Original Article

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An *In Silico* Analysis Identified Members of the Pleckstrin Homology-Like Domain, Family B (PHLDB family) as Potential Prognostic and Predictive Biomarkers of Treatment Response in Breast Cancer Patients

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ABSTRACT

Objective: Breast cancer is the leading cause of morbidity and mortality in women worldwide. This malignant neoplasm can be classified into four clinically relevant subtypes according to the expression of a number of biomarkers. However, these tumors show considerable intratumoral heterogeneity and multidrug resistance. Members of the pleckstrin homology-like domain, family B (PHLDB) play a critical role in the regulation of p53 and AKT signaling pathways, important for cancer and cellular metabolism. The present study was performed to evaluate the expression pattern of PHLDB family members in breast cancer and its potential prognostic and predictive value for therapeutic response using bioinformatics tools.

Materials and Methods: This in silico analysis was performed using several online repositories, including UALCAN, GEPIA2, bc-GenExMiner, KM Plotter, PrognoScan and ROC Plotter.

Results: PHLDB family genes were found to be differentially expressed in tumor samples when compared to healthy breast tissue samples. Furthermore, epigenetic regulation may be one of the regulatory mechanisms for the expression of these markers. The PHLDB family of genes proved to be potential markers for predicting the development of lymph node metastasis ($p<0.0001$) and poor clinical outcome. All members of the PHLDB family were significantly correlated with hormone receptors. High levels of PHLDBs expression were associated with worse overall survival and recurrence-free survival in breast cancer patients. Finally, our data demonstrate that members of the PHLDB family can be promising markers in the stratification of patients who may or may not respond to different available therapies.

Conclusion: Our cumulative results demonstrate that PHLDB family members may be promising biomarkers for predicting prognosis and therapeutic response in breast cancer patients.

Keywords: Breast cancer, PHLDB, *in silico* analysis, biomarkers

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Key Points

- The pleckstrin homology-like domain, family b (PHLDB) family of genes are differentially expressed in tumor and normal breast tissues.
- Members of the PHLDB family are potential markers for predicting the development of lymph node metastasis and poor clinical outcome.
- Reduced expression of PHLDB 1, 2, and 3 mRNA was associated with decreased overall and recurrence-free survival rates in breast cancer patients.
- There is a possible relationship between PHLDB family member expression and response to endocrine therapy and to anti-HER2 antibodies.

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Introduction

Breast cancer is the malignant neoplasm with the highest rates of occurrence and mortality among women worldwide (1). Currently, it is known that breast cancer represents a phenotypically and biologically heterogeneous collection of diseases, culminating in different clinical patterns, prognosis and response to usual treatments (2).

Based on the expression of molecular biomarkers, breast cancer can be classified into four main subtypes widely accepted and used in clinical practice: Luminal A, Luminal B, human epidermal growth factor receptor 2 (HER2+) and triple negative breast cancer (TNBC) (3). The segregation of these molecular subtypes is due to genes responsible for the expression of hormone receptors for estrogen (ER) and progesterone (PR), HER2 and the cell proliferation marker, Ki-67 (4).

Although the sum of current clinical, pathological and molecular indicators favors a contribution in establishing the prognosis and predicting the therapeutic response of patients, the investigation of new, more robust, sensitive, specific and well-validated biomarkers is occurring, partially in response to the trend towards personalized medicine (5).

In this context, the Pleckstrin Homology-like Domain (PHLD) multifunctional protein class has been attracting interest for its role in the regulation of p53 and AKT signaling pathways, both of which are important for cancer and cellular metabolism (6). The PHLD protein class is organized into two separate families, PHLDA and PHLDB, each of which is composed of three members (6). All members of the PHLD families code for proteins that have a functional domain called PH (pleckstrin homology) (6). PH-like domains consist of 100 to 120 amino acid residues and are found in a wide range of proteins involved in intracellular signaling, and may also participate in cytoskeletal rearrangement and membrane trafficking (7). Furthermore, proteins with the PH domain have been well categorized as phosphatidylinositol-binding molecular modules located internally in the cell membrane, as well as other proteins with varying specificity (8, 9). The two PHLD protein families, A and B, differ from each other by the position of their PH domain in the N- or C-terminal region or in the length of the protein (6). Although identified nearly three decades ago, the PHLD class of proteins remains understudied in the oncological context, with members of the PHLDB family receiving the least attention in recent research.

Therefore, the present study was carried out to evaluate the expression pattern of PHLDB family members in breast cancer and its potential prognostic and predictive value for therapeutic response, through public datasets deposited in online repositories.

Materials and Methods

UALCAN and GEPIA2: UALCAN (http://ualcan.path.uab.edu/) is a free online platform to access and assess the expression profile of biomarkers in different types of cancers (10). UALCAN was used to investigate gene expression levels of PHLDB family members in normal and tumor samples from the breast, as well as in tumor subgroups and at different clinical stages. The level of methylation of the promoter region of the PHLDB family in breast cancer samples and normal tissues was also investigated using this same platform. Additionally, GEPIA2 (http://gepia2.cancer-pku.cn/) was accessed. GEPIA2 is a new improved web server to analyze RNA sequencing expression data from 9,736 tumors and 8,587 normal samples from the TCGA project (The Cancer Genome Atlas) and Genotype-Tissue Expression (GTEx) (11).

bc-GenExMiner: The Breast Cancer Gene-Expression Miner v4.5 (http://bcgenex.centregauducheau.fr/) is an online mining tool for properly annotated breast cancer transcriptomic data (12). For this study, we considered only the microarray data to analyze the expression of the PHLDB family with clinic pathological parameters, regarding the classic breast cancer biomarkers and the different molecular subtypes. The median expression was used as the cut-off point.

KM Plotter: The Kaplan–Meier Plotter (https://kmplot.com/analysis/) is a practical, easy-to-use survival analysis platform that hosts data from 21 different types of cancers (13). We investigated the expression of PHLDB family members according to overall survival (OS) and recurrence-free survival (RFS). The dataset included cDNA microarrays from the TCGA available in the KM Plotter online database. The validated probes were chosen according to the best automatic cut selection criteria. Follow-up time was adjusted to 120 months. Log-rank *p*-values and hazard ratio (HR) with 95% confidence interval (CI) were automatically determined.

PrognoScan: The PrognoScan online database (http://www.prognoscan.org/) provides a powerful platform to assess biological relationships between gene expression and cancer patient prognosis information, including overall survival (OS), relapse-free survival (RFS), distant metastasis-free survival (DMFS), and disease-specific survival (DSS) (14). PrognoScan includes public cDNA microarray datasets with clinical annotations of gene expression and prognosis from Gene Expression Omnibus (GEO) and ArrayExpress, for example. Cox *p*-values and hazard ratio (HR) with 95% confidence intervals (CI) were calculated automatically.

ROC Plotter: The ROC Plotter (http://www.rocplot.org/) is an interactive and user-friendly online tool (15). With transcriptomic data from 3104 breast cancer patients treated and not treated with endocrine therapy, anti-HER2 therapy, or chemotherapy. Here, we quickly evaluated the expression pattern of PHLDB family genes in the face of the treatment received by the patient.

Results

PHLDB Family Expression and Methylation Status in Samples From Breast Cancer Patients

Using TCGA data analyzed by the UALCAN platform, it was found that *PHLDB1* and *PHLDB2* had reduced expression in breast cancer tumor tissues when compared to adjacent normal tissues (Figures 1a and 2a; $p<0.0001$, respectively) and in a larger cohort the same pattern was observed (Supplementary Figures 1a and 1b; $p = 0.01$, respectively). Furthermore, hyper-methylation of the *PHLDB1* promoter region was observed in breast cancer tissues in relation to healthy tissues (Figure 1b; $p<0.0001$), indicating a possible direct relationship of this epigenetic regulatory mechanism with the reduction of expression in samples of breast cancer. Meanwhile, the highest level of methylation of the *PHLDB2* promoter region was observed in healthy breast tissues compared to tumor tissues (Figure 2b; $p<0.0001$). Contrary to what was observed for *PHLDB1* and *PHLDB2*, *PHLDB3* gene expression was higher in breast tumor samples when compared to healthy tissue samples (Figure 3a; $p<0.0001$) and, again, the same pattern was observed in a larger cohort, although this was not statistically significant (Supplementary Figure 1C). The highest level of methylation of

the *PHLDB3* promoter region was observed in breast cancer tissues compared to normal tissues (Figure 3b; $p<0.0001$).

Additionally, the expression patterns of PHLDB family members in relation to molecular classification was investigated. The Luminal type exhibited an increased transcriptional distribution in relation to the TNBC and HER2+ subtypes (Figures 1c, 2c and 3c; $p<0.0001$, respectively). Furthermore, patients with the most advanced clinical stage of breast cancer tended to express lower levels of *PHLDB1*, although this was not statistically significant when compared to the other stages of the disease (Figure 1d). However, there was no association between the differential expression of *PHLDB2* and *PHLDB3* with the different clinical stages of patients with breast tumors (Figures 2d and 3d, respectively).

Association of the Expression of PHLDB Family Members With Clinical-Pathological Characteristics

The open-source tool, bc-GenExMiner, was used for this analysis. The sample evidence suggested that there was a statistically significant association between the status of *PHLDB1* expression with all variables tested (Table 1). Significant associations were observed between *PHLDB2* expression and nodal status (p = 0.0228), Scarff-Bloom-Richardson (SBR) classification ($p<0.0001$), Nottingham Prognostic Index (NPI) (p = 0.0002), the statuses of ER ($p<0.0001$), PR (p = 0.0061), HER2 (p = 0.0147), and TP53 ($p<0.0001$) and molecular classification ($p<0.0001$) (Table 1). For the last member of the PHLDB family, statistically significant associations were found between differential expression of *PHLDB3* and patient age ($p<0.0001$), SBR classification ($p<0.0001$), NPI ($p<0.0001$), TP53 mutational status ($p<0.0001$), the expression of ER ($p<0.0001$), PR ($p<0.0001$), and HER2 ($p<0.0001$) and molecular subtype ($p<0.0001$) (Table 1).

Expression of PHLDB Family Members and Prognostic Value in Breast Cancer Patients

Next, the prognostic value of PHLDB family genes using the KM Plotter platform was investigated. Most notably, reduced levels of mRNA expression of PHLDB family members were significantly correlated with poor prognosis for overall survival (PHLDB1 p = 0.0044; PHLDB2 p = 0.0040 and PHLDB3 p = 0.0046) (Figures 4a, 4b and 4c, respectively) and recurrence-free survival (PHLDB1 $p<0.0001$; PHLDB2 p = 0.0013 and PHLDB3 $p<0.0001$) (Figures 4d, 4e and 4f, respectively). Additionally, the PrognoScan database showed that down-regulation of PHLDB family expression was significantly associated with reduction in cumulative rates of overall survival (OS), recurrence-free survival (RFS), distant metastasis-free survival (DMFS) and disease-free survival (DFS) (Table 2).

We also investigated the prognostic role of PHLDB family members in different intrinsic molecular subtypes. Kaplan-Meier curves indicated that high PHLDB1 level was significantly associated with lower cumulative rates of RFS in the TNBC subtype (p = 0.0330) and OS in the TNBC (p = 0.0330) and HER2 subtypes (p = 0.0067)

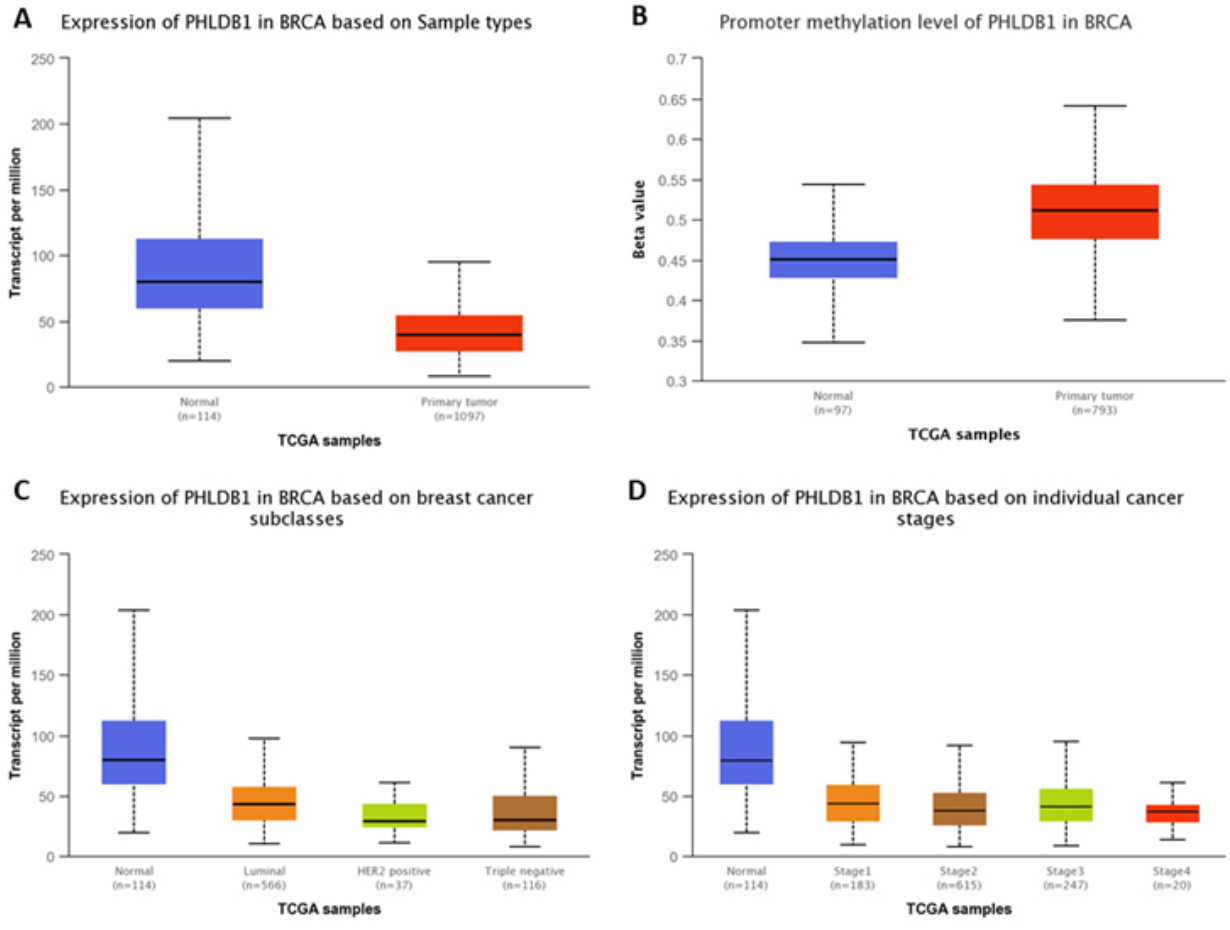

Figure 1. Expression of PHLDB1 in breast cancer patients. **a)** Expression of PHLDB1 in tumor and normal breast samples. **b)** Methylation profile of the PHLDB1 promoter region in tumor and normal breast samples. **c)** PHLDB1 expression in different molecular subtypes of breast cancer. **d)** PHLDB1 expression based on the different stages of the diseasePHLDB: pleckstrin homology-like domain family B

(Supplementary Figures 2D, 2A and 5A, respectively). Meanwhile, reduced levels of PHLDB1 showed lower RFS in Luminal A ($p < 0.0001$) and Luminal B ($p < 0.0001$) and OS in Luminal A ($p = 0.0008$) subtypes in breast cancer patients (Supplementary Figures 3D, 4D and 3A, respectively). We found that upregulation of PHLDB2 expression was significantly correlated with worse rates of RFS in the TNBC ($p = 0.0014$) and HER2 ($p = 0.0210$) subtypes and OS in the HER2 subtype ($p = 0.0240$) (Supplementary Figures 2E, 5E and 5B, respectively). In contrast, reduced levels of PHLDB2 mRNA expression were significantly correlated with reduced RFS in Luminal A ($p = 0.0002$) and Luminal B ($p = 0.0170$) and OS in Luminal A subtype ($p = 0.0025$) (Supplementary Figures 3E, 4E and 3B, respectively). Finally, the Kaplan-Meier curves indicated that the highest level of PHLDB3 correlated with preferable RFS in TNBC ($p = 0.0260$), Luminal A ($p < 0.0001$) and Luminal B ($p = 0.0009$) and OS subtypes in the Luminal A subtype ($p = 0.0260$) (Supplementary Figures 2F, 3F, 4F and 3C, respectively). Meanwhile, high PHLDB3 level was significantly associated with lower cumulative OS rates in the HER2 subtype ($p = 0.0150$) (Supplementary Figure 5C).

Predictive Value of PHLDB Family Members for Treatment Response

Considering the reports of some previous studies indicating PHLD family members as potential biomarkers for response to different treatments (16, 17), we conducted an analysis with the ROC Plotter web tool. Our results showed that among patients with hormone-dependent tumors, those who did not respond to hormone treatment had significantly reduced expression of *PHLDB1* in cases classified as Luminal A ($p = 0.040$) (Figure 5a), but there was no relationship in Luminal B tumors ($p = 0.054$) (Figure 5b). In the evaluation of PHLDB2 related to response rates to endocrine treatment, there was no statistical association in cases subtyped as Luminal A ($p = 0.300$) and Luminal B ($p = 0.054$) (Figures 6a and 6b, respectively). Finally, for the last family member, a significant relationship was found between high levels of PHLDB3 for patients who responded to endocrine treatment with tamoxifen or anastrozole (Luminal A, $p = 0.047$ and Luminal B, $p = 0.012$) (Figures 7a and 7b, respectively). Furthermore, reduced *PHLDB3* expression in HER2+ tumors was correlated with low response rates to anti-HER2 treatment ($p = 0.029$) (Figure 7c). However, PHLDB1 and PHLDB2 showed no relationship in the response rates of patients with tumors that overexpress HER2 when treated with monoclonal antibodies targeting this receptor ($p = 0.710$ and $p = 0.320$, respectively) (Figures 5c and 6c, respectively). Contrary to the effect observed for hormone-dependent and HER2-overexpressing tumors, patients with TNBC-type tumors that did not respond to chemotherapy had significantly increased rates of PHLDB1 ($p = 0.009$) (Figure 5d) and PHLDB2 ($p = 0.034$) (Figure 6d), in this particularly more aggressive form of breast cancer. However, for the third family member, no relationship between *PHLDB3* differential expression with response to chemotherapeutic treatments was observed in TNBC cases ($p = 0.730$) (Figure 7d).

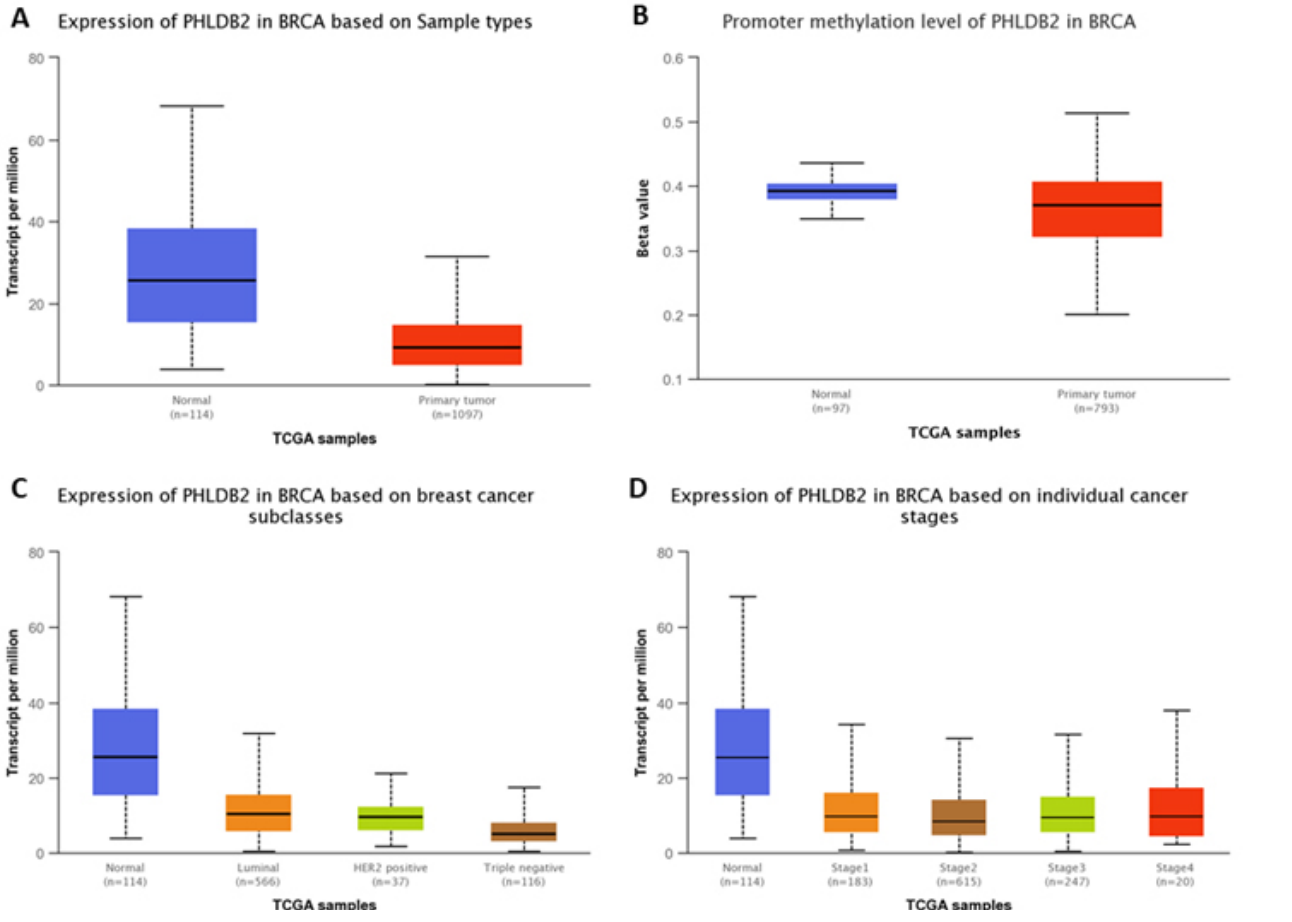

Figure 2. Expression of PHLDB2 in breast cancer patients. **a)** Expression of PHLDB2 in tumor and normal breast samples. **b)** Methylation profile of the PHLDB2 promoter region in tumor and normal breast samples. **c)** PHLDB2 expression in different molecular subtypes of breast cancer. **d)** PHLDB2 expression based on the different stages of the diseasePHLDB: pleckstrin homology-like domain family B

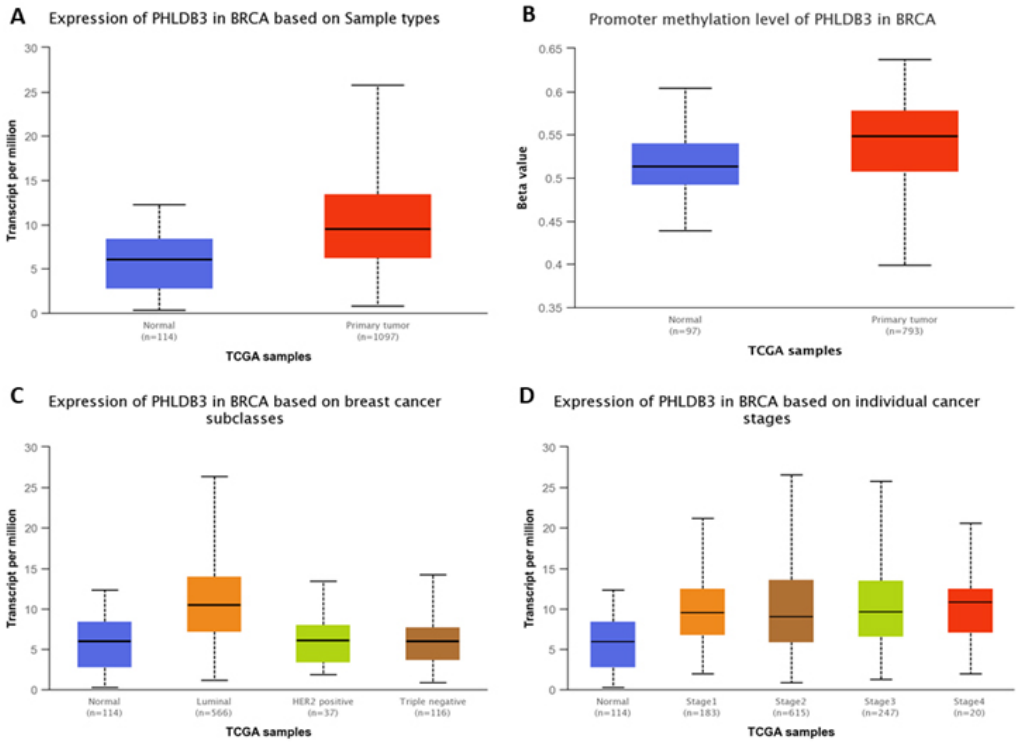

Figure 3. Expression of PHLDB3 in breast cancer patients. **a)** Expression of PHLDB3 in tumor and normal breast samples. **b)** Methylation profile of the PHLDB3 promoter region in tumor and normal breast samples. **c)** PHLDB3 expression in different molecular subtypes of breast cancer. **d)** PHLDB3 expression based on the different stages of the diseasePHLDB: pleckstrin homology-like domain family B

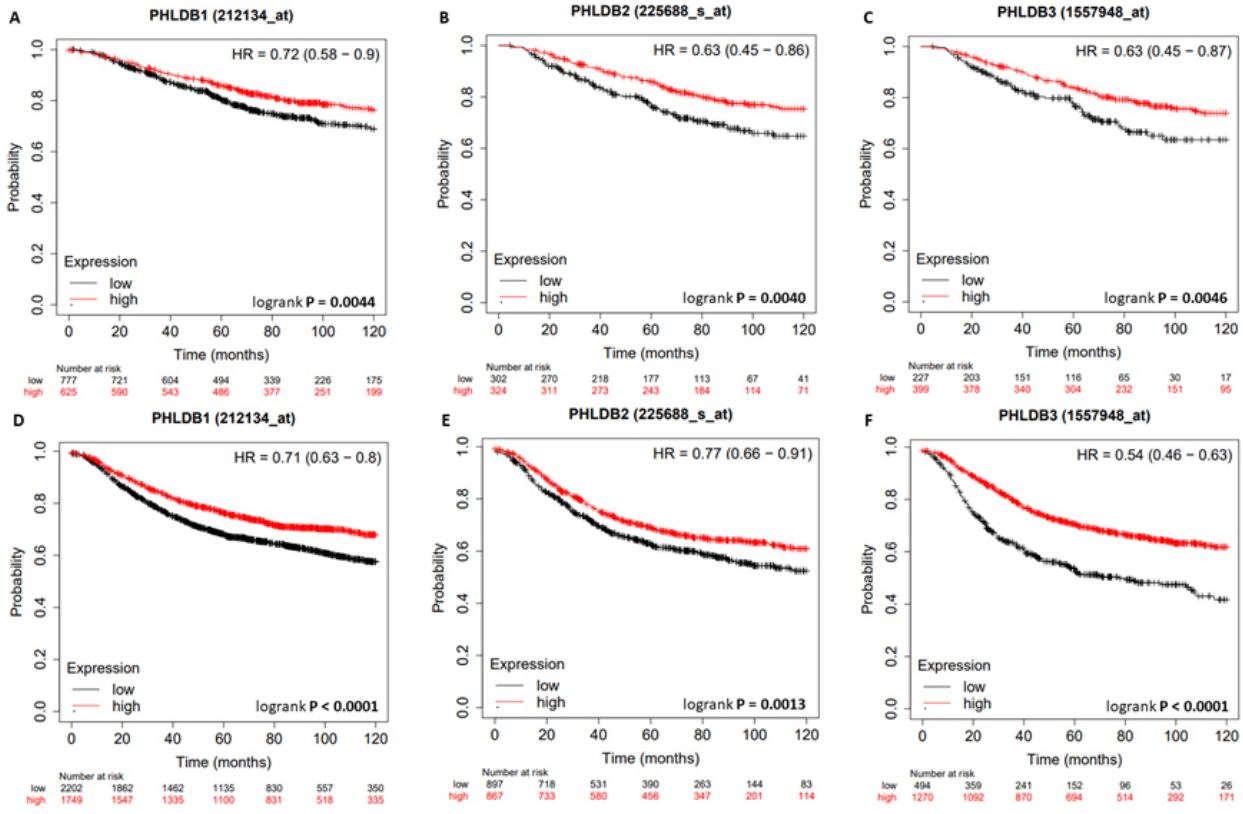

Figure 4. Survival curves derived from the Kaplan–Meier Plotter evaluating the prognostic significance of members of the PHLDB family. Overall survival for breast cancer patients stratified by expression of PHLDB1 **(a)**, PHLDB2 **(b)**, and PHLDB3 **(c)**; Relapse-free survival of patients stratified by the expression of PHLDB1 **(d)**, PHLDB2 **(e)** and PHLDB3 **(f)**PHLDB: pleckstrin homology-like domain family B

Discussion and Conclusion

Despite great advances in the diagnosis, prognosis, prevention and treatment of breast cancer, this type of malignant tumor remains the most prevalent and lethal in women globally (3). In this context, hundreds of other biomarker candidates are being studied for potential implications for improving diagnosis and personalized therapy. In view of this, our study aimed to investigate the expression profile of members of the PHLDB family and the potential prognostic and clinically useful value in breast cancer using bioinformatics tools, taking into account the limitation of studies of members of the PHLDB family in the context of breast oncology and the attractive relationship of these markers as direct and indirect targets of p53 at its transcriptional levels and as competitive modulators of AKT activity by directly interfering in the binding of this oncoprotein to phosphatidylinositol (6).

The PH domain shared by all members of the PHLD family has the ability to anchor itself transiently on the surface of the intracellular

Table 1. Relationship between the expression of PHLDB family members and clinical parameters of breast cancer patients using the bc-GenExMiner database.

Variables	Number of the patients	PHLDB1 microarray	*p*-value	Number of the patients	PHLDB2 microarray	*p*-value	Patient Number	PHLDB3 microarray	*p*-value
Age									
≤51	2813	Increased	**0.0011**	2296	-	0.1212	2209	-	**<0.0001**
>51	4692	-		4292	-		4084	Increased	
Nodal status									
Negative	4431	Increased	**<0.0001**	3259	Increased	**0.0228**	3095	-	0.1373
Positive	3457	-		3052	-		2934	-	
SBR									
1	915	-		820	-		779	-	
2	3025	Decreased		2609	Decreased		2486	Decreased	
3	3033	Decreased	**<0.0001**	2653	Decreased	**<0.0001**	2527	Decreased	**<0.0001**
NPI									
1	1234	-		998	-		917	-	
2	2119	Decreased		1823	Decreased		1714	Decreased	**<0.0001**
3	675	Decreased	**<0.0001**	662	Decreased	**0.0002**	650	Decreased	
Status TP53									
Wild-type	638	Increased	**0.0008**	578	Increased	**<0.0001**	578	Increased	**<0.0001**
Mutated	284	-		264	-		264	-	
Estrogen receptor									
Negative	2362	-	**<0.0001**	1822	-	**<0.0001**	1707	-	**<0.0001**
Positive	6531	Increased		5006	Increased		4828	Increased	
Progesterone receptor									
Negative	2509	-	**<0.0001**	2761	-	**0.0061**	2123	-	**<0.0001**
Positive	3224	Increased		2184	Increased		2712	Increased	
HER2									
Negative	4120	Increased	**0.0407**	3362	-	**0.0147**	3279	-	**<0.0001**
Positive	683	-		642	Increased		639	Increased	
Molecular subtypes									
Luminal A	3103	Increased		2517	Increased		2467	-	
Luminal B	2809	Decreased		2274	Decreased		2228	Increased	
HER2	1156	-		837	-		821	-	**<0.0001**
Triple negative	1867	-	**<0.0001**	1465	Decreased	**<0.0001**	1417	Decreased	

Significant p-values are shown in bold.

PHLDB: pleckstrin homology-like domain family B; SBR: Scarff-Bloom-Richardson; NPI: Nottingham Prognostic Index; HER2: human epidermal growth factor receptor 2

membrane and participate in multiple signal transduction processes, being the subject a number of studies (9, 18). To date, the expression pattern in patient samples and the potential prognostic and predictive value of response to different accepted therapies provided by investigating PHLDB family members remain unclear in breast cancer.

Initially, we analyzed the expression profile of the members of the PHLDB family using the UALCAN and GEPIA2 databases. PHLDB1 and PHLDB2 were expressed less in breast tumor samples when compared to healthy tissue. Meanwhile, PHLDB3 was expressed more highly in breast cancer samples. To date, no study has investigated the

Table 2. PHLDB family expression and survival data from breast cancer patients using the PrognoScan database

Gene name	Dataset	Probe name	End point	Patient number	Cox p-value	HR
PHLDB1	GSE11121	212134_at	Distant Metastasis Free Survival	200	**0.019867**	0.37 (0.16–0.86)
PHLDB1	GSE1456-GPL96	212134_at	Overall Survival	159	**0.008066**	0.21 (0.07–0.67)
PHLDB2	GSE1456-GPL97	225688_s_at	Relapse Free Survival	159	**0.011724**	0.57 (0.36–0.88)
PHLDB2	GSE1456-GPL97	225688_s_at	Disease Specific Survival	159	**0.031768**	0.56 (0.34–0.95)
PHLDB2	GSE1456-GPL97	238419_at	Relapse Free Survival	159	**0.030639**	0.68 (0.48–0.96)
PHLDB2	GSE4922-GPL97	238419_at	Disease Free Survival	249	**0.049142**	1.32 (1.00–1.73)
PHLDB3	GSE12276	236082_at	Relapse Free Survival	204	**0.034811**	0.77 (0.60–0.98)
PHLDB3	GSE12276	1557948_at	Relapse Free Survival	204	**0.001562**	0.66 (0.51–0.85)
PHLDB3	GSE1456-GPL97	236082_at	Overall Survival	159	**0.011543**	3.63 (1.33–9.87)
PHLDB3	GSE1456-GPL97	236082_at	Disease Specific Survival	159	**0.028974**	3.70 (1.14–11.97)

Significant values are shown in bold.
PHLDB: pleckstrin homology-like domain family B; HR: hazard ratio

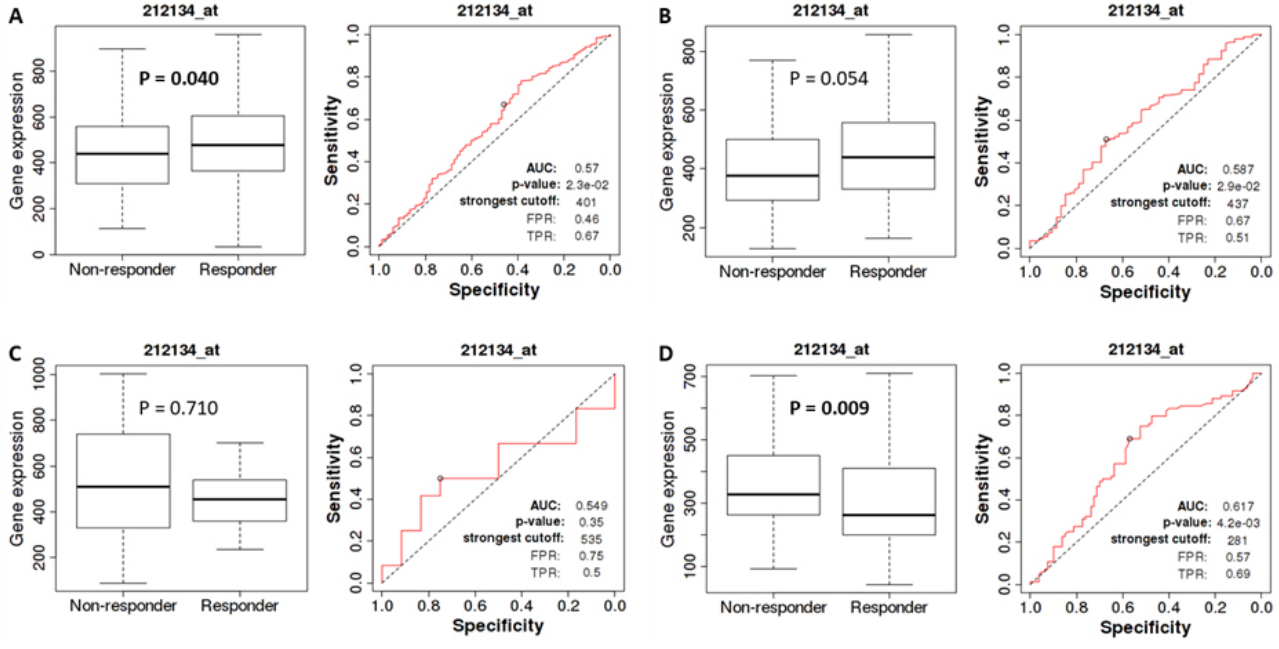

Figure 5. PHLDB1 expression pattern in patients receiving different therapies. 5-year recurrence-free survival among responders and non-responders to endocrine therapy in patients with tumors classified as Luminal A **(a)** and Luminal B **(b)**. 5-year recurrence-free survival among responders and non-responders to anti-HER2 therapy in patients with HER2+ tumors **(c)**. 5-year recurrence-free survival among chemotherapy responders and non-responders in patients with TNBC tumors **(d)**PHLDB: pleckstrin homology-like domain family B; HER2: human epidermal growth factor receptor 2

gene expression profile of the PHLDB family in healthy and tumor samples from the breast and therefore the current study is a pioneer in this sense. Furthermore, our results indicate that the methylation process can serve to repress or activate PHLDB family gene expression in breast tumor samples. It is known that the loss of balance in the methylation of specific regions of DNA can lead to increased predisposition to various diseases and abnormalities, including cancer (19). Another study identified PHLDB2 mRNA as differentially

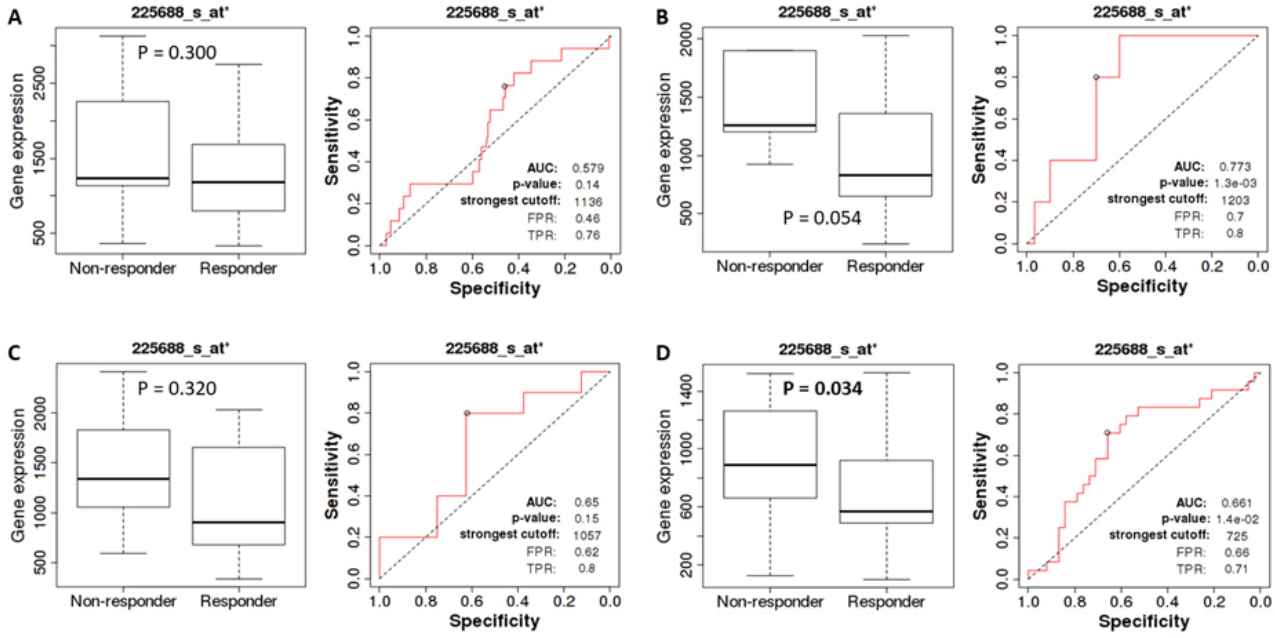

Figure 6. PHLDB2 expression pattern in patients receiving different therapies. 5-year recurrence-free survival among responders and non-responders to endocrine therapy in patients with tumors classified as Luminal A **(a)** and Luminal B **(b)**. 5-year recurrence-free survival among responders and non-responders to anti-HER2 therapy in patients with HER2+ tumors **(c)**. 5-year recurrence-free survival among chemotherapy responders and non-responders in patients with TNBC tumors **(d)**PHLDB: pleckstrin homology-like domain family B; HER2: human epidermal growth factor receptor 2

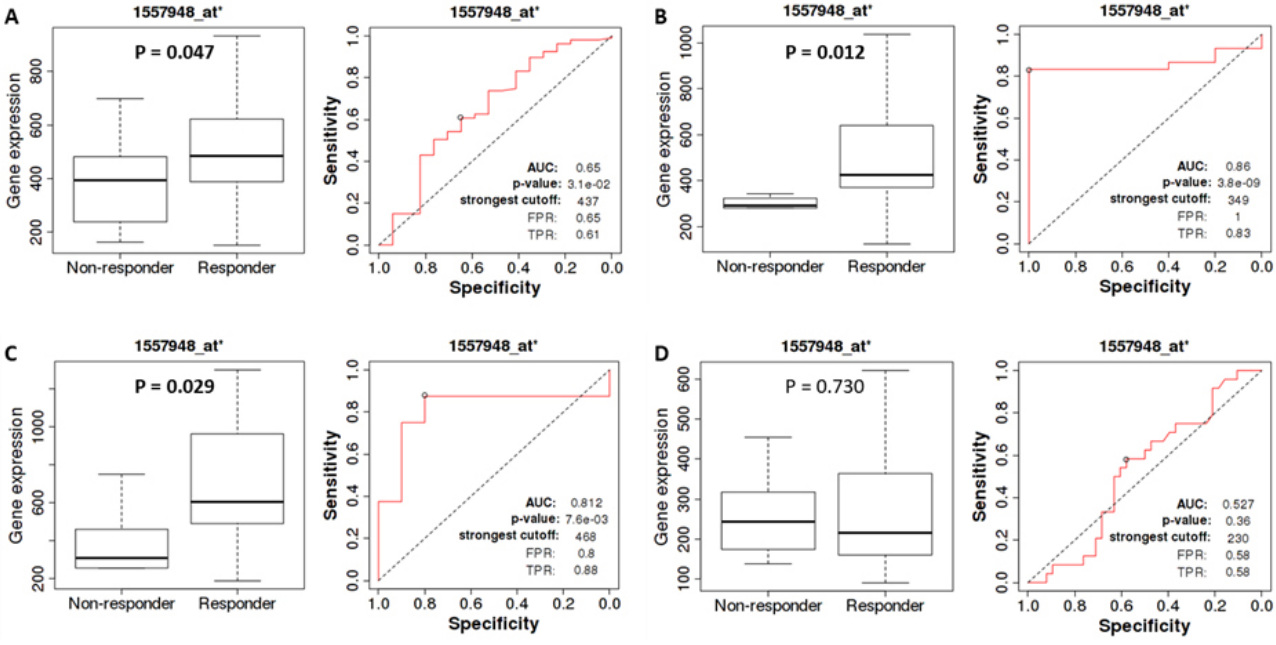

Figure 7. PHLDB3 expression pattern in patients receiving different therapies. 5-year recurrence-free survival among responders and non-responders to endocrine therapy in patients with tumors classified as Luminal A **(a)** and Luminal B **(b)**. 5-year recurrence-free survival among responders and non-responders to anti-HER2 therapy in patients with HER2+ tumors **(c)**. 5-year recurrence-free survival among chemotherapy responders and non-responders in patients with TNBC tumors **(d)** PHLDB: pleckstrin homology-like domain family B; HER2: human epidermal growth factor receptor 2

expressed, driven by methylation in uterine corpus endometrial carcinoma (UCEC) samples (20). Together, these observations may indicate that DNA methylation may be an important mechanism of epigenetic regulation of the PHLDB family in breast cancer, requiring further investigation.

Next, the relevance of the expression of PHLDB family members to different clinic pathological characteristics of breast cancer patients was analyzed. It was found that increased expression of the three members of the PHLDB family was significantly correlated with several variables, including lower rates of lymph node involvement and with the lowest degree of SBR and NPI. Routinely in clinical practice, the presence and extent of lymph node metastases are indicators of an aggressive phenotype, generally with an inverse relationship with prognosis (21). Thus, the genes of the PHLDB family, based on this *in silico* study, are shown to be potential markers for predicting the development of lymph node metastasis and unsatisfactory clinical outcome.

Additionally, our work showed a statistically significant correlation between the increased expression of *PHLDB1*, *2* and *3* with wild-type *TP53* and hormone receptor positivity (ER and PR) and, inevitably, with Luminal subtype tumors. In addition, PHLDB2 and 3 were more highly expressed in tumors with positive HER2 receptor tyrosine kinase classification, while PHLDB1 was inversely correlated compared to its paralogs. Interestingly, in addition to our findings, in previous studies it was observed that MCF-7 malignant breast cells treated with E2 (17β-estradiol) showed a large increase in the expression of *PHLDA1* transcripts compared to untreated cells (22) and that ER and NF-κB act synergistically for the direct transcriptional activation of *PHLDA1* (23). As for HER2, the picture remains unclear between the relationship between the PHLD family and this tyrosine kinase. However, previous work has already identified that *PHLDA2* expression is reduced at transcriptional and protein levels immediately and significantly by suppression of EGFR/HER2 oncogenic signaling in multiple HER2+ breast cancer cell lines (24, 25). These data indicate that members of the PHLD family can act as downstream targets of the EGFR/HER2 oncogenic signaling pathway. Finally, PHLD class proteins have been suggested as direct and indirect targets of p53 at its transcriptional levels by different studies (26, 27), demonstrating a potential critical role in tumorigenesis.

Subsequently, the prognostic significance of PHLDB family members in breast cancer was investigated using the public Kaplan–Meier Plotter and PrognoScan databases. It was found that reduced expression of PHLDB1, 2 and 3 mRNA was associated with decreased rates of OS and RFS in breast cancer patients. Supporting our previous data, the reduced expression of PHLDB family members was identified as critical for OS, RFS, DMFS and DFS reduction by the meta-analysis performed with the PrognoScan online repository. No study to date has evaluated the possible prognostic role of the PHLDB family in breast cancer. However, other works have already convincingly demonstrated that among the paralogs of the PHLDB family, members of the PHLDA family have a possible tumor suppressor role in breast cancer (28-30). Regarding the prognostic impact on different molecular subtypes, we identified that the reduced expression of PHLDB family members was associated with significantly reduced rates of OS and RFS in patients with Luminal-type tumors. For TNBC subtype tumors, an inverse role was observed, where the increased expression of PHLDB1 and 2 seems to favor a worse prognosis. Finally, among patients with tumors classified as HER2+, increased expression of PHLDB1 and 3 was responsible for worse OS. However, when evaluating these data, we have to take into account that the curves generated for OS and RFS of patients with breast cancer of molecular subtypes TNBC and HER2+ was based on smaller data sets when compared to Luminal-type tumors. Furthermore, we already know that many members of the PHLD family have a pleiotropic mechanism that will depend on the cell, tissue and molecular type and context. These findings provide evidence that PHLDB family members can serve as predictive markers for breast cancer prognosis.

Finally, our results for predicting therapeutic response showed that among patients with tumors classified as hormone-dependent and who were not responsive to endocrine treatment, these cases had lower gene expression for *PHLDB1* and *PHLDB3*. For HER2+ cases, reduced expression of *PHLDB3* was observed in samples from patients who did not respond to anti-HER2 antibody therapy. Finally, for the TNBC subtype, high expression of *PHLDB1* and *PHLDB2* was identified in samples from patients who did not respond to chemotherapeutic agents. So far, we do not know how these markers may be acting in TNBC cases, and *in vitro* studies are needed to confirm the relationship between PHLDB1 and 2 in the rates of patients' responses to chemotherapy.

Whereas, the PI3K/AKT/mTOR signaling pathway has been consistently implicated in resistance to several therapies in breast cancer (31) and that proteins with the PH domain can bind to phosphatidylinositol coupled to the surface of the intracellular membrane for suppression of this important oncogenic signaling pathway (9), we can hypothesize that PHLDB1 and 3 appear to be promising molecules to stratify patients who may or may not respond to hormone therapy and anti-HER2 agents. In addition to our findings, other studies have already demonstrated a possible relationship between the members of the PHLD family for therapeutic response in cases of Luminal and HER2+ breast cancer (16, 17, 24, 32).

In summary, this pioneering research revealed that members of the PHLDB family may be promising biomarkers for predicting prognosis and therapeutic response in breast cancer patients. It is important to highlight that *in silico* and data mining analyzes may have certain limitations, such as the extent and quality of information in publicly available databases, non-pairing of samples and, sometimes, small cohort size. However, our research was able to provide a stimulus, we hope, for possible further *in vitro* and *in vivo* studies, necessary for an application in the context of translational medicine in oncology.

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Informed Consent: Informed consent was not required for this study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: R.G.N, J.M., D.O.C.; Design: R.G.N, J.M., D.O.C.; Data Collection and/or Processing: R.G.N, J.M.; Analysis and/or Interpretation: R.G.N, D.O.C., S.J.J.; Literature Search: R.G.N, J.M., D.O.C., S.J.J.; Writing: R.G.N, J.M., D.O.C., S.J.J.

Conflict of Interest: No conflict of interest was declared by the authors.

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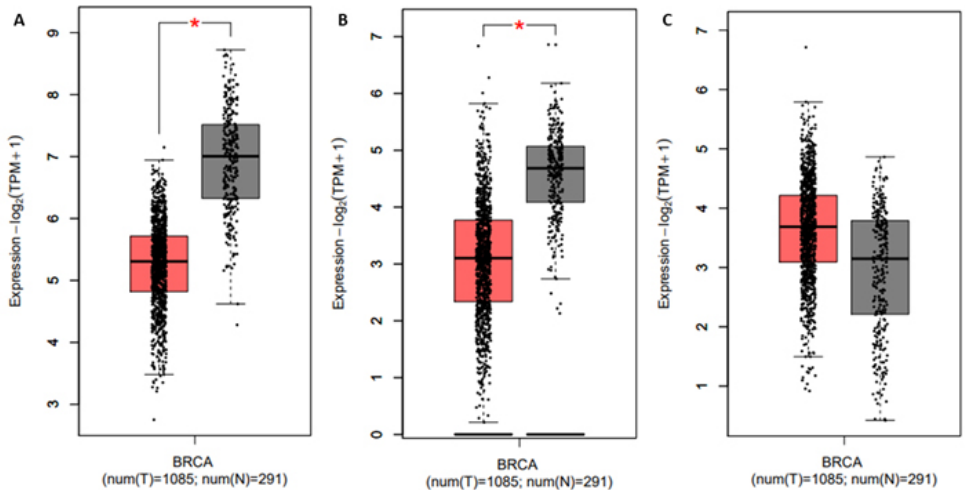
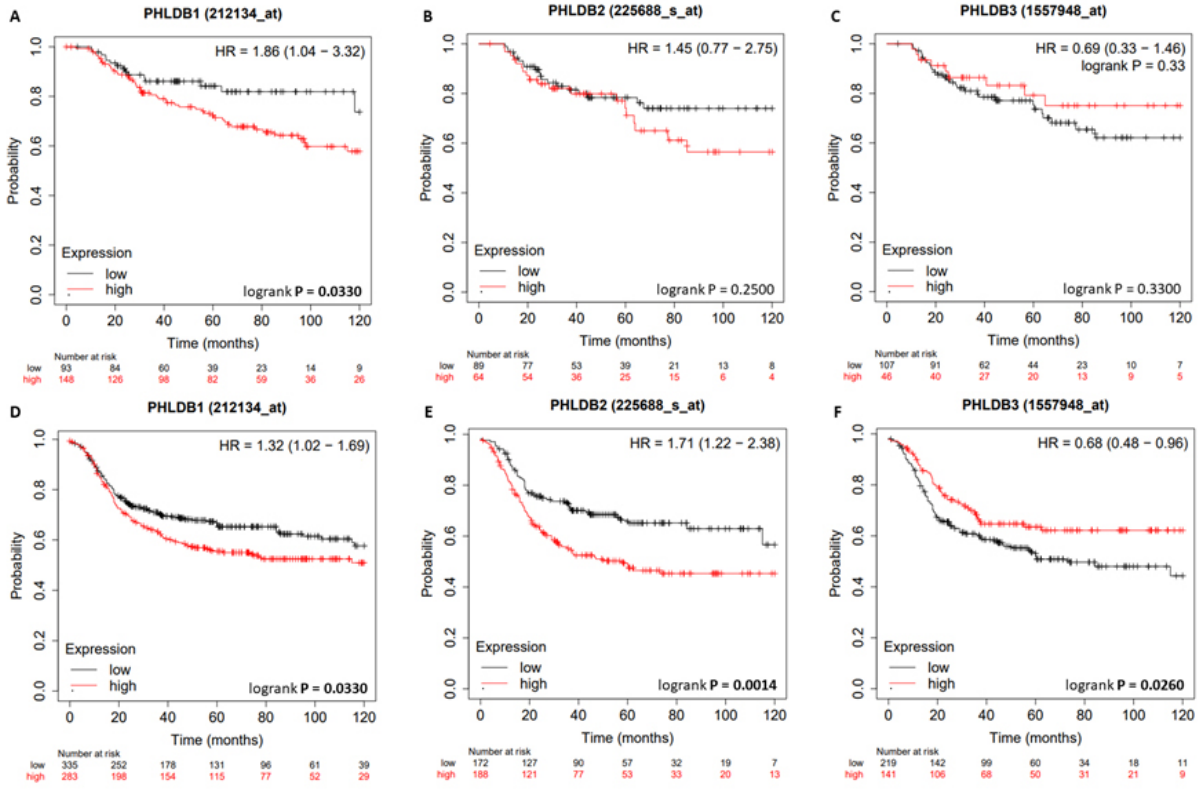
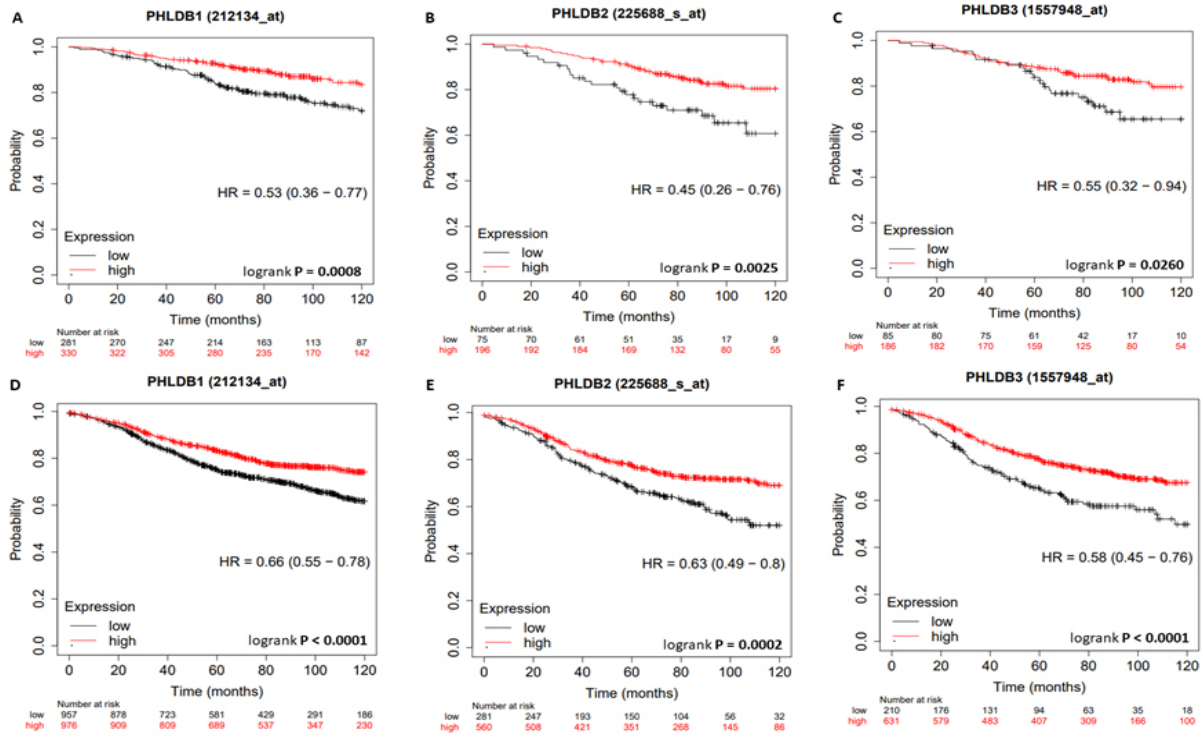


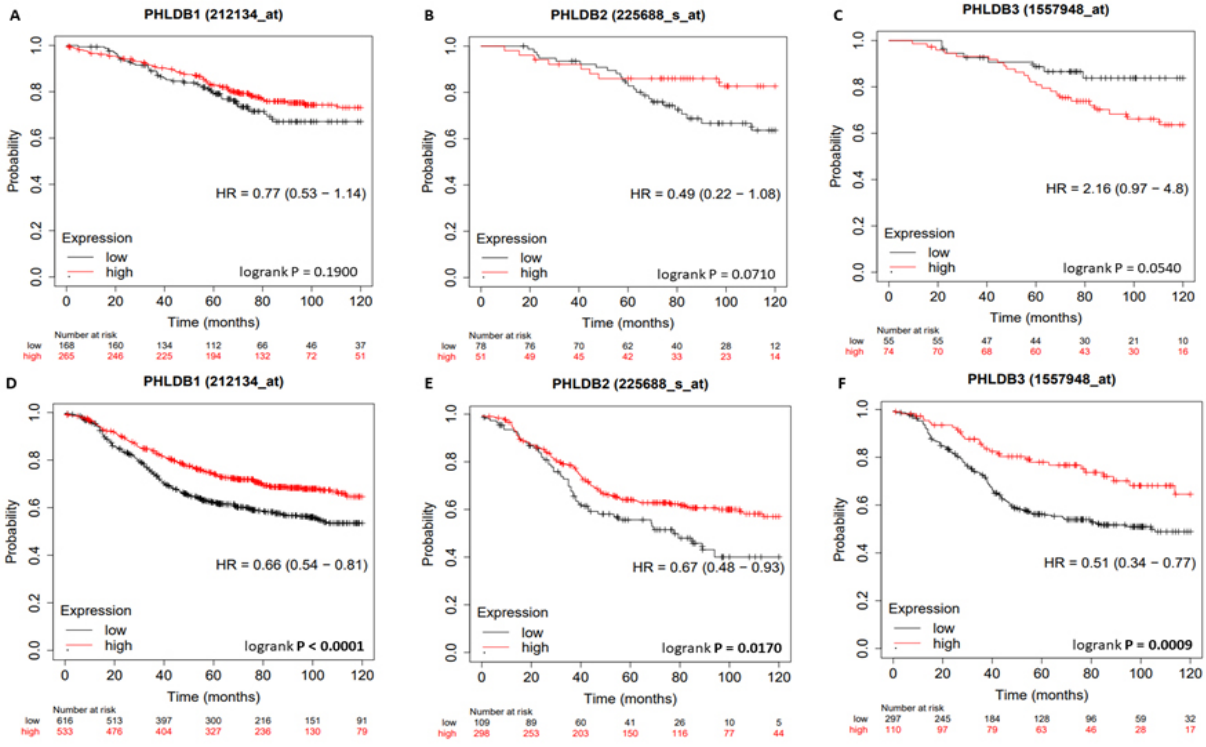
Supplementary Figure 1. Expression of PHLDB family members in normal and tumor samples of the breast. Gene expression in normal and tumor breast tissue samples for PHLDB1 **(a)**, PHLDB2 **(b)** and PHLDB3 **(c)** using the GEPIA2 databasePHLDB: pleckstrin homology-like domain family B



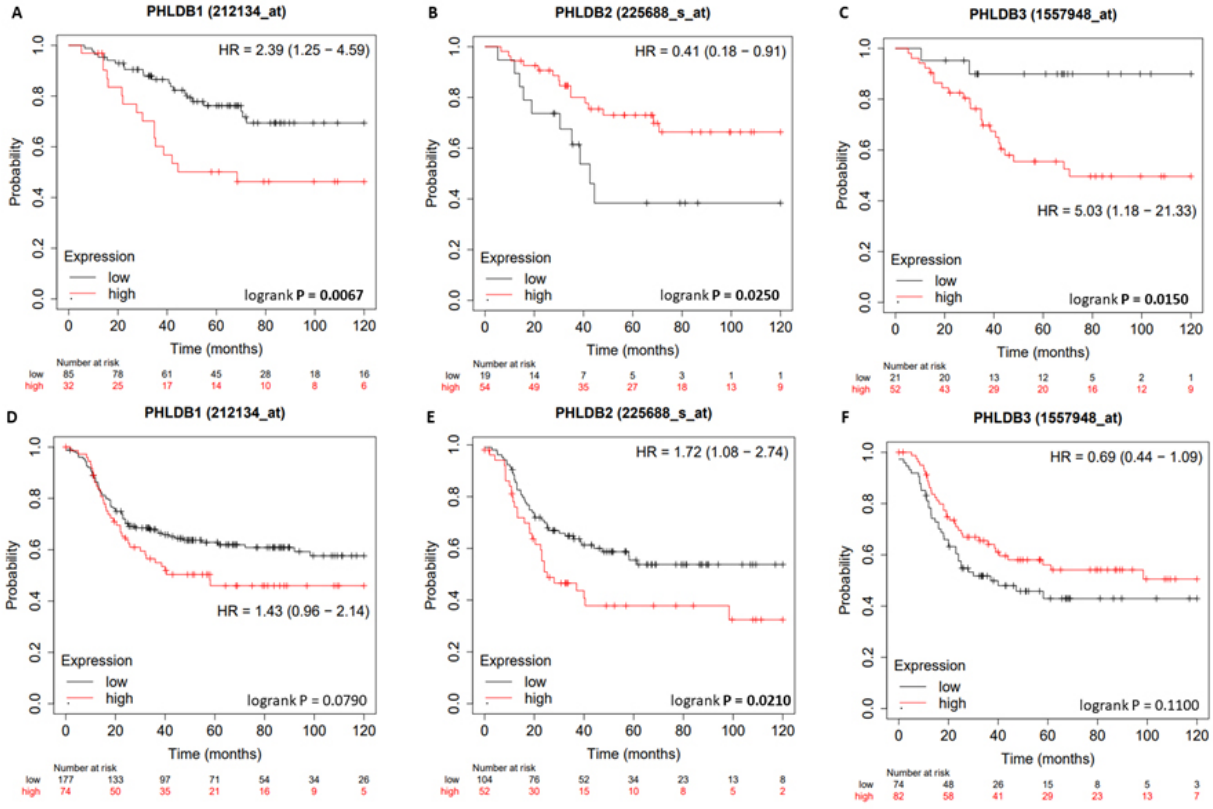
Supplementary Figure 2. Survival curves derived from the Kaplan–Meier Plotter evaluating the prognostic significance of PHLDB family members in TNBC subtype tumors. Overall survival for breast cancer patients stratified by expression of PHLDB1 **(a)**, PHLDB2 **(b)**, and PHLDB3 **(c)**; Relapse-free survival of patients stratified by the expression of PHLDB1 **(d)**, PHLDB2 **(e)** and PHLDB3 **(f)**PHLDB: pleckstrin homology-like domain family B; TNBC: triple negative breast cancer



Supplementary Figure 3. Survival curves derived from the Kaplan–Meier Plotter evaluating the prognostic significance of PHLDB family members in Luminal A subtype tumors. Overall survival for breast cancer patients stratified by the expression of PHLDB1 **(a)**, PHLDB2 **(b)** and PHLDB3 **(c)**; Relapse-free survival of patients stratified by the expression of PHLDB1 **(d)**, PHLDB2 **(e)** and PHLDB3 **(f)**PHLDB: pleckstrin homology-like domain family B



Supplementary Figure 4. Survival curves derived from the Kaplan–Meier Plotter evaluating the prognostic significance of PHLDB family members in Luminal B subtype tumors. Overall survival for breast cancer patients stratified by the expression of PHLDB1 **(a)**, PHLDB2 **(b)** and PHLDB3 **(c)**; Relapse-free survival of patients stratified by the expression of PHLDB1 **(d)**, PHLDB2 **(e)** and PHLDB3 **(f)**PHLDB: pleckstrin homology-like domain family B



Supplementary Figure 5. Survival curves derived from the Kaplan–Meier Plotter evaluating the prognostic significance of PHLDB family members in HER2+ subtype tumors. Overall survival for breast cancer patients stratified by expression of PHLDB1 **(a)**, PHLDB2 **(b)**, and PHLDB3 **(c)**; Relapse-free survival of patients stratified by the expression of PHLDB1 **(d)**, PHLDB2 **(e)** and PHLDB3 **(f)** PHLDB: pleckstrin homology-like domain family B; HER2: human epidermal growth factor receptor 2