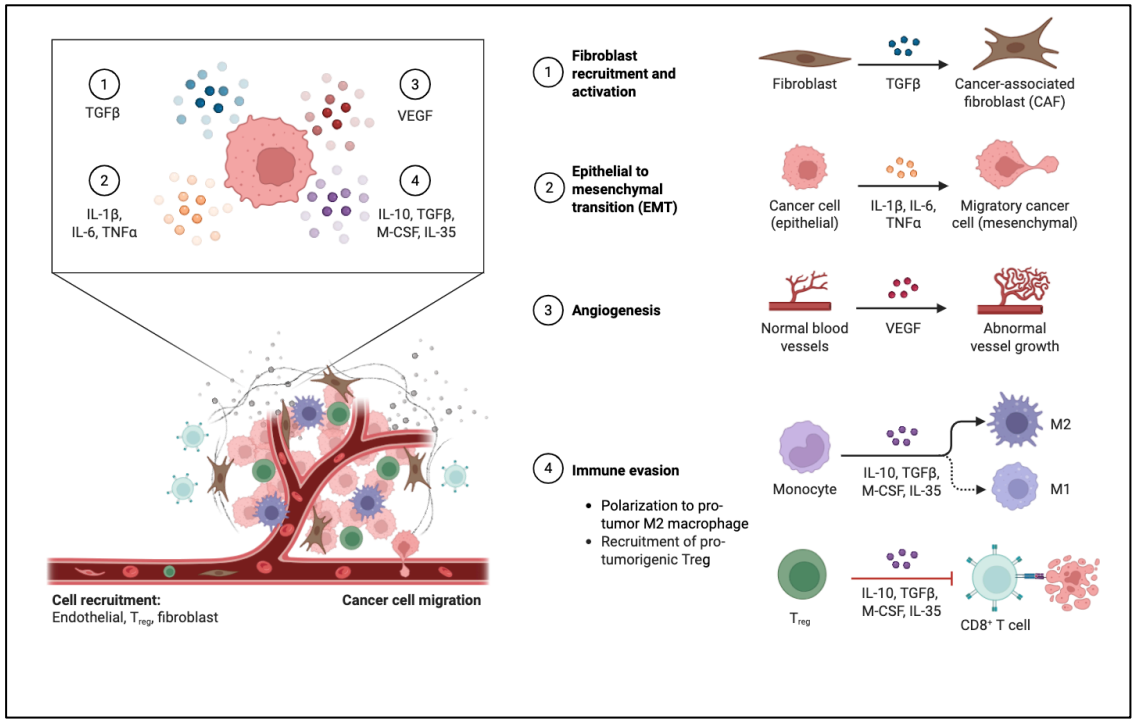

Figure 5. *Components of tumor microenvironment. Created by Biorender.*

Tertiary lymphoid structures (TLSs) are emerging as important prognostic biomarkers in BC. Multiple studies have shown that TLS presence is associated with improved overall survival and disease-free survival (29,36,37). TLSs are positively correlated with early tumor stage, high tumor-infiltrating lymphocytes, HER2 and Ki-67 expression, nevertheless are negatively associated with estrogen and progesterone receptor status (36,37). They are particularly common in triple-negative breast cancer and may predict better outcomes regardless of other factors like PDL-1 expression.

B lymphocytes (CD20+ cells) are adaptive immune cells responsible for humoral immunity through the production and secretion of antibodies. They also contribute to the immune response by presenting antigens and co-stimulatory molecules to T lymphocytes.

In BC, B lymphocytes and immunoglobulin gene expression have been shown favourable outcomes in retrospective studies (38,39). While this suggests a protective anti-tumor role for B cells and antibodies, other research has associated them with poor prognostic indicators in BC. B cells can induce the production of transforming growth factor beta (TGF-β), a cytokine that promotes the conversion of CD4+ T cells into immunosuppressive T regulatory cells (40).

Invasive tumors show significant immune infiltration, with the composition and density of immune cells impacting prognosis. Higher levels of CD8+ T cells, M1 macrophages, and mature dendritic cells are associated with better outcomes. Recent single-cell analyses have identified that exhausted CD8+ T cells including diverse subpopulations as immune checkpoint blockade-permissive stem-like cells and immune checkpoint blockade-refractory terminally differentiated cell. These populations are in distinct niches and are characterized by specific transcriptional processes (41).

1.6. Immunotherapy in breast cancer

As a promising approach for breast cancer treatment, immunotherapy has emerged particularly for triple-negative subtype as a standard of treatment in early and metastatic setting (42). In HER2+ subtype it is not a standard of treatment however, there is a strong biological rationale supporting it. HR+/HER2- subtype presents challenge for immunotherapy strategies due to lower immunogenicity and TILs (43).

Immune checkpoint inhibitors, especially those targeting PD-1/PD-L1, have shown efficacy in clinical trials when combined with chemotherapy (44,45). (**Table 1)**.

Table 1. *Breast cancer and immunotherapy clinical trials*

Study	Phase	Population	Intervention	Key Findings
GIADA Trial	II	Early-stage HT+/HER2- premenopausa l BC patients	Chemotherapy + nivolumab	Luminal-B BC with a Basal molecular subtype and/or a state of immune activation may respond. More research is needed.
I-ISPY 2.2	II	Early-stage, high-risk breast cancer	Dato-Deruxtecan + durvalumab	EFS/DFS/OS N/A. Not powered for statistical significance. Predictive subtypes immune response: RPS3 (HER2-/immune+): PCR 72%. Larger

				confirmatory trial is needed.
Checkmate 7FL	III	Early-stage, high-risk RH+/HER2- BC	Chemotherapy ± Nivolumab	More PCR in immunotherapy arm especially in G3, N+ and PDL-1+
Keynote-522	III	Early-stage TNBC	Pembrolizumab + chemotherapy	OS at 5 years: 86.6% vs. 81.7%; EFS at 5 years: 81.2% vs. 72.2%; consistent safety profile.
GeparNuevo NCT02685059	II	Early-stage TNBC	Chemotherapy ± Durvalumab	Durvalumab added to NACT in TNBC significantly improved survival despite a modest PCR increase and no adjuvant component of durvalumab. Additional studies are needed.
NeoTRIP NCT002620280	III	Early-stage TNBC	Nab-paclitaxel + Carboplatin (AUC2) ± Atezolizumab	Addition of atezolizumab to nab-paclitaxel and carboplatin did not significantly increase the rate of pCR. PD-L1 expression was the most significant factor influencing the rate of pCR.
Keynote-028	I	Advanced PD-L1+ solid tumors, including breast cancer (ER+/HER2-)	Pembrolizumab	ORR: 12% (95% CI, 2.5%-31.2%); Manageable safety profile.
PACE	II	HR+/HER2- breast cancer, PD CDKi + IA.	Palbociclib + Fulvestrant ± Avelumab	Adding immunotherapy to Palbociclib + Fulvestrant showed potential benefit; further validation required.
IMpassion 031	III	Early stage TNBC	Atezolizumab/placebo + Chemotherapy	Atezolizumab increased PCR rate by 17%. No statistical significance in long-term survival endpoints.
IMpassion 050	III	Early stage HER2+ BC	Atezolizumab/placebo + Chemotherapy + Trastuzumab-pertuzumab	Atezolizumab did not increase PCR rates versus placebo in the ITT or PD-L1-positive populations
Keynote-355	III	Metastatic TNBC	Pembrolizumab + chemotherapy	OS median (CPS ≥10): 23.0 months vs. 16.1 months.

IMpassion 130	III	Metastatic TNBC	Atezolizumab ± nab-paclitaxel	OS median (PD-L1+): 25.0 months vs. 15.5 months (HR=0.62); PFS median: 7.5 months vs. 5 months.
Impassion 131	III	1L Metastatic TNBC	Paclitaxel ± Atezolizumab	The combination of atezolizumab and paclitaxel failed to demonstrate survival benefits.
Keynote 150 ENHANCE-1	Ib/II	Metastatic TNBC. ≤2 prior systemic treatment	Eribulin + Pembrolizumab	Well tolerated and showed promising antitumor activity in mTNBC. Efficacy outcomes appeared influenced by line of therapy and PD-L1 status.
Keynote 119	III	Metastatic TNBC. ≤1-2 prior systemic treatment	Pembrolizumab or Chemotherapy	Not significantly improve OS in patients with previously treated metastatic TNBC
Safir-02 Breast immuno trial	II	Metastatic HER2-/HR+ or TN breast cancer. ≤1 prior systemic treatment	Maintenance with durvalumab vs Chemotherapy	Durvalumab not improve OS, PFS in overall population. Exploratory analysis: Improve OS in TNBC subgroup.
ENCORE-602	II	Metastatic TNBC previously treated.	Atezolizumab ± Entinostat	PFS was not prolonged with the combination and resulted in greater toxicity
Keynote 086	II	Metastatic TNBC ≥1 prior systemic treatment.	Pembrolizumab	Pembrolizumab monotherapy demonstrated durable antitumor activity in a subset of patients with previously treated mTNBC and had a manageable safety profile

CPS Combined positive score
DFS Disease free survival
EFS Event free survival
ITT Intention to treat
mTNBC Metastatic triple negative breast cancer
N/A Not applicable
NACT Neoadyuvant chemotherapy
ORR Objective response rate
OS Overall survival
PCR Pathologic complete response
PFS Progression free survival

Other immunotherapeutic strategies are under investigation; cancer vaccines, adoptive T cell therapy, oncolytic viruses and the combination of ICI with antibody drug conjugates will be a promising approach (46,47).

However, barriers remain on immunosuppressive tumor microenvironment and limited efficacy of immunotherapy in monotherapy (30,44). Nanomedicine approaches are being explored to improve immunotherapy delivery and efficacy while reducing derived toxicities (48). Ongoing research focuses on combination therapies and personalized strategies to overcome resistance (46).

1.7. Immune gene expression

Tumors' gene expression profiling has facilitated the identification of prognostic gene signatures and patients selection for targeted therapies (49).

Recent studies have investigated immune gene profiles and biomarkers to predict immunotherapy response in solid tumors. Some prognostic signatures have been described such as the NK, B cells, monocytes, macrophages and dendritic cells functions (50). Specific immune cell clusters as TREM2hi macrophages and γδ T cells, have shown resistance to ICI (51). On the other hand, Axelrod et al and Galili et al have identified immune-related signatures associated with better prognosis in TNBC (52,53). In addition, immune checkpoint genes, as CTLA-4 and PD-L1, show variable expression across BC subtypes (54).

TME plays essential role in cancer progression with stromal and immune infiltration (55). Innate immune cells, contribute to the complex interactions within the tumor microenvironment (56). In breast cancer, CD47 correlates with immune activation and favourable prognosis in certain subtypes (57). Additionally, peripheral immune cell populations and parameters (lymphocyte counts, neutrophil-to-lymphocyte ratio (NLR) and lymphocyte-to-monocyte ratio (LMR) are variable in BC patients and may serve as potential biomarkers for disease progression and therapy response (58).

The implementation of these tools will require a validation and the use of robust and reproducible genomic-based platforms. BC is predominantly transcriptional disease rather than one driven by mutational patterns if we compared with other solid tumors as melanoma and lung cancer. Considering the above-mentioned information, we must assume that we are at the beginning of a new paradigm in breast cancer therapy.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

1. Hypothesis

Very young women (≤35 years) with hormone receptor-positive, HER2-negative breast cancer have distinct transcriptomic profile that differentiates them from older patients (≥50 years) and may underlie their worse prognosis and distinct therapeutic vulnerabilities.

2. Objectives

2.1. Main objectives

To characterize the transcriptomic landscape of hormone receptor-positive, HER2-negative (HR+/HER2-) breast cancer in very young patients (≤35 years) compared older patients (≥50 years).

- Perform RNA sequencing analysis to identify differentially expressed genes and gene signatures between the two age groups.
- Determine whether specific transcriptomic features are associated with the more aggressive clinical behaviour observed in very young patients.

2.2. Secondary objectives

To explore the relationship between clinical characteristics and transcriptomic profiles across age groups.

- Integrate clinicopathological data with transcriptomic findings to uncover age-related patterns.
- Better understand how transcriptomic differences may influence in tumor phenotype, treatment response and breast cancer behaviour.

To identify biological processes that may underlie the aggressive phenotype of HR+/HER2- breast cancer in very young patients.

- Use gene expression data to propose relevant molecular pathways and biologic processes that could be implicate in this setting.

MATERIAL AND METHODS

MATERIAL AND METHODS

1. Clinical samples

This study included 66 non-consecutive BC patients over the age of 18 years recruited at Hospital Clínico Universitario de Valencia (Spain), with ethical approval from the institutional ethical committee of HCUV (2024/387). All procedures adhered to the Declaration of Helsinki, and signed informed consent was obtained from all participants. For the RNA-seq studies, formalin-fixed paraffin-embedded (FFPE) samples were collected before any treatment from 33 VYBC patients (≤ 35 years old) and 33 OBC patients (≥ 50 years old). Twenty-two VYBC and 27 OBC samples met the quality criteria to carry out RNA-seq analysis (clinicopathological characteristics detailed in **Table 2**.

***Table 2**. Clinicopathological characteristics patient's cohort.*

Characteristics		VYBC	OBC
Total, n		22	27
Median age, years (range)		32.5 (22–35)	66 (50–94)
Molecular subtype, n (%)			
	Luminal	12 (54.5%)	17 (63%)
	TNBC	6 (27.3%)	6 (22.2%)
	HER2	4 (18.2%)	4 (14.8%)
Grade, n (%)			
	1	4 (18.2%)	5 (18.5%)
	2	11 (50%)	13 (48.1%)
	3	7 (31.8%)	9 (33.3%)
Stage, n (%)			
	I	11 (50%)	25 (92.6%)
	II	0 (0%)	1 (3.7%)
	III	11 (50%)	1 (3.7%)
Pathological T stage, n (%)			
	pT1	7 (31.8%)	22 (81.5%)
	pT2	12 (54.5%)	5 (18.5%)
	pT3	2 (9.1%)	0 (0%)
	pT4	1 (4.5%)	0 (0%)
Regional lymph node metastasis, n (%)			
	No	12 (54.5%)	24 (88.9%)
	Yes	10 (45.5%)	3 (11.1%)
Distant metastasis, n (%)			
	No	22 (100%)	27 (100%)
	Yes	0 (0%)	0 (0%)
PAM50 intrinsic subtype			
	Luminal A	7 (31.8%)	9 (33.3%)
	Luminal B	5 (22.7%)	4 (14.8%)

Basal-like	6 (27.3%)	6 (22.2%)
HER2-Enriched	2 (9.1%)	4 (14.8%)
Normal-like	2 (9.1%)	4 (14.8%)

*TNBC, triple-negative breast cancer

To validate our findings, a second cohort of BC patients was included, comprising FFPE tumor samples obtained from surgery, ensuring all patients had not received treatment prior to surgery. This validation cohort included 12 HR+/HER2-VYBC patients and 29 HR+/HER2-OBC patients, with clinicopathological characteristics detailed in **Table 3**. Patients were not carriers of genetic mutations related to hereditary BC.

Table 3. *Clinicopathological characteristics of HR+/HER2- patient samples in the validation cohort.*

Characteristics		Cohort	
		VYBC	OBC
Total, n		12	29
Median age, years (range)		33 (33–35)	62 (50-79)
Grade, n (%)			
	1	4 (33.3%)	6 (20.7%)
	2	8 (66.7%)	20 (69.0%)
	3	0 (0%)	1 (3.4%)
	n.a.	0 (0%)	2 (6.9%)
Stage, n (%)			
	I	5 (41.7%)	18 (62.0%)
	II	6 (50%)	7 (24.1%)
	III	1 (8.3%)	0 (0%)
	n.a.	0 (0%)	4 (13.7%)
Pathological T stage, n (%)			
	pT1	8 (66.7%)	15 (51.7%)
	pT2	3 (25%)	10 (34%)
	pT3	0 (0%)	0 (0%)
	pT4	1 (8.3%)	0 (0%)
	n.a.	0 (0%)	4 (13.7%)
Regional lymph node metastasis, n (%)			
	No	7 (58.3%)	26 (89.7%)
	Yes	5 (41.7%)	3 (3%)
Distant metastasis, n (%)			
	No	12 (100%)	29 (100%)
	Yes	0 (0%)	0 (0%)

*TNBC, triple-negative breast cancer; n.a., not available; HR, hormone receptor; HER2, human epidermal growth factor receptor 2; BC, breast cancer.

2. RNAseq analysis

FFPE tumor samples from treatment naïve core needle biopsies were analyzed. Following RNA extraction, a preanalytic sample selection was performed to assess and

ensure RNA quality. RNA sequencing was conducted using the HiSeq X Series platform (Illumina, San Diego, CA, USA). Quality control, alignment, and quantification of RNA-seq data were carried out using standard bioinformatics pipelines. PCA was used to assess samples similarity, with the expectation that all observations within group would cluster together. Differential expression analysis was carried out using the Bioconductor package DESeq2. Initially, genes with zero counts in all samples were excluded and then raw counts were normalized by DESeq2. For multiple testing correction, p was adjusted using the false discovery rate (FDR) method. Genes with FDR below 5% were considered significant. Differentially expressed genes (DEG) between VYBC and OBC were identified based on absolute log2 fold change ≥ 2 and FDR $< 5\%$.

Heatmap dendrograms were generated using Euclidean distance and the complete clustering method within a hierarchical clustering algorithm. To analyze the change in the mean levels of each gene signature in VYBC and OBC patients, a T-test was performed if the normality assumption was met; otherwise, the nonparametric Wilcoxon rank-sum test was used. Normalized counts of genes comprising a gene signature were averaged for this analysis.

3. Immunohistochemical analysis

Immunohistochemical staining was performed on FFPE tissue sections using VENTANA BenchMark XT (Roche, Basel, Switzerland). Primary antibodies against ER (clone SP1, Ventana), PR (clone 1E2, Ventana), HER2 (clone 4B5, Ventana), and Ki67 (clone 30-9, Ventana), and UltraView Universal DAB Detection Kit (760-500, Ventana) were used following manufacturer's protocols. Evaluation was carried out by an expert pathologist.

4. PAM50 intrinsic subtyping

All tumors were assigned to an intrinsic molecular subtype of BC (Luminal A, Luminal B, HER2-E, Basal-like) and the normal-like group using the research-based PAM50 subtype predictor. The PAM50 subtyping was performed using the RNA-seq gene expression data from the PAM50 genes. Intrinsic subtypes were determined by the PAM50 predictor after applying the HER2/estrogen receptor subgroup-specific gene normalization method as previously described (59,60).

5. PAM50-Based calculation of ROR and CES scores

The PAM50 ROR was calculated using a previously reported and validated formula. ROR was analyzed both as a continuous variable, and as categorical groups based on previously established cutoffs with low-, moderate- and high-risk scores (59,60).

The PAM50-based CES score was calculated as previously reported (61,62). The correlation coefficients (CC) of each sample to the PAM50 Luminal A and Basal-like subtype centroids were computed, and the difference between these values was used to determine the CES (CES=CC to Luminal A –CC to Basal-like). CES was analyzed both as a continuous variable, and as categorical groups (CES-E [endocrine sensitive], CES-U [uncertain], and CES-C [chemo-sensitive]) based on cutoffs based on tertile groups determined in our dataset.

6. Gene set enrichment analysis

GSEA was conducted using the Bioconductor package ClusterProfiler for the genes overexpressed in VYBC luminal samples (63). GSEA was used to identify different groups of DEGs associated with different biological processes.

7. Deconvolution analysis

Computational deconvolution methods were applied to infer the cellular composition of the TME from bulk RNA-seq data. For this purpose, the immunodeconv R package (an integrative framework that provides access to multiple established deconvolution algorithms) was employed to estimate the relative abundance of immune and stromal cell populations within the tumor samples (64).

8. Histological examination

FFPE tumor specimens obtained from surgical resection were sectioned and stained with hematoxylin and eosin. An expert pathologist, blinded to the study arms, evaluated the presence and characteristics of TLS within the TME using established criteria (65). TLS included the recognition of lymphocytes in zones with high endothelial venules. The immune cells formed aggregates in solid and rounded configurations with or without germinal centers. TLS were scored when located within tumor stroma, defined as direct contact or within 5 mm of the tumor. The absolute number of TLS was recorded.

The pathologist also determined the percentage of TILs within the tumor samples using the same hematoxylin and eosin-stained sections, as previously established (66). TILs were defined as the mean percentage of stroma of an invasive carcinoma infiltrated by lymphocytes in 5% increments; 1% criteria were used if less than 5% of stroma was infiltrated by TILs.

9. Statistical analysis

All statistical analyses were conducted using R software (version 4.3) (R Core Team, 2024). Continuous variables were compared using the Student's t-test for normally distributed data, as assessed by Shapiro-Wilk test; when normality was not met, the nonparametric Wilcoxon rank-sum test was utilized. Categorical variables were assessed using the chi-square test or Fisher's exact test, as appropriate based on expected frequencies. Statistical significance was a set at a two-sided p-value < 0.05 for all comparisons.

RESULTS

RESULTS

1. Patient characteristics

To investigate age-associated molecular differences, this study enrolled 66 BC patients from the Hospital Clínico of Valencia. Patients were stratified into two distinct cohorts base on age: VYBC patients, defined as those aged 35 or younger and OBC patients, defined as those aged 50 years and older. This age-based stratification aimed to exclude the intermediate age group and enhance the contrast between biologically distinct subpopulation.

Among them, 22 samples from premenopausal VYBC patients (median age at diagnosis: 32.5 years; range 22-35), and 27 samples from postmenopausal OBC patients (median age at diagnosis: 66 years; range 50-94) met the quality criteria required for RNA-sequencing analysis. All samples were obtained from treatment-naïve primary tumors to avoid therapeutic biases in gene expression profiles. The clinicopathological characteristics of the included patients are summarized in **Table 2**.

2. Differential gene expression analysis

Transcriptome analyses were conducted on untreated primary tumors. The Principal Component Analysis (PCA) illustrated increased heterogeneity among VYBC samples (**Figure 6**). The examination of differentially expressed genes revealed 82 significantly downregulated and 12 significantly upregulated genes in VYBC samples (log2 fold change ≥ |2|; adjusted p < 89 0.05 (**Figure 7**, **Figure 8**, **Table 4**).

To further explore age-related differences in BC, gene signatures related to tumor aggressiveness and cellular composition of the TME were examined. Our data revealed an overrepresentation of signatures associated with proliferation and chromosome instability in the VYBC cohort. Besides, VYBC samples exhibited characteristics of more immunoreactive tumors, presenting significantly higher scores in immune-related gene signatures such as T cell markers, inflammation, IFN gamma, M1 macrophages, B cell markers, and immunoglobulin G (**Figure 9**).

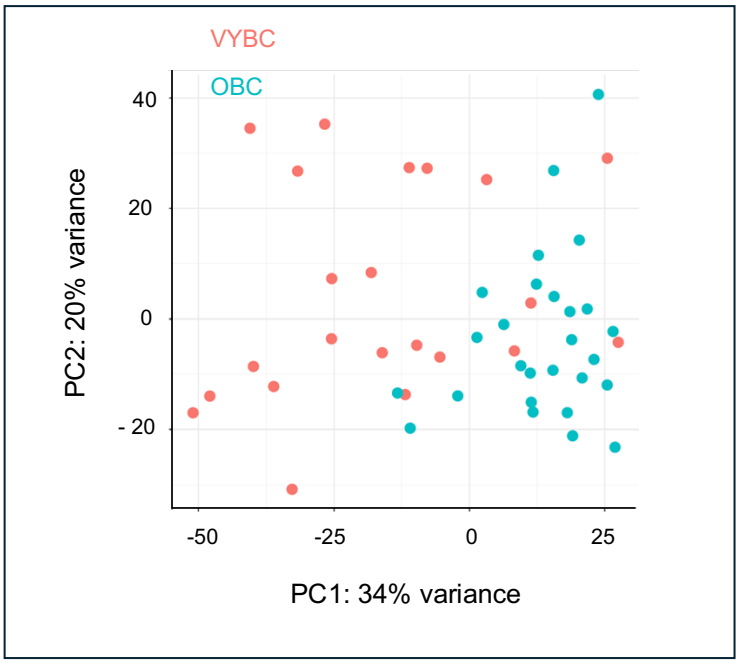

***Figure 6**. RNA-seq differential expression analysis between VTBC and OBC patients. PCA plot for VYBC samples (red) vs. OBC samples (blue).*

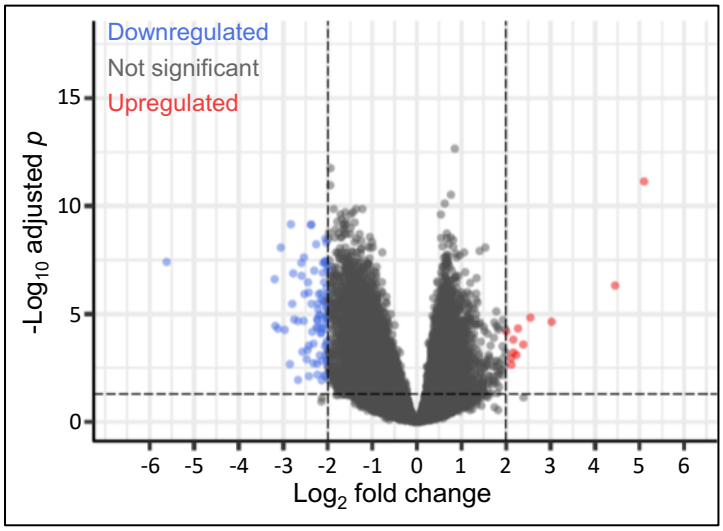

***Figure 7.** RNA-seq differential expression analysis between VYBC and OBC patients. Volcano plot representing RNA-seq differential expression results between VYBC and OBC. Colored dots represent genes significantly upregulated (red) or downregulated (blue) in VYBC with |log2FoldChange|> 2 and adjusted p < 0.05.*

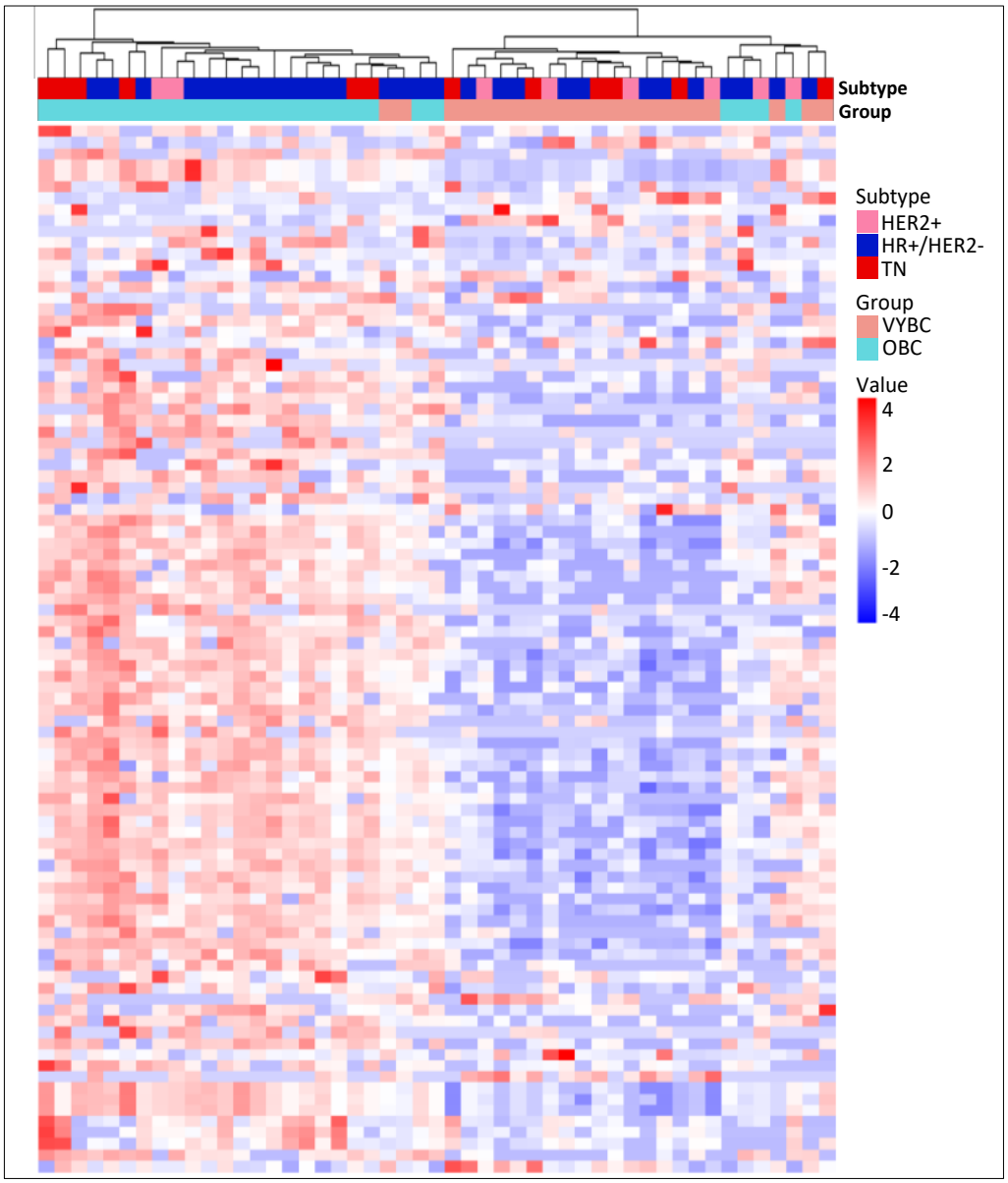

Figure 8. *RNA-seq differential expression analysis between VYBC and OBC patients. Hierarchical heatmap of the differentially expressed genes between VYBC and OBC patients. Each row represents a gene, and each column represents a patient sample.*

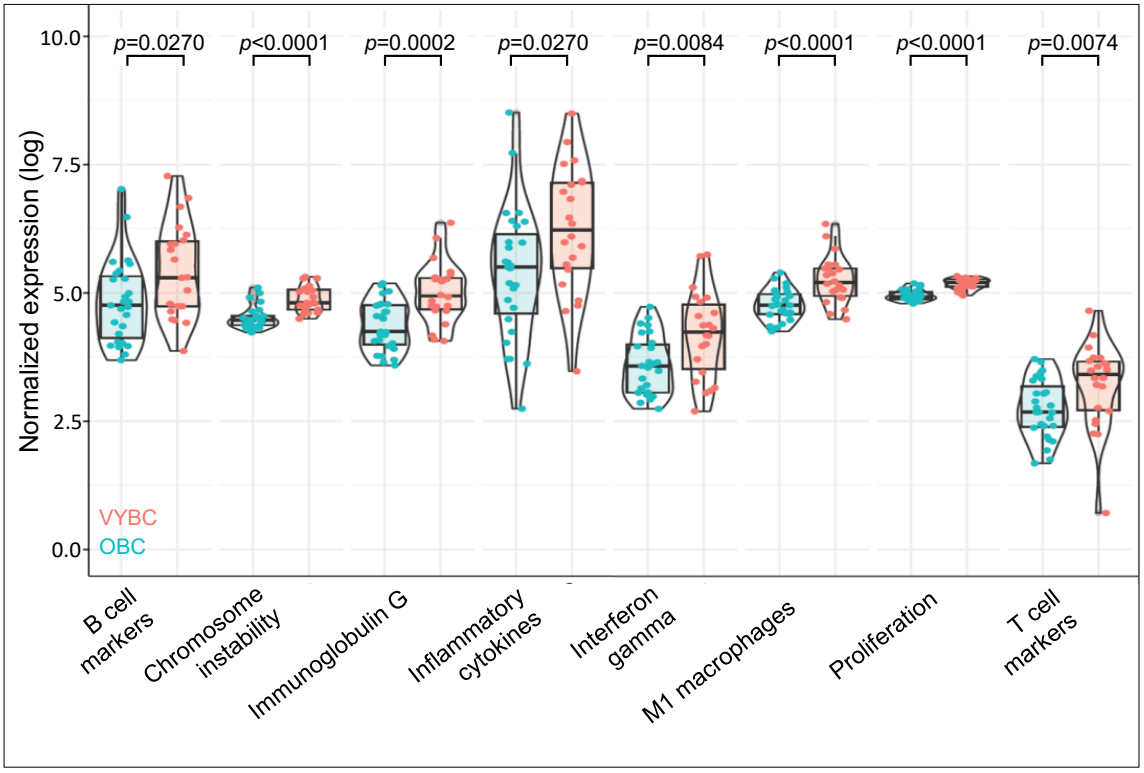

Figure 9. *RNA-seq differential expression analysis between VYBC and OBC patients. Violin plots and boxplots of normalized expression scores of gene signatures related to tumor aggressiveness and cellular composition of the TME in VYBC (red) and OBC (blue) samples.*

Table 4. *List of genes differentially deregulated in VYBC vs OBC patient samples (Fold Change ±2; adjusted P value < 0.05).*

Gene name	Fold Change (log 2)	Adjusted P value
DCD	-5,619	3,91E-08
CYP4F8	-3,194	2,48E-07
NDP	-3,179	3,56E-05
PIP	-3,119	4,80E-05
RNA5SP70	-3,056	8,42E-09
UGT2B28	-2,967	5,41E-05
LINC00613	-2,851	2,11E-03
RNA5SP331	-2,829	7,06E-10
RNA5SP40	-2,798	3,39E-06
RNA5SP157	-2,777	1,32E-07
GCNT1P4	-2,756	1,80E-05
TERB2	-2,672	2,11E-05
CYB5AP5	-2,668	1,13E-02
RNA5SP416	-2,592	4,39E-08
RNA5SP163	-2,588	1,72E-07
RNA5SP31	-2,570	5,62E-04
SQSTM1P1	-2,542	2,10E-05
RNA5SP292	-2,536	2,42E-08
RNA5SP105	-2,524	1,19E-06
UGT2B11	-2,475	1,24E-03

CYP2B7P	-2,455	4,72E-04
LINC02247	-2,446	3,42E-07
RNA5SP420	-2,428	1,02E-06
UGT2B24P	-2,425	2,91E-04
SCGB2A1	-2,421	7,60E-03
TRIM49C	-2,376	7,36E-10
TRIM49	-2,372	7,36E-10
RNU6-627P	-2,364	3,39E-06
VENTXP7	-2,349	6,62E-05
MIR6089	-2,325	1,88E-03
RNU6-904P	-2,321	2,36E-04
RNA5SP364	-2,304	9,92E-08
TRIM49B	-2,260	5,94E-09
RNA5SP213	-2,252	4,15E-05
MIR1180	-2,243	2,01E-03
LINC01986	-2,236	1,92E-05
RNA5SP461	-2,236	1,74E-05
MIR4634	-2,232	6,47E-03
RNA5SP285	-2,220	4,40E-05
RNA5SP471	-2,218	5,92E-06
MIR320E	-2,213	3,26E-06
RNA5SP371	-2,203	1,21E-06
TCL1B	-2,195	1,17E-05
MRPS33P4	-2,179	8,15E-04
LINC02106	-2,163	7,91E-05
FOSB	-2,163	1,09E-05
RNA5SP341	-2,153	1,08E-06
RNA5SP146	-2,139	7,17E-05
OR8C1P	-2,137	1,17E-02
RNA5SP197	-2,137	2,10E-06
CLCA2	-2,136	1,46E-03
RNA5SP480	-2,135	4,57E-05
RNA5SP91	-2,111	1,25E-07
RNA5SP106	-2,103	4,33E-08
RNA5SP176	-2,101	2,84E-05
CFC1	-2,084	2,48E-06
RNA5SP401	-2,083	3,40E-07
CFC1B	-2,081	2,72E-06
SRSF1P1	-2,079	6,74E-03
RNA5SP466	-2,070	4,17E-08
RBPMS2P1	-2,065	3,66E-08
SNORD31B	-2,063	3,14E-04
RNA5SP46	-2,057	3,30E-09
RNA5SP164	-2,051	7,75E-03
RNA5SP90	-2,049	7,68E-04
RNA5SP258	-2,048	3,59E-08
RNA5SP117	-2,046	4,78E-05
HMHB1	-2,041	1,34E-05
LINC00587	-2,037	2,42E-04
RNA5SP133	-2,035	1,45E-05
RNA5SP396	-2,032	5,02E-09

AKR1B10P1	-2,027	1,32E-03
RNA5SP241	-2,025	4,34E-06
RNA5SP27	-2,025	5,06E-06
RNA5SP271	-2,022	5,94E-07
OR11G1P	-2,016	1,72E-04
HPGD	-2,014	1,85E-05
CDY18P	-2,011	2,28E-03
RNA5SP425	-2,007	6,07E-08
RNA5SP497	-2,004	1,17E-06
RNA5SP303	-2,003	1,26E-03
SHISAL2B	-2,001	8,89E-08
ELOCP21	2,007	6,42E-05
SIMALR	2,112	1,11E-03
TRAJ31	2,123	2,24E-03
AREG	2,163	6,09E-04
PRSS2	2,163	1,54E-04
GABRP	2,234	7,78E-04
ZFP57	2,269	4,70E-05
IL20	2,388	2,58E-04
CXCL13	2,547	1,48E-05
TCN1	3,026	2,31E-05
CSN3	4,448	4,82E-07
CPB1	5,100	7,36E-12

3. Subtype-specific analyses of classical markers, intrinsic subtype, risk of recurrence, and chemoendocrine sensitivity

To better understand the age-related differences between VYBC and OBC, subtype-specific analyses were conducted. Progesterone and estrogen receptors expression were evaluated by immunohistochemistry (IHC), revealing no differences at the protein level (**Figure 10**). In contrast, gene expression analyses showed lower expression of PGR and estrogen receptor (ESR1) expression at mRNA level in HR+/HER2-VYBC compared to OBC patients (**Figure 11**). Proliferation was assessed by measuring Ki67 levels through IHC, revealing significant overexpression in VYBC samples within HR+/HER2-and TN subtypes. This was confirmed by RNA-sequencing analysis only in the HR+/HER2-subset (**Figure 10**, **Figure 11**).

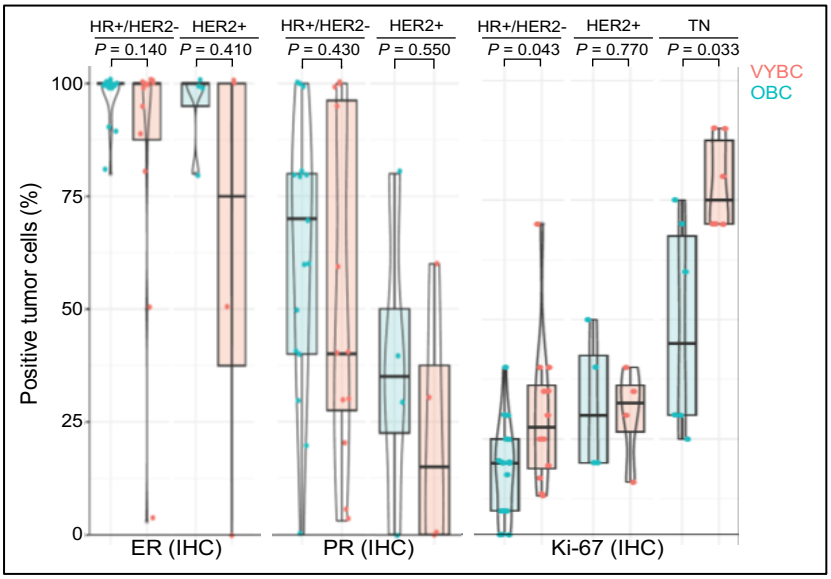

Figure 10. *Subtype-specific analyses of classical BC markers. Violin plots and boxplots of the percentage of positive tumor cells by IHC and by BC subtypes. VYBC are represented in red, and OBC samples in blue.*

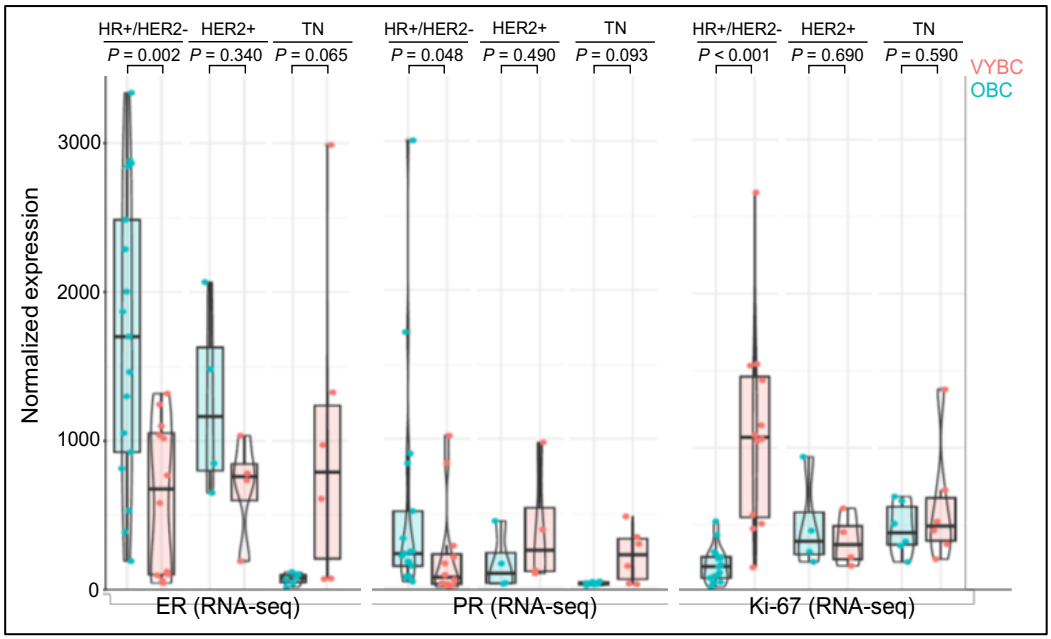

Figure 11. *Subtype-specific analyses of classical BC markers. Violin plots and boxplots of the percentage of normalized expression data from RNA-seq by BC subtype. VYBC are represented in red, and OBC samples in blue.*

The distribution of the PAM50-based intrinsic subtype classification in the VYBC samples was 31.8% Luminal A, 22.7% Luminal B, 27.3% Basal-like, 9.1% HER2-Enriched, and 9.1% Normal-like. In the OBC samples, the subtypes were distributed as

33.3% Luminal A, 14.8% Luminal B, 120 22.2% Basal-like, 14.8% HER2-Enriched, and 14.8% Normal-like (**Table 2**). Among HR+/HER2-patients, the distribution in VYBC versus OBC was 25.0% versus 47.1% for 122 luminal A, 25.0% versus 17.6% for luminal B, 41.7% versus 5.9% for basal-like, and 8.3% versus 17.6 for Normal-like. HER2-Enriched was observed in 11.8% of OBC patients (**Table 5**).

Table 5. *Clinicopathological characteristics of HR+/HER2- patient samples.*

Characteristics	**VYBC**	**OBC**
	12	17
Median age, years (range)	33 (28–35)	66 (49–79)
Grade group, n (%)		
1	3 (25%)	5 (29.4%)
2	7 (58.3%)	11 (64.7%)
3	2 (16.7%)	1 (5.9%)
Stage, n (%)		
I	9 (75%)	17 (100%)
II	0 (0%)	0 (0%)
III	3 (25%)	0 (0%)
Pathological T stage, n (%)		
pT1	6 (50%)	13 (76.5%)
pT2	6 (50%)	4 (23.5%)
pT3	0 (0%)	0 (0%)
pT4	0 (0%)	0 (0%)
Regional lymph node metastasis, n (%)		
No	8 (66.7%)	16 (94.1%)
Yes	4 (33.3%)	1 (5.9%)
Distant metastasis, n (%)		
No	12 (100%)	17 (100%)
Yes	0 (0%)	0 (0%)
Intrinsic subtype		
Luminal A	3 (25.0%)	8 (47.1%)
Luminal B	3 (25.0%)	3 (17.6%)
Basal-like	5 (41.7%)	1 (5.9%)
HER2-Enriched	0 (0.0%)	2 (11.8%)
Normal-like	1 (8.3%)	3 (17.6%)

The PAM50-based ROR score was also calculated, showing a higher ROR in VYBC compared to OBC, exclusively in the HR+/HER2-subset of samples (p = 0.027). Our results revealed that in HR+/HER2-VYBC group, 50.0% of the cases were classified as ROR-high, 33.3% 128 as ROR-medium, and 16.7% as ROR-low, while in HR+/HER2-OBC group ROR-high, ROR-medium, and ROR-low represented 11.8%, 47.1%, and 41.2%, respectively (**Figure 12**, **Figure 13**).

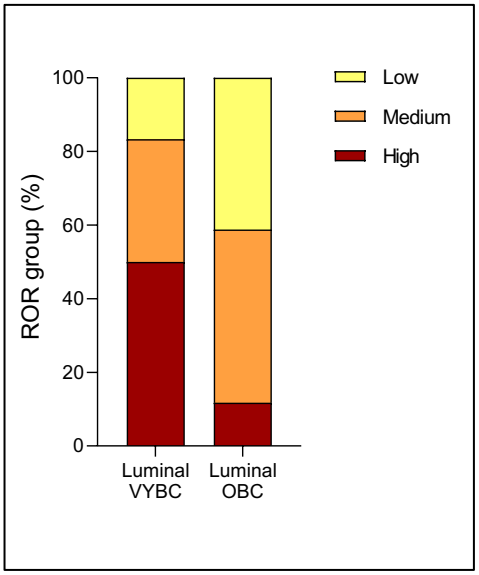

Figure 12. *Stacked bar charts of the distribution of the ROR groups in VYBC and OBC luminal samples.*

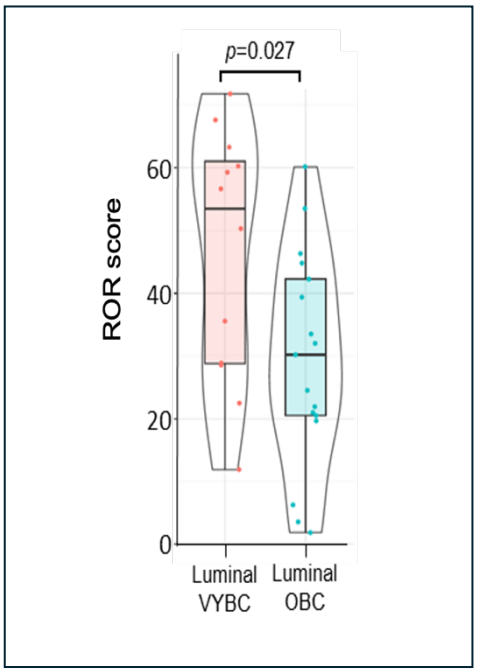

Figure 13. *Violin plots and boxplots of the ROR score in luminal samples. VYBC samples are represented in red, and OBC samples in blue.*

The PAM50-based CES was also determined for HR+/HER2-samples. CES is a genomic signature that estimates chemoendocrine sensitivity in BC. Thus, samples were classified as chemotherapy-sensitive (CES-C), uncertain (CES-U), or endocrine-sensitive (CES-E). Interestingly, the distribution in VYBC samples was: 58.3% CES-C, 16.7% CES-U, and 25.0% CES-E. In contrast, OBC samples showed a distribution of 23.5% CES-C, 29.4% CES-U, and 136 41.1% CES-E. Additionally, the analysis of CES as a continuous variable revealed significant differences between HR+/HER2-VYBC and OBC samples. VYBC patients presented lower levels of CES, indicating a better response to chemotherapy compared to OBC patients ($p = 0.017$). This finding suggests that VYBC patients were more resistant to endocrine therapy than OBC patients (**Figure 14**, **Figure 15**).

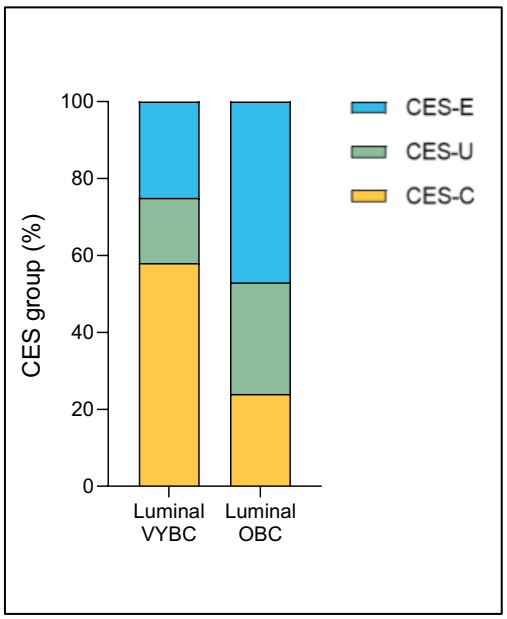

***Figure 14**. Stacked bar charts of distribution of CES groups in HR+/HER2-VYBC and OBC samples.*

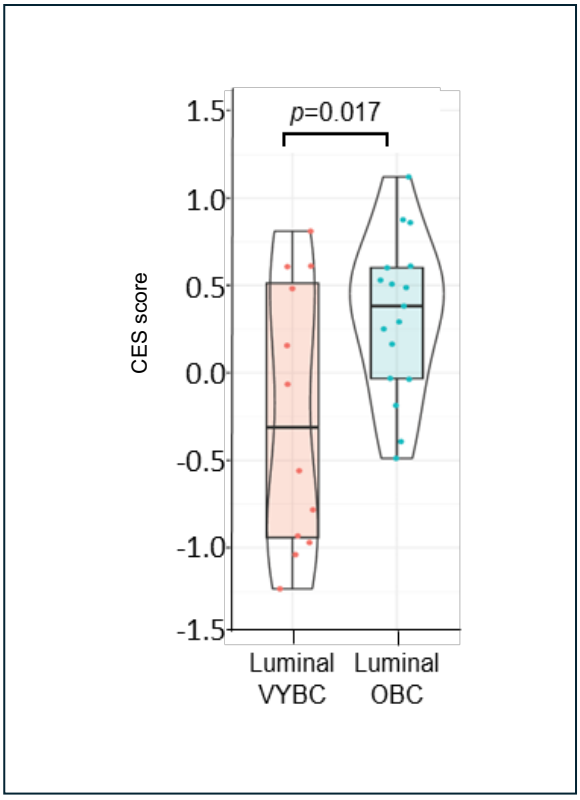

Figure 15. *Violin plots and boxplots of CES score in HT+/HER2- samples. VYBC are represented in red and OBC samples in blue.*

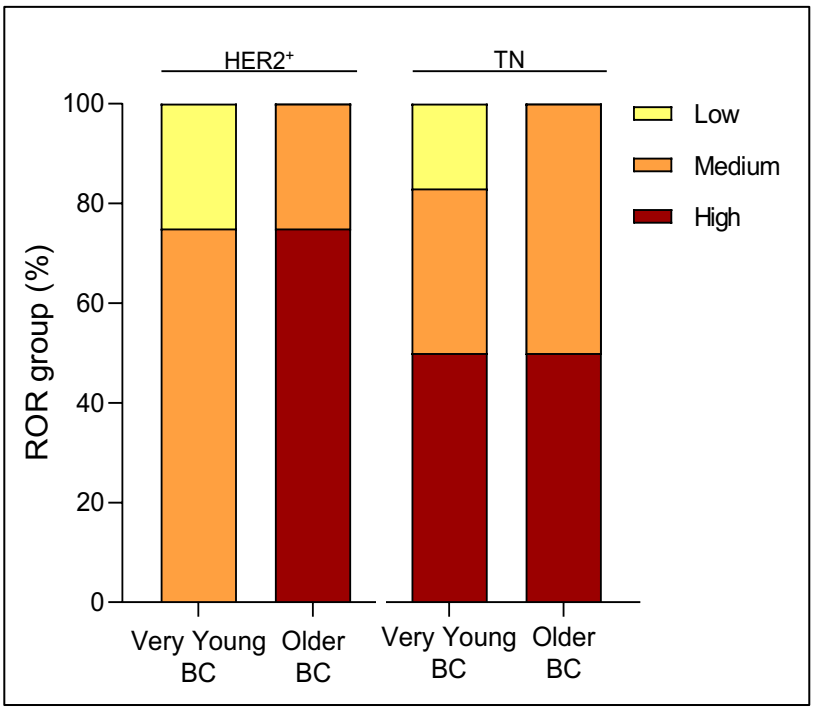

Figure 16. *PAM50 risk of recurrence (ROR) analysis. Stacked bar charts of distribution of ROR groups in VYBC and OBC HER2 (left) and TNBC (right) samples.*

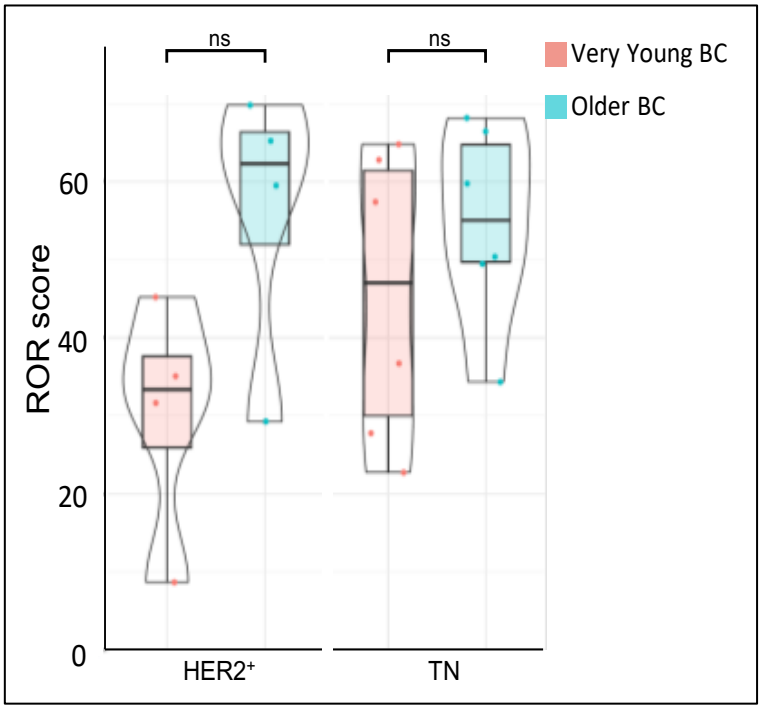

Figure 17. *PAM50 risk of recurrence (ROR) analysis. Violin plots and boxplots of ROR Score in HER2 (left) and TNBC (right) samples. VYBC are represented in red and OBC samples in blue.*

4. Differential gene expression analysis of HR+/HER2-tumors

Considering that the observed differences were specific to HR+/HER2- BC, we performed a transcriptomic analysis to identify age-associated gene expression variations within this cohort. This analysis included 12 samples from VYBC and 17 samples from OBC patients clinicopathological data in (**Table 5**). Notably, most diagnoses in both groups were at stage I.

PCA confirmed an increased heterogeneity among HR+/HER2-VYBC compared to OBC (**Figure 18**). Differential gene expression analysis identified 133 significantly downregulated and 106 upregulated genes in HR+/HER2-VYBC patients (log2 fold change ≥ |2|; adjusted $p < 0.05$) (**Figure 19**, **Table 6**). Remarkably, hierarchical clustering grouped VYBC and OBC patients separately, underscoring substantial differences between these age-defined subgroups (**Figure 20**). Moreover, Gene Ontology (GO) classification of differentially expressed genes showed an overrepresentation of categories related to immune activity in VYBC patients, including adaptive immune response, immunoglobulin production, immunoglobulin-mediated immune response, lymphocyte-mediated immunity, and production of molecular mediators of the immune response. Furthermore, categories associated with increased proliferation such as mitotic sister chromatid segregation, mitotic nuclear division, and sister chromatid segregation were also upregulated in VYBC patients (**Figure 21**, **Figure 22**)

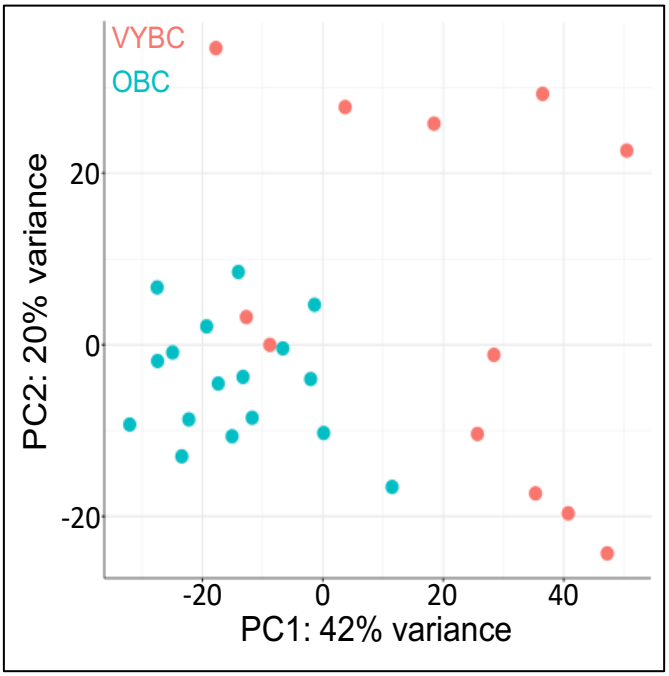

Figure 18. *RNA-seq differential expression analysis between VYBC and OBC HR+/HER2- patients. PCA plot for HR+/HER2-VYBC samples (red) vs. HR+/HER2-OBC samples (blue).*

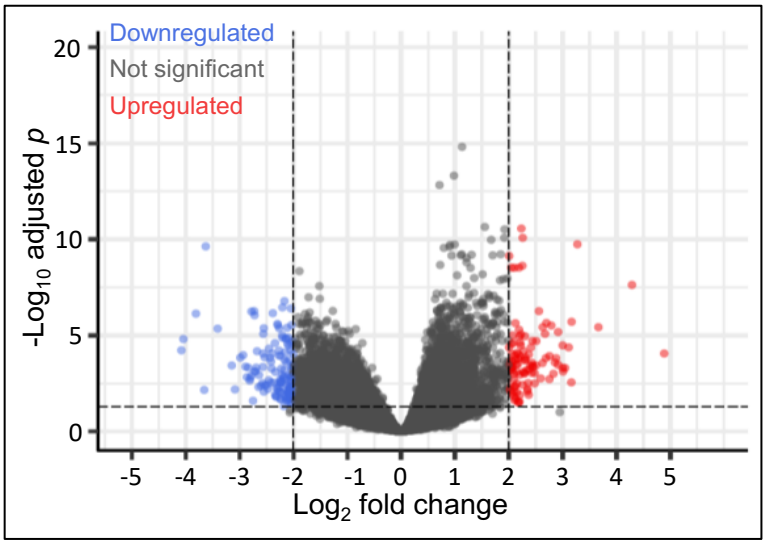

Figure 19. *RNA-seq differential expression analysis between VYBC and OBC HR+/HER2- patients. Volcano plot representing RNA-seq differential expression results between HR+/HER2- VYBC and OBC. Colored dots represent genes significantly upregulated (red) or downregulated (blue) in HR+/HER2-VYBC with log2 fold change ≥ |2|and adjusted p < 0.05*

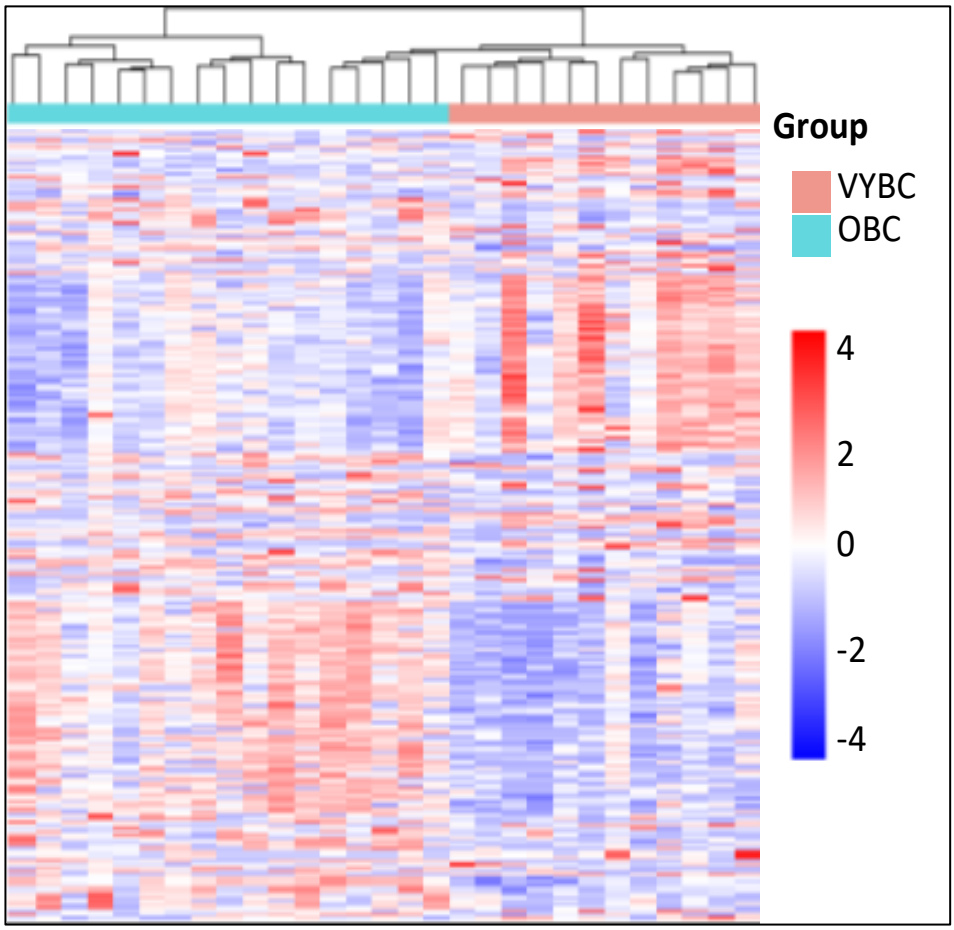

Figure 20. *RNA-seq differential expression analysis between VYBC and OBC HR+/HER2-patients. Hierarchical heatmap of the differentially expressed genes between HR+/HER2-VYBC and OBC patients. Each row represents a gene, and each column represents a patient samples.*

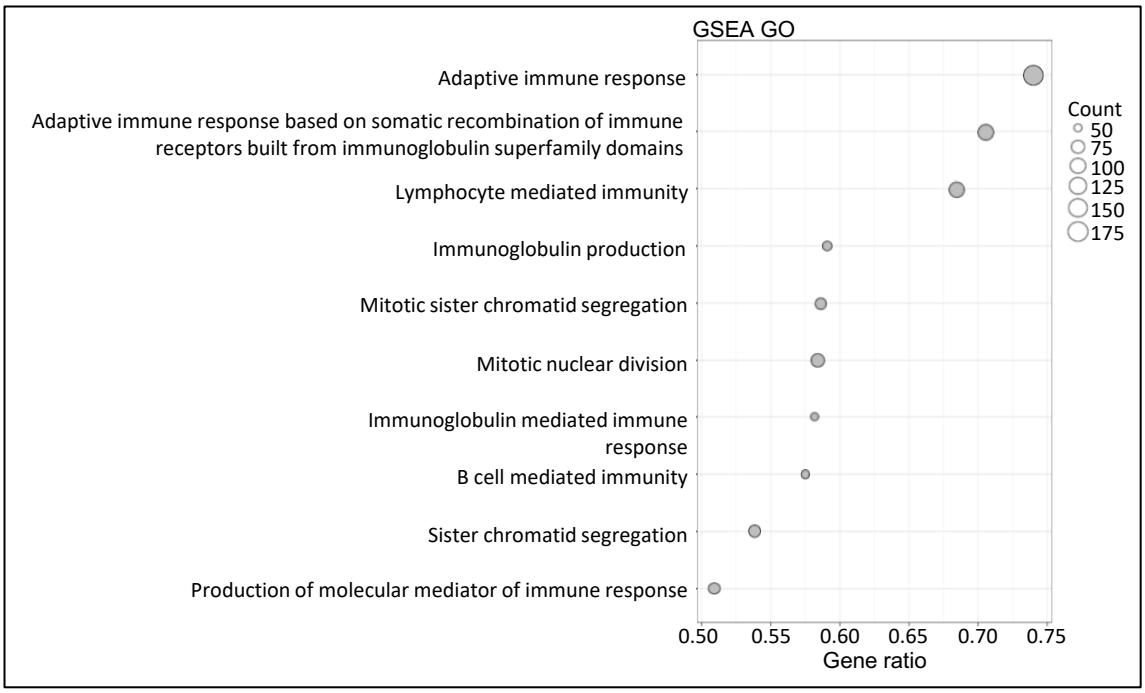

Figure 21. *RNA-seq differential expression analysis between VYBC and OBC HR+/HER2-patients. Gene set enrichment analysis (GSEA) of genes overexpressed in HR+/HER2-VYBC patients using the GO Biological Processes. The name of the biological pathway (Y-axis) and gene count in each item (X-axis) are shown (p = 1.75e−09).*

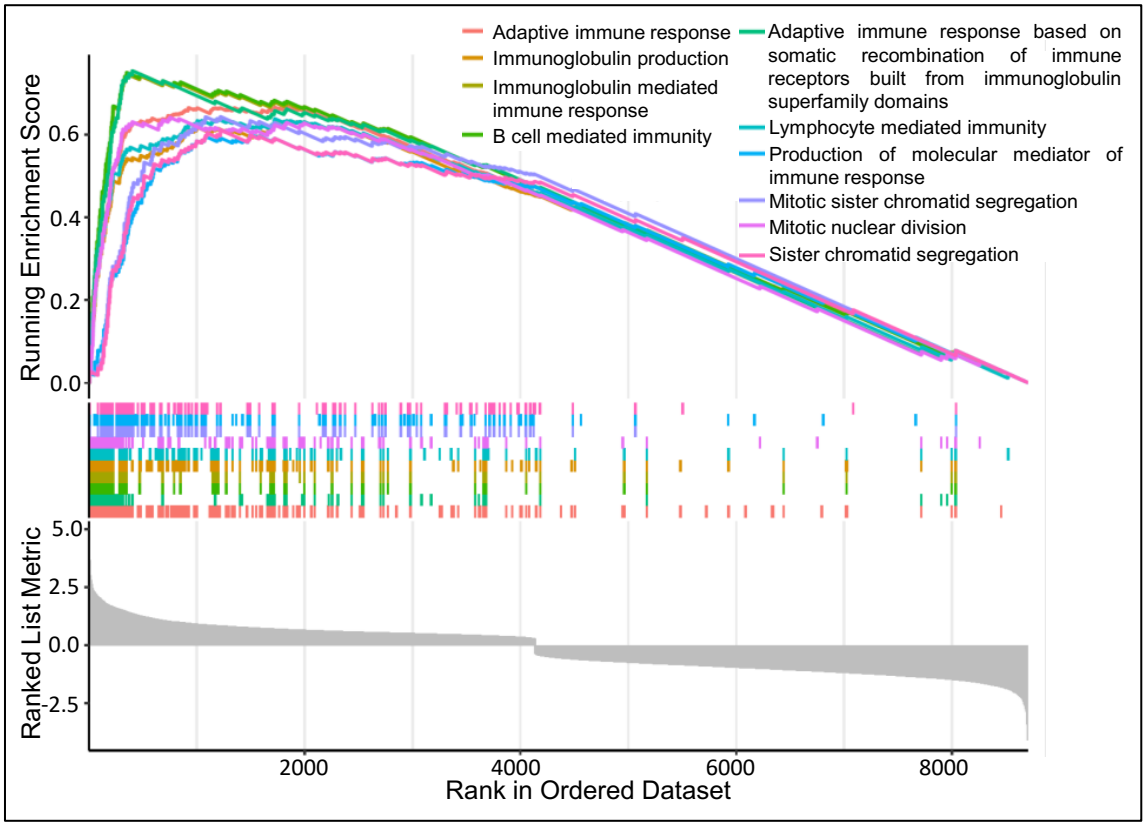

Figure 22. *RNA-seq differential expression analysis between VYBC and OBC HR+/HER2-patients. GSEA plot for enriched biological pathways in HR+/HER2-VYBC vs. HR+/HER2-OBC samples showing the Running Enrichment Score profile and positions of gene set members on the ranked list.*

Table 6. *List of genes differentially the regulated in VYBC vs OBC luminal patient samples (Fold Change ±2; adjusted P value < 0.05)*

Gene name	Fold Change (log 2)	Adjusted *P* value
PIP	-4,08	5,96457E-05
NDP	-4,04	1,54894E-05
CYP4Z1	-3,81	7,38102E-07
CYB5AP5	-3,66	0,006867842
CLIC6	-3,63	2,33764E-10
CYP2B6	-3,41	4,43404E-06
SCGB2A2	-3,15	0,000367414
LINC00613	-3,09	0,006505245
CYP4Z2P	-2,99	0,000144394
CYP4F8	-2,93	0,000106513
RNA5SP213	-2,89	0,000422441
RNU6-324P	-2,86	0,001492726
NDP-AS1	-2,84	0,000472176
UGT2B11	-2,81	0,00226475
UGT2B24P	-2,81	0,001738586
RNU6-904P	-2,78	0,001281919
TUBAP9	-2,78	5,70002E-07
DCD	-2,75	0,025228167
CYP4X1	-2,73	5,44262E-07
UGT2B28	-2,72	0,000756617
TUBAP8	-2,72	9,00777E-07
TCL1B	-2,70	0,000744917
RNA5SP105	-2,69	0,000473843
RNU6-627P	-2,68	6,63642E-05
RNU6-551P	-2,65	0,004897539
RNA5SP40	-2,64	0,001160899
LINC02116	-2,60	0,004157167
RNU6-819P	-2,57	0,003020894
RNU4-83P	-2,57	0,000438846
RNA5SP70	-2,56	8,72056E-05
RNY4P14	-2,56	8,3974E-06
FOSB	-2,55	4,22032E-06
CYP2B7P	-2,55	0,008542588
SHISA2	-2,53	0,000138368
RNU6-1258P	-2,53	0,004071615
SNORD31B	-2,50	0,003894513
RNU4-72P	-2,47	0,006114375
RNU6-648P	-2,46	0,002138501
RNA5SP292	-2,45	4,84802E-05
RNA5SP416	-2,43	0,000145279
RNY1P8	-2,42	0,002886481
RNY4P28	-2,42	0,000230537

PTPRT-AS1	-2,40	0,000526589
RNA5SP331	-2,40	8,74113E-05
RNY3P5	-2,38	6,99346E-07
CARTPT	-2,36	0,002861854
RNA5SP503	-2,34	2,5127E-05
MIR1180	-2,34	0,012094057
RNA5SP133	-2,34	0,000799945
UGT2B25P	-2,33	0,01469262
SNORA75B	-2,31	0,015770909
GCNT1P4	-2,31	0,005603623
TRIM49	-2,30	2,51875E-06
TRIM49C	-2,29	2,98379E-06
RNU4-63P	-2,29	0,005938262
RNU6-1207P	-2,27	0,008195472
RNU4-81P	-2,27	6,08266E-05
NOVA1	-2,27	0,000112733
RNY1P7	-2,27	0,004622582
TRIM49B	-2,26	4,22475E-06
RNU6-926P	-2,25	0,002581423
RNU6-714P	-2,25	0,00845458
RNU5E-5P	-2,24	0,001978467
RNA5SP468	-2,24	0,002795785
RNU6-793P	-2,24	0,020985721
LINC02888	-2,24	0,020516113
PTPRT	-2,23	0,000116093
RNA5SP115	-2,23	0,00290384
RNA5SP157	-2,23	0,000532905
RNU6-811P	-2,21	0,002818448
NR4A1AS	-2,21	0,001160899
KCNE4	-2,21	9,77375E-06
RNY3P8	-2,20	3,49378E-07
RNU6-573P	-2,20	1,32403E-05
KRTAP4-11	-2,20	0,009710044
EPYC	-2,18	0,001577457
RNA5SP461	-2,18	0,00135336
ARHGAP16P	-2,17	0,04853386
RNU6-541P	-2,17	0,000634225
RNA5SP41	-2,17	0,001887894
RNY4P19	-2,17	1,64323E-07
RNA5SP364	-2,16	0,000196691
MAOB	-2,15	7,4905E-05
OR10Q2P	-2,15	0,013988903
RNU6-760P	-2,15	0,018306047
MIOXP1	-2,14	1,5163E-05
FOS	-2,14	1,44356E-05
HNF4GP1	-2,13	0,024758337

RNU6-259P	-2,13	0,006938207
RNY4P16	-2,13	0,000248182
TFAP2B	-2,12	0,003722806
RASD1	-2,12	4,00753E-06
RNA5SP106	-2,11	2,77124E-05
UGT2B27P	-2,11	0,018878585
C11orf97	-2,10	0,009098066
VTRNA1-2	-2,10	0,0103597
SLITRK6	-2,10	0,025824222
RNU5A-6P	-2,10	1,30431E-05
TERB2	-2,09	0,009073781
KCND3-AS1	-2,08	3,33918E-05
RNA5SP401	-2,08	0,000180153
SNORD65B	-2,08	0,021219372
MRPS30-DT	-2,08	0,005985346
SCGB1D1	-2,08	0,016868364
RNA5SP420	-2,08	0,00116599
SLC6A4	-2,07	0,010725981
RNU6-1213P	-2,07	0,00468361
KRTAP4-8	-2,07	0,007699482
FBXL12P1	-2,06	0,005815021
RNA5SP163	-2,06	0,003222575
RNA5SP197	-2,05	0,001845794
RNU5A-2P	-2,05	6,18867E-05
RNU4-35P	-2,05	3,93738E-07
RNA5SP341	-2,05	0,001286059
RNA5SP176	-2,05	0,004526939
SLC5A8	-2,04	0,002446946
MRPS22P1	-2,04	0,013814778
RNA5SP361	-2,04	0,016533618
IGSF21	-2,04	2,49703E-05
RNA5SP220	-2,04	0,00040687
RNU6-521P	-2,04	0,008327021
RNA5SP285	-2,04	0,004903671
LINC01986	-2,04	0,00137498
RNU4-70P	-2,04	2,90627E-06
RNA5SP529	-2,03	0,034563776
RNA5SP533	-2,03	0,034563776
MRPS33P4	-2,03	0,015799182
SNORD105	-2,03	0,001447987
CST5	-2,03	0,000588883
NR4A1	-2,01	1,96511E-05
PTPRT-DT	-2,01	0,000432916
RNA5SP46	-2,00	8,75534E-05
RNU6-349P	-2,00	0,000371681
IGHV3-7	2,00	4,20103E-05

MELK	2,01	7,32365E-10
IGKV3-11	2,01	0,00035017
ELF5	2,02	0,00476262
LINC01152	2,02	0,006500583
IGHV1-69	2,03	0,000419847
IGHV3-48	2,04	0,000538019
IGKV1-13	2,05	0,000211382
CKAP2L	2,05	2,97253E-09
IGKV1-6	2,05	0,00048094
TRBV20-1	2,05	0,010376525
CDH3	2,05	2,78941E-05
PRAME	2,06	9,14722E-05
KRT86	2,06	0,000700593
CHODL	2,07	0,000439881
PADI2	2,07	0,000237643
CDCA7	2,07	9,86854E-06
IGLC3	2,09	2,33084E-05
IGHV3-20	2,09	0,00195194
ELOVL2	2,09	0,008425662
IGHV1-2	2,09	0,013843922
IGLV1-36	2,10	0,001021746
CENPF	2,10	3,02159E-09
FDCSP	2,11	0,024005526
MIR3680-1	2,11	0,004765511
CXCL9	2,11	0,000109273
IGHV3OR16-9	2,12	0,004413483
CDCA2	2,12	2,31248E-06
KCNJ3	2,13	0,019614894
IGHV1-46	2,13	0,000189594
IGHV3-21	2,14	0,000334314
IGHV1OR15-1	2,14	0,000785643
GNG13	2,15	0,009141492
IGLV3-19	2,15	0,003258932
RDH10-AS1	2,15	8,06804E-05
IGHV3-53	2,17	0,00030689
SPANXC	2,17	0,027705842
IGHV4-55	2,17	0,000163391
SPANXA2	2,18	0,032066815
IGKV1D-13	2,18	9,38825E-05
ASPM	2,19	2,97253E-09
IGLC2	2,19	5,06799E-06
IGKV1D-39	2,20	1,1396E-05
SPANXA1	2,20	0,028084937
IGHV3-66	2,20	0,001127022
MIR3679	2,20	0,012403653
RDH10	2,21	2,42197E-05

IGHV4-39	2,22	0,000130928
CXCL13	2,22	3,32963E-05
IGKV1-39	2,23	8,84143E-06
MKI67	2,23	2,74034E-11
FOXM1	2,25	2,37833E-09
ANLN	2,26	8,35287E-11
IGHV1-3	2,26	0,003750404
IGHV4OR15-8	2,27	0,000772237
IGKV1-12	2,28	6,57608E-05
IGHV2-26	2,29	0,00095373
ZFP57	2,29	0,000926917
IGKV1-8	2,29	0,000198336
IGLV3-21	2,30	1,10934E-05
IGHV3-69-1	2,31	0,000187862
IGHV5-51	2,31	0,000146383
IGKV3D-15	2,34	0,000634225
AREG	2,35	0,008082349
MUC5B	2,35	0,006294403
IGKV6D-21	2,36	0,003028096
HMGB3P32	2,37	0,012423043
IGLV1-47	2,37	9,76264E-05
IGLV1-44	2,38	0,000591081
IGHV1OR15-3	2,40	0,000376971
IGLV3-1	2,41	4,26053E-05
KLRC3	2,43	0,000888881
IGHV3-64	2,43	0,000265651
IGHG1	2,44	1,93897E-05
MMP7	2,44	0,00037247
IGKV3-15	2,45	0,000436768
MMP1	2,48	0,000920641
IGHJ3P	2,48	0,003164524
IGHV1-18	2,51	0,000367236
IGKV1D-12	2,51	1,96511E-05
FAM83B	2,56	5,44745E-07
TCN1	2,59	0,001727173
IGHV4-61	2,63	0,000275333
CXCL10	2,63	3,7489E-06
IGHV4-31	2,67	8,27095E-06
GSDMC	2,69	0,000151853
IGHV4-28	2,70	2,23826E-06
SIMALR	2,75	0,001887894
IGHV4-59	2,75	0,000117965
IGLV1-40	2,79	3,05547E-06
IGLV1-50	2,83	0,000991003
MUC16	2,86	0,000254444
C21orf91-OT1	2,88	0,000152257

IGHV4-34	2,92	6,67301E-06
IGHV3-38	2,92	0,000337376
AARD	3,00	3,3047E-05
LINC01956	3,00	0,000489804
VGLL1	3,01	0,000759834
IGHV3-71	3,05	0,000496007
PROM1	3,11	4,20103E-05
CPB1	3,16	0,002777033
IGHV4-4	3,17	1,95926E-06
MYBL2	3,27	1,81701E-10
IGLV6-57	3,66	3,7489E-06
GABRP	4,29	2,36277E-08
CSN3	4,89	8,75534E-05

5. Evaluation of differences in immunological signatures and tumor microenvironment composition

Differential gene expression analysis revealed differences in pathways related to TME. Consequently, we decided to investigate these differences in TME composition using multiple approaches. First, we analyzed immune-related gene signatures in HR+/HER2-patients, revealing significant overrepresentation in VYBC tumors (**Figure 23**).

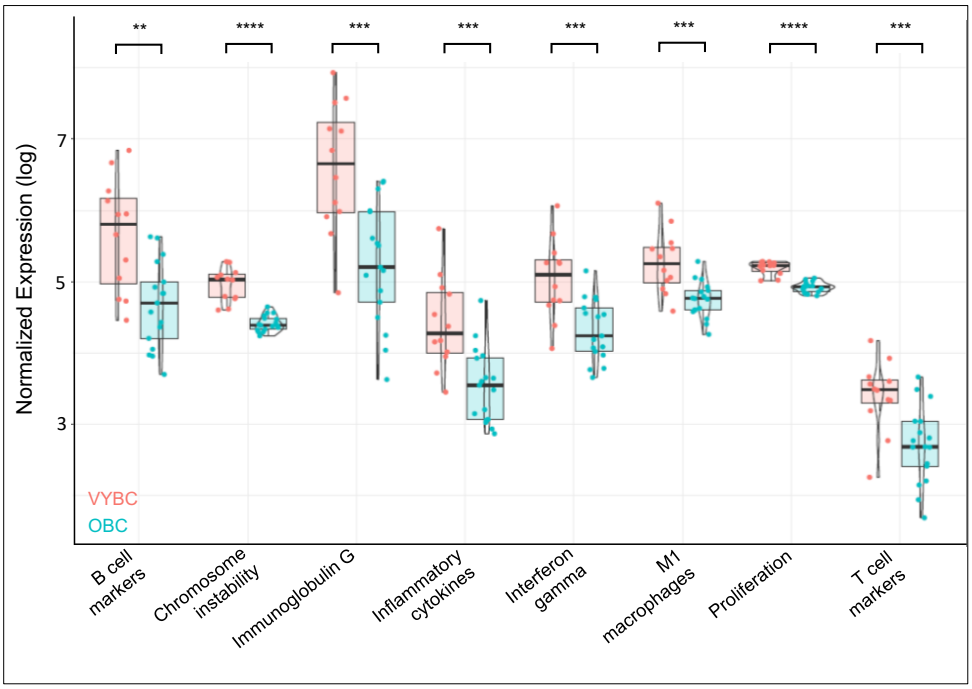

Figure 23. Analyses of differences in TME composition and expression of immune checkpoints in HR+/HER2-VYBC and OBC samples. *Violin plots and boxplots of normalized expression scores of gene signatures related to tumor aggressiveness and cellular composition of the TME in HR+/HER2-samples.*

Higher scores in chromosome instability and proliferation signatures were also found in this subset of patients. Moreover, different angiogenesis signatures were up-regulated in HR+/HER2-VYBC patients (data not shown). Conversely, no differences were found in HER2+ or TN subtypes (**Figure 24**, **Figure 25**).

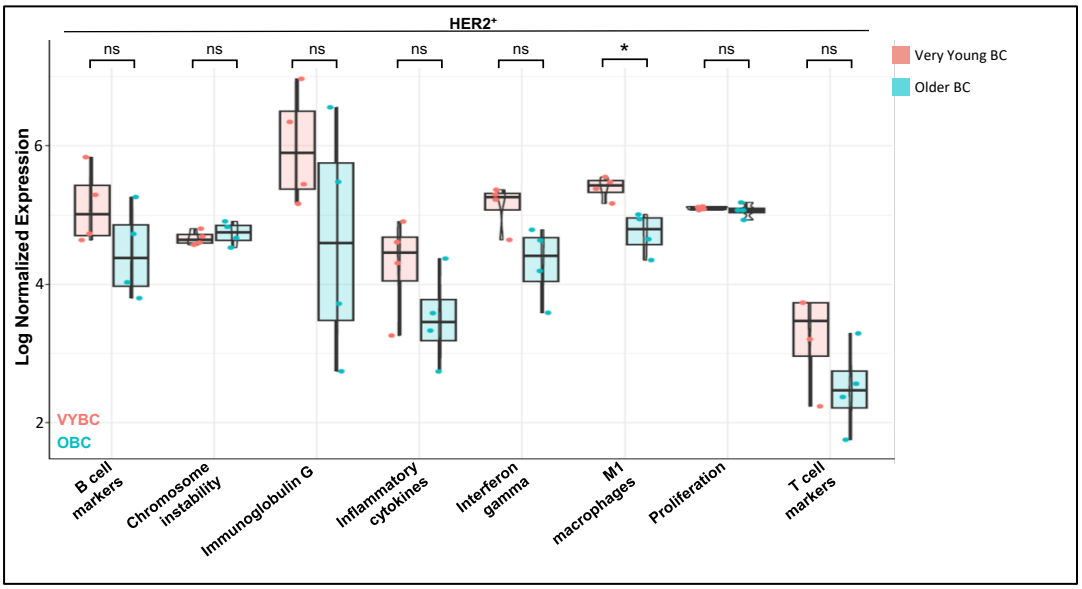

Figure 24. *Violin plots and boxplots of normalized expression scores of gene signatures related to tumor aggressiveness and cellular composition of the tumor microenvironment in HER2 samples. VYBC are represented in red and OBC samples in blue.*

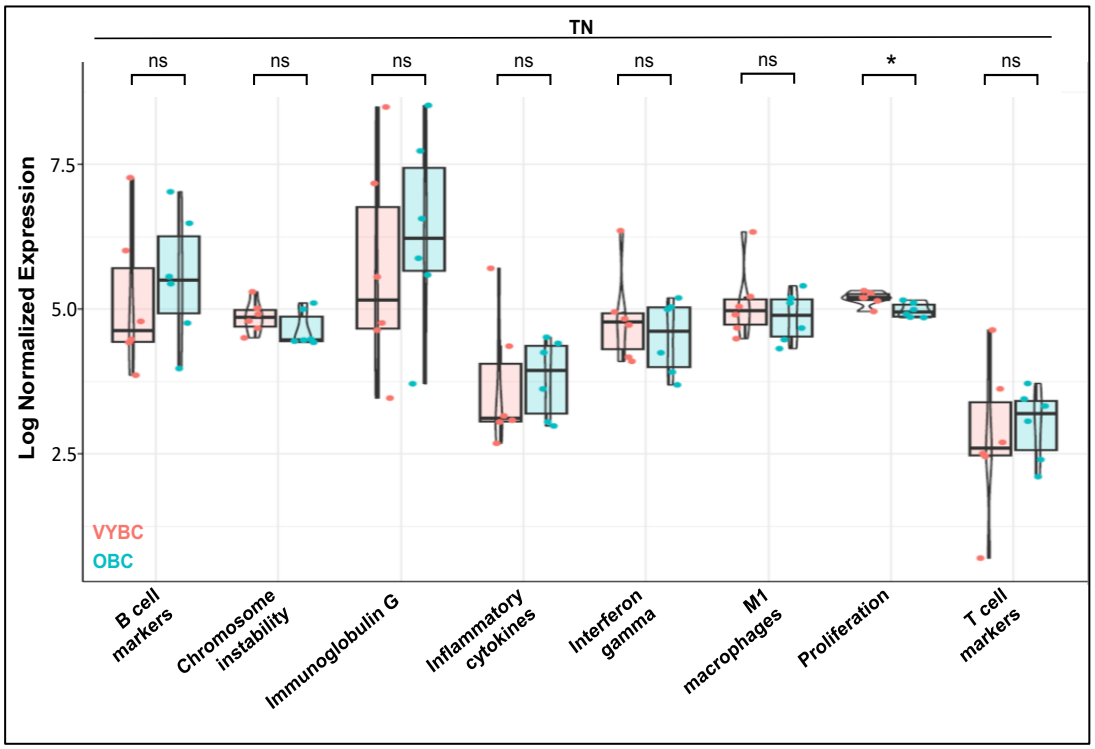

Figure 25. *Violin plots and boxplots of normalized expression scores of gene signatures related to tumor aggressiveness and cellular composition of the tumor microenvironment in HER2 (A) and TNBC (B) samples. VYBC are represented in red and OBC samples in blue.*

Subsequently, a computational analysis of TME was conducted. TME components were quantified by the Microenvironment Cell Population-counter (MCP-counter) algorithm. This marker-based analysis allows for the calculation of immune cell infiltration in tumors based on bulk-RNA-seq data, and inter-sample comparison. MCP-counter analysis estimated higher infiltration of immune cells in HR+/HER2-VYBC tumors; in particular, B cells, macrophages, monocytes, myeloid dendritic cells, neutrophils, NK cells, and T cells including CD8+ T cells ($p < 197\ 0.05$) (**Figure 26**).

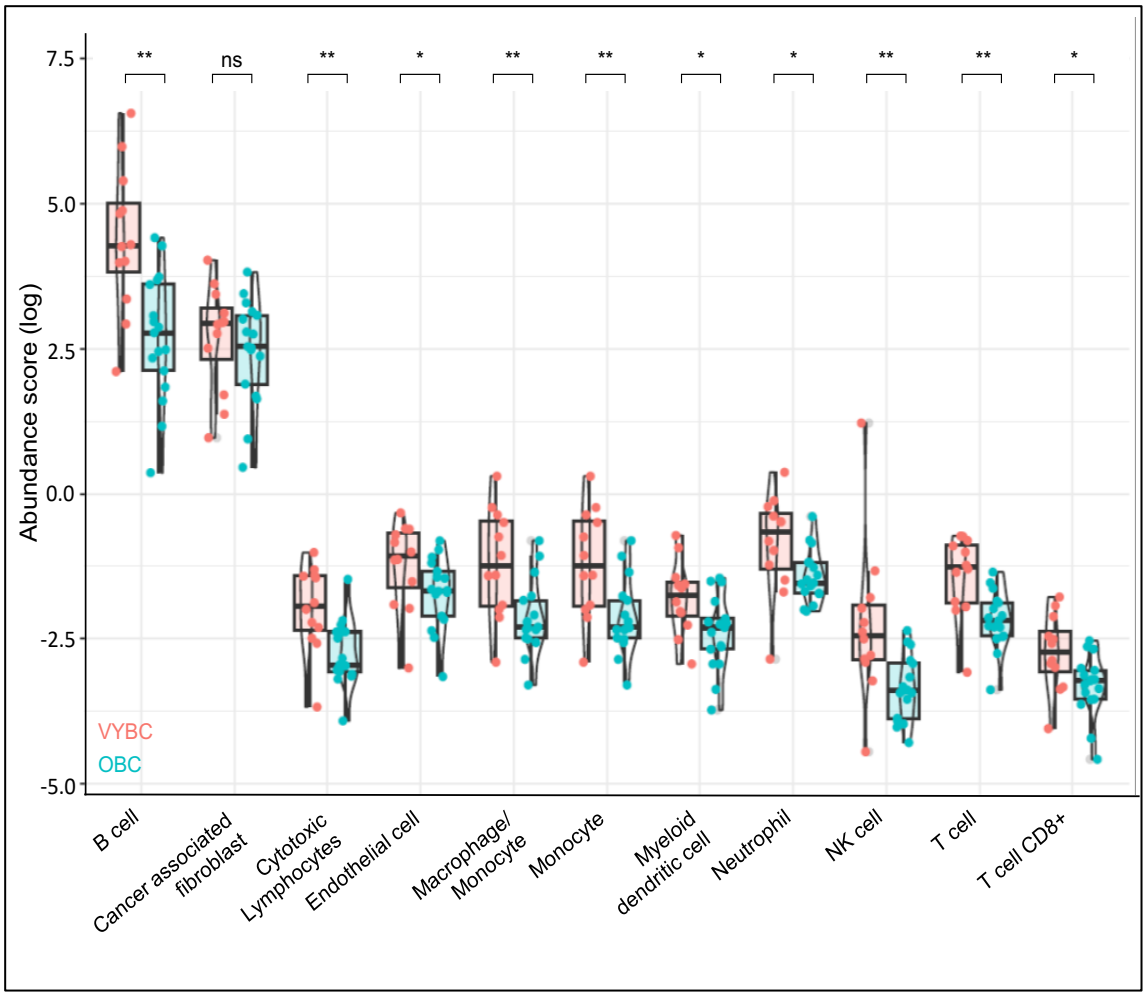

Figure 26. *Analyses of differences in TME composition and expression of immune checkpoints in HR+/HER2- VYBC and OBC samples. Violin plots and boxplots of the abundance score of TME cells determined by MCP-counter.*

This overrepresentation of immune cells in HR+/HER2-VYBC tumors was further validated by using the quanTIseq (quantification of the Tumor Immune contexture from human RNA-seq data) algorithm, which uses restricted least-squares regression to estimate the absolute proportions of the infiltration levels of immune cell types from RNA-seq data (67). Consistent with previous results, quanTIseq analysis estimated higher infiltration of B cells, macrophages, NK cells, and T cells including CD8+ T cells in

VYBC patients (**Figure 27**). These findings highlight age-dependent differences in TME composition with a notable upregulation of immune infiltrates in VYBC tumors.

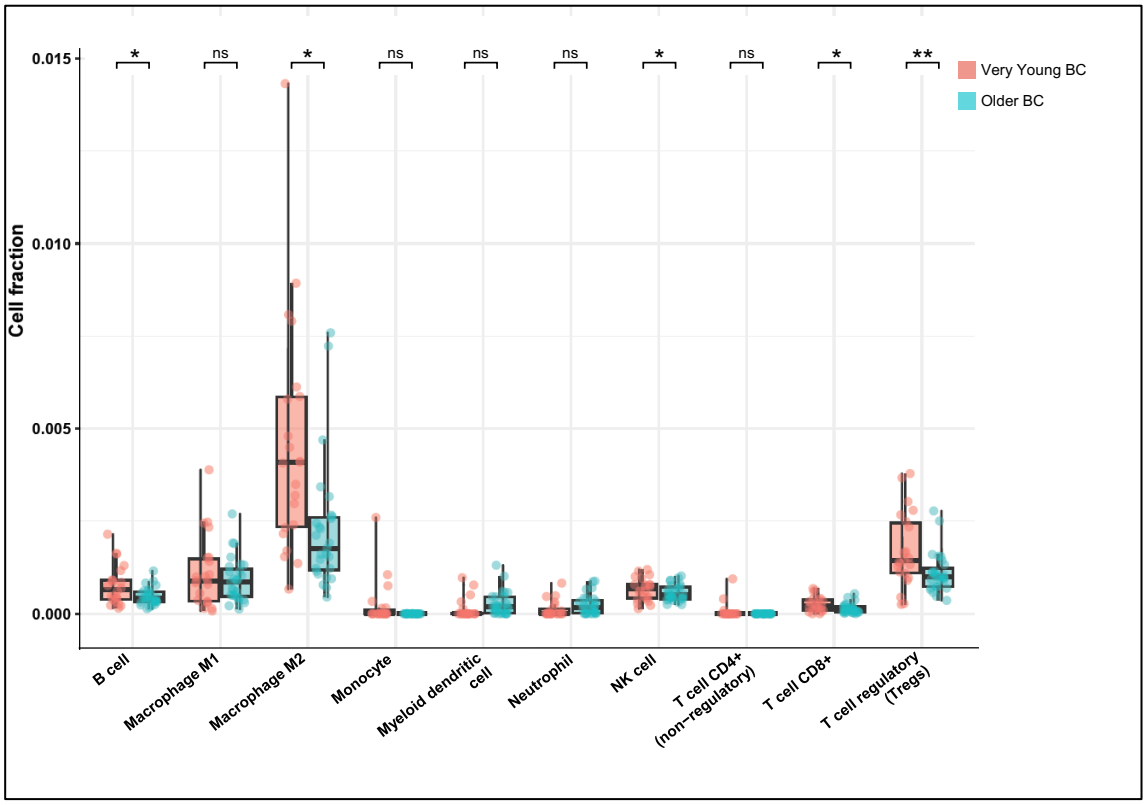

Figure 27. *Violin plots and boxplots of cell fraction of TME cells determined by MCP-counter in luminal VYBC and OBC samples. VYBC are represented in red, and OBC samples in blue.*

Based on these findings, we further evaluated the potential application of immunotherapy for treating HR+/HER2-VYBC patients by different approaches. First, we explored the expression of immunological checkpoints and found that HR+/HER2-VYBC patients exhibited higher expression of these immune checkpoint members (**Figure 28**). Consequently, our findings support that HR+/HER2-VYBC tumors are enriched in characteristics of "hot" tumors (**Figure 29**).

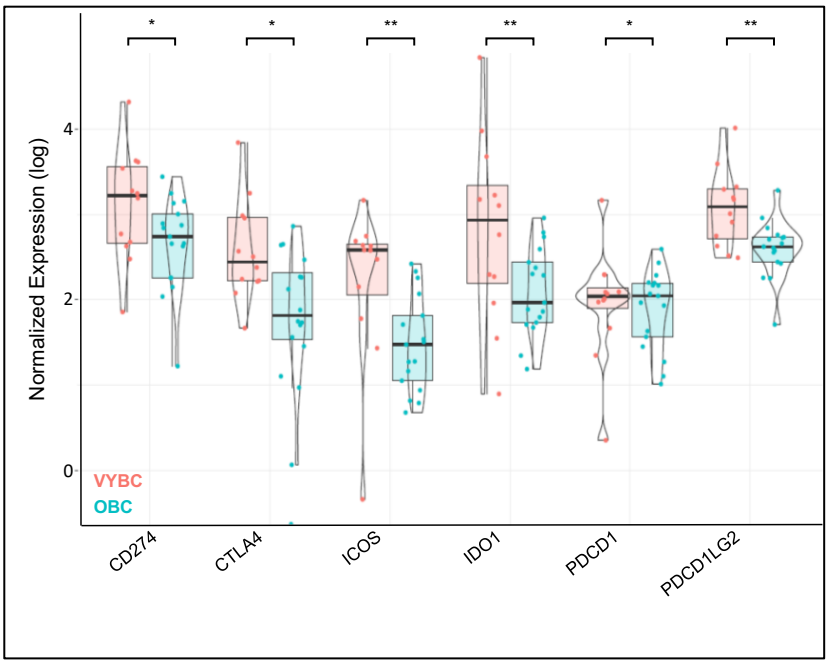

Figure 28. *Analyses of differences in TME composition and expression of immune checkpoints in HR+/HER2-VYBC and OBC samples. Violin plots and boxplots of normalized expression data of immune checkpoints from RNA-seq.*

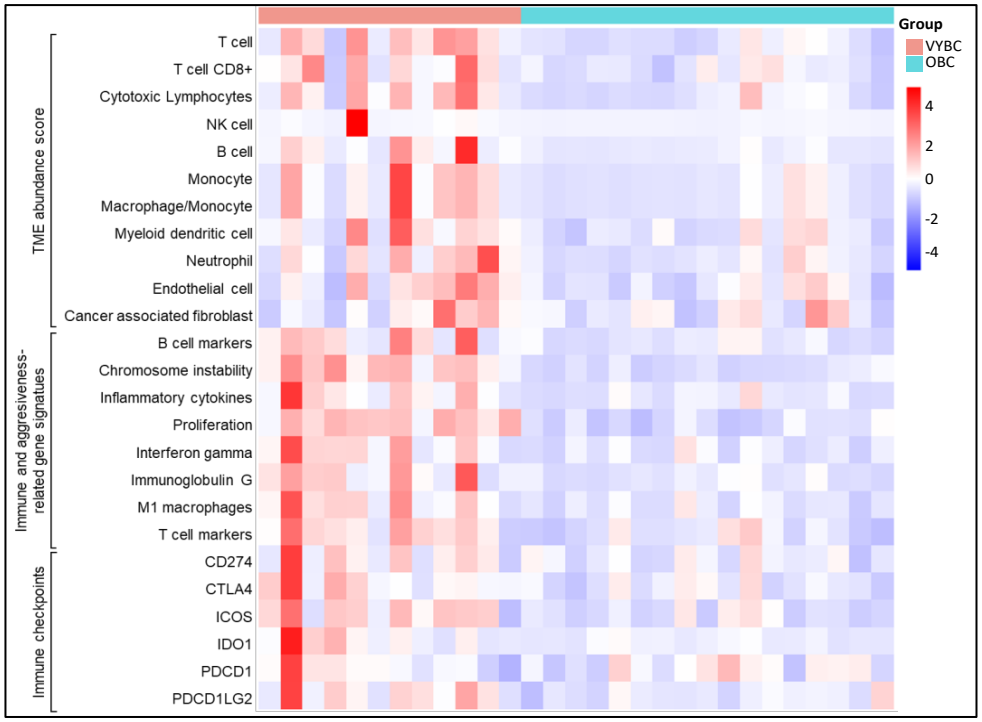

Figure 29. *Analyses of differences in TME composition and expression of immune checkpoints in HR+/HER2-VYBC and OBC samples. Heatmap illustrating differences in TME composition between HR+/HER2-VYBC and OBC samples. TME abundance scores obtained using MCP-counter, immune and aggressiveness-related signature scores, and expression of immune checkpoints are included and represented in rows. Each column represents a patient sample. VYBC samples are represented in red and OBC samples in blue.*

6. Histological validation

To further validate our findings, we conducted a histological evaluation of tumors from HR+/HER2-VYBC and OBC patients to assess the tumor-infiltrating lymphocytes (TILs) and tertiary lymphoid structures (TLSs), both indicators of potential immunotherapy response. This validation was performed on treatment-naïve tumors from a validation cohort consisting of 12 VYBC patients and 29 OBC patients (clinicopathological data in **Table 3**).

Consistent with transcriptomic results, HR+/HER2-VYBC tumors exhibited significantly higher percentages of TILs than tumors from OBC patients ($p = 0.0196$) (**Figure 30**, **Figure 31**). Additionally, TLS abundance was also examined, revealing that in the VYBC group, 58.3% of samples presented 0-1 TLS, 16.7% had 2-3, and 25% had more than 5 TLS. In contrast, in the OBC group, 75.9% of samples presented 0-1 TLS, 17.2% had 2-3, 6.9% had 4-5, and none had more than 5 TLS. Despite the limited size of this cohort, we confirmed that HR+/HER2-VYBC tumors presented a higher abundance of TLS compared to OBC tumors ($p= 0.0376$) (**Figure 32**). These findings were robust and consistent with the results obtained from RNA-seq analysis.

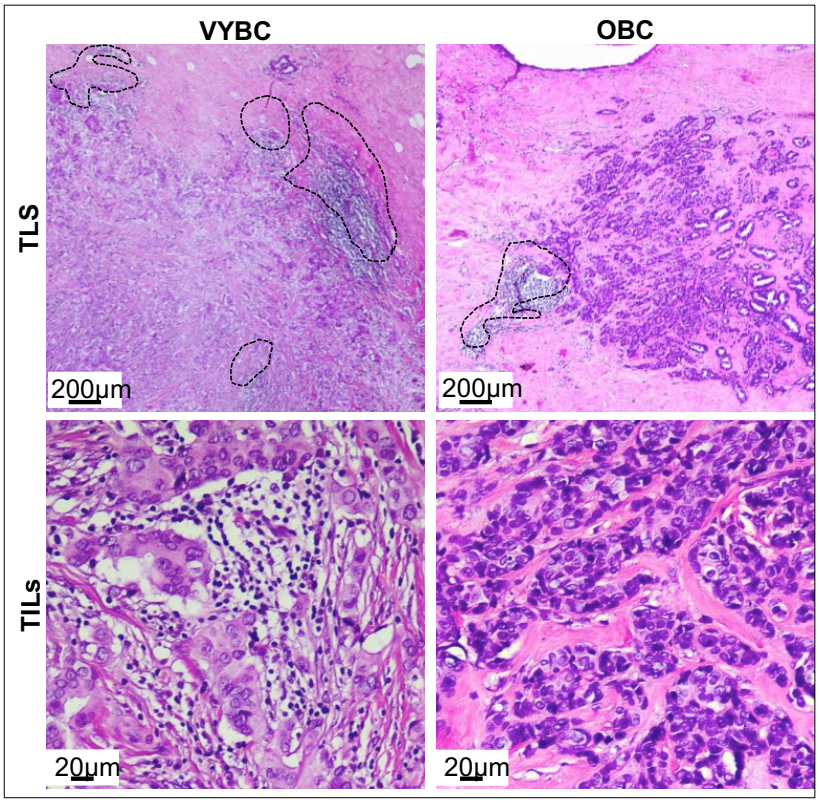

Figure 30. *TLS and TILs histological properties of HR+/HER2-VYBC and OBC patients. Representative images of TLS (up, scale bar: 200 µm) and TILs (down, scale bar: 20 µm) in HR+/HER2-VYBC and OBC patients.*

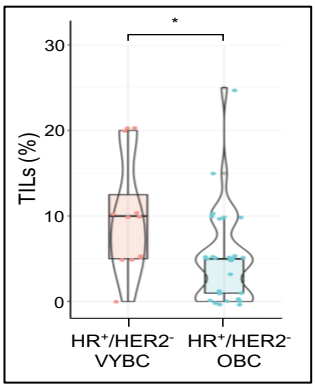

***Figure 31**. TILs, histological properties of HR+/HER2- VYBC and OBC patients. Violin plots and boxplots of enrichment of TILs in tumors from HR+/HER2- VYBC and OBC.*

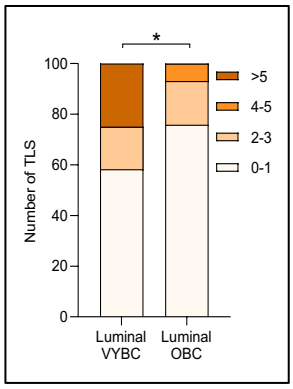

***Figure 32**. TLS, histological properties of HR+/HER2-VYBC and OBC patients. Stacked bar charts of the distribution of TLS groups in HR+/HER2-VYBC and OBC samples.*

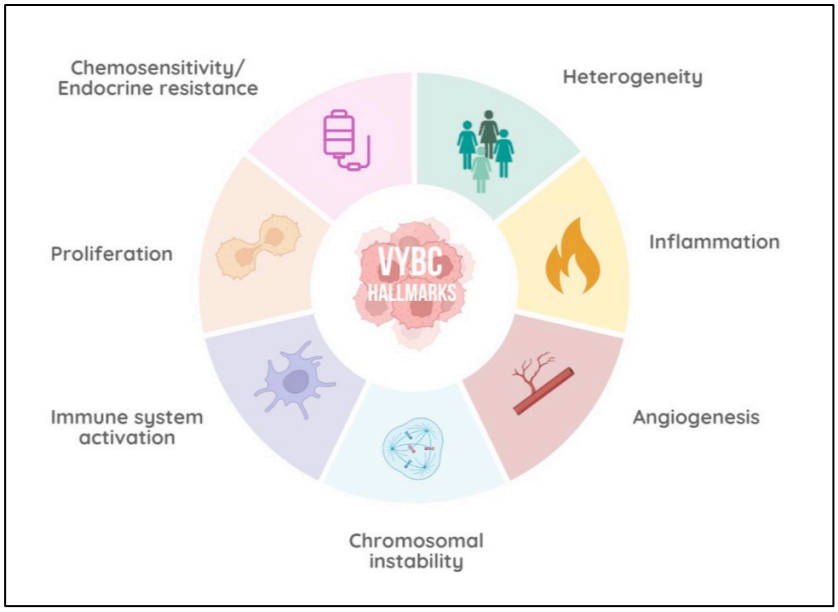

***Figure 33.** Very Young Breast Cancer Hallmarks.*

DISCUSSION

DISCUSSION

The diminished survival rates observed in very young BC patients cannot be solely attributed to late diagnoses or the higher prevalence of HER2+ and TN subtypes, both associated with unfavourable outcomes. These findings suggest that age itself may be considered an independent prognostic factor, specifically in HR+/HER2- disease. Several studies have demonstrated that women under 40 with BC experience worse survival outcomes, particularly within the HR+/HER2-subtype, focusing on the role of intrinsic biological differences in prognosis. Notably, these unfavourable outcomes are not consistent across all subtypes, with no significant correlation observed in other BC subtypes (14,68–70).

Systemic treatments significantly affect the quality of life in young BC patients. These treatments are associated with physical and psychological side effects including fertility concerns and emotional distress, which can be particularly challenging for younger women. Addressing these challenges is critical for optimizing long-term survivorship care (71).

Furthermore, several authors have explored age-associated genomic alterations at the DNA level. Somatic alterations occur at different frequencies in young versus old luminal BC patients. Indeed, GATA3 and ARID1A somatic mutations are more frequent in younger patients, wheres older patients have higher frequency of PIK3CA, CDH1 and MAP3K1 mutations. Additionally, copy number alterations and deficient homologous recombination are more frequently observed in young patients (20,72).

Refining adjuvant therapy in premenopausal women remains a critical area of ongoing research. The balance between providing adequate treatment to minimize recurrence and avoiding overtreatment is particularly challenging in this population. Some strategies as tailored endocrine therapies and ovarian function suppression, combined with risk stratification based on genomic assays, are being explored to achieve this balance (73–75) TAILORx and RxPONDER trials provided further evidence regarding the molecular differences between tumors from old and young BC patients. These prospectives clinical trials compared the clinical benefit of endocrine therapy alone and chemoendocrine therapy in luminal BC patients. Interestingly, chemoendocrine therapy showed benefits in terms of invasive disease-free survival and distant relapse-

free survival only in premenopausal women, while combinatorial treatment did not show benefit in postmenopausal women (76,77).

The PAM50 intrinsic subtyping and ROR score are established tools for risk assessment in postmenopausal women with HR+/HER2- tumors. However, their prognostic value in premenopausal women remains uncertain. Brown *et al.* studied of PAM50 intrinsic subtypes and ROR scores in premenopausal HR+/HER2- breast cancer, finding a higher prevalence of luminal B and non-luminal subtypes in younger women, alongside significantly higher ROR scores, particularly in node-negative patients (78). These findings not only underscore the aggressive tumor biology characteristic of younger women but also explain the predictive capacity of higher ROR and CES scores in anticipating better responses to chemotherapy. In line with these results, our study confirmed that HR+/HER2-VYBC patients presented higher PAM50-based ROR and CES, which predicts better response to chemotherapy than HR+/HER2- OBC patients. To the best of our knowledge, this study represents the largest dataset of RNA sequencing in very young women diagnosed with BC.

In recent years, the tumor immune microenvironment has emerged as an area of interest in BC research. We compared the transcriptomic landscape of BC tumors from VYBC and OBC patients, focusing on premenopausal women under 35 and postmenopausal women over 50 to elucidate unique insights into the age-associated differences. This deliberate exclusion of the intermediate age range contributed to the strength of our study, enabling a more targeted exploration of age-related variations in tumor profiles. Our results indicated that age-associated differences are mainly found within the HR+/HER2-subtype.

We conducted a comprehensive deconvolution analysis of the samples by using MCP-counter and quanTIseq algorithms. This approach allowed us to evaluate the tumor cellular composition using bulk RNA-seq data (67). We found that HR+/HER2- tumors in very young women exhibited significantly higher immune cell infiltration, categorizing them as "hot" tumors (an unexpected finding as this subtype is typically considered “cold”). Importantly, “hot” tumors are more likely to respond to immune checkpoint inhibitors, in contrast to “cold” tumors (79,80).

Based on these finding, we explored the potential application of immunotherapy in luminal VYBC. First, we confirmed that tumors from HR+/HER2-VYBC patients overexpress immune checkpoint molecules, supporting our hypothesis. Then, tumors were histologically assessed to determine differences in predictors of immunotherapy response, specifically TILs and TLS (81–83). TLSs are ectopic aggregates of lymphocytes resembling lymph nodes that can be found embedded in the tumor mass or at the tumor margin. These structures contain B cells, T cells, and dendritic cells, and have been described to play a crucial role in determining response to immunotherapy (79,83,84). Several studies have indicated the applicability of TLS as valuable predictors of immunotherapy response in BC (83,85). Thus, the histological evaluation revealed that tumors from HR+/HER2-VYBC patients present more TILS and TLS than those from OBC patients, highlighting the potential applicability of immunotherapy to this subset of patients.

Even though luminal BC are generally considered "cold" tumors, several clinical trials have demonstrated that immunotherapy could benefit some of these patients. Interestingly, the Phase II GIADA Trial enrolled premenopausal patients with Luminal B BC subtype that were treated with chemotherapy and immunotherapy. The trial found that in Basal molecular subtype, TILs and immune-related gene signatures were found to be associated with pathological complete response (86). Additionally, the phase-II I-SPY2 and the phase-III KEYNOTE-756 clinical trials demonstrated the clinical benefit of immune checkpoint inhibitors in a subset of luminal BC patients (87,88). Therefore, it is evident that some HR+/HER2-patients might benefit from immunotherapy. In this scenario, our work provides a deeper understanding of a subset of luminal patients that could benefit from this treatment.

We anticipate that our findings will significantly contribute to a deeper understanding of BC biology and clinical behaviour in very young patients. Our transcriptomic analyses revealed upregulated immunological signatures and increased immune infiltrate, findings that were corroborated histologically by higher TILs and TLS presence. This dual confirmation strengthens the case for investigating immunotherapy in this subgroup and provides a foundation for the development of more personalized therapeutic strategies.

In conclusion, our comprehensive transcriptomic analysis highlights the distinctive molecular landscape of HR+/HER2-BC in very young women. This subtype is marked by high heterogeneity, increased proliferation, chromosomal instability, angiogenesis, elevated immune cell infiltration, and more endocrine resistance and susceptibility to chemotherapy. These findings suggest significant potential for immunotherapy in this patient subgroup and a crucial opportunity to refine targeted therapeutic strategies. Elucidating the unique biological underpinnings could lead to significant improvements in clinical outcomes for HR+/HER2-BC in very young women. Continued research in this area is essential to deepen our understanding of the disease and, ultimately, improve patient outcomes. The insights from this work may lead to personalized treatments to address the specific needs of young patients with HR+/HER2-BC.

CONCLUSIONS

CONCLUSIONS

- Our study provides a comprehensive characterization of the transcriptomic landscape of HR+/HER2- breast cancer in very young women, revealing distinct molecular features compared to older breast cancer patients.

- No significant differences were observed in classical biomarkers between very young and older patients, highlighting the need for deeper molecular profiling.

- Age-related transcriptomic differences were identified not only in tumor cells but also in the tumor microenvironment.

- HR+/HER2- tumors in very young women showed greater heterogeneity, increased proliferation, chromosomal instability and enriched immune infiltration (features associated with hot tumors).

- These findings suggest that HR+/HER2- tumors in very young patients could be more endocrine-resistant but potentially more immunoreactive.

- Taken together, the results underscore the need for personalized therapeutic strategies in this subgroup of patients.

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APPENDIX

RESUMEN EN LENGUA OFICIAL UV

Título: Descifrando el perfil transcriptómico del cáncer de mama en mujeres jóvenes: Nuevas perspectivas.

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INTRODUCCIÓN:

El cáncer de mama (CM) es el cáncer más común en mujeres y representa la segunda causa principal de muertes relacionadas con el cáncer a nivel mundial.

La incidencia global de cáncer de mama fue aproximadamente de 2.3 millones de nuevos casos en 2020, lo que resultó en alrededor de 685,000 muertes. En los últimos 30 años, la incidencia global de cáncer de mama ha aumentado un 57.8%, con una tasa de incremento anual del 0.5%. Se estima que para el año 2050, la tasa de incidencia estandarizada por edad (ASIR, por sus siglas en inglés) será de 59.63/100,000, lo que representará un aumento del 32.13% en comparación con 2019. Además, Xu et al. estimaron que para el año 2050, la tasa de mortalidad estandarizada por edad (ASDR, por sus siglas en inglés) en mujeres será de 16.42/100,000, un aumento del 4.69% en comparación con 2019. Estos resultados resaltan la necesidad de un mayor conocimiento de la enfermedad en el subgrupo de pacientes con cáncer de mama muy jóvenes y enfatizan la magnitud de la situación actual.

El CM es una enfermedad compleja y heterogénea que afecta a mujeres de diversos grupos de edad. Aunque generalmente se reconoce como una enfermedad predominantemente detectada en mujeres mayores, la incidencia de CM entre mujeres muy jóvenes (definidas como aquellas diagnosticadas a los 35 años o menos) está aumentando y presenta desafíos clínicos y biológicos únicos. Este subgrupo de pacientes suele mostrar un fenotipo más agresivo y peores resultados en comparación con las mujeres mayores, independientemente del estadio al momento del diagnóstico.

Varios factores contribuyen a estos resultados desfavorables, incluidos los retrasos en el diagnóstico, las características patológicas desfavorables y la falta de adherencia al tratamiento. Es importante señalar que existe una mayor proporción de cánceres de mama HER2-positivos (HER2+) y triples negativos (TN) entre las mujeres más jóvenes, que son los subtipos de CM asociados con un pronóstico más pobre. Sin embargo, el subtipo más frecuente sigue siendo el de receptor hormonal positivo/HER2-negativo (HR+/HER2-). Aunque el CM HR+ generalmente tiene un pronóstico más favorable, sigue siendo un desafío clínico en mujeres jóvenes. De hecho, las mujeres diagnosticadas con cáncer de mama luminal menores de 40 años tienen un riesgo estadísticamente significativo de muerte por CM superior al de las pacientes posmenopáusicas. Curiosamente, este aumento en la tasa de mortalidad no se observó entre las mujeres con subtipos triple negativo o HER2-positivo. Estas observaciones destacan la necesidad de comprender más a fondo la enfermedad en las pacientes muy jóvenes, lo que podría influir en las estrategias de manejo clínico para este subgrupo. Además, este subgrupo de pacientes está subrepresentado en los ensayos clínicos.

En los últimos años, la aparición de tecnologías de alto rendimiento ha revolucionado la investigación del cáncer, permitiendo un análisis integral del paisaje genómico y molecular de los tumores. Actualmente, la plataforma Oncotype DX se considera una herramienta valiosa para personalizar el tratamiento adyuvante en pacientes con cáncer de mama HR+ en etapas tempranas. No obstante, los resultados del ensayo clínico RxPONDER sugieren que Oncotype DX predice en menor medida los beneficios de la quimioterapia en pacientes premenopáusicas comparado con mujeres posmenopáusicas. El perfil transcriptómico ha surgido como una herramienta poderosa para desentrañar las alteraciones moleculares y los procesos biológicos clave, así como para identificar nuevos subtipos moleculares y potenciales terapias dirigidas.

Las pacientes con cáncer de mama muy joven (VYBC, por sus siglas en inglés) presentan una biología tumoral más agresiva y peores resultados en comparación con las pacientes mayores, como se mencionó anteriormente. Tasas de proliferación más altas, fenotipos más agresivos y perfiles genómicos distintos son características diferenciadoras de estos tumores. Waks et al. revelaron diferencias mutacionales en PIK3CA, GATA3 y alteraciones en ARID1A entre pacientes jóvenes y mayores con cáncer de mama luminal A. Las pacientes VYBC poseen firmas genómicas de riesgo intermedio a alto y mutaciones en genes que codifican proteínas estructurales. Además, se identificaron

patrones transcriptómicos que reflejan una mayor proliferación y señalización oncogénica en este subgrupo, independientemente de los subtipos moleculares. Nuevos hallazgos han sido descritos en este subgrupo como una nueva firma de 6 genes, asociada con características tumorales agresivas, proliferación tumoral y menor supervivencia, la cual se vinculó con características clínicas y patológicas más agresivas y tuvo valor pronóstico independiente para predecir los resultados.

A través de múltiples subtipos, estas pacientes presentan menores tasas de mutación, pero están enriquecidas con mutaciones impulsoras y alteraciones en el número de copias, además de presentar características de senescencia prematura. Probablemente también exhiben características únicas en su microambiente inmunológico, lo que puede afectar las respuestas a la inmunoterapia.

Estos hallazgos subrayan la importancia de considerar la edad biológica en el manejo clínico para mejorar los resultados en las pacientes jóvenes con cáncer de mama. Se necesita más investigación para desarrollar biomarcadores específicos por edad que definan las estrategias de tratamiento para estas pacientes.

El cáncer de mama está significativamente influenciado por el microambiente inmunológico. El sistema inmune juega un papel fundamental en el mantenimiento de la homeostasis tisular a través de la vigilancia y la activación de respuestas inflamatorias de las células inmunitarias innatas y adaptativas.

Estudios recientes han identificado diferentes subtipos inmunológicos entre los tumores de mama, caracterizados por funciones biológicas, firmas de células inmunitarias y resultados clínicos. Los inhibidores de puntos de control inmunológico (ICI) han mostrado ser prometedores en el tratamiento del cáncer de mama triple negativo avanzado. Sin embargo, el microambiente tumoral inmunosupresor (TME, por sus siglas en inglés) sigue siendo una barrera significativa para la efectividad del tratamiento. Se requieren algunas estrategias para superar esta resistencia, incluyendo terapias combinadas y la focalización de poblaciones específicas de células inmunitarias. Comprender la relación entre las respuestas inmunitarias sistémicas y locales es crucial para desarrollar nuevos enfoques en el tratamiento del cáncer de mama.

El perfil de expresión génica de los tumores ha facilitado la identificación de firmas génicas pronósticas y la selección de pacientes para terapias dirigidas.

Estudios recientes han investigado perfiles génicos inmunológicos y biomarcadores para predecir la respuesta a la inmunoterapia en tumores sólidos. Se han descrito algunas firmas pronósticas, como las funciones de células NK, células B, monocitos, macrófagos y células dendríticas. Clústeres específicos de células inmunitarias, como los macrófagos TREM2hi y las células T γδ, han mostrado resistencia a los inhibidores de puntos de control inmunológico. Por otro lado, Axelrod et al. y Galili et al. han identificado firmas inmunológicas asociadas con un mejor pronóstico en el cáncer de mama triple negativo. Además, los genes de puntos de control inmunológico, como CTLA-4 y PD-L1, muestran una expresión variable entre los subtipos de cáncer de mama.

El microambiente tumoral desempeña un papel esencial en la progresión del cáncer con infiltración estromal e inmunitaria. Las células inmunitarias innatas contribuyen a las interacciones complejas dentro del microambiente tumoral. En el cáncer de mama, CD47 se correlaciona con la activación inmunitaria y un pronóstico favorable en ciertos subtipos. Además, las poblaciones y parámetros de células inmunitarias periféricas (recuentos de linfocitos, relación neutrófilo-linfocito (NLR) y relación linfocito-monocito (LMR)) son variables en pacientes con cáncer de mama y pueden servir como biomarcadores potenciales para la progresión de la enfermedad y la respuesta a la terapia.

La implementación de estas herramientas requerirá una validación y el uso de plataformas genómicas robustas y reproducibles. El cáncer de mama es predominantemente una enfermedad transcripcional en lugar de una impulsada por patrones mutacionales, si lo comparamos con otros tumores sólidos como el melanoma y el cáncer de pulmón. Considerando la información mencionada anteriormente, debemos asumir que estamos al comienzo de un nuevo paradigma en la terapia del cáncer de mama.

OBJETIVOS:

El **objetivo principal** de esta tesis doctoral es caracterizar el perfil transcriptómico del cáncer de mama con receptores hormonales positivos y HER2 negativo (HR+/HER2-) en mujeres muy jóvenes (≤35 años), en comparación con

pacientes de edad más avanzada (≥50 años). Para ello, se plantean los siguientes objetivos específicos:

- Realizar un análisis de secuenciación de ARN (RNA-seq) con el fin de identificar genes diferencialmente expresados y firmas transcriptómicas específicas entre ambos grupos etarios.

- Determinar si existen características transcriptómicas concretas que se asocien con el comportamiento clínico más agresivo que se ha observado habitualmente en las pacientes muy jóvenes diagnosticadas con este subtipo de cáncer de mama.

Además, se plantean los siguientes **objetivos secundarios** orientados a contextualizar y ampliar los hallazgos transcriptómicos obtenidos:

- Explorar la relación entre las características clínicas y los perfiles transcriptómicos a través de un enfoque comparativo entre los distintos grupos de edad.

- Para ello, se pretende integrar datos clínico-patológicos con los resultados transcriptómicos, con el fin de identificar patrones moleculares relacionados con la edad que puedan tener un impacto clínico relevante.

- Asimismo, se busca comprender cómo las diferencias transcriptómicas pueden influir en el fenotipo tumoral, la respuesta al tratamiento y el comportamiento biológico del cáncer de mama en función de la edad de la paciente.

- Identificar procesos biológicos que puedan estar en la base del fenotipo agresivo que caracteriza al cáncer de mama HR+/HER2- en mujeres muy jóvenes.

Para ello, se utilizarán los datos de expresión génica para proponer rutas moleculares y procesos biológicos relevantes que podrían estar implicados en el desarrollo y progresión tumoral en este subgrupo poblacional.

METODOLOGÍA:

1. Muestras.

Este estudio observacional retrospectivo se diseñó con el propósito de caracterizar las diferencias transcriptómicas del cáncer de mama HR+/HER2- entre pacientes jóvenes

y pacientes de edad avanzada. Para ello, se incluyeron un total de 66 pacientes no consecutivas con diagnóstico histopatológico de carcinoma de mama invasivo que cumplían los criterios de inclusión y exclusión previamente definidos. Todas las pacientes eran mayores de 18 años y fueron diagnosticadas y tratadas en el Hospital Clínico Universitario de Valencia (HCUV). El estudio fue aprobado por el Comité de Ética de Investigación Clínica del centro (código 2024/387), y se llevó a cabo conforme a los principios éticos establecidos en la Declaración de Helsinki. Todas las participantes firmaron un consentimiento informado por escrito antes de la recolección de las muestras.

Las muestras tumorales fueron obtenidas antes de la administración de cualquier tratamiento sistémico o local, lo que garantiza que el perfil molecular analizado no se encontraba influenciado por intervenciones terapéuticas previas. Las muestras analizadas fueron procesadas mediante los protocolos estándar de fijación en formol e inclusión en parafina (FFPE). Se seleccionaron dos grupos de pacientes claramente diferenciados por edad: por un lado, 33 pacientes jóvenes (≤35 años, denominadas VYBC - Very Young Breast Cancer), y por otro, 33 pacientes mayores (≥50 años, denominadas OBC - Older Breast Cancer).

De este total, solo se consideraron para análisis transcriptómico aquellas muestras que cumplían estrictos criterios de calidad de ARN, resultando finalmente en 22 muestras del grupo VYBC y 27 del grupo OBC. Con el objetivo de validar los hallazgos transcriptómicos obtenidos, se incorporó una segunda cohorte de pacientes, también con muestras FFPE, obtenidas tras cirugía y sin exposición a tratamiento previo. Esta cohorte de validación incluyó 12 pacientes HR+/HER2- VYBC y 29 pacientes HR+/HER2- OBC, todas sin mutaciones germinales en genes asociados al cáncer de mama hereditario (BRCA1/2, PALB2, TP53, entre otros).

2. Análisis transcriptómico (RNA-seq)

El análisis transcriptómico fue realizado a partir de muestras FFPE provenientes de biopsias de aguja gruesa. La extracción de ARN se llevó a cabo utilizando kits comerciales optimizados para tejidos FFPE, seguidos de una evaluación exhaustiva de calidad mediante espectrofotometría y bioanálisis. Solo se seleccionaron aquellas muestras con valores adecuados de integridad y pureza de ARN (RIN y A260/280). La

secuenciación del ARN se realizó empleando la plataforma HiSeq X Series de Illumina (San Diego, CA, EE.UU.), que proporciona lecturas de alta profundidad y precisión.

Los datos obtenidos fueron procesados mediante un pipeline bioinformático estándar: control de calidad con FastQC, alineamiento de secuencias con STAR, y cuantificación de genes con HTSeq-count. Posteriormente, se utilizó el paquete DESeq2 de Bioconductor para llevar a cabo el análisis de expresión génica diferencial. Se excluyeron aquellos genes con conteo cero en todas las muestras, y los conteos fueron normalizados mediante la metodología de varianza estabilizada (VST). Para controlar la inflación del error tipo I derivado de comparaciones múltiples, se aplicó el método de tasa de descubrimiento falso (False Discovery Rate, FDR). Se consideraron estadísticamente significativos los genes con FDR < 0,05 y una magnitud de cambio ≥2 (log2 fold change).

El análisis de componentes principales (PCA) fue empleado para visualizar la agrupación de las muestras en función del perfil transcriptómico, permitiendo identificar patrones según el grupo de edad. Para representar los datos, se generaron dendrogramas tipo heatmap, utilizando distancia euclídea y clustering jerárquico completo. Las firmas génicas fueron analizadas mediante el cálculo del promedio de expresión de los genes componentes, y su comparación entre grupos se realizó utilizando pruebas paramétricas (T de Student) o no paramétricas (prueba de Wilcoxon), según la distribución de los datos (evaluada mediante la prueba de Shapiro-Wilk).

3. Análisis inmunohistoquímico

Se realizaron tinciones inmunohistoquímicas en secciones de tejido FFPE utilizando el sistema automatizado VENTANA BenchMark XT (Roche, Basilea, Suiza). Se emplearon anticuerpos primarios contra receptores de estrógenos (ER; clon SP1), progesterona (PR; clon 1E2), HER2 (clon 4B5) y Ki67 (clon 30-9), junto con el sistema de detección UltraView Universal DAB (760-500), siguiendo los protocolos establecidos por el fabricante. La evaluación fue llevada a cabo por un patólogo experto.

4. Subtipado intrínseco PAM50

A cada tumor se le asignó un subtipo molecular intrínseco (Luminal A, Luminal B, HER2-Enriquecido, Basal-like) mediante el clasificador de investigación PAM50, utilizando los datos de expresión génica obtenidos por RNA-seq. La asignación se realizó

tras aplicar una normalización específica basada en subgrupos HER2/receptor de estrógenos, según metodologías previamente descritas.

5. Cálculo de ROR y CES a partir de PAM50

La puntuación de Recurrence of Risk (ROR) se calculó mediante una fórmula validada previamente. El ROR se analizó como variable continua y como variable categórica según puntos de corte establecidos (riesgo bajo, intermedio y alto).

La puntuación CES (Chemo-Endocrine Score) basada en PAM50 se calculó según lo descrito en la literatura. Se determinaron los coeficientes de correlación (CC) de cada muestra con los centroides de los subtipos Luminal A y Basal-like, y se calculó el CES como la diferencia entre ambos (CES = CC a Luminal A – CC a Basal-like). El CES se analizó como variable continua y categórica, definiendo tres grupos: CES-E (endocrino-sensible), CES-U (incierto) y CES-C (quimiosensible), basándose en los terciles de distribución en nuestra cohorte.

6. Análisis de enriquecimiento génico (GSEA)

El análisis de enriquecimiento de conjuntos de genes (GSEA) se realizó utilizando el paquete ClusterProfiler de Bioconductor, aplicado a los genes sobreexpresados en las muestras luminales del grupo VYBC. El objetivo fue identificar procesos biológicos asociados a los genes diferencialmente expresados (DEG).

7. Análisis de deconvolución

Se aplicaron métodos computacionales de deconvolución para inferir la composición celular del microambiente tumoral (TME) a partir de los datos de RNA-seq. Para ello, se utilizó el paquete immunodeconv de R, que integra varios algoritmos de deconvolución reconocidos, permitiendo estimar la abundancia relativa de poblaciones inmunes y estromales presentes en cada muestra.

8. Evaluación histológica

Se analizaron muestras tumorales FFPE obtenidas por resección quirúrgica, las cuales fueron seccionadas y teñidas con hematoxilina y eosina. Un patólogo experto,

ciego respecto al grupo asignado, evaluó la presencia y características de las estructuras linfoides terciarias (TLS) dentro del microambiente tumoral, siguiendo criterios previamente establecidos. Las TLS se definieron por la presencia de linfocitos organizados en zonas con vénulas de endotelio alto en agregados sólidos o redondos, con o sin centros germinales. Se consideraron TLS aquellas ubicadas en el estroma tumoral, en contacto directo o situadas a menos de 5 mm del tumor. También se determinó el número absoluto de TLS presentes en cada muestra.

Además, el patólogo, cuantificó el porcentaje de TILs en las secciones teñidas, según los criterios establecidos, definiéndose como el porcentaje medio de estroma de un carcinoma invasivo infiltrado por linfocitos con incrementos del 5%. En aquellos casos que presentaban una infiltración <5%, se aplicaron intervalos del 1%.

9. Análisis estadístico

Todos los análisis estadísticos se realizaron utilizando el software R (versión 4.3) (R Core Team, 2024). Las variables continuas se compararon mediante la prueba T de Student si cumplían la normalidad (verificada con el test de Shapiro-Wilk); en caso contrario, se utilizó la prueba de rangos de Wilcoxon. Las variables categóricas se analizaron mediante la prueba chi-cuadrado o el test exacto de Fisher, según la frecuencia esperada. En todos los análisis, se consideró estadísticamente significativo un p valor < 0,05.

RESULTADOS:

Características Clínicas y Patológicas

El estudio incluyó un total de 49 pacientes, 22 de las cuales pertenecían al grupo de mujeres muy jóvenes (VYBC, ≤35 años) y 27 al grupo de mujeres mayores (OBC, ≥50 años). Las pacientes jóvenes tuvieron una mediana de edad de 32.5 años, con un rango de 22 a 35 años, mientras que las mujeres mayores presentaron una mediana de 66 años, con un rango de 50 a 94 años. Además, se observó que el 55% de las mujeres jóvenes tenían tumores luminales, mientras que el 45% restante presentaban subtipos no luminales, lo que contrasta con la mayor prevalencia de tumores luminales (63%) en el grupo de mujeres mayores. A pesar de estas diferencias en la distribución de subtipos, ambos grupos mostraron una frecuencia similar de tumores HER2 positivos.

Análisis de Expresión Génica Diferencial

El análisis transcriptómico de las muestras de tumores primarios no tratados reveló diferencias significativas en la expresión génica entre los dos grupos de pacientes. La Análisis de Componentes Principales (PCA) mostró una mayor heterogeneidad en las muestras de las mujeres jóvenes, lo que sugiere una mayor diversidad molecular en sus tumores en comparación con las mujeres mayores. En total, 82 genes fueron regulados a la baja y 12 genes fueron regulados al alza en los tumores de las mujeres jóvenes (log2 fold change ≥ |2|, p ajustado < 0.05).

Entre los genes que mostraron una regulación diferencial, se encontraron varios relacionados con la proliferación celular, la estabilidad cromosómica y la respuesta inmune. En particular, los tumores luminales en las pacientes jóvenes mostraron una mayor firma de proliferación (p = 5.17e-05), lo que se correlacionó con una mayor expresión de ki67 en las muestras inmunohistoquímicas (p = 0.021). Esto indica que los tumores de las mujeres jóvenes tienen una mayor capacidad proliferativa, factor relacionado con la agresividad del cáncer.

Inestabilidad Cromosómica y Firmas de Inmunidad

El análisis también identificó una mayor inestabilidad cromosómica (CIN70, p = 1e-06) en los tumores de las mujeres jóvenes en comparación con los tumores de las mujeres mayores. La inestabilidad cromosómica es un signo de mayor agresividad tumoral y está asociada con un peor pronóstico. Estos hallazgos sugieren que los tumores de las mujeres jóvenes presentan una mayor susceptibilidad a sufrir alteraciones genéticas que podrían promover su evolución y resistencia a los tratamientos.

En términos de la respuesta inmune, los tumores de las mujeres jóvenes mostraron una mayor expresión de firmas inmunológicas, en particular aquellas relacionadas con la activación de linfocitos T citotóxicos, macrófagos M1, y células B. Estos resultados indican que los tumores en mujeres jóvenes pueden ser más inmunorreactivos que los tumores en mujeres mayores, lo que sugiere un mayor potencial de respuesta a terapias que modulen la inmunidad, como los inhibidores de puntos de control inmunológico (ICI). La presencia de estas firmas inmunológicas fue respaldada por un mayor número de linfocitos infiltrantes en los tumores de las mujeres jóvenes, un hallazgo que refuerza la clasificación de estos tumores como "calientes" desde el punto de vista inmunológico.

Infiltración Inmunitaria y Estructuras Linfoides Terciarias

El análisis de deconvolución realizado con los algoritmos MCP-counter y quanTIseq reveló una mayor infiltración de células inmunitarias en los tumores de las mujeres jóvenes, lo que sugiere que estos tumores están caracterizados por un microambiente inmunológico más activo. Las células inmunitarias predominantes fueron los linfocitos T citotóxicos, que juegan un papel clave en la eliminación de células tumorales. Estos resultados son coherentes con la sobreexpresión de moléculas de puntos de control inmunológico, como PD-1 y su ligando PD-L1, que se asociaron con la mayor infiltración inmunitaria observada.

La validación histológica de estos hallazgos confirmó la presencia de un mayor número de linfocitos infiltrantes en los tumores de las mujeres jóvenes. Además, se observó un aumento en la cantidad de estructuras linfoides terciarias (TLS) en los tumores de las mujeres jóvenes. Estas estructuras, que se encuentran dentro del microambiente tumoral, están asociadas con una respuesta inmune más activa y se correlacionan con un mejor pronóstico en diversos tipos de cáncer. La mayor presencia de TLS en los tumores de las mujeres jóvenes sugiere que este subgrupo de pacientes podría beneficiarse de terapias inmunológicas más agresivas.

Puntuación Quimioendocrina (CES) y Sensibilidad a la Quimioterapia

En relación con la quimioterapia, los análisis de la puntuación quimioendocrina (CES) basada en el subtipado PAM50 mostraron que las muestras luminales de las mujeres jóvenes presentaban puntuaciones CES significativamente más altas que las de las mujeres mayores. Las puntuaciones CES más altas están asociadas con una mayor sensibilidad a la quimioterapia, lo que sugiere que las pacientes jóvenes podrían obtener mayores beneficios de la quimioterapia adyuvante en comparación con las mujeres mayores. Esto también refuerza la necesidad de desarrollar estrategias de tratamiento personalizadas, ya que las mujeres jóvenes con cáncer de mama HR+/HER2- parecen ser menos sensibles a la terapia endocrina pero más susceptibles a la quimioterapia.

CONCLUSIONES:

Nuestro estudio ofrece una caracterización integral del perfil transcriptómico del cáncer de mama HR+/HER2- en mujeres muy jóvenes, revelando características moleculares diferenciadas en comparación con pacientes de mayor edad.

No se observaron diferencias significativas en los biomarcadores clásicos entre pacientes jóvenes y mayores, lo que pone de manifiesto la necesidad de realizar un perfilado molecular más profundo.

Se identificaron diferencias transcriptómicas relacionadas con la edad, tanto en las células tumorales como en el microambiente tumoral.

Los tumores HR+/HER2- en mujeres muy jóvenes presentaron mayor heterogeneidad molecular, aumento de la proliferación, inestabilidad cromosómica y una mayor infiltración inmunitaria, características asociadas a los denominados "tumores calientes".

Estos hallazgos sugieren que los tumores HR+/HER2- en pacientes muy jóvenes podrían mostrar una menor sensibilidad a la terapia endocrina, pero un mayor grado de inmunorreactividad.

En conjunto, estos resultados refuerzan la necesidad de desarrollar estrategias terapéuticas personalizadas para este subgrupo de pacientes.

DISCUSIÓN:

Las tasas de supervivencia reducidas observadas en mujeres muy jóvenes diagnosticadas con cáncer de mama no pueden atribuirse únicamente a diagnósticos en estadios avanzados ni a la mayor prevalencia de subtipos tumorales desfavorables, como HER2+ o triple negativo (TN). Estos hallazgos sugieren que la edad en sí misma podría actuar como un factor pronóstico independiente, especialmente en el contexto del cáncer de mama HR+/HER2-. Diversos estudios han demostrado que las mujeres menores de 40 años presentan peores desenlaces en términos de supervivencia, particularmente dentro del subtipo HR+/HER2-, lo cual refuerza la hipótesis de que existen diferencias

biológicas intrínsecas que condicionan el pronóstico en este grupo de edad. Es relevante señalar que estas asociaciones desfavorables no son consistentes en todos los subtipos de cáncer de mama, ya que no se han observado correlaciones significativas en subtipos distintos al HR+/HER2-.

Los tratamientos sistémicos tienen un impacto considerable en la calidad de vida de las mujeres jóvenes con cáncer de mama. Estos tratamientos están asociados a efectos adversos físicos y psicológicos, entre los que destacan los problemas relacionados con la fertilidad y el distrés emocional, que representan un reto añadido en pacientes de menor edad. Abordar estas cuestiones es fundamental para optimizar el seguimiento y cuidado a largo plazo de las supervivientes.

Paralelamente, varios autores han explorado las alteraciones genómicas relacionadas con la edad a nivel de ADN. Se ha evidenciado que ciertas mutaciones somáticas ocurren con frecuencias diferentes entre pacientes jóvenes y mayores con tumores luminales. En concreto, las mutaciones en GATA3 y ARID1A son más frecuentes en mujeres jóvenes, mientras que las mutaciones en PIK3CA, CDH1 y MAP3K1 predominan en mujeres de mayor edad. Además, se ha observado una mayor frecuencia de alteraciones en el número de copias y deficiencia en la recombinación homóloga en pacientes jóvenes.

La optimización de la terapia adyuvante en mujeres premenopáusicas continúa siendo un área prioritaria de investigación. Encontrar el equilibrio entre evitar el infratratamiento y prevenir el sobretratamiento constituye un desafío particular en esta población. Algunas estrategias, como la individualización de la terapia endocrina, la supresión de la función ovárica y la estratificación del riesgo basada en herramientas genómicas, están siendo evaluadas para alcanzar este objetivo.

Los ensayos clínicos TAILORx y RxPONDER han aportado evidencia adicional sobre las diferencias moleculares entre los tumores de pacientes jóvenes y mayores. Estos estudios prospectivos evaluaron el beneficio clínico de la terapia endocrina en monoterapia frente a la quimioendocrina en pacientes con cáncer de mama luminal. De forma interesante, la terapia combinada mostró beneficios significativos en supervivencia libre de enfermedad invasiva y en reducción de recurrencias a distancia únicamente en mujeres premenopáusicas, sin mostrar ventajas claras en mujeres postmenopáusicas.

Las herramientas como el subtipado intrínseco PAM50 y la puntuación ROR (Risk of Recurrence) están ampliamente validadas para la estratificación del riesgo en mujeres postmenopáusicas con tumores HR+/HER2-. Sin embargo, su valor pronóstico en mujeres premenopáusicas aún no está bien establecido. En un estudio de Brown et al., se analizaron los subtipos PAM50 y las puntuaciones ROR en mujeres premenopáusicas con cáncer de mama HR+/HER2-, observándose una mayor prevalencia de subtipos Luminal B y no luminales en mujeres jóvenes, así como puntuaciones ROR significativamente más altas, especialmente en pacientes sin afectación ganglionar. Estos hallazgos no solo reflejan una biología tumoral más agresiva en mujeres jóvenes, sino que también explican la capacidad predictiva de puntuaciones elevadas de ROR y CES (Chemo-Endocrine Score) para anticipar una mejor respuesta a la quimioterapia.

En consonancia con estos resultados, nuestro estudio confirmó que las pacientes jóvenes HR+/HER2- presentaron puntuaciones significativamente más altas de ROR y CES basadas en PAM50, lo cual predice una mayor probabilidad de beneficio con quimioterapia adyuvante en comparación con las pacientes mayores HR+/HER2-.

El presente trabajo constituye el mayor conjunto de datos de secuenciación de ARN (RNA-seq) en mujeres muy jóvenes con cáncer de mama descrito hasta la fecha. En los últimos años, el microambiente inmunológico tumoral ha surgido como un área de interés creciente en la investigación en cáncer de mama. En este contexto, comparamos el panorama transcriptómico de tumores de mama entre pacientes jóvenes y mayores, centrando el análisis en mujeres premenopáusicas menores de 35 años frente a mujeres postmenopáusicas mayores de 50 años, con el objetivo de dilucidar diferencias moleculares específicas relacionadas con la edad. La exclusión deliberada del rango etario intermedio fortaleció nuestro diseño, permitiendo una exploración más precisa y dirigida de las variaciones biológicas relacionadas con la edad.

Nuestros resultados indican que las diferencias más relevantes asociadas a la edad se concentran fundamentalmente en el subtipo HR+/HER2-. Realizamos un análisis de deconvolución computacional exhaustivo mediante los algoritmos MCP-counter y quanTIseq, lo que nos permitió evaluar la composición celular del tumor a partir de datos de RNA-seq masivos. Detectamos que los tumores HR+/HER2- en mujeres muy jóvenes presentan una infiltración inmunitaria significativamente mayor, clasificándolos como tumores "calientes", un hallazgo inesperado, dado que este subtipo se considera

tradicionalmente "frío" desde el punto de vista inmunológico. Es relevante destacar que los tumores "calientes" presentan mayor probabilidad de responder a inhibidores de puntos de control inmunitario, en contraste con los tumores "fríos".

A partir de estos hallazgos, exploramos la posible aplicación de la inmunoterapia en pacientes jóvenes con cáncer de mama luminal HR+/HER2-. En primer lugar, confirmamos que los tumores de pacientes jóvenes HR+/HER2- presentan una sobreexpresión de moléculas inmunorreguladoras (checkpoints inmunitarios), lo cual respalda nuestra hipótesis inicial. Posteriormente, se realizó una evaluación histológica con el objetivo de identificar diferencias en predictores de respuesta a inmunoterapia, centrándonos específicamente en la presencia de linfocitos infiltrantes del tumor (TILs) y estructuras linfoides terciarias (TLS).

Las TLS son agregados ectópicos de linfocitos que se asemejan a ganglios linfáticos, y pueden encontrarse embebidas dentro del tumor o en sus márgenes. Estas estructuras contienen linfocitos B, linfocitos T y células dendríticas, y se ha descrito que desempeñan un papel clave en la modulación de la respuesta a la inmunoterapia. Varios estudios han indicado que las TLS pueden actuar como biomarcadores predictivos valiosos de la respuesta a inmunoterapia en el cáncer de mama. En nuestro estudio, la evaluación histológica reveló que los tumores HR+/HER2- de pacientes muy jóvenes presentaban un mayor número de TILs y TLS en comparación con los tumores de pacientes de mayor edad, lo que refuerza el potencial terapéutico de la inmunoterapia en este subgrupo específico.

A pesar de que los tumores luminales se consideran tradicionalmente "fríos" desde el punto de vista inmunológico, varios ensayos clínicos han demostrado que ciertos pacientes con este subtipo podrían beneficiarse de tratamientos con inhibidores del punto de control inmunitario. De forma destacada, el ensayo clínico fase II GIADA incluyó pacientes premenopáusicas con cáncer de mama Luminal B, tratadas con quimioterapia en combinación con inmunoterapia. En dicho estudio, en el subtipo molecular basal se observó una asociación entre la presencia de TILs y firmas génicas inmunorrelacionadas con la respuesta patológica completa.

Asimismo, los ensayos clínicos fase II I-SPY2 y fase III KEYNOTE-756 han demostrado beneficios clínicos del uso de inhibidores de puntos de control inmunitario

en un subgrupo de pacientes con cáncer de mama luminal. En este contexto, se hace evidente que algunas pacientes HR+/HER2- podrían beneficiarse de la inmunoterapia.

Nuestro trabajo aporta una comprensión más profunda de un subconjunto de pacientes luminales con características inmunorreactivas, que podrían responder positivamente a la inmunoterapia. Anticipamos que estos hallazgos contribuirán de forma significativa al entendimiento de la biología tumoral y el comportamiento clínico del cáncer de mama en mujeres muy jóvenes.

Nuestros análisis transcriptómicos revelaron una sobreexpresión de firmas inmunológicas y un aumento de la infiltración inmunitaria, hallazgos que fueron confirmados histológicamente mediante la identificación de un mayor número de TILs y TLS. Esta doble validación molecular e histológica refuerza la hipótesis del potencial beneficio de la inmunoterapia en este subgrupo de pacientes y proporciona una base sólida para el desarrollo de estrategias terapéuticas más personalizadas.