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LETTER TO THE JOURNAL

# Deciphering the transcriptomic landscape of early HR$^+$/HER2$^-$ breast cancer in very young women

Although breast cancer (BC) predominantly affects women over 50, about 5% of cases occur in very young women, aged 35 years or younger, presenting significant clinical challenges and poor outcomes. To explore age-associated molecular differences in BC, we evaluated the transcriptomic profiles of tumors from very young and older patients, focusing on the tumor microenvironment (TME). Methods are described in the Supplementary file.

This study enrolled 66 patients from Hospital Clínico Universitario of Valencia, stratified into very young (≤35 years, premenopausal) and older (>50 years, postmenopausal) BC patients. RNA-sequencing was performed on untreated primary tumors, and data were collected from 22 very young (median: 32.5 years; range: 22-35 years) and 27 older (median: 66 years; range: 50-94 years) BC patients who met quality criteria (clinicopathological characteristics in Supplementary Table S1). Principal component analysis illustrated increased heterogeneity among very young HR$^+$/HER2$^-$ BC samples (Figure 1A). Differential expression analysis revealed 82 downregulated and 12 upregulated genes in very young BC samples ($\log_2$ fold change $\geq |2|$; adjusted $P < 0.05$) (Figure 1B-C, Supplementary Table S2). Besides, gene signatures related to tumor aggressiveness and the TME revealed increased proliferation, chromosome instability, and heightened immunoreactivity, presenting higher scores in T cell markers, inflammation, interferon gamma, M1 macrophages, B cell markers, and immunoglobulin G in very young BC patients compared to older BC patients (Figure 1D).

Subtype-specific analyses revealed no differences in protein levels of hormone receptors (HRs) by immunohistochemistry (IHC) (Figure 1E). However, mRNA levels of HRs were lower in HR$^+$/human epidermal growth factor receptor 2 (HER2)$^-$ very young BC compared to older BC (Figure 1F). Ki-67 IHC revealed higher proliferation in very young BC patients than in older BC patients within HR$^+$/HER2$^-$ and triple-negative (TN) subtypes, confirmed by RNA-sequencing in HR$^+$/HER2$^-$ (Figure 1E-F). The PAM50-based intrinsic subtype was determined. The PAM50-based risk of recurrence score was higher in very young BC compared to older BC, exclusively in the HR$^+$/HER2$^-$ subtype ($P = 0.027$, Figure 1G-H, Supplementary Figure S1). The PAM50-based chemoendocrine score of HR$^+$/HER2$^-$ samples was lower in very young BC ($P = 0.017$, Figure 1I-J), suggesting better chemotherapy response but greater resistance to endocrine therapy compared to older BC.

Considering that the observed differences were specific to HR$^+$/HER2$^-$ BC, we conducted a transcriptomic analysis to identify age-associated gene expression variations within this subtype. This analysis included 12 very young BC and 17 older BC samples (clinicopathological data in Supplementary Table S3). Principal component analysis confirmed greater heterogeneity in HR$^+$/HER2$^-$ very young BC compared to older BC (Figure 1K). Differential gene expression analysis identified 133 downregulated and 106 upregulated genes in HR$^+$/HER2$^-$ very young BC patients (Figure 1L, Supplementary Table S4). Hierarchical clustering grouped very young BC and older BC patients separately, underscoring substantial differences between these subgroups (Figure 1M). Moreover, Gene Ontology analysis of differentially expressed genes showed an overrepresentation of immune-related categories in very young BC, including adaptive immune response, immunoglobulin production, immunoglobulin-mediated immune response, lymphocyte-mediated immunity, and production of molecular mediators of the immune response (Figure 1N). Furthermore, proliferation-related categories such as mitotic sister chromatid segregation, mitotic nuclear division, and sister chromatid segregation were also upregulated in very young BC (Figure 1O).

**Abbreviations:** BC, breast cancer; HER2, human epidermal growth factor receptor 2; HR, hormone receptors; IHC, immunohistochemistry; MCP-counter, Microenvironment Cell Population-counter; NK, natural killer; TILs, tumor-infiltrating lymphocytes; TLSs, tertiary lymphoid structures; TME, tumor microenvironment; TN, triple-negative.

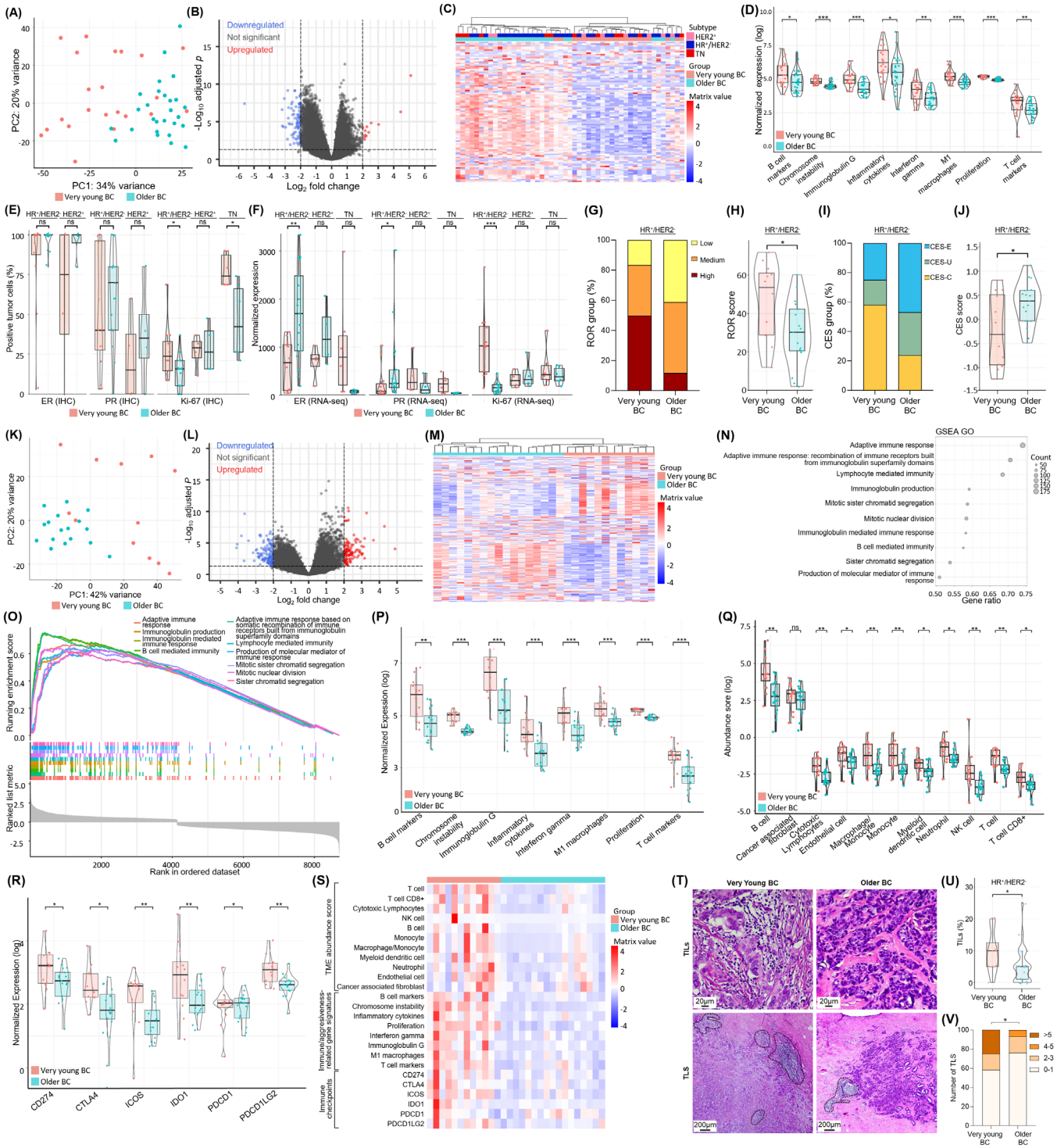


**FIGURE 1** Characterization of molecular differences between very young and older HR$^+$/HER2$^-$ BC patients. (A) Principal component analysis plot for HR$^+$/ HER2$^-$ very young BC samples ($n = 22$, red) *vs.* older BC samples ($n = 27$, blue). (B) Volcano plot representing RNA-sequencing differential expression results between very young BC and older BC. Colored dots represent genes significantly upregulated (red) or downregulated (blue) in very young BC with $\log_2$ fold change $\geq |2|$ and adjusted $P < 0.05$. (C) Hierarchical heatmap of the differentially expressed genes between very young and older BC patients. Each row represents a gene, and each column represents a patient sample. (D) Violin plots and boxplots of normalized expression scores of gene signatures related to tumor aggressiveness and cellular composition of the TME in very young BC (red) and older BC (blue) samples. (E-F) Violin plots and boxplots of the percentage of positive tumor cells by IHC (E) and normalized expression data from RNA-sequencing (F) by BC subtype. (G) Stacked bar charts of the distribution of ROR groups in very young BC ($n = 12$) and older BC ($n = 17$) HR$^+$/HER2$^-$samples. (H) Violin plots and boxplots of the ROR score in HR$^+$/HER2$^-$ samples. (**I**) Stacked bar charts of distribution of CES groups in HR$^+$/HER2$^-$ very young BC and older BC samples. (J) Violin

Given the observed differences in TME-related pathways, TME composition was investigated using multiple approaches. Immune-related gene signatures were found significantly overrepresented in very young HR$^+$/HER2$^-$ BC tumors, along with elevated scores in chromosome instability, and proliferation (Figure 1P). No differences were found in HER2$^+$ or TN subtypes, except for M1 macrophages in the HER2$^+$ subtype and proliferation in the TN subtype (Supplementary Figure S2). Subsequently, TME components were quantified by the Microenvironment Cell Population-counter (MCP-counter) algorithm, which allows for the calculation of immune cell infiltration in tumors and inter-sample comparison. MCP-counter analysis revealed higher immune cell infiltration in very young HR$^+$/HER2$^-$ BC tumors compared to older counterparts, particularly B cells, macrophages, monocytes, dendritic cells, neutrophils, natural killer (NK) cells, and T cells including CD8$^+$ T cells (Figure 1Q). This was validated with quanTIseq, confirming increased infiltration of B cells, M2 macrophages, NK cells, and CD8$^+$ T cells (Supplementary Figure S3). These findings highlight age-dependent differences in TME composition with increased immune infiltrates in very young HR$^+$/HER2$^-$ BC tumors.

Based on these findings, we evaluated the potential of immunotherapy for very young HR$^+$/HER2$^-$ BC patients. First, we explored the expression of immunological checkpoint genes, which were elevated in very young HR$^+$/HER2$^-$ BC (Figure 1R), supporting their classification as "hot" tumors (Figure 1S). Subsequently, we conducted histological evaluations of tumors from HR$^+$/HER2$^-$ patients to assess the tumor-infiltrating lymphocytes (TILs) and tertiary lymphoid structures (TLSs), both are indicators of potential immunotherapy response. This validation was performed on treatment-naïve tumors obtained from surgical specimens of 12 very young HR$^+$/HER2$^-$ BC and 29 older HR$^+$/HER2$^-$ BC patients (clinicopathological data in Supplementary Table S5). Very young HR$^+$/HER2$^-$ BC tumors exhibited higher TIL percentages than older BC tumors (Figure 1T-U); 25% of very young BC tumors had more than 5 TLSs, compared to none in older BC tumors, which predominantly had 0-1 TLS (Figure 1T, V). Despite the limited size of this cohort, these findings support RNA-sequencing results.

The diminished survival rates among young BC patients cannot be solely attributed to late diagnoses or the increased prevalence of HER2$^+$ and TN subtypes, associated with unfavorable outcomes [1, 2]. Clinical trials such as TAILORx [3] and RxPONDER [4] demonstrated that chemotherapy showed benefits only in premenopausal women, highlighting the need for a deeper understanding of the age-associated molecular differences, particularly in HR$^+$/HER2$^-$ BC, which could lead to personalized treatments for young patients. In line with this, we confirmed that very young HR$^+$/HER2$^-$ BC patients presented higher PAM50-based risk of recurrence and chemoendocrine scores, suggesting a better chemotherapy response compared to older HR$^+$/HER2$^-$ BC patients [5].

The exclusion of the intermediate age range (36-49 years) strengthened our study, focusing on age-related variations in tumor profiles. Our findings unveiled a heightened infiltration of immune cells in HR$^+$/HER2$^-$ very young BC tumors, categorizing them as "hot" tumors, which typically respond better to immune checkpoint inhibitors [6, 7]. Besides, we confirmed overexpression of

plots and boxplots of CES score in HR$^+$/HER2$^-$ samples. (K) Principal Component Analysis plot for HR$^+$/HER2$^-$ very young BC samples (red) *vs*. HR$^+$/HER2$^-$ older BC samples (blue). (L) Volcano plot representing RNA-sequencing differential expression results between HR$^+$/HER2$^-$ very young BC and older BC. Colored dots represent genes significantly upregulated (red) or downregulated (blue) in HR$^+$/HER2$^-$ very young BC with $\log_2$ fold change $\geq |2|$ and adjusted $P < 0.05$. (M) Hierarchical heatmap of the differentially expressed genes between HR$^+$/HER2$^-$ very young BC and older BC patients. Each row represents a gene, and each column represents a patient sample. (N) GSEA of genes overexpressed in HR$^+$/HER2$^-$ very young BC patients using the GO Biological Processes. (O) GSEA plot for enriched biological pathways in HR$^+$/HER2$^-$ very young BC vs. HR$^+$/HER2$^-$ older BC samples showing the Running Enrichment Score profile and positions of gene set members on the ranked list. (P) 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. (Q) Violin plots and boxplots of the abundance score of TME cells determined by MCP-counter. (R) Violin plots and boxplots of normalized expression data of immune checkpoint genes from RNA-sequencing. (S) Heatmap illustrating differences in TME composition between HR$^+$/HER2$^-$ very young BC and older BC 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. Very young BC samples are represented in red and older BC samples in blue. (T) Representative images of TILs and TLS in HR$^+$/HER2$^-$ very young BC ($n = 12$) and older BC patients ($n = 29$). (U) Violin plots and boxplots of enrichment of TILs in tumors from HR$^+$/HER2$^-$ very young BC and older BC patients. (V) Stacked bar charts of the distribution of TLS groups in HR$^+$/HER2$^-$ very young BC and older BC samples. Abbreviations: BC, breast cancer; CES, chemoendocrine score; CES-C: chemotherapy-sensitive; CES-U: uncertain; CES-E: endocrine-sensitive; GSEA, Gene set enrichment analysis; GO, gene ontology; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; HR, hormone receptors; ns, non-significant; NK, natural killer; PR, progesterone receptor; ROR, risk of recurrence; TILs, tumor-infiltrating lymphocytes; TLSs, tertiary lymphoid structures; TME, tumor microenvironment; TN, triple-negative; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

immune checkpoint molecules and higher levels of TILs and TLS in these tumors compared to older BC patients, suggesting that HR$^+$/HER2$^-$ very young BC patients could benefit from immunotherapy [8]. Clinical trials like I-SPY2 [9] and KEYNOTE-756 [10] supported this possibility, as demonstrated by the clinical benefit of immune checkpoint inhibitors in a subset of HR$^+$/HER2$^-$ BC patients. Our research enhances understanding of BC biology in very young patients, identifying upregulated immunological signatures and increased immune infiltrates. These findings could lead to the development of targeted therapies to improve outcomes and survival rates for this underexplored and vulnerable population. Although our results show promising clinical applicability, a limitation of this study was the limited sample size, which warrants further validation and additional prospective studies to confirm our findings.

In conclusion, our comprehensive analysis highlights the distinctive molecular landscape of HR$^+$/HER2$^-$ BC in very young women, including high heterogeneity, increased proliferation, chromosomal instability, immune cell infiltration, endocrine resistance, and susceptibility to chemotherapy. These findings suggest a potential for immunotherapy in this patient subgroup and a crucial opportunity to refine targeted therapeutic strategies, which could improve clinical outcomes for HR$^+$/HER2$^-$ BC in very young women.

## AUTHOR CONTRIBUTIONS

Conceptualization: IGC, MT, ALl, AP, BB, MTM, and JMC. Formal analysis: IGC, MT, JC, CP, FBM. Methodology: IGC, MT, JC, CP, STR, AAR, and ALa. Data curation: IGC, MT, CT, OB, and CH. Resources, supervision, project administration: MTM and JMC. All authors contributed to writing and editing the manuscript and approved the final version.

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## CONFLICT OF INTEREST STATEMENT

Authors have no conflict of interest to declare

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## DATA AVAILABILITY STATEMENT

The data generated in this study are available in Figshare (https://doi.org/10.6084/m9.figshare.28523627.v1).

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The use of clinical data and tumor samples was approved by the institutional ethical committee of INCLIVA (2019/144). The study included BC patients over the age of 18 years recruited at Hospital Clínico Universitario de Valencia (Spain). All procedures adhered to the Declaration of Helsinki, and signed informed consent was obtained from all participants.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.