Original Article

Eur J Breast Health 2023; 19(2): 148-158
DOI: 10.4274/ejbh.galenos.2023.2022-12-10

Altered Expression of CYSLTR1 is Associated With Adverse Clinical Outcome in Triple Negative Breast Tumors: An *In Silico* Approach

Andrés Galindo Céspedes[1], Mércia Patrícia Ferreira Conceição[2], Daniel Rodrigues de Bastos[2], Gabriela Ávila de Grazia[2], Jean Michel Rocha Sampaio Leite[3], Renan Gomes do Nascimento[4], Matthew Thomas Ferreira[2], Rossana Mendoza Lopez[2]

[1]Clinical Pathology Service, Almanzor Aguinaga Asenjo National Hospital, Chiclayo, Peru
[2]Instituto do Cancer do Estado de Sao Paulo ICESP, Hospital das Clinicas da Faculdade de Medicina da Universidade de São Paulo FMUSP HC, São Paulo, Brazil
[3]Department of Nutrition, School of Public Health, University of São Paulo, São Paulo, Brazil
[4]Department of Clinical Oncology Pharmacy, São Camilo Hospital, São Paulo, Brazil

ABSTRACT

Objective: Triple negative breast cancer (TNBC) has high relapse rates due to dysregulated inflammatory signaling pathways and significant changes in the tumor microenvironment, probably influencing the failure of several therapies. The Cysteinyl Leukotriene Receptor 1 (CYSLTR1), a leukotriene modulator of inflammation, has been shown to play an important role in cancer pathogenesis and survival but few studies have been reported on its role in breast cancer.

Materials and Methods: The present work was conducted using publicly available platforms that have omics data to assess the clinical potential of CYSLTR1 expression and its prognostic validation in large cohorts of samples from breast cancer patients. Web platforms containing clinical information, RNA-seq and protein data were selected to perform *in silico* analyses of the potential marker CYLSTR1. Added together, the platforms included modules for correlation, expression, prognosis, drug interactions, and construction of gene networks.

Results: Kaplan–Meier curves revealed that reduced levels of CYSLTR1 corresponded to an unfavorable outcome for overall survival (p<0.005) as well as relapse-free survival (p<0.001) in the basal subtype. Additionally, CYSLTR1 was downregulated in breast tumor samples compared to adjacent healthy tissue (p<0.01) and the basal subtype exhibited the lowest expression of CYSLTR1 relative to the other subtypes (p<0.0001). Furthermore, gene networking analysis showed strong associations of CYSLTR1 with two protein-coding genes (*P2RY10* and *XCR1*) when tested on a TNBC dataset.

Conclusion: Our data highlighted the relevance of CYSLTR1 since it may play an important role in TNBC therapy. However, further *in vitro* and *in vivo* studies should be directed towards validating our findings in an effort to improve our understanding of TNBC pathology.

Keywords: CYSLTR1, leukotriene, mediators of inflammation, triple-negative breast cancer

Cite this article as: Céspedes AG, Conceição MPF, de Bastos DR, de Grazia GÁ, Leite JMRS, do Nascimento RG, Ferreira MT, Lopez RM. Altered Expression of CYSLTR1 is Associated With Adverse Clinical Outcome in Triple Negative Breast Tumors: An *In Silico* Approach. Eur J Breast Health 2023; 19(2): 148-158

Key Points

- CYSLTR1 is downregulated in breast tumors.
- TNBC exhibited less CYSLTR1 than Luminals and HER2 subtypes.
- Low CYSLTR1 expression was associated with worse survival in breast cancer patients.
- Low CYSLTR1 expression was associated with worse survival in TNBC.

Introduction

It is widely known that the severity of breast cancer (BCa) results from a multitude of extrinsic and intrinsic factors, including tumor heterogeneity, which has been identified as the most relevant cause of poor outcome in patients with different subtypes of BCa (1). BCa can be stratified in hormone-dependent tumors, with receptor of human epidermal growth factor 2 (HER2) overexpression or triple negative (TNBC) according to immunohistochemical (IHC) staining for estrogen receptor (ER), progesterone receptor (PR), HER2, and the cell proliferation marker Ki-67. Another form of classification widely used in clinical practice is based on the transcriptomic profiles in Luminal A, Luminal B, HER2+, basal-like, normal-like, and claudin-low (2-4). This molecular classification has been confirmed by several research groups in different populations of patients with BCa (5-7). Patients with TNBC do not benefit from hormone therapy or targeted

Corresponding Author:
Rossana Mendoza Lopez; rossana.veronica@hc.fm.usp.br

Received: 11.01.2023
Accepted: 15.02.2023
Available Online Date: 01.04.2023

therapies commonly used in luminal and HER2+ cases. This lack of therapeutic options increases the chances of tumor recurrence, leading to a high mortality rate (8, 9).

On the other hand, BCa is strongly associated with inflammation and the release of signaling molecules derived from arachidonic acid, such as leukotrienes, as well as G protein-coupled receptors in the tumor microenvironment (TMev), which results in mediation of allergic, infectious, and inflammatory reactions through a phosphatidylinositol-calcium second messenger cascade (10-12). Among these messengers, Cysteinyl Leukotriene Receptor 1 (CYSLTR1) is implicated in mediating bronchoconstriction and asthma, and its dysregulation may be of concern in inflammation-related neoplasms (13). For example, in colorectal tumor cells, overexpression of CYSLTR1 is associated with proliferation, survival, and migration, as well as a poor prognosis in patients with colorectal adenocarcinoma (14). Furthermore, in patients with breast tumors, high expression levels of CYSLTR1 and low levels of CYSLTR2 were correlated with high mortality rates in univariate analyses for 144 patients (15). Another study suggests that CYSLTR1 is positively correlated with clinical features, such as tumor size, histologic type, lymph node metastasis, and TNM staging in a BCa population of 90 subjects (16).

Data mining represents a useful aproach to strengthen the knowledge and status of malignant neoplasms. With the era of omics, there is an increasing amount of genomic, transcriptomic, proteomic, and epigenetic data generated by high performance technologies available in public databases. Many of the studies deposited on these platforms have helped to characterize intrinsic cancer subtypes, predict survival, and therapeutic responses, generating a large amount of molecular biomarkers for BCa. There are only a few studies, with limited samples, that have explored the role of CYSLTR1 in women diagnosed with BCa. Therefore, the central objective of this study was to explore the status of CYSLTR1 according to expression levels and its potential prognostic value in BCa using datasets deposited in public repositories.

Materials and Methods

UALCAN and GENT2

UALCAN is a user-friendly online platform that provides easy access to OMICS cancer data. Thus, it allows for easy expression profiling of possible biomarkers, in associations with survival and gene regulation data, rendering a robust profile analysis (17). With this tool, we identified the difference between the CYSLTR1 expression levels of normal and breast tumor tissues. Moreover, in order to confirm our results, we accessed GENT2, a new tool focused on the expression analysis of normal and tumor tissue samples (18).

cBioPortal

The TCGA database (Firehose Legacy) was accessed through the cBioPortal platform to select mRNA expression Z-scores related to 1.108 samples (log RNA Seq V2 RSEM) with a ±2 threshold (19, 20). Clinical pathological data were obtained and cross-linked with CYSLTR1 expression data. Male cases (n = 16) and those who had no information of CYSLTR1 levels (n = 4) were excluded, resulting in 1.088 patients to be assessed.

bc-GenExMiner

The bc-GenExMiner v.5 is a microarray and RNA-seq data-mining tool containing data of BCa patients only. Three analysis modules were explored: Correlation, expression, and prognosis (21, 22). For this study, we considered only RNA-seq data, excluding samples from TCGA.

Kaplan–Meier Plotter

Kaplan–Meier (KM) Plotter is a publicly available platform that hosts data of 21 different types of cancer and contains Affymetrix gene signatures (probes of 20.129 genes) of 3.421 patients (23). For this study, we selected the best probe option corresponding to the *CYSLTR1* gene: 230866_at; *P2RY10* gene: 236280_at; and *XCR1* gene: 221468_at. The overall survival (OS) and relapse-free survival (RFS), adjusted for 120 months' total follow-up time, were available for all of them. The patients were also stratified by high and low expression of the target gene as the best cut-off between the lower and upper quartile was selected. Analyses were performed according to all deposited cases and only with the basal-like subtype, considering the prognostic value and its impact on poor clinical outcome.

Metascape

Metascape is a user-friendly tool for omics data analysis (24). Here, we accessed CYSLTR1 co-expressed genes previously obtained on bc-GenExMiner for interaction analysis. The protein network data was downloaded and analyzed on Cytoscape v.8.0 (25).

Geo Database

The Geo Database is a microarray and RNA-seq data deposit platform. In order to analyze the expression profile of *CYSLTR1* in different subtypes of breast tumors, we accessed the GSE76275 and GSE96058 files (26).

Gene Co-expression Network

The co-expression analysis was conducted using RNA-seq data of TNBC from the bc-GenExMiner v.5 database. A correlation value >0.7 was used as a cut-off, then the data was accessed using the String platform to generate CYSLTR1 co-expressed genes network data and to export it to Cytoscape v8.0 software to select the genes with close interactions with CYLSTR1. In addition, the co-expressed gene list was also accessed using the Metascape software in order to conduct enrichment analyses.

Comparative Toxicogenomics Database

The Comparative Toxicogenomics Database (CTD) is a publicly available tool for manually curated information about chemical interactions with genes, proteins, and chemical relationships with diseases (27). The CTD was accessed to obtain potential drugs capable of interacting positively or negatively with CYSLTR1.

mirTarBase Repository

By using the mirTarBase database we accessed the prediction of experimentally validated miRNAs targeting CYSTLR1 and significant co-expressed genes (28). In addition, we carried out survival analyses of both genes and best-predicted miRNAs in TNBC population.

Statistical Analysis

For platforms with integrated statistical capabilities, the analyzes were performed as described in the topics above. For additional data, analyses were conducted with the Statistical Package for Social Sciences (SPSS) version 25.0 (IBM Inc., Armonk, NY USA) or GraphPad v.7 (California, USA). The chi-square or Fisher's exact test was applied to compare categorical variables. For univariate and multivariate analysis, the Cox regression method was used. All groups were tested for

Gaussian distribution. The Mann–Whitney or t test was used to assess the difference between two groups, and ANOVA (analysis of variance) or Kruskal–Wallis for more than two groups. For survival analysis, survival curves were performed by KM method and compared using log-rank test; additionally, Cox regression univariate and multivariate were performed calculating hazard ratio (HR) with 95% confident interval. A significance level of 5% was adopted.

Results

CYSLTR1 is Downregulated in Breast Tumors and Correlated with Clinical Pathological Parameters

Our study evaluated data from different platforms (Firehose Legacy, cBioPortal and TCGA) which together represented a massive cohort of 1.097 tumor and 114 non-tumor breast samples. Our findings showed that samples from patients with breast tumors had low levels of CYSLTR1 mRNA compared to adjacent healthy tissues (p<0.01) (Figure 1A) and, in a larger cohort, we observed the same profile (p = 0.01) (Supplementary Figure 1A). In addition, significant associations were observed between differential expression of CYSLTR1 with patient age (p = 0.01), histological subtype (p<0.0001), *TP53* mutational status (p<0.0001), ER status (p<0.0001), PR status (p<0.0001), and molecular subtype (p<0.0001) (Table 1).

Using the TCGA dataset, we performed analyses to identify the expression profile of CYSLTR1 according to clinicopathological parameters. Initially, we observed significant differences between the histological subtypes, where in invasive ductal carcinoma tumors showed low expression of CYSLTR1 when compared to invasive lobular carcinoma (p<0.0001) (Figure 1B). We also describe expression patterns in accordance with the PAM50 subtype classification, where the basal-like type exhibited a decreased transcriptional distribution of CYSLTR1 compared to the other subtypes (Basal-like *vs* HER2, p<0.0001; Basal-like vs Luminal A, p<0.0001; Basal-like *vs* Luminal B, p<0.0001; Basal-like vs Normal-like, p<0.0001) (Figure 1C). In addition, patients whose tumors were negative for hormone receptors (ER and PR) and HER2 had lower levels of CYSLTR1 compared to those with positive expression for these receptors (ER+ vs ER-, p<0.0001; PR+ vs PR-, p<0.0001; HER2- vs HER2+, p = 0.02) (Figures 1D-F).

In order to confirm our findings in a larger cohort, we performed an analysis on bc-GenExMiner database. Consequently, a similar profile was observed in terms of expression levels according to the PAM50 classification (p<0.0001) (Supplementary Figure 1B), as well as estrogen (p<0.0001) (Supplementary Figure 2A), progesterone (p<0.0001) (Supplementary Figure 2B), and HER2+ receptors (p<0.0001) (Supplementary Figure 2C). Moreover, within the TCGA cohort, we assessed the possible differences between Basal-like and non-Basal-like (p<0.0001) (Supplementary Figure 2D), TNBC and non-TNBC (p<0.0001) (Supplementary Figure 2E), Basal-like and TNBC *vs* non- Basal-like and non-TNBC (p<0.0001) (Supplementary Figure 2F); thus, we confirmed that CYSLTR1 transcription levels were downregulated in samples with negative expression for hormone receptors.

Interestingly, the Basal-like immune-suppressed (BLIS) samples showed lower CYSLTR1 expression compared to Basal-like immune-activated samples (p = 0.004) (Figure 2B), while luminal androgen receptor (LAR) (p = 0.001) (Figure 2B) and mesenchymal (MES) samples (p<0.0001) (Figure 2B) exhibited higher levels when compared to BLIS. Similarly, Basal-like 1 and Basal-like 2 triple-negative tumors levels were lower in the cohort from TCGA/UALCAN (p<0.01 and p<0.001, respectively) (Supplementary Figure 3).

Low CYSLTR1 Expression was Associated with Worse Prognosis

Reduced CYSLTR1 mRNA expression levels were significantly correlated with unfavorable prognosis for both OS (Figure 3A; Supplementary Figure 4A-C) and RFS (Figures 3C; Supplementary Figure 4D-F) for all intrinsic BCa subtypes, but especially in basal subtype (Figure 3B, D).

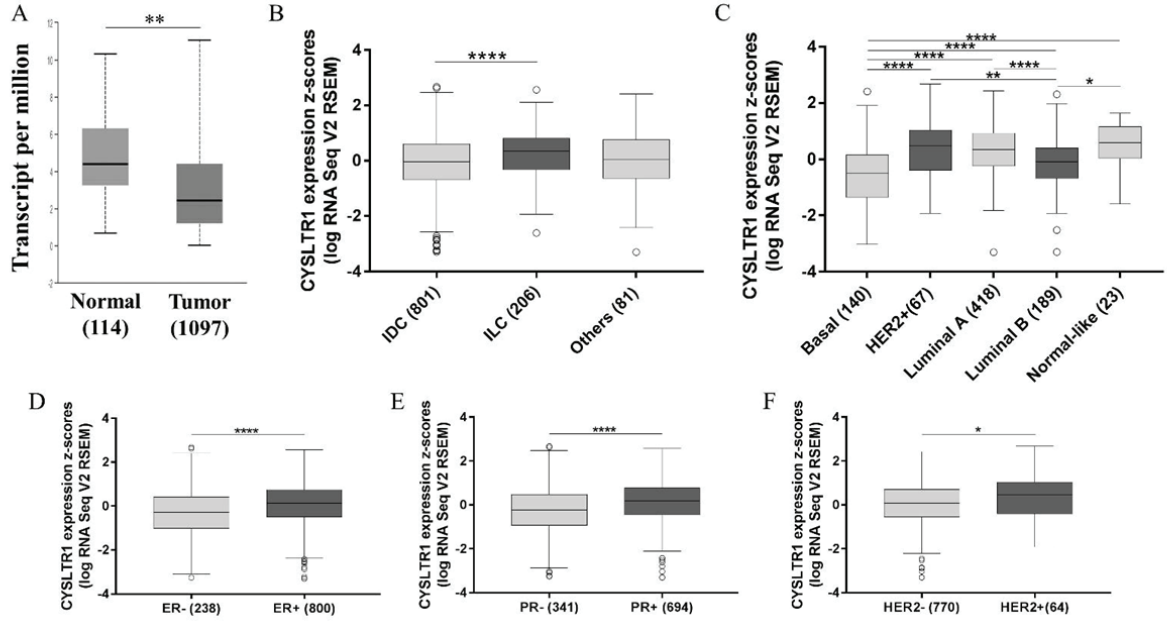


Figure 1. Expression of *CYSLTR1*. Expression of *CYSLTR1* in **A.** Normal and tumor breast tissue; **B.** According to histological subtype; **C.** PAM50 classification; **D.** Estrogen receptor; **E.** Progesterone receptor and **F.** HER2. Data obtained from the Firehose Legacy, cBioPortal, TCGA. *P* values indicate significance according to Wilcoxon or ANOVA tests: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001

Table 1. Associations between clinic-pathological parameters and *CYSLTR1* expression

Parameters	High		Low		*p*-value
	n	%	n	%	
Age					
≤50	147	27.0	186	34.2	0.010*
>50	397	73.0	358	65.8	
Menopause status					
Peri	17	3.1	23	4.2	
Post	366	67.3	339	62.3	0.325
Pre	110	20.2	120	22.1	
NA	51	9.4	62	11.4	
Cancer type					
IDC	372	68.4	429	78.9	
ILC	131	24.1	75	13.8	<0.0001*
Other	41	7.5	40	7.4	
TP53					
Mutated	125	23.0	175	32.2	
Wild type	373	68.6	297	54.6	<0.0001*
Not profiled	46	8.5	72	13.2	
TNM					
Stage I/II	403	74.1	390	71.7	
Stage III/IV	130	23.9	141	25.9	0.692
Stage X	6	1.1	7	1.3	
NA	5	0.9	6	1.1	
ER Status By IHC					
Negative	89	16.4	149	27.4	
Positive	437	80.3	363	66.7	<0.0001*
NA	18	3.3	32	5.9	
PR status by IHC					
Negative	134	24.6	207	38.1	
Positive	391	71.9	303	55.7	<0.0001*
NA	19	3.5	34	6.3	
HER2 status by IHC					
Negative	287	52.8	271	49.8	
Positive	84	15.4	77	14.2	0.868
NA	173	31.8	196	36.0	
TN/nTN					
nTN	490	90.1	434	79.8	
TN	37	6.8	79	14.5	<0.0001*
NA	17	3.1	31	5.7	
PAM50Call_RNAseq					
Basal	39	7.2	101	18.6	
Her2	44	8.1	23	4.2	
Luminal A	264	48.5	154	28.3	<0.0001*
Luminal B	79	14.5	110	20.2	
Normal-like	17	3.1	6	1.1	
NA	101	18.6	150	27.6	

NA: not available; ER: estrogen receptor; PR: progesterone receptor; TN: triple-negative; nTN: non-triple negative. Data obtained from TCGA – Firehose Legacy, cBioPortal database (*p<0.05)

We employed the GSE96058 dataset to execute univariate and multivariate regression analyses. The low expression of CYSLTR1 was an independent factor associated with lower OS in women with Bca (HR = 1.40, p = 0.002) (Table 2). Tumor size, lymph node status, and age were also related to high risk of the disease.

Gene-interaction and Enrichment Analyses

A list of correlated genes (cut-off ≤ or ≥0.7) within the basal subtype is available in Supplementary Table 1. Among the CYLSTR1 co-expressed genes, it was mainly observed that *P2RY10* and *XCR1* proteins interact directly with CYSLTR1 (Figure 4A-B). The Gene Ontology enrichment analyses demonstrated that several genes co-expressed with CYLSTR1 are involved in the immune system response and immune cell processing and activation (Figure 4C).

Identification and Prognostic Value of Predicted Genes and MicroRNAs

According to KM plotter repository, *P2RY10* and *XCR1* were assessed for RFS and OS of transcripts. *P2RY10* demonstrated a lower but significant expression associated to poor outcome in all subtypes, as well as in the basal subtype [Supplementary Figure 5A-B (RFS) and 5C-D (OS), respectively]. Moreover, by using the mirTarBase repository, three microRNAs: has-miR-335-5p, has-miR-3130-3p, and has-miR-3607-3p were identified as potential regulatory elements of *CYSLTR1*, *P2RY10* and *XCR1*, respectively. However, OS in the same TNBC patients according to miRNAs (Figure 5) and transcript expression levels (Supplementary Figure 6) were not significantly associated.

Modulation of CYSLTR1 Expression

Through the CTD, we obtained a list of six drugs capable of interacting with CYSLTR1. Leukotrienes C4, D4, and E4 can bind to CYSLTR1 and increase its activity (Figure 6). Other effects might occur depending on the drug in use; for example, the administration of leukotrienes C4 and E4 results in an abundance of calcium, while D4 increases the expression of widely studied proteins such as interleukin (IL)-6, tumor necrosis factor (TNF), and CXCL8. However, Montelukast, Pobilukast, and Zafirlukast (composts of leukotrienes receptors antagonist, LTRAs) induced a reduction in CYSLTR1 protein activity.

Discussion and Conclusion

CYSLTR1 belongs to the cysteinyl leukotriene synthesis pathway and codes for a transmembrane protein receptor that when coupling with many ligands, triggers inflammation-related signaling which leads to a determined phenotype or disease state (15, 29, 30). Yet, there is no consistent evidence to link CYSLTR1 with underlying Bca pathogenesis or even with prognostic values in individuals with aggressive breast tumors.

According to our findings, breast tumor tissue samples showed reduced levels of CYSLTR1 compared to healthy tissue samples. To date, little is known about the profile of CYSLTR1 transcripts in BCa. A study performed by Wang et al. (16) using the RT-qPCR technique, showed that CYSLTR1 was significantly upregulated in tumor samples (n = 90) vs. paraneoplastic breast tissues (n = 30) (16). However, we have to be careful when comparing our findings to this data due to the sample type and size, and approach utilized. Additionally, we observed that a decrease in CYSLTR1 transcripts leads to an unfavorable survival outcome in patients with TNBC tumors, being the first study that evaluated two different datasets and with a relevant sample size.

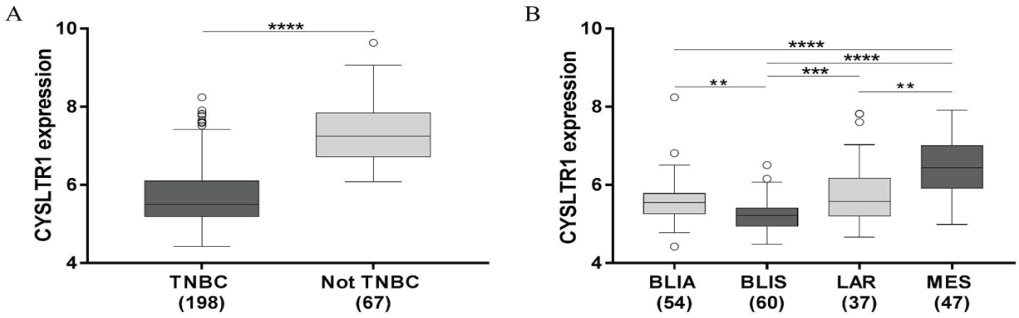


Figure 2. Expression of *CYSLTR1* in TNBC. **A.** *CYSLTR1* expression profile in TNBC in a population from GSE76275 and in **B.** TNBC subtypes. BLIA: basal-like immune-activated; BLIS: basal-like immune-suppressed; LAR: luminal androgen receptor; MES: mesenchymal. *P* values indicate significance according to Wilcoxon or ANOVA tests: ***p*<0.01, ****p*<0.001 and *****p*<0.0001

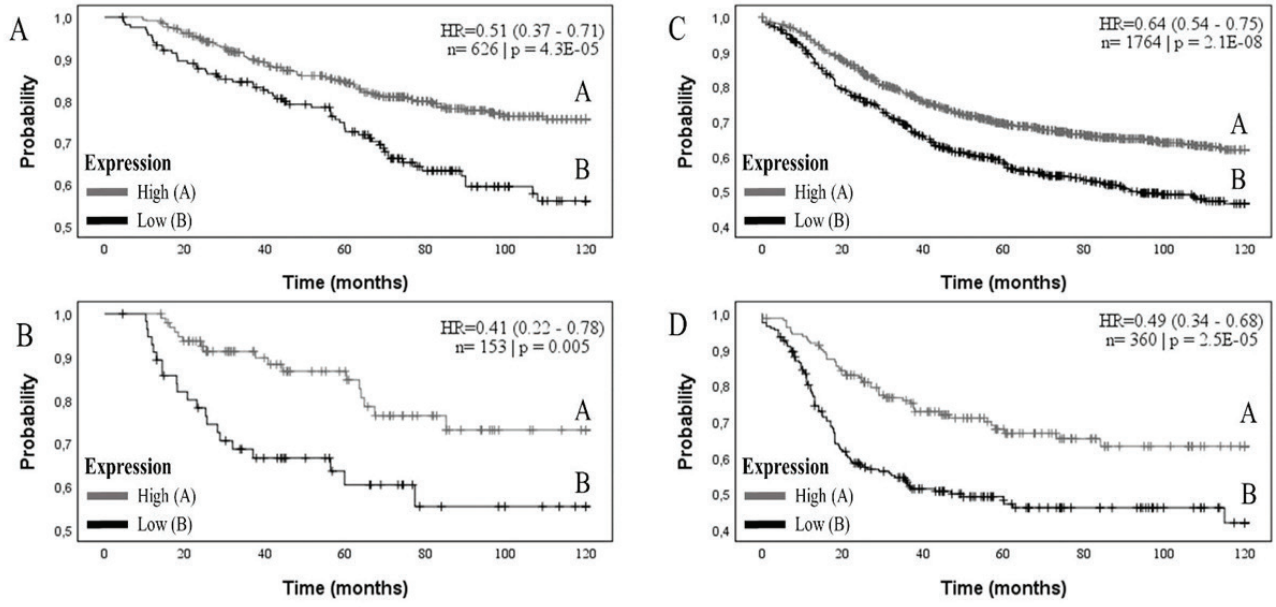


Figure 3. Survival probability of BCa patients stratified by *CYSLTR1* relative expression. Overall survival of **A.** all subtypes and **B.** Basal. Relapse-free survival of **C.** all subtypes and **D.** Basal. Data obtained from the KM Plotter online platform using the 230866_at probe

Table 2. Univariate and multivariate regression analysis of BCa patients for overall survival

Variables*	Univariate		Multivariate	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Ki67 (Ki67+ vs Ki67-)	1.89 (1.24–2.88)	0.003		
Basal status (Basal vs nBasal)	2.37 (1.83–3.07)	<0.0001		
TN status (TN vs nTN)	2.52 (1.72–3.70)	<0.0001		
Age (>50 vs ≤50 vs)	3.88 (2.54–5.93)	<0.0001	4.05 (2.60–6.30)	<0.0001
Tumor size (>20 mm vs ≤20 mm)	2.74 (2.22–3.39)	<0.0001	2.58 (2.06–3.23)	<0.0001
Lymph status (N+ vs N-)	1.54 (1.24–1.91)	<0.0001	1.25 (1.00–1.56)	0.047
CYSLTR1 (High vs Low)	1.40 (1.13–1.73)	0.002	1.46 (1.17–1.82)	0.001

*In all analyzed categories, the reference extract is the second group into the parenthesis. nBasal: non-basal; TN: triple-negative; nTN: non-triple negative; N+: positive lymph-node status; N-: negative lymph-node status; CI: confidence interval; HR: hazard ratio

However, it is necessary to reinforce the idea of working with different tumor stages and treatment cohorts to better understand this possible relationship of CYSLTR1 as a potential biomarker in cancer.

Our univariate prognostic analysis according to the Cox proportional hazards regression model confirmed the results observed in the Kaplan–Meier curves as a function of the differential expression of CYSLTR1. Furthermore, the high CYSLTR1 expression group

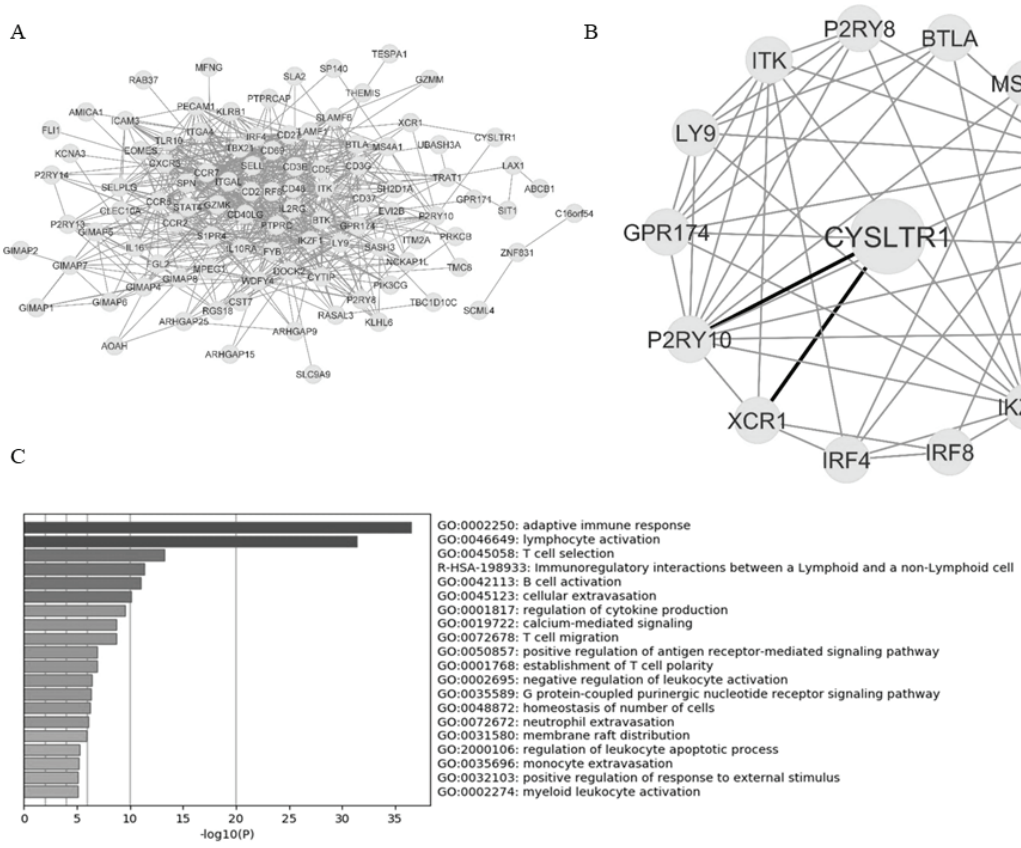


Figure 4. Network and Gene Ontology enrichment of main co-expressed proteins with CYLSTR1 in TNBC subtype. **A.** Representative network of CYSLTR1 co-expressed proteins and **B.** representative of protein with the closest interactions with CYLSTR1; **C.** Bar charts represent enriched pathways categories in which *CYLSTR1* co-expressed genes participates

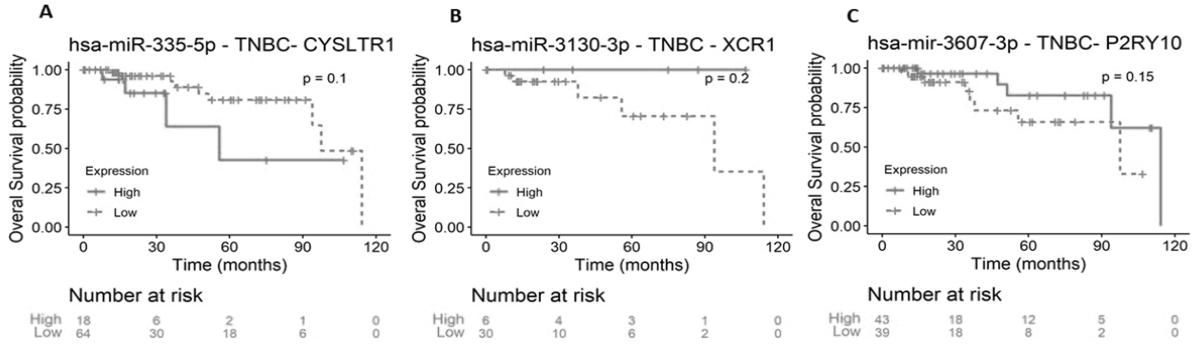


Figure 5. Overall survival in TNBC. Patients stratified by predicted miRNAs expression targeting **A.** *CYSLTR1*, **B.** *XCR1*, and **C.** *P2RY10* genes. Data obtained from the Firehose Legacy, cBioPortal, TCGA

remained an independent prognostic factor in relation to the risk of cancer-specific death, when adjusted for age, tumor size, and lymph node involvement. Our results differ from the study by Magnusson et al. (15) who did not observe a statistical association between the differential expression of CYSLTR1 and the prognosis of patients with BCa. We have to emphasize that the study evaluated the immunoreactivity of the CYSLTR1 protein and had a small set of samples (n = 139).

Considering that *CYLSTR1* gene expression in the basal subtype was significantly decreased when compared to the other subtypes, we were led to carry out an in-depth investigation into this clinically more aggressive molecular subtype of BCa. Therefore, we evaluated the expression patterns of CYSLTR1 in the four stable TNBC subtypes, characterized by the expression of distinct molecular profiles that present different prognoses, proposed through studies by the Burstein and Lehman groups (26, 31). Our results showed that CYSLTR1 is consistently expressed in the MES subgroup. Here, we hypothesize that it is possible that CYSLTR1 is more actively involved in epithelial mesenchymal transition and angiogenesis, than in the processes of tumor differentiation and immune activity. This may be supported by the role of cysteinyl leukotrienes (cys-LTs) since they are pro-inflammatory mediators that modulate vascular leakage, permeability and microvasculature response via other leukotriene molecules (32-34). Furthermore, CYSLTR1 transcripts were also expressed to a greater extent in tumors of the LAR subgroup, which is highly expressed on

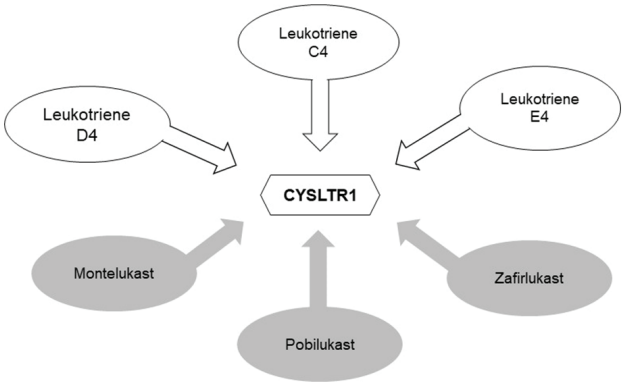


Figure 6. Drug interactions with *CYSLTR1* obtained from the Comparative Toxicogenomic Database. The image shows six drugs capable of modulating the expression of *CYSLTR1* positively or negatively. For example, leukotriene C4 binds to *CYSLTR1* and increases its activity (white). On the other hand, Montelukast binds to *CYSLTR1* inhibiting its activity (gray). White: increases CYLSTR1 activity; Gray: decreases CYLSTR1 activity

the nuclear androgen receptor receptor. Consequently, as CYSLTR1 is linked to kinase activity (35, 36), this could lead us to hypothesize that CYSLTR1 could participate in blocking androgen-dependent signaling and PIK3. To date, no work has focused on studying its possible role in tumorigenesis in TNBC cases. Thus, our work brings unprecedented data about this aggressive type of BCa.

Our analyses of signaling and enrichment pathways for CYSLTR1 have indicated some immunological mechanisms related to inflammation in which toll-like family genes and cytokines may participate. It is noteworthy that two protein-coding genes: *P2RY10* and *XCR1* exhibited a positive correlation with CYSLTR1 in a TNBC dataset. Eosinophils can be found in the TMev, as they secrete different types of leukotrienes as part of the induction of inflammatory processes (10). Furthermore, they also generate significant amounts of platelet activating factor and promote the production of characteristic cytokines such as TNFα and IL-5 (37, 38). *P2RY10* is a G-couple protein receptor that participates in the inflammatory response, stimulated by many molecules such as chemokines, lysophospholipids and prostanoids. Its biological role has not been fully elucidated, but it may participate in eosinophil maturation and eosinophilopoiesis *in vitro* (38). On the other hand, Yang et al. (39) suggest that XCR1 may act as a progression factor in ER-responsive Bca cell lines through the MAPK/ERK and PI3K/AKT/mTOR pathways that promote migration and invasion by significantly decreasing the protein level of β-catenin (40). Regarding the possible prognostic role, patients with TNBC cases who had high gene expression of *XCR1* and *P2RY10* exhibited a trend towards greater survival, further confirmed by an independent dataset.

As for epigenetic mechanisms of gene regulation, has-miR-355-5p showed a certain tendency to downregulate CYSLTR1 expression in a TNBC setting. To date, there is no evidence that describes consistent associations between these genes and their Bca-targeted miRNAs. Therefore, we suggest IHC studies to unravel mechanisms underlying survival and immunological processes in TNBC.

Regarding possible drug interactions, we observed that Montelukast, Zafirlukast and Pobilucast played a role in reducing CYSLTR1 expression levels in our *in silico* experiments. Based on the above, Suknuntha et al.'s (30) group observed that MDA-MB-231 BCa cells, when treated with Montelukast and Zafirlukast molecules, can inhibit cell proliferation and apoptosis, but only Zafirlukast can induce cell cycle arrest. On the other hand, leukotrienes appear as possible positive modulators of CYSLTR1 expression. Both strategies are promising and need to be carefully investigated.

Study Limitations

Some limitations of the analysis performed here must be acknowledged. First, we employed different expression analysis methods compared to other studies examining CYSLTR1 in Bca. Second, as seen in *in silico* analyses, RNA-seq based expression data were not complemented with protein data to corroborate our findings. Third, many studies available in public databases were deficient in clinicopathological information, and the most important, TNBC studies only accounted for up to 15% of the Bca population, so it is difficult to reach significant conclusions. Nonetheless, based on our findings, we can provide insights into the possible role of CYSLTR1 in BCa disease survival, particularly in TNBC cases.

Our study showed that CYSLTR1 is transcriptionally less expressed in breast tumors compared to adjacent tissue. Additionally, among the tumor subtypes, TNBC had lower levels of CYSLTR1. Low CYSLTR1 expression was associated with worse survival in BCa patients and especially in TNBC. CYSLTR1 is co-expressed with genes that participate in the adaptive immune response and lymphocyte activation. Finally, we suggest that CYSLTR1 may not be working alone, but with linked proteins and miRNAs that could serve as new possible targets for other therapies in Bca, especially in TNBC subtypes.

Ethics Committee Approval: Not necessary.

Informed Consent: Not necessary.

Peer-review: Externall and internally peer-reviewed.

Authorship Contributions

Concept: M.P.F.C., D.R.d.B.; Design: M.P.F.C., D.R.d.B.; Data Collection or Processing: A.G.C., M.P.F.C., D.R.d.B.; Analysis or Interpretation: A.G.C., M.P.F.C., D.R.d.B.; Literature Search: A.G.C., M.P.F.C., D.R.d.B.; Writing: A.G.C., M.P.F.C., D.R.d.B., G.Á.d.G., J.M.R.S.L., R.G.d.N., M.T.F., R.M.L.

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

Financial Disclosure: The authors declared that this study received no financial support.

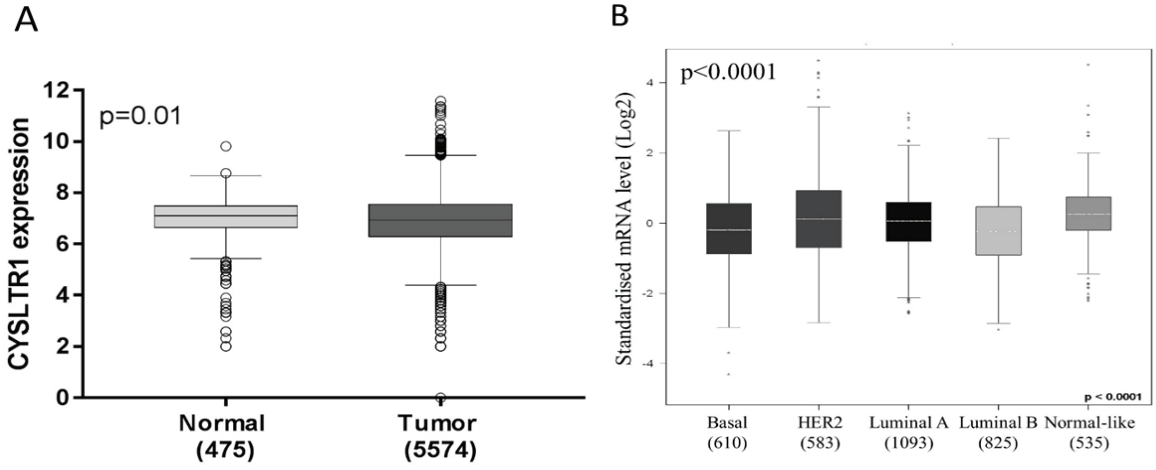
References

1. Turashvili G, Brogi E. Tumor heterogeneity in breast cancer. Front Med (Lausanne) 2017; 4: 227. (PMID: 29276709) [Crossref]
2. Hu Z, Fan C, Oh DS, Marron JS, He X, Qaqish BF, et al. The molecular portraits of breast tumors are conserved across microarray platforms. BMC Genomics 2006; 7: 96. (PMID: 16643655) [Crossref]
3. Sorlie T et al, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001; 98: 10869-10874. (PMID: 11553815) [Crossref]
4. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature 2000; 406: 747-752. (PMID: 10963602) [Crossref]

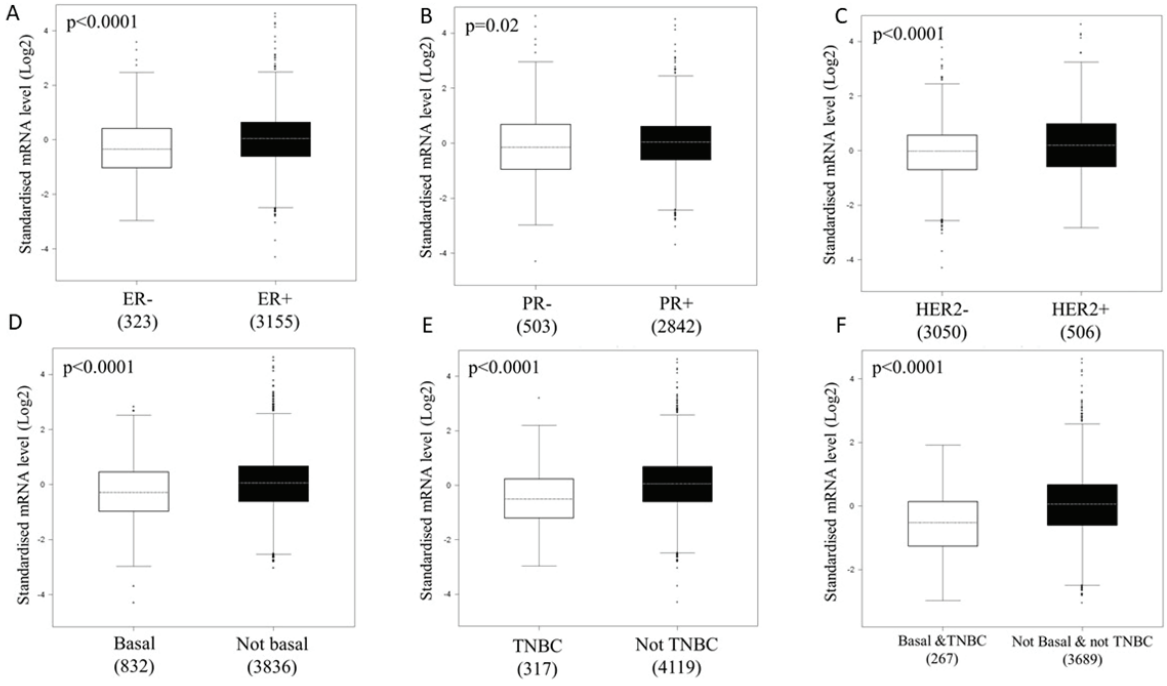
5. Bertucci F, Finetti P, Rougemont J, Charafe-Jauffret E, Cervera N, Tarpin C, et al. Gene expression profiling identifies molecular subtypes of inflammatory breast cancer. Cancer Res 2005; 65: 2170-2178. (PMID: 15781628) [Crossref]
6. Di Cosimo S, Baselga J. Management of breast cancer with targeted agents: importance of heterogeneity. [corrected]. Nat Rev Clin Oncol 2010; 7: 139-147. Epub 2010 Feb 2. Erratum in: Nat Rev Clin Oncol 2010; 7: 184. (PMID: 20125090) [Crossref]
7. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A 2003; 100: 8418-8423. (PMID: 12829800) [Crossref]
8. Ellsworth RE, Blackburn HL, Shriver CD, Soon-Shiong P, Ellsworth DL. Molecular heterogeneity in breast cancer: State of the science and implications for patient care. Semin Cell Dev Biol 2017; 64: 65-72. (PMID: 27569190) [Crossref]
9. Lehmann BD, Jovanović B, Chen X, Estrada MV, Johnson KN, Shyr Y, et al. Refinement of Triple-Negative Breast Cancer Molecular Subtypes: Implications for Neoadjuvant Chemotherapy Selection. PLoS One 2016; 11: e0157368. (PMID: 27310713) [Crossref]
10. Savari S, Vinnakota K, Zhang Y, Sjölander A. Cysteinyl leukotrienes and their receptors: bridging inflammation and colorectal cancer. World J Gastroenterol 2014; 20: 968-977. (PMID: 24574769) [Crossref]
11. Lukic A, Wahlund CJE, Gómez C, Brodin D, Samuelsson B, Wheelock CE, et al. Exosomes and cells from lung cancer pleural exudates transform LTC4 to LTD4, promoting cell migration and survival via CysLT1. Cancer Lett 2019; 444: 1-8. (PMID: 30508568) [Crossref]
12. Cheng Y, Pereira M, Raukar N, Reagan J, Quesenberry M, Goldberg L, et al. Inflammation-related gene expression profiles of salivary extracellular vesicles in patients with head trauma. Neural Regen Res 2020; 15: 676-681. (PMID: 31638091) [Crossref]
13. Massoumi R, Sjölander A. The role of leukotriene receptor signaling in inflammation and cancer. ScientificWorldJournal 2007; 7: 1413-1421. (PMID: 17767358) [Crossref]
14. Ohd JF, Nielsen CK, Campbell J, Landberg G, Löfberg H, Sjölander A. Expression of the leukotriene D4 receptor CysLT1, COX-2, and other cell survival factors in colorectal adenocarcinomas. Gastroenterology 2003; 124: 57-70. (PMID: 12512030) [Crossref]
15. Magnusson C, Liu J, Ehrnström R, Manjer J, Jirström K, Andersson T, et al. Cysteinyl leukotriene receptor expression pattern affects migration of breast cancer cells and survival of breast cancer patients. Int J Cancer 2011; 129: 9-22. (PMID: 20824807) [Crossref]
16. Wang X, Liu W, Han Y, Feng Y, Li D. Significance of cysteinyl leukotriene receptor in breast cancer. Chinese Journal of Clinical Oncology 2012; 39: 1522-1526. [Crossref]
17. Chandrashekar DS, Bashel B, Balasubramanya SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi BVSK, et al. UALCAN: A Portal for Facilitating Tumor Subgroup Gene Expression and Survival Analyses. Neoplasia 2017; 19: 649-658. (PMID: 28732212) [Crossref]
18. Park S-J, Yoon B-H, Kim S-K, Kim S-Y. GENT2: an updated gene expression database for normal and tumor tissues. BMC Medical Genomics 2019; 12(Suppl 5): 101. (PMID: 31296229) [Crossref]
19. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio Cancer Genomics Portal: An open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012; 2: 401-404. (PMID: 22588877) [Crossref]
20. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 2013; 6: pl1. (PMID: 23550210) [Crossref]
21. Jézéquel P, Campone M, Gouraud W, Guérin-Charbonnel C, Leux C, Ricolleau G, et al. Bc-GenExMiner: An easy-to-use online platform for gene prognostic analyses in breast cancer. Breast Cancer Res Treat 2012; 131: 765-775. (PMID: 21452023) [Crossref]
22. Jézéquel P, Frénel JS, Campion L, Guérin-Charbonnel C, Gouraud W, Ricolleau G, et al. bc-GenExMiner 3.0: New mining module computes breast cancer gene expression correlation analyses. Database (Oxford) 2013; 2013. (PMID: 23325629) [Crossref]
23. Győrffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res and Treat 2010; 123: 725-731. (PMID: 20020197) [Crossref]
24. Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat Commun 2019; 10: 1523. (PMID: 30944313) [Crossref]
25. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. Genome Res 2003; 13: 2498-2504. (PMID: 14597658) [Crossref]
26. Burstein MD, Tsimelzon A, Poage GM, Covington KR, Contreras A, Fuqua SAW, et al. Comprehensive genomic analysis identifies novel subtypes and targets of triple-negative breast cancer. Clin Cancer Res 2015; 21: 1688-1698. (PMID: 25208879) [Crossref]
27. Mattingly CJ, Rosenstein MC, Colby GT, Forrest JN, Boyer JL. The Comparative Toxicogenomics Database (CTD): a resource for comparative toxicological studies. J Exp Zool A Comp Exp Biol 2006; 305: 689-692. (PMID: 16902965) [Crossref]
28. Chou CH, Chang NW, Shrestha S, Hsu S Da, Lin YL, Lee WH, et al. miRTarBase 2016: Updates to the experimentally validated miRNA-target interactions database. Nucleic Acids Res 2016; 44: D239-247. (PMID: 26590260) [Crossref]
29. Trinh HKT, Lee SH, Cao TBT, Park HS. Asthma pharmacotherapy: an update on leukotriene treatments. Expert Rev Respir Med 2019; 13: 1169-1178. (PMID: 31544544) [Crossref]
30. Suknuntha K, Yubolphan R, Krueaprasertkul K, Srihirun S, Sibmooh N, Vivithanaporn P. Leukotriene receptor antagonists inhibit mitogenic activity in triple negative breast cancer cells. Asian Pac J Cancer Prev 2018; 19: 833-837. (PMID: 29582642) [Crossref]
31. Lehmann BD, Bauer JA, Schafer JM, Pendleton CS, Tang L, Johnson KC, et al. PIK3CA mutations in androgen receptor-positive triple negative breast cancer confer sensitivity to the combination of PI3K and androgen receptor inhibitors. Breast Cancer Res 2014; 16: 406. (PMID: 25103565) [Crossref]
32. Colazzo F, Gelosa P, Tremoli E, Sironi L, Castiglioni L. Role of the Cysteinyl Leukotrienes in the Pathogenesis and Progression of Cardiovascular Diseases. Mediators Inflamm 2017; 2017: 2432958. (PMID: 28932020) [Crossref]
33. Maekawa A, Frank Austen K, Kanaoka Y. Targeted gene disruption reveals the role of cysteinyl leukotriene 1 receptor in the enhanced vascular permeability of mice undergoing acute inflammatory responses. J Biol Chem 2002; 277: 20820-20824. (PMID: 11932261) [Crossref]
34. Duah E, Adapala RK, Al-Azzam N, Kondeti V, Gombedza F, Thodeti CK, et al. Cysteinyl leukotrienes regulate endothelial cell inflammatory and proliferative signals through CysLT 2 and CysLT 1 receptors. Sci Rep 2013; 3: 1-6. (PMID: 24253666) [Crossref]
35. Omarini C, Guaitoli G, Pipitone S, Moscetti L, Cortesi L, Cascinu S, et al. Neoadjuvant treatments in triple-negative breast cancer patients: Where we are now and where we are going. Cancer Manag Res 2018; 10: 91-103. (PMID: 29391830) [Crossref]
36. Kondeti V, Duah E, Al-Azzam N, Thodeti CK, Boyce JA, Paruchuri S. Differential Regulation of Cysteinyl Leukotriene Receptor Signaling by

Protein Kinase C in Human Mast Cells. PloS One 2013; 8: e71536. (PMID: 23977066) [Crossref]

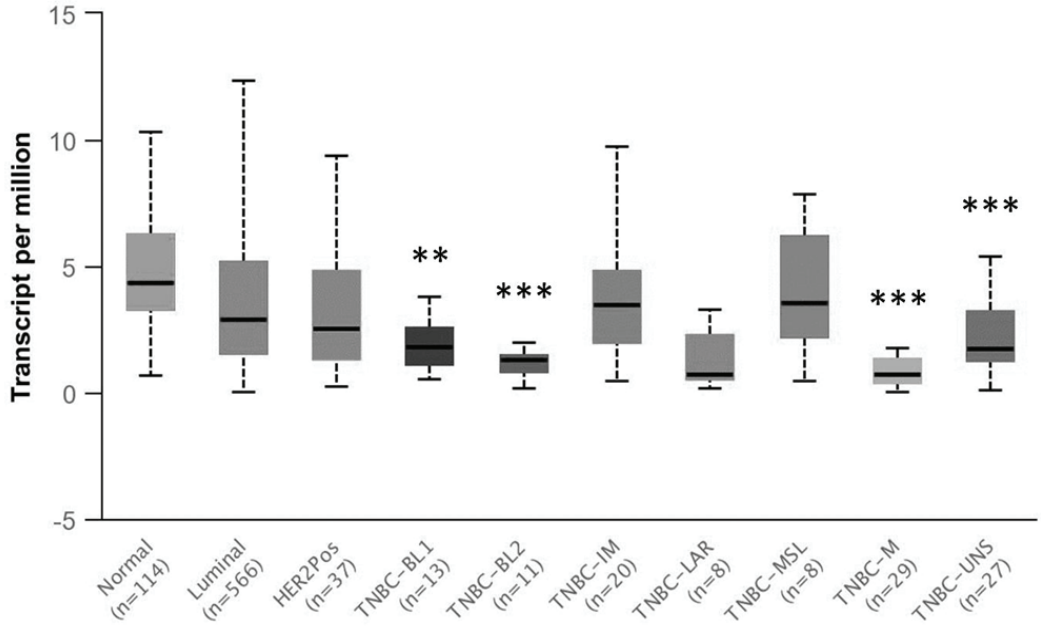
37. Gurusamy M, Tischner D, Shao J, Klatt S, Zukunft S, Bonnavion R, et al. G-protein-coupled receptor P2Y10 facilitates chemokine-induced CD4 T cell migration through autocrine/paracrine mediators. Nat Commun 2021; 12: 6798. (PMID: 34815397) [Crossref]
38. Hwang SM, Kim HJ, Kim SM, Jung Y, Park SW, Chung IY. Lysophosphatidylserine receptor P2Y10: A G protein-coupled receptor that mediates eosinophil degranulation. Clin Exp Allergy 2018; 48: 990-999. (PMID: 29700886) [Crossref]
39. Yang XL, Qi LG, Lin FJ, Ou ZL. The role of the chemokine receptor XCR1 in breast cancer cells. Breast Cancer (Dove Med Press) 2017; 9: 227-236. (PMID: 28408852) [Crossref]
40. Gantsev SK, Umezawa K, Islamgulov DV, Khusnutdinova EK, Ishmuratova RS, Frolova VY, et al. The role of inflammatory chemokines in lymphoid neoorganogenesis in breast cancer. Biomed Pharmacother 2013; 67: 363-366. (PMID: 23602049) [Crossref]



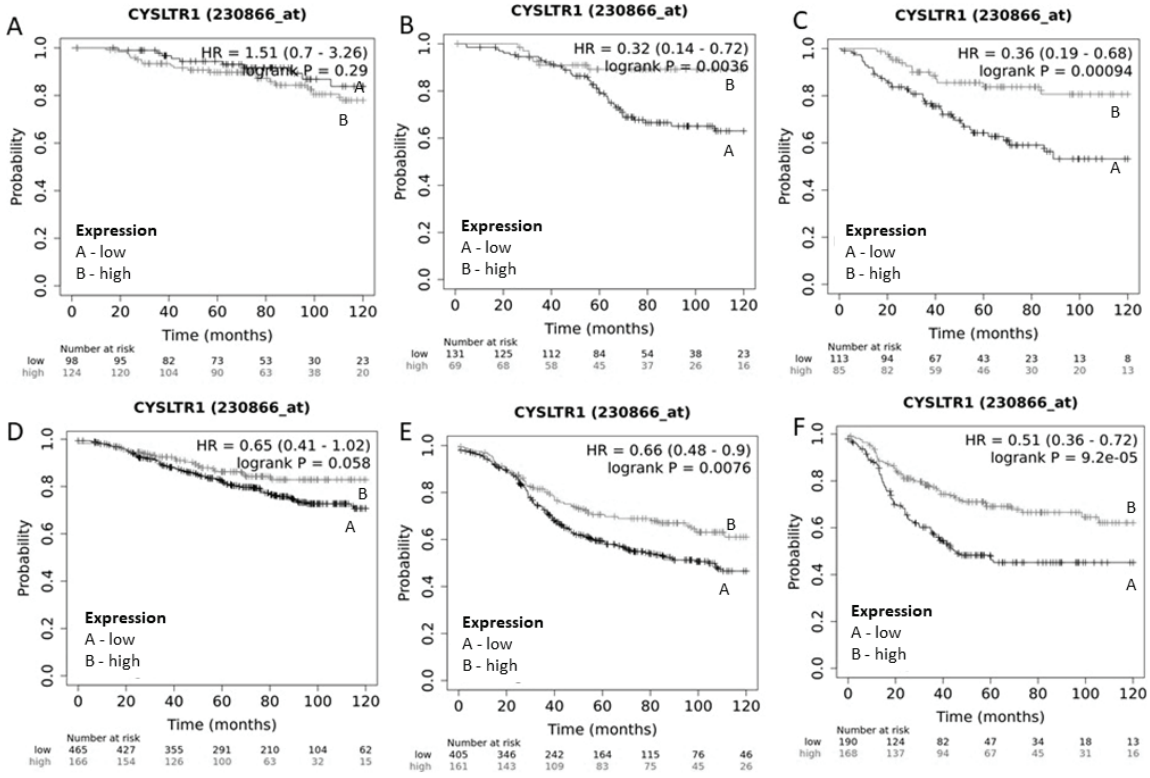
Supplementary Figure 1. CYSLTR1 transcript expression levels in breast cancer patients. According to: A. Sample type and B.- PAM50 classification. Data obtained from bcGenExMiner database. *P* values indicate significance according to Wilcoxon or ANOVA tests: **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001



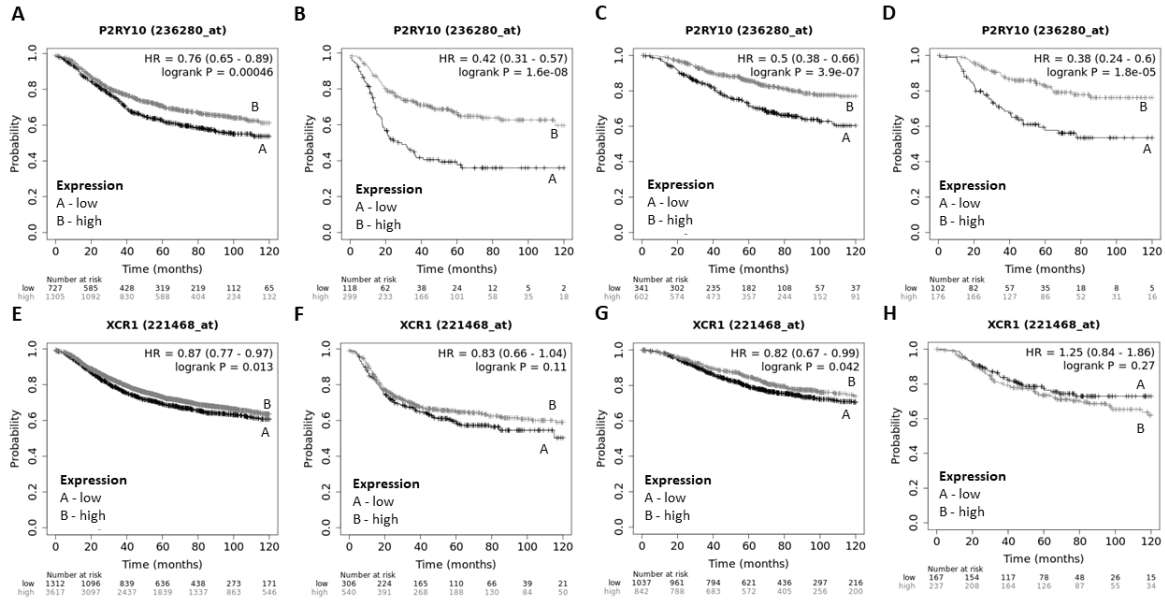
Supplementary Figure 2. *CYSLTR1* mRNA levels. According to: **A.** ER receptor, **B.** PR receptor, **C.** HER2 Status, data obtained from bcGenExMiner platform. Basal and Not Basal subtype, **E.** TNBC and Not TNBC subtype, **F.** Basal/TNBC and Not Basal/Not TNBC subtype, Data obtained from BRCA-TCGA dataset. *P* values indicate significance according to Wilcoxon or ANOVA tests: **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001



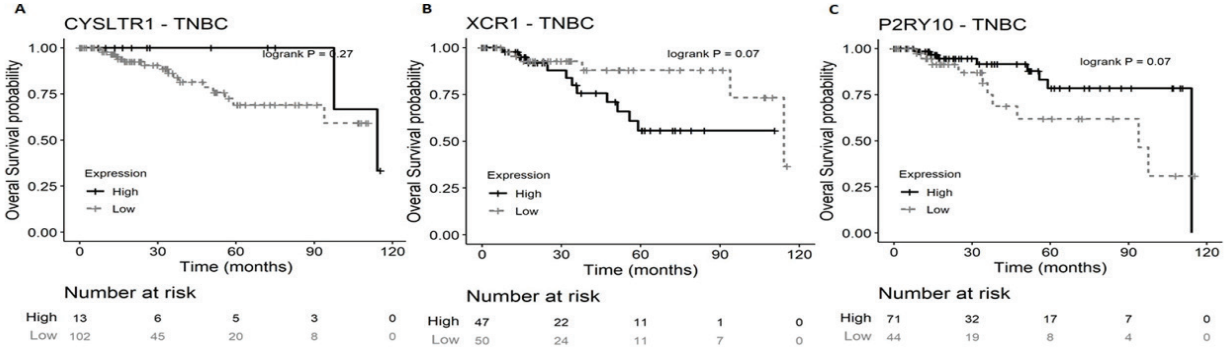
Supplementary Figure 3. Correlation of *CYSLTRL1* expression according to Lehmann et al. TNBC subtypes. *P* values indicate significance according to Wilcoxon or ANOVA tests: **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001



Supplementary Figure 4. Survival probability of BCa patients stratified by the relative expression of *CYSLTR1*. OS in accord with **A.** Luminal **A, B.** Luminal **B, C.** HER2 subtypes. RFS according to **D.** Luminal **A, E.** Luminal B, and **C.** HER2 subtypes. Data obtained from KM Plotter platform with follow up threshold adjusted for 120 months



Supplementary Figure 5. Survival probability of patients stratified by the relative expression of *P2RY10* and *XCR1*. RFS considering all BCa subtypes **(A, E);** basal subtype **(B, F).** OS according to all BCa subtypes **(C, G);** basal subtype **(D, H).** Data obtained from KM Plotter platform with follow up threshold adjusted for 120 months



Supplementary Figure 6. Overall survival of TNBC patients. Stratified by the relative expression of **A.** *CYSLTR1*; **B.** *XCR1*; and **C.** *P2RY10*. Data obtained from TCGA repository with follow up threshold adjusted for 120 months