

Original Article

Running Title: *BACE1-AS* and *SIRT1* Expression in Breast Cancer Leukocytes

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The Expression of Long Non-Coding RNA *BACE1-AS* and *SIRT1* gene in Peripheral Blood Leukocytes of Breast Cancer Patients: A Computational and Molecular Study

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Abstract

Background: Breast cancer is molecularly heterogeneous, highlighting the need to identify circulating molecular alterations. This study aimed to investigate the expression of Beta-site amyloid precursor protein-cleaving enzyme-1 antisense RNA (*BACE1-AS*) and Sirtuin 1 (*SIRT1*) in peripheral blood leukocytes of patients with breast cancer and to explore their molecular characteristics and potential clinical relevance using molecular and bioinformatic analyses.

Method: This case-control study included 30 treatment-naïve breast cancer patients and 30 healthy controls. *BACE1-AS* and *SIRT1* expression were quantified by quantitative real-time polymerase chain reaction. The Cancer Genome Atlas Breast Invasive Carcinoma (TCGA-BRCA) data from 1,098 tumors and 114 adjacent normal tissues were analyzed for expression, molecular

subtypes, survival, correlations, diagnostic performance, and co-expression. Statistical analyses used R version 4.4 and GraphPad Prism version 9.5. A two-sided $P < 0.05$ was considered to be statistically significant.

Results: Leukocyte *BACE1-AS* (fold change = 10.18, $P < 0.001$) and *SIRT1* (fold change = 5.05, $P < 0.001$) were significantly higher in patients than controls. Their leukocyte expression was not significantly correlated ($r = 0.18$, $P = 0.53$). In TCGA-BRCA, *BACE1-AS* showed subtype-dependent expression, a weak positive correlation with *SIRT1* ($r = 0.14$), and good tissue-based diagnostic performance (area under the curve (AUC) = 0.79). Co-expression analysis indicated enrichment of networks related to cellular stress adaptation and genome maintenance. Leukocyte *BACE1-AS* showed poor diagnostic performance (AUC = 0.57).

Conclusion: *BACE1-AS* was associated with subtype-dependent expression and stress- and genome-maintenance-related networks but does not support standalone blood-based biomarker use. Further mechanistic studies are warranted.

Keywords: *BACE1-AS*; *SIRT1*; TCGA-BRCA; Breast neoplasms; RNA, Long noncoding

Introduction

Although advances in breast cancer screening and diagnostic technologies have improved early detection, important diagnostic limitations remain in specific clinical settings, underscoring the need for complementary molecular biomarkers.^{1, 2} Advances in genomic technologies have revealed extensive transcriptional activity across the human genome, giving rise to a diverse repertoire of non-coding RNAs (ncRNAs), which constitute a major component of the transcriptome.³ The pathophysiology of many diseases, including lung, breast, colorectal, and bladder cancers, is closely linked to dysregulation of ncRNAs, according to a wealth of evidence.⁴ Long non-coding RNAs (lncRNAs), which are longer than 200 nucleotides and have little or no potential for protein coding, have drawn special attention among these ncRNAs.⁵ RNA polymerase II transcription, 5'-capping, polyadenylation, and alternative splicing are among the molecular traits that lncRNAs and mRNAs have in common. Because of their three-dimensional structures, they can interact with proteins and nucleic acids to control transcriptional and post-transcriptional aspects of gene expression.⁶ A growing body of research indicates that

lncRNAs are crucial for basic biological processes like cell division, apoptosis, tumor initiation, growth, and metastasis.⁷ Because of these regulatory properties, lncRNAs have attracted interest as potential therapeutic targets and biomarkers for cancer diagnosis and prognosis.⁵ The stability and detectability of some lncRNAs in biological fluids, such as blood and urine, have increased interest in their potential use as non-invasive biomarkers. Disease-specific changes in lncRNA expression may therefore have potential applications in diagnosis, disease monitoring, and assessment of treatment response.⁸ Aberrant expression of certain lncRNAs in breast cancer has been associated with oncogenic or tumor-suppressive functions, indicating their involvement in important cancer-related pathways.⁹ Their stability in circulation and tissue specificity further supports their potential as useful biomarkers. HOX transcript antisense RNA (*HOTAIR*), plasmacytoma variant translocation 1 (*PVT1*), nuclear paraspeckle assembly transcript 1 (*NEAT1*), and *BACE1-AS* are among the lncRNAs that have been frequently linked to a variety of cancer types.^{10, 11}

BACE1 is a protein-coding gene located on chromosome 11q23.3 and encodes an aspartyl protease primarily involved in amyloid precursor protein processing.¹² Its canonical transcript (RefSeq: NM_012104) encodes the major *BACE1* protein isoform, while additional transcript variants have also been described. *BACE1-AS* is a long non-coding antisense RNA transcribed from the opposite strand of the *BACE1* locus.¹³ *BACE1* expression is regulated at both transcriptional and post-transcriptional levels by transcription factors, inflammatory mediators, microRNAs, and antisense transcripts. *BACE1-AS* has been reported to form RNA–RNA duplexes with *BACE1* mRNA, thereby enhancing *BACE1* mRNA stability and increasing *BACE1* expression, representing an important post-transcriptional regulatory mechanism.¹⁴⁻¹⁹

SIRT1, located on chromosome 10q21.3, encodes a highly conserved NAD⁺-dependent class III histone deacetylase. The canonical transcript (RefSeq: NM_012238) is accompanied by multiple transcript variants generated through alternative promoter usage and alternative splicing. *SIRT1* regulates several transcription factors and signaling pathways, including tumor protein p53, Forkhead Box O proteins, Nuclear Factor Kappa B (NF-κB), Hypoxia-Inducible Factor 1-α, and Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-α, thereby influencing apoptosis, DNA repair, oxidative stress responses, metabolism, inflammation, and cellular senescence. Emerging evidence indicates that *SIRT1* also modulates the expression of several lncRNAs through epigenetic mechanisms, whereas lncRNAs can regulate *SIRT1* expression through competing endogenous RNA (ceRNA)-dependent and transcriptional mechanisms, suggesting a reciprocal regulatory network involved in cancer progression.²⁰⁻²³

Among cancer-associated lncRNAs, *BACE1-AS* has attracted increasing attention because of its ability to regulate the expression of its sense counterpart, *BACE1*, through post-transcriptional mechanisms. Although initially characterized in Alzheimer's disease, accumulating evidence suggests that *BACE1-AS* may contribute to several cancer-related processes, including apoptosis resistance, angiogenesis, proliferation, invasion, and metastasis, although its effects appear to be context-dependent. Dysregulated expression of *BACE1* and *BACE1-AS* has been reported in several malignancies, supporting their involvement in cancer-associated molecular processes and motivating further investigation of their potential clinical relevance.^{11, 24}

Beyond its established role in cellular homeostasis, *SIRT1* exhibits context-dependent functions in cancer and may act either as a tumor promoter or a tumor suppressor depending on tumor type and molecular context.²² Importantly, both *SIRT1* and *BACE1-AS* have been implicated in biological processes related to oxidative stress, inflammation, apoptosis, and epigenetic regulation. *SIRT1* suppresses NF-κB signaling and modulates chromatin accessibility through histone deacetylation, thereby influencing the transcription of numerous coding and non-coding genes, including cancer-associated lncRNAs. Conversely, lncRNAs have been shown to regulate *SIRT1* expression through ceRNA-dependent and epigenetic mechanisms.^{20, 22} Although a direct interaction between *BACE1-AS* and *SIRT1* has not been established in breast cancer, their involvement in partially overlapping biological processes provides a rationale for investigating their expression simultaneously. This approach is exploratory and does not imply a direct functional interaction or established biomarker utility. The present study investigated *BACE1-AS*

and *SIRT1* expression at two complementary levels: peripheral blood leukocytes from breast cancer patients and transcriptomic data from the The Cancer Genome Atlas Breast Invasive Carcinoma (TCGA-BRCA) cohort, in order to characterize their expression patterns and explore their molecular and clinical associations.

Materials and Methods

Study design and participants

This case–control study was conducted between 2022 and 2024 at Mazandaran University of Medical Sciences. A total of 30 newly diagnosed, treatment-naïve female patients with histopathologically confirmed invasive breast carcinoma and 30 healthy female controls were initially enrolled. Patients were recruited from Shahid Rajaei Hospital. Healthy volunteers were selected during routine health examinations and were frequency-matched to patients according to age and menopausal status. Participants with a history of other malignancies, autoimmune disorders, chronic inflammatory diseases, active infections, pregnancy, or immunosuppressive therapy were excluded from both groups. The study protocol was approved by the Ethics Committee of Mazandaran University of Medical Sciences (Approval No.: IR.MAZUMS.REC.1404.541) and written informed consent was obtained from all participants prior to enrollment.

Sample size determination

No formal a priori statistical power calculation was performed. The initial sample size was determined based on a previously published case–control study investigating circulating long non-coding RNA expression in breast cancer, which included 30 participants in each group.²⁵ Given the exploratory nature of the present study and the feasibility of participant recruitment during the study period, 30 breast cancer patients and 30 healthy controls were

initially recruited. Following predefined RNA quality and quantitative real-time polymerase chain reaction (qRT-PCR) quality-control criteria, samples with undetectable cycle threshold values or unsuccessful amplification after repeated assays were excluded from the final gene expression analysis. Consequently, the final analysis included 14 breast cancer patients and 22 healthy controls.

Peripheral blood collection and leukocyte isolation

Peripheral venous blood samples (5 mL) were collected into ethylenediaminetetraacetic acid -containing tubes from all participants. Total leukocytes were isolated using ammonium–chloride–potassium (ACK) red blood cell lysis buffer. Briefly, 1 mL of whole blood was mixed with 10 mL of ice-cold 1× ACK lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA; pH 7.2–7.4) and incubated on ice for 10 min. Samples were then centrifuged at 400 × g for 10 min at 4°C. The resulting leukocyte pellet was washed twice with phosphate-buffered saline. Immediately after isolation, leukocytes were processed for RNA extraction to minimize RNA degradation and preserve transcript integrity.²⁶ No further immunophenotypic separation or quantification of individual leukocyte subsets (e.g., neutrophils, lymphocytes, monocytes, or other immune-cell populations) was performed. Therefore, the expression measurements represent bulk leukocyte RNA and may be influenced by differences in leukocyte subset composition between participants.

RNA extraction and cDNA synthesis

Total RNA was extracted from isolated leukocytes using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA). Only RNA

samples with an A260/A280 ratio ≥ 1.9 and an A260/A230 ratio ≥ 2.0 were used for subsequent analyses. cDNA was synthesized from 1 μ g of total RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio Inc., Japan) according to the manufacturer's instructions.

qRT-PCR

qRT-PCR was performed using TB Green Premix Ex Taq II (Takara Bio Inc., Japan) on a StepOnePlus Real-Time PCR System (Applied Biosystems, USA). All reactions were performed in triplicate, and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the endogenous reference gene for normalization. Relative gene expression levels were calculated using the comparative $2^{-\Delta\Delta C_t}$ method. Amplification efficiency for each primer pair was evaluated using standard curve analysis, and only primer pairs with efficiencies ranging from 95 to 105% were accepted. Samples showing undetectable cycle threshold (C_t) values or unsuccessful amplification after repeated qRT-PCR assays were excluded from the final gene expression analysis according to the predefined quality-control criteria. The final qRT-PCR analysis therefore comprised 14 breast cancer patients and 22 healthy controls. The *BACE1-AS* primer pair was specifically used to quantify the *BACE1-AS* transcript and was not intended to measure *BACE1* mRNA. The primer sequences used for amplification of *GAPDH*, *BACE1-AS*, and *SIRT1* are presented in table 1.

Bioinformatics Analysis of TCGA-BRCA Cohort

Data acquisition and preprocessing

Gene expression and corresponding clinical data for invasive breast carcinoma were retrieved from the TCGA-BRCA project using the TCGAbiolinks R package (v2.30.0). RNA Sequencing data generated by the STAR-Counts pipeline included 1,098 primary tumor samples and 114 adjacent

normal breast tissue samples. Unstranded read counts were extracted from the Summarized Experiment object. After filtering out genes with low expression (mean counts < 10 across all samples), the genes of primary interest (*BACE1-AS* and *SIRT1*) were retained for further analyses. Variance-stabilizing transformation (VST) was performed using DESeq2 (v1.42.0), and counts per million values were calculated using edgeR. Clinical annotations, including sample type (tumor or normal), age, clinical stage, vital status, survival time, and Prediction Analysis of Microarray 50 (PAM50) molecular subtype (Luminal A, Luminal B, Human Epidermal Growth Factor Receptor 2 (HER2)-enriched, Basal-like, and Normal-like), were obtained from the colData slot.

Differential expression and diagnostic performance

Expression levels of *BACE1-AS* and *SIRT1* were compared between tumor and normal tissues using VST-transformed values. Diagnostic performance was evaluated by receiver operating characteristic (ROC) curve analysis using the pROC package. The area under the curve (AUC) and corresponding 95% confidence intervals (95% CI) were reported.

Correlation and subtype-specific analyses

Pearson and Spearman correlation analyses were performed to evaluate the association between *BACE1-AS* and *SIRT1* expression levels in the overall TCGA-BRCA cohort and within individual PAM50 molecular subtypes. Correlation coefficients and corresponding *P*-values were reported.

Survival analysis

Overall survival (OS) was defined as the interval from the date of initial diagnosis to death from any cause or the last follow-up. Patients who were alive at the last follow-up were treated as censored observations. Kaplan–Meier survival curves were generated after stratifying patients into high-

and low-expression groups according to the median expression value of each gene. Statistical differences between survival curves were evaluated using the log-rank test. All survival analyses were performed using the TCGA-BRCA cohort.

Identification of co-expressed genes and functional enrichment

Pearson correlation analysis was performed between *BACE1*-AS expression and all other genes in the TCGA-BRCA dataset. The top 200 positively correlated genes were selected for downstream functional enrichment analyses. Genes meeting the predefined significance criteria ($|r| > 0.30$ and false discovery rate (FDR) < 0.05) were highlighted in volcano plots, whereas the top 30 correlated genes were visualized using hierarchical clustering heatmaps generated with the pheatmap package.

The top 200 genes most strongly correlated with *BACE1*-AS were submitted to the Enrichr platform for functional enrichment analysis using the Reactome_2022 and Gene Ontology (GO)_Biological_Process_2023 databases. Only pathways and biological processes with adjusted *P*-values < 0.05 were considered to be statistically significantly enriched. Terms with adjusted *P*-values ≥ 0.05 were not interpreted as significant enrichment, and were excluded from the presentation of significant pathways. For each database, statistically significant enriched terms were visualized using horizontal bar plots based on $-\log_{10}$ (adjusted *P*-value).

Statistical analysis

Statistical analyses were performed using R software (version 4.4.0; R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism (version 9.5.0; GraphPad Software, San Diego, CA, USA). Data distribution was assessed using the Shapiro–Wilk test. Depending on data distribution, comparisons between groups were performed using either the unpaired two-tailed Student's

t-test or the Mann–Whitney U test. Correlations were evaluated using Pearson's or Spearman's correlation coefficients, as appropriate. ROC curve analysis was used to assess diagnostic performance. Survival analyses were performed using Kaplan–Meier survival curves and the log-rank test. Data are presented as mean $\pm$ standard error of the mean (SEM), as appropriate. All statistical tests were two-sided, and a *P*-value < 0.05 was considered to be statistically significant. For functional enrichment analyses, an adjusted *P*-value < 0.05 was considered to be statistically significant.

Results

Expression profile and diagnostic significance of BACE1-AS

In the TCGA-BRCA cohort comprising 1,212 samples (1,098 primary tumors and 114 adjacent normal breast tissues), *BACE1*-AS expression was lower in primary tumor tissues than in adjacent normal tissues; however, the difference was not statistically significant. Although the difference in *BACE1*-AS expression between primary tumor and adjacent normal tissues was not statistically significant, ROC analysis showed good discriminatory performance, with an AUC of 0.79 (95% CI: 0.79–0.81) (Figure 1A–B). A non-significant group-level comparison does not necessarily preclude discriminatory performance across the full distribution of expression values.

BACE1-AS and SIRT1 expression across PAM50 molecular subtypes

BACE1-AS expression varied significantly among PAM50-defined molecular subtypes (Kruskal–Wallis test, $P < 0.05$). The Basal-like and Luminal A tumors exhibited the highest expression levels (median VST ≈ 8.2 and 7.9 , respectively), while HER2-enriched and Luminal B tumors showed lower expression levels (median ≈ 7.7) (Figure 2). *SIRT1* expression also differed significantly across PAM50-defined molecular subtypes

(Kruskal–Wallis test, $P < 0.05$), with the highest expression observed in Luminal A tumors and lower expression levels observed in basal-like and normal-like tumors (Figure 2).

Prognostic relevance of *BACE1-AS*

Kaplan–Meier survival analysis showed no significant association between *BACE1-AS* expression and OS in the TCGA-BRCA cohort (log-rank $P = 0.19$) (Figure 3A). In contrast, *SIRT1* expression was significantly associated with OS, with a significant difference between the high- and low-expression groups (log-rank $P = 0.043$) (Figure 3B). The combined *BACE1-AS/SIRT1* expression signature was not significantly associated with OS (log-rank $P = 0.24$) (Figure 3C).

Correlation between *BACE1-AS* and *SIRT1*

Across the overall TCGA-BRCA cohort, *BACE1-AS* expression showed a weak positive correlation with *SIRT1* expression (Pearson's $r = 0.14$, $P = 8.47 \times 10^{-7}$; Spearman's $\rho = 0.14$, $P = 1.87 \times 10^{-6}$). Subtype-specific analyses showed a weak positive correlation in basal-like tumors (Pearson's $r = 0.16$, $P = 0.02$) and a moderate positive correlation in normal-like breast tissues ($r = 0.34$, $P = 0.03$). No statistically significant correlation was observed in Luminal A, Luminal B, or HER2-enriched tumors (Table 2). These findings indicate a weak overall association between *BACE1-AS* and *SIRT1* expression and do not establish a functional interaction between the two genes (Figure 4; Table 2).

Co-expression network of *BACE1-AS*

Pearson correlation analysis identified several genes strongly positively correlated with *BACE1-AS* expression. Among the strongest correlations were observed for alpha-1,2-mannosyltransferase (*ALG9*) ($r = 0.66$, FDR-adjusted $P = 5.83 \times 10^{-148}$), ring finger protein 214 (*RNF214*) ($r = 0.56$, FDR-adjusted $P = 1.36 \times 10^{-98}$), and lysine methyltransferase 2A (*KMT2A*) ($r = 0.55$,

FDR-adjusted $P = 1.32 \times 10^{-92}$), along with several long non-coding RNAs ($r > 0.80$, FDR-adjusted $P < 10^{-100}$) (Figure 5A; Table 3). Hierarchical clustering of the top 30 genes correlated with *BACE1-AS* showed distinct expression patterns across the analyzed samples (Figure 5B). These findings indicate that *BACE1-AS* expression is associated with specific transcriptional co-expression patterns in the TCGA-BRCA cohort.

Enrichment and functional pathway analyses

Functional enrichment analysis of the top 200 positively correlated genes identified statistically significant enrichment in biological processes and pathways related to DNA damage response, ubiquitin-mediated proteolysis, and autophagy. In the Gene Ontology Biological Process 2023 analysis, enriched terms included autophagy of peroxisome (GO: 0030242, adjusted $P = 0.001$) and positive regulation of telomere maintenance (GO: 0032206, adjusted $P = 0.02$) (Table 4). The genes contributing to these enriched processes included *ATM* (ATM serine/threonine kinase), *PJVK* (Pejvakin), *MRE11* (MRE11 homolog, double-strand break repair nuclease), and *NFRKB* (nuclear factor related to kappa-B binding protein). Reactome 2022 analysis identified significant enrichment in sensing of DNA double-strand breaks (R-HSA-5693548, adjusted $P = 0.001$) and deubiquitination (R-HSA-5688426, adjusted $P = 0.01$), involving genes such as *MAVS* (mitochondrial antiviral signaling protein), *PSMB5* (proteasome 20S subunit beta 5), *USP28* (ubiquitin specific peptidase 28), and *OGT* (O-GlcNAc transferase) (Table 5). These findings indicate that genes co-expressed with *BACE1-AS* are enriched in biological processes related to DNA damage response, ubiquitin-mediated proteolysis, and autophagy; however, co-expression analysis does not establish a direct functional role for *BACE1-AS* in these pathways.

Expression Levels of BACE1-AS and SIRT1 in Peripheral Blood Leukocytes

The study initially included 30 breast cancer patients and 30 healthy controls. The mean age of patients and controls was 50.06 ± 11.36 and 43.35 ± 9.22 years, respectively. No significant differences were observed between the groups regarding demographic characteristics. During qRT-PCR analysis, 24 samples (16 patients and 8 controls) were excluded because they did not meet the predefined analytical quality criteria, including undetectable Ct values or unsuccessful amplification. Consequently, the final gene expression analysis was performed on 36 samples, comprising 14 breast cancer patients and 22 healthy controls. RT-PCR analysis demonstrated significantly increased expression levels of both *SIRT1* and *BACE1-AS* in peripheral blood leukocytes of breast cancer patients compared with healthy controls (Figure 6). *SIRT1* expression showed a mean fold change of 5.05 ($P < 0.0001$). Similarly, *BACE1-AS* expression was significantly elevated, with a mean fold change of 10.18 ($P < 0.0001$). Correlation analysis performed on the 36 evaluable samples revealed no significant association between *BACE1-AS* and *SIRT1* expression levels ($r = 0.18$, $P = 0.53$) (Figure 7). ROC curve analysis was performed to evaluate the discriminative value of leukocyte *BACE1-AS* expression. The analysis yielded an AUC of 0.57 (95% CI: 0.36–0.77), indicating limited discriminatory performance for distinguishing breast cancer patients from healthy controls (Figure 8).

Discussion

The present study investigated the expression and potential clinical relevance of *BACE1-AS* and *SIRT1* in breast cancer using complementary computational and experimental approaches. In the TCGA-BRCA cohort, *BACE1-AS* expression was

slightly lower in breast tumor tissues than in adjacent normal tissues, although the difference was not statistically significant, while tissue-based ROC analysis showed an AUC of 0.795. In peripheral blood leukocytes, *BACE1-AS* and *SIRT1* were significantly upregulated in patients with breast cancer, with fold changes of 10.18 and 5.05, respectively. However, leukocyte *BACE1-AS* showed limited discriminatory performance (AUC = 0.57, 95% CI: 0.36–0.77), and no significant correlation was observed between *BACE1-AS* and *SIRT1* in peripheral blood. In TCGA-BRCA, *BACE1-AS* and *SIRT1* showed a weak positive correlation, whereas *BACE1-AS* was not significantly associated with overall survival. Both genes also showed differential expression across PAM50 molecular subtypes, and co-expression and enrichment analyses identified associations with cellular stress responses, genome maintenance, ubiquitin-mediated processes, and autophagy. Overall, these findings indicate that *BACE1-AS* and *SIRT1* exhibit compartment- and subtype-dependent expression patterns, while the present data do not support *BACE1-AS* as an established diagnostic or prognostic biomarker in breast cancer.

The first major finding was the slightly lower expression of *BACE1-AS* in breast tumor tissues, although this difference was not statistically significant. This observation differs from reports describing oncogenic functions of *BACE1-AS* in several malignancies.^{20,27} Such discrepancies may reflect differences in cancer type, molecular subtype, disease stage, and cellular composition. Because TCGA expression represents bulk tissue, the measured signal reflects malignant, stromal, and immune-cell populations. *BACE1-AS* may therefore exhibit cell-specific or context-dependent regulation that is obscured in bulk measurements. Thus, the absence of

significant overexpression in bulk breast tumor tissue should not be interpreted as evidence against previously reported biological functions of *BACE1-AS*, but rather as evidence that its expression may depend on cellular and molecular context.

A second finding was the relatively good discriminatory performance of tissue *BACE1-AS* (AUC = 0.795, 95% CI: 0.791–0.810), despite the absence of significant differential expression. Differential-expression testing and ROC analysis assess different statistical properties; therefore, a non-significant group-level difference does not necessarily preclude classification ability. Nevertheless, the tissue-based AUC should be interpreted as exploratory because it was obtained from a retrospective TCGA dataset without external clinical validation. Accordingly, this result should not be considered as the evidence of established clinical biomarker utility and requires validation in independent cohorts.

A third finding was the heterogeneous expression of *BACE1-AS* and *SIRT1* across PAM50 subtypes, with relatively higher expression in Basal-like and Luminal A tumors and lower expression in HER2-enriched and Luminal B tumors. This pattern may reflect differences in hormone receptor status, proliferative activity, cellular stress, and DNA damage-related programs.^{11,22,28,29} However, these associations are descriptive and do not establish that either molecule directly regulates molecular subtype. Therefore, molecular subtype should be considered when interpreting *BACE1-AS* and *SIRT1* expression rather than assuming a uniform pattern across breast cancer.

Another important finding was the absence of a significant association between *BACE1-AS* and overall survival (log-rank $P = 0.19$). Although previous studies have linked *BACE1-AS* with malignant progression or clinical outcomes in other cancers,^{20,27,30} the present results do not support a prognostic

role in the overall TCGA-BRCA cohort. In contrast, *SIRT1* expression was significantly associated with overall survival in the TCGA-BRCA cohort (log-rank $P = 0.043$), suggesting that the clinical relevance of these two molecules may differ in breast cancer. However, this association should be interpreted cautiously because survival outcomes in retrospective public datasets may be influenced by molecular heterogeneity and unmeasured clinicopathological factors. The apparent separation of survival curves for *BACE1-AS* may therefore reflect random variation or unmeasured clinicopathological factors. Overall, the present findings do not support *BACE1-AS* as a prognostic marker, whereas the observed association between *SIRT1* expression and overall survival warrants further validation in larger, independent cohorts and through multivariable and subtype-specific analyses.

Co-expression analysis identified genes strongly associated with *BACE1-AS*, including *ALG9*, *RNF214*, *KMT2A*, *MSANTD2*, *NKAPD1*, and *CEP164*, with enrichment of processes related to cellular stress, genome maintenance, ubiquitin-mediated regulation, and autophagy.^{31–33} These findings may indicate shared transcriptional programs; however, co-expression cannot establish direct interaction or causality. Thus, these results should be regarded as hypothesis-generating and provide candidate pathways for subsequent mechanistic investigation rather than demonstrating a direct biological role for *BACE1-AS*.

Correlation analysis showed a weak positive association between *BACE1-AS* and *SIRT1* in TCGA-BRCA (Pearson $r = 0.140$; Spearman $\rho = 0.136$). Despite statistical significance, the small effect size suggests limited biological relevance and may partly reflect the large TCGA sample size. No significant correlation was observed in peripheral blood

leukocytes ($r = 0.18$, $P = 0.53$). The weak tissue correlation and absence of correlation in blood therefore do not support a consistent expression-based or direct regulatory relationship between *BACE1-AS* and *SIRT1*. In peripheral blood, both *BACE1-AS* and *SIRT1* were significantly upregulated, with fold changes of 10.18 and 5.05, respectively. This pattern contrasts with the tissue-level findings and emphasizes that tumor tissue and circulating leukocytes represent distinct biological compartments. Systemic inflammatory signaling, tumor-derived mediators, extracellular vesicles, and tumor-immune interactions could contribute to altered leukocyte transcription.^{20,31,34,35} However, an important alternative explanation is variation in leukocyte subset composition, including changes in the neutrophil-to-lymphocyte ratio. Because differential leukocyte counts were unavailable, this possibility cannot be excluded. Accordingly, increased leukocyte expression should be interpreted as a breast cancer-associated peripheral blood molecular pattern rather than direct evidence of increased expression within tumor cells. The significant increase in leukocyte *SIRT1* is also noteworthy because previous studies have primarily investigated *SIRT1* in breast tumor tissue.^{21,22,28,29,36–40} The observed peripheral increase may reflect systemic immune or cellular stress responses rather than tumor-specific regulation. Similarly, the absence of correlation between leukocyte *BACE1-AS* and *SIRT1* argues against a strong common regulatory axis, although indirect or cell-specific interactions cannot be excluded. Further studies incorporating leukocyte subset characterization and functional analyses are therefore required to clarify the significance of these findings. Importantly, despite significantly higher leukocyte *BACE1-AS* expression, its diagnostic performance was limited (AUC = 0.57, 95% CI: 0.36–0.77). Thus, statistical

upregulation should not be equated with diagnostic utility, and the present findings do not support leukocyte *BACE1-AS* as a clinically useful standalone biomarker. Future studies should determine whether it provides additional value when combined with other molecular or clinicopathological variables.

Our study combined qRT-PCR analysis of peripheral blood leukocytes with independent TCGA-BRCA transcriptomic analysis, enabling evaluation of *BACE1-AS* and *SIRT1* across distinct biological compartments. *BACE1-AS* was assessed through differential expression, molecular subtype distribution, ROC analysis, survival, correlation, co-expression, and enrichment analyses. The experimental assays incorporated technical triplicates and quality-control procedures, while computational analyses used established statistical approaches. The integration of experimental and computational evidence provides a multidimensional assessment of these molecules and highlights compartment-specific expression patterns.

Several limitations should be acknowledged in the present study. First, the relatively small case-control sample size for qRT-PCR, particularly after quality-control exclusions, may have limited statistical power and generalizability. Second, the observational TCGA analyses cannot establish causality. Third, both datasets were based on bulk RNA measurements and therefore cannot resolve cell-specific expression. Fourth, differential leukocyte counts were unavailable, preventing assessment of leukocyte subset effects. Fifth, protein-level and functional validation were not performed. Sixth, the tissue ROC analysis lacked independent external validation, while the leukocyte AUC was insufficient to support clinical diagnostic application. Finally, co-expression and enrichment analyses were exploratory and cannot establish direct molecular

interactions. Future studies should therefore include larger independent cohorts, detailed leukocyte immunophenotyping or isolated cell subsets, longitudinal and multivariable analyses, external diagnostic validation, cell-specific transcriptomic approaches, and protein-level and functional experiments to clarify the biological and clinical significance of *BACE1-AS* and *SIRT1*.

Conclusion

The present study identified subtype-related variation in *BACE1-AS* expression and co-expression patterns associated with biological processes related to DNA damage response, genome maintenance, ubiquitin-mediated regulation, and autophagy in the TCGA-BRCA cohort. However, these co-expression findings do not establish a direct functional role for *BACE1-AS* in these pathways. No significant association was observed between *BACE1-AS* expression and OS, and its correlation with *SIRT1* was weak, providing no evidence of a biologically significant or direct regulatory relationship between the two molecules. Although *BACE1-AS* and *SIRT1* were significantly upregulated in peripheral blood leukocytes from breast cancer patients, leukocyte *BACE1-AS* showed limited diagnostic discrimination (AUC = 0.57) and therefore does not support its use as a standalone blood-based diagnostic biomarker in the present cohort. The opposite expression patterns observed between tumor tissue and peripheral blood leukocytes may reflect differences between biological compartments, including potential differences in leukocyte composition, and should not be interpreted as the evidence of a systemic regulatory mechanism. Overall, these findings support further investigation of the biological role and cellular context of *BACE1-AS* in breast cancer rather than its immediate clinical application as a peripheral blood biomarker.

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Authors' Contribution

Hadis Musavi: Conceptualization, Methodology, Investigation, Writing – Original Draft, Visualization; Fereshteh Ahmadipour: Methodology, Validation, Formal analysis, Investigation, Writing – Review and Editing; Tayebbeh Azramezani Kop: Investigation, Data curation, Software, Writing – Review and Editing; Zahra Yazdani Kachooi: Investigation, Resources, Validation, Writing – Review and Editing; Fatemeh Gorjinezhad: Methodology, Investigation, Formal analysis, Writing – Original Draft; Fariba Ghodrati: Investigation, Data curation, Software, Writing – Review and Editing; Abouzar Bagheri: Conceptualization, Methodology, Supervision, Project administration, Writing – Review and Editing; Masoumeh Karimi: Methodology, Investigation, Validation, Writing – Review and Editing; Abbas Khonakdar-Tarsi: Conceptualization, Methodology,

Supervision, Project administration, Writing – Review and Editing, Corresponding author.

Conflict of Interest

None declared.

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Table 1. Primer sequences used for qRT-PCR

Gene	Direction	Sequence (5'→3')
GAPDH	Forward	ACTCTGGTAAAGTGGATATTGTTGC
	Reverse	GGAAGATGGTGGTGGGATTTC
BACE1-AS	Forward	GTGCCTCAGTTCCTTCATC
	Reverse	AGTGCCTTCTCTCTGCTG
SIRT1	Forward	TAGGCGGCTTGATGGTAATC
	Reverse	GGCATGTCCCACTATCACTG

qRT-PCR: Quantitative real-time polymerase chain reaction

Table 2. Pearson's and Spearman's correlation coefficients between BACE1-AS and SIRT1 expression across PAM50 molecular subtypes in the TCGA-BRCA cohort

Subtype	N	Pearson's r	P value	Spearman's ρ	P value
Luminal A (LumA)	571	0.08	0.07	0.03	0.51
Luminal B (LumB)	209	0.05	0.49	0.05	0.51
HER2-enriched	82	0.03	0.77	0.08	0.50
Basal-like	197	0.16	0.02	0.11	0.12
Normal-like	40	0.34	0.03	0.34	0.03

BACE1-AS: β -site amyloid precursor protein cleaving enzyme 1 antisense RNA; SIRT1: Sirtuin 1; PAM50: Prediction Analysis of Microarray 50; TCGA-BRCA: The Cancer Genome Atlas Breast Invasive Carcinoma; HER2: Human epidermal growth factor receptor 2; LumA: Luminal A; LumB: Luminal B; N: Number of samples; r: Correlation coefficient; P: Probability value.

Table 3. Top 10 protein-coding genes and long non-coding RNAs positively correlated with BACE1-AS expression in the TCGA-BRCA cohort

Gene symbol	Pearson's r	P value	FDR-adjusted P value
ALG9	0.66	5.05×10^{-152}	5.83×10^{-148}
RNF214	0.56	1.76×10^{-102}	1.36×10^{-98}
AL160006.1	0.55	1.43×10^{-99}	8.27×10^{-96}
KMT2A	0.55	2.87×10^{-96}	1.32×10^{-92}
MSANTD2	0.54	9.19×10^{-96}	3.54×10^{-92}
AL158212.3	0.54	5.75×10^{-95}	1.90×10^{-91}
NKAPD1	0.54	1.24×10^{-92}	3.57×10^{-89}
CENATAC	0.52	7.06×10^{-85}	1.81×10^{-81}
AC024075.2	0.51	1.28×10^{-83}	2.96×10^{-80}
CEP164	0.51	4.76×10^{-83}	1.00×10^{-79}

BACE1-AS: β -site amyloid precursor protein cleaving enzyme 1 antisense RNA; TCGA-BRCA: The Cancer Genome Atlas Breast Invasive Carcinoma; FDR: False discovery rate; r: Pearson correlation coefficient; P: Probability value; ALG9: Alpha-1,2-mannosyltransferase 9; RNF214: Ring finger protein 214; KMT2A: Lysine methyltransferase 2A; MSANTD2: Myb/SANT DNA binding domain containing 2; NKAPD1: NKAP domain containing 1; CENATAC: CENATAC complex subunit 1; CEP164: Centrosomal protein 164

Table 4. Gene ontology biological process enrichment analysis of genes co-expressed with BACE1-AS in the TCGA-BRCA cohort

GO biological process	GO ID	Adjusted P value	Associated genes
Autophagy of peroxisome	GO:0030242	0.001	ATM; PJVK
Positive regulation of telomere maintenance	GO:0032206	0.02	MRE11; ATM; NFRKB
Negative regulation of TOR signaling	GO:0032007	0.02	SZT2; TSC1; ATM
Regulation of organelle organization	GO:0033043	0.02	MAVS; TMED9; PJVK; NFRKB
Signal transduction in response to DNA damage	GO:0042770	0.02	ATM; USP28; HINFP
mRNA splicing, via spliceosome	GO:0000398	0.02	DDX17; GPKOW; CWF19L2; CENATAC; RNPC3; SRSF9
Selective autophagy	GO:0061912	0.02	PJVK; ATM; OGT
Telomere organization	GO:0032200	0.03	MRE11; ATM; NFRKB
Telomere maintenance	GO:0000723	0.03	MRE11; ATM; NFRKB

GO: Gene ontology; BACE1-AS: β -site amyloid precursor protein cleaving enzyme 1 antisense RNA; ATM: ATM serine/threonine kinase; PJVK: Pejvakin; MRE11, MRE11 homolog, double-strand break repair nuclease; NFRKB: Nuclear factor related to kappa-B binding protein; SZT2: Seizure threshold 2 homolog; TSC1: TSC complex subunit 1; MAVS: Mitochondrial antiviral signaling protein; TMED9: Transmembrane p24 trafficking protein 9; USP28: Ubiquitin-specific peptidase 28; HINFP: Histone H4 transcription factor; DDX17: DEAD-box helicase 17; GPKOW: G-patch and KOW motifs; CWF19L2: CWF19 like cell cycle control protein 2; CENATAC: CENATAC complex subunit 1; RNPC3: RNA-binding region-containing protein 3; SRSF9: Serine and arginine-rich splicing factor 9; OGT: O-GlcNAc transferase

Table 5. Top enriched reactome pathways associated with genes co-expressed with BACE1-AS in the TCGA-BRCA cohort

Term	Adjusted <i>P</i> value	Genes
Sensing of DNA double strand breaks R-HSA-5693548	0.001	MRE11; ATM
Deubiquitination R-HSA-5688426	0.001	MAVS; PSMB5; TAF10; FOXK1; USP28; NFRKB; OGT; OTUD3
UCH proteinases R-HSA-5689603	0.01	PSMB5; FOXK1; NFRKB; OGT
Impaired BRCA2 binding to PALB2 R-HSA-9709603	0.02	MRE11; ATM
Defective HDR thru homologous recombination (HRR) due to BRCA1 loss-of-function R-HSA-9701192	0.02	MRE11; ATM
Macroautophagy R-HSA-1632852	0.02	MTMR3; ATG101; TSC1; ATM
Resolution of D-loop structures thru synthesis-dependent strand annealing (SDSA) R-HSA-5693554	0.03	MRE11; ATM
Autophagy R-HSA-9612973	0.04	MTMR3; ATG101; TSC1; ATM
Resolution of D-loop structures thru holliday junction intermediates R-HSA-5693568	0.04	MRE11; ATM
Resolution Of D-Loop Structures R-HSA-5693537	0.04	MRE11; ATM

BACE1-AS: β -site amyloid precursor protein cleaving enzyme 1 antisense RNA; TCGA-BRCA: The Cancer Genome Atlas Breast Invasive Carcinoma; MRE11: MRE11 homolog, double-strand break repair nuclease; ATM: ATM serine/threonine kinase; MAVS: Mitochondrial antiviral signaling protein; PSMB5: Proteasome 20S subunit beta 5; TAF10: TATA-box binding protein-associated factor 10; FOXK1: Forkhead box K1; USP28: Ubiquitin-specific peptidase 28; NFRKB: Nuclear factor related to kappa-B binding protein; OGT: O-GlcNAc transferase; OTUD3: OTU deubiquitinase 3; MTMR3: Myotubularin-related protein 3; ATG101: Autophagy-related 101; TSC1: TSC complex subunit 1; BRCA1: BRCA1 DNA repair associated; BRCA2: BRCA2 DNA repair associated; PALB2: Partner and localizer of BRCA2; HDR: Homology-directed repair; HRR: Homologous recombination repair; SDSA: Synthesis-dependent strand annealing

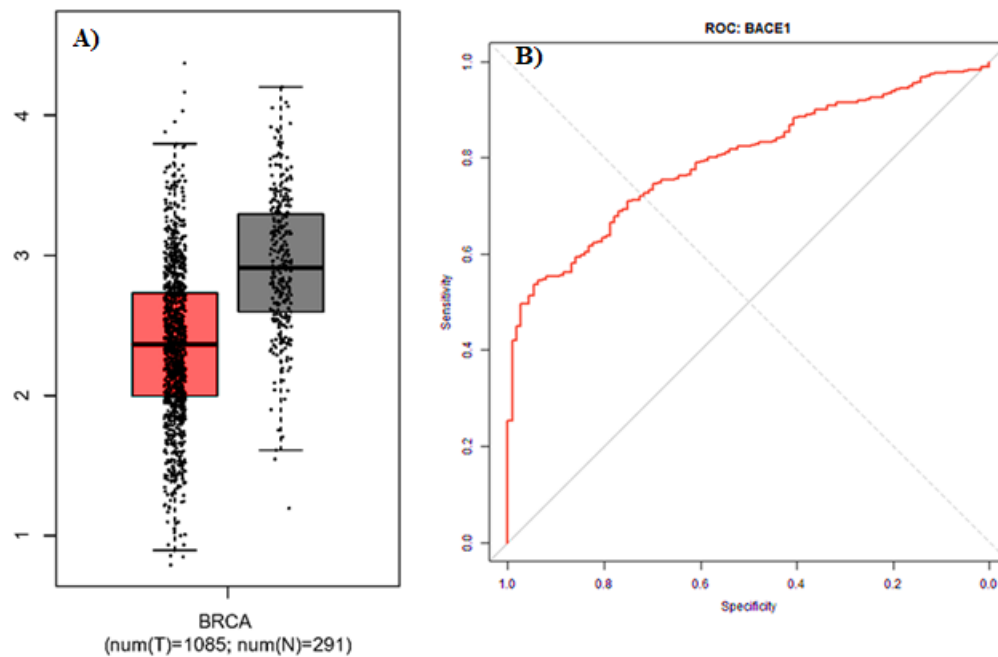
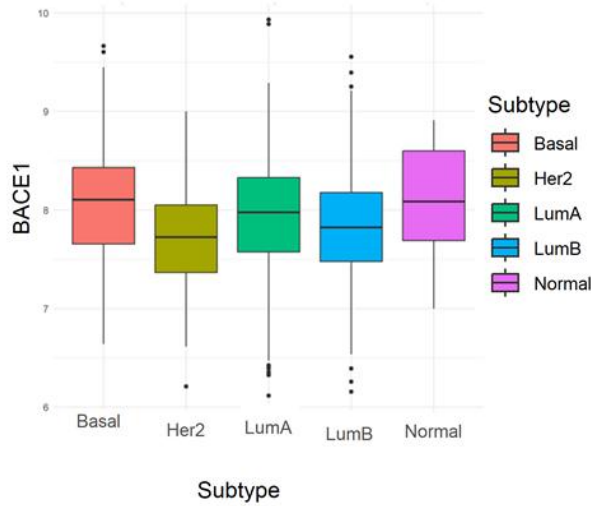


Figure 1. Differential expression and diagnostic performance of BACE1-AS in the TCGA-BRCA cohort. (A) BACE1-AS expression levels in primary breast tumor tissues compared with adjacent normal breast tissues. (B) ROC curve showing the discriminatory performance of BACE1-AS expression for distinguishing primary breast tumor tissues from normal breast tissues.

BACE1-AS: β -site amyloid precursor protein cleaving enzyme 1 antisense RNA; TCGA-BRCA: The Cancer Genome Atlas Breast Invasive Carcinoma; ROC: Receiver operating characteristic

A) BACE1 Expression by PAM50 Subtype



B) SIRT1 Expression by PAM50 Subtype

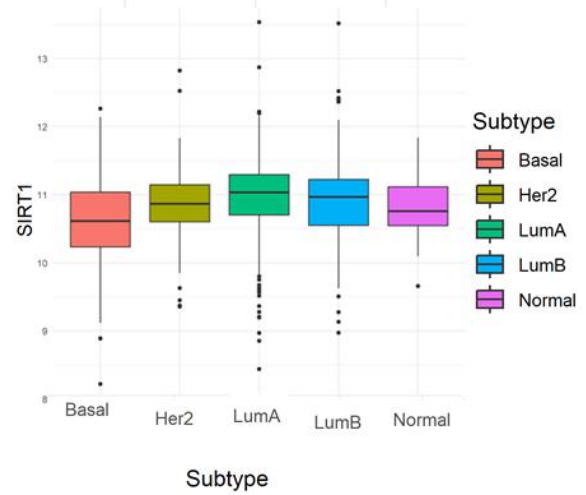
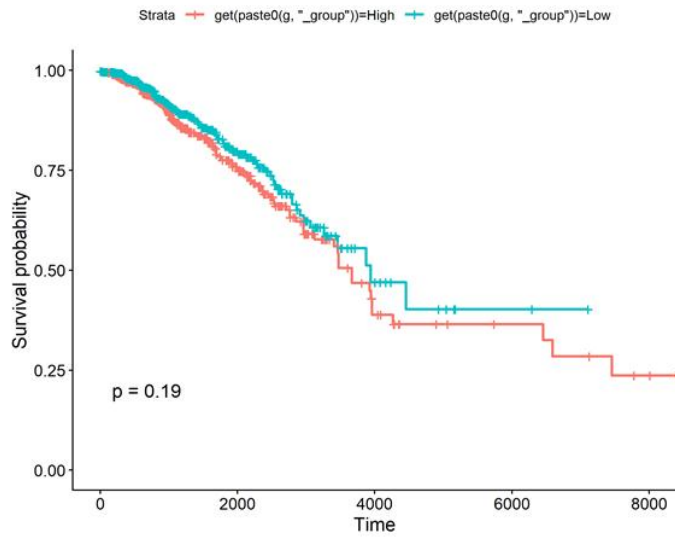


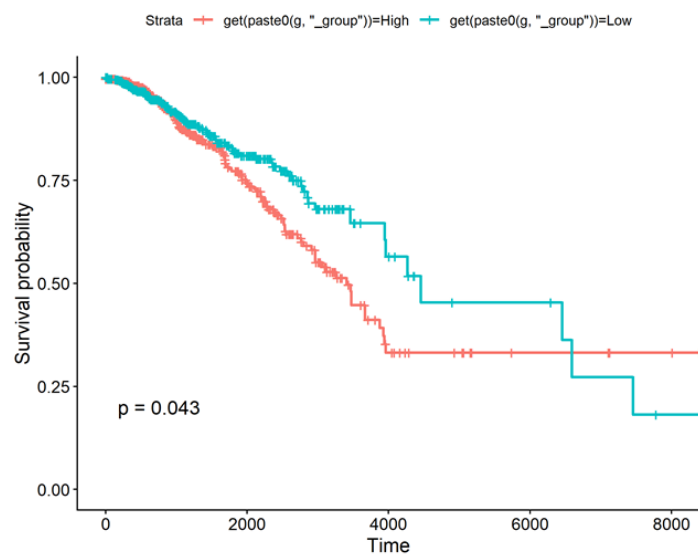
Figure 2. This figure shows the expression patterns of BACE1-AS and SIRT1 across PAM50 molecular subtypes in the TCGA-BRCA cohort. (A) Distribution of BACE1-AS expression levels across the Luminal A, Luminal B, HER2-enriched, Basal-like, and normal-like breast cancer molecular subtypes. (B) Distribution of SIRT1 expression levels across the Luminal A, Luminal B, HER2-enriched, Basal-like, and normal-like breast cancer molecular subtypes.

BACE1-AS: β -site amyloid precursor protein cleaving enzyme 1 antisense RNA; SIRT1: Sirtuin 1; PAM50: Prediction Analysis of Microarray 50; TCGA-BRCA: The Cancer Genome Atlas Breast Invasive Carcinoma; HER2: Human epidermal growth factor receptor 2

A) KM: BACE1



B) KM: SIRT1



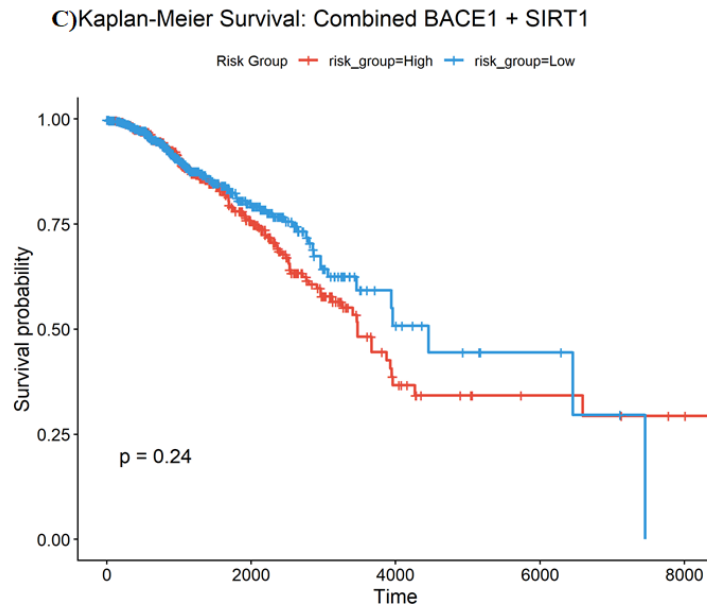


Figure 3. This figure shows the Kaplan–Meier overall survival curves according to BACE1-AS and SIRT1 expression in the TCGA-BRCA cohort. (A) Overall survival according to BACE1-AS expression. (B) Overall survival according to SIRT1 expression. (C) Overall survival according to the combined BACE1-AS/SIRT1 expression signature.

BACE1-AS: β -site amyloid precursor protein cleaving enzyme 1 antisense RNA; SIRT1: Sirtuin 1; TCGA-BRCA: The Cancer Genome Atlas Breast Invasive Carcinoma; OS: Overall survival

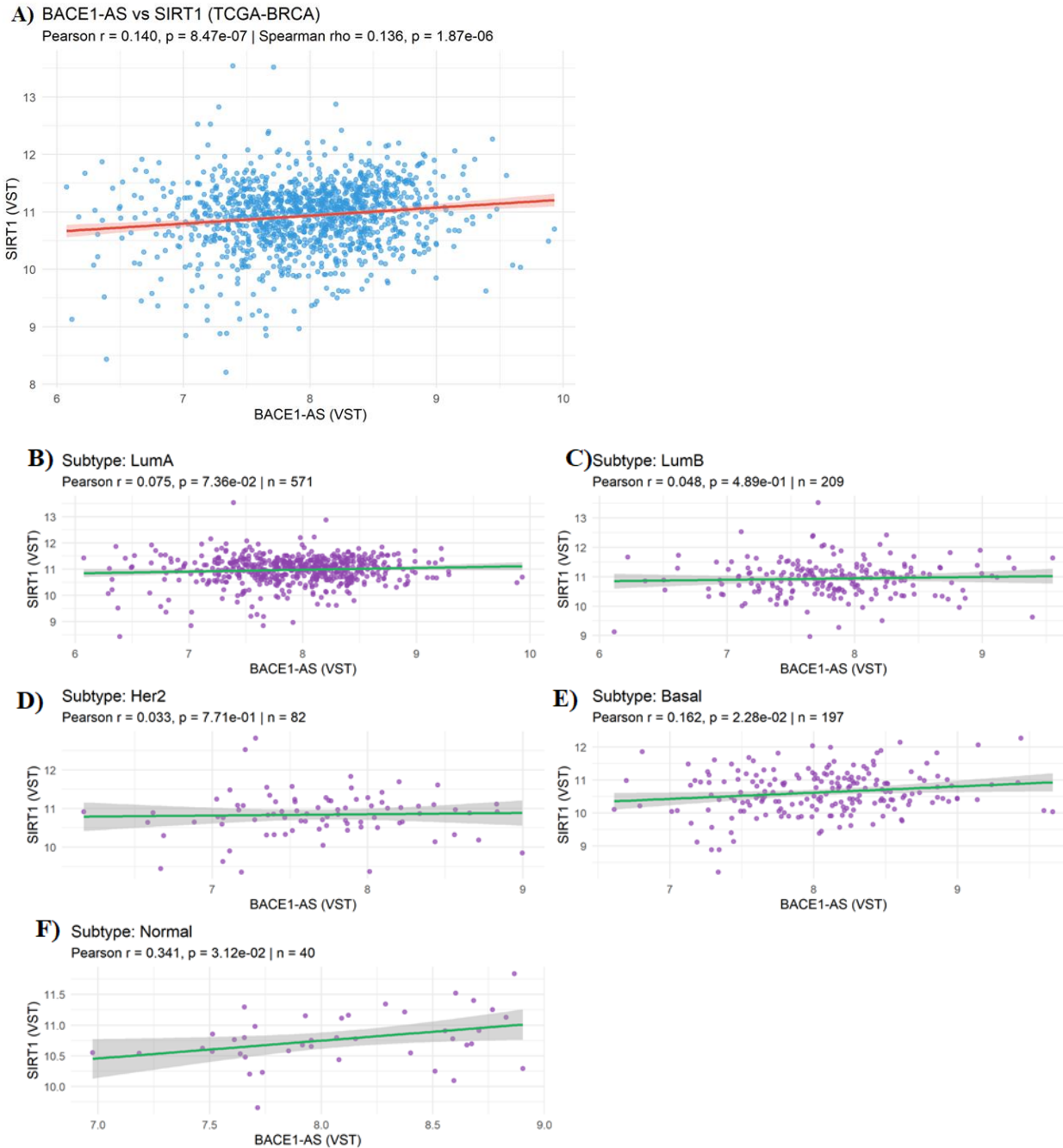


Figure 4. This figure shows the correlation between BACE1-AS and SIRT1 expression in the TCGA-BRCA cohort. (A) Correlation between BACE1-AS and SIRT1 expression in the overall TCGA-BRCA cohort, assessed using Pearson and Spearman correlation analyses. (B–F) Correlation analyses stratified by PAM50 molecular subtypes, including LumA, LumB, HER2-enriched (HER2), Basal-like (Basal), and Normal-like (Normal) subtypes. Each scatter plot shows the expression levels of BACE1-AS and SIRT1, with the fitted regression line and 95% confidence interval. Pearson correlation coefficients (r), corresponding P -values, and sample sizes (n) are indicated in each panel.

LumA: Luminal A; LumB: Luminal B; BACE1-AS: β -site amyloid precursor protein cleaving enzyme 1 antisense RNA; SIRT1: Sirtuin 1; TCGA-BRCA: The Cancer Genome Atlas Breast Invasive Carcinoma; PAM50: Prediction Analysis of Microarray 50; HER2: Human epidermal growth factor receptor 2

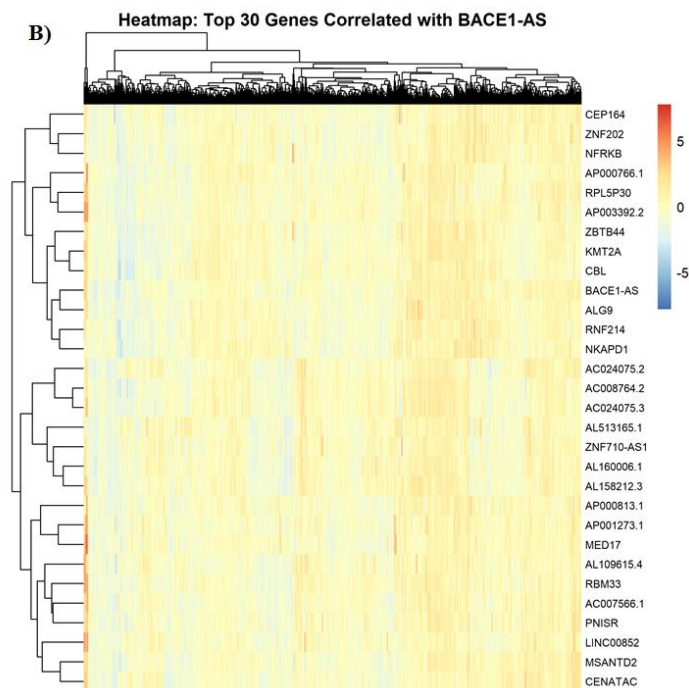
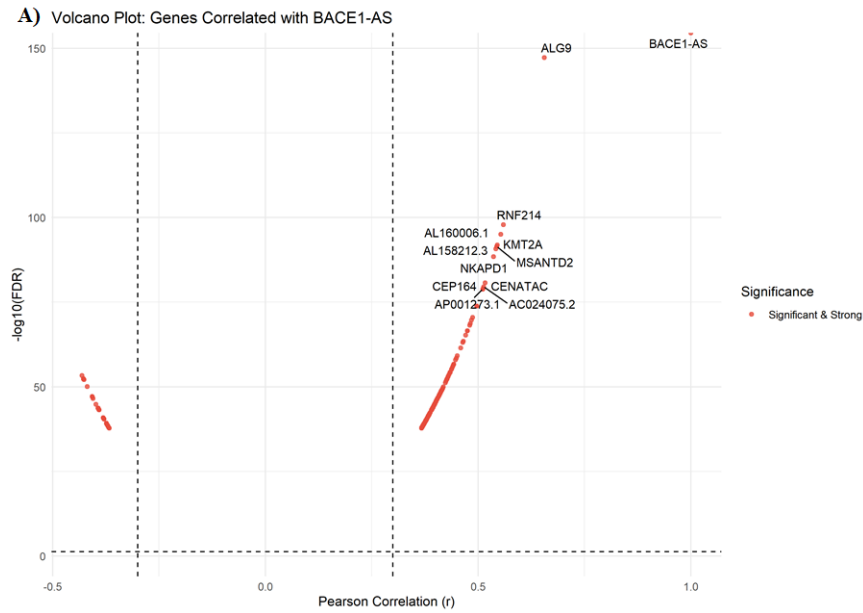


Figure 5. This figure shows the genome-wide co-expression analysis of BACE1-AS in the TCGA-BRCA cohort. (A) Volcano plot showing the Pearson correlation coefficients and FDR-adjusted significance values for genes associated with BACE1-AS expression. (B) Heatmap showing the expression patterns of the top 30 genes most strongly correlated with BACE1-AS expression across the analyzed samples.

BACE1-AS: β -site amyloid precursor protein cleaving enzyme 1 antisense RNA; TCGA-BRCA: The Cancer Genome Atlas Breast Invasive Carcinoma; FDR: False discovery rate

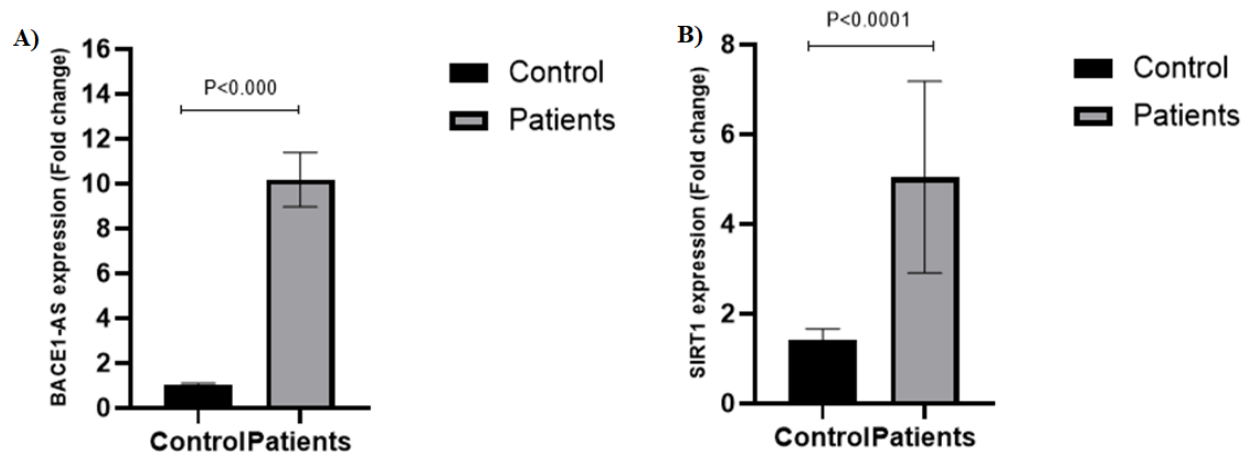


Figure 6. This figure shows the relative expression levels of BACE1-AS and SIRT1 in peripheral blood leukocytes from breast cancer patients and healthy controls. Relative expression levels of (A) BACE1-AS and (B) SIRT1 were measured in peripheral blood leukocytes from breast cancer patients (n = 14) and healthy controls (n = 22) using quantitative real-time polymerase chain reaction (qRT-PCR).

BACE1-AS: β -site amyloid precursor protein cleaving enzyme 1 antisense RNA; SIRT1: Sirtuin 1; qRT-PCR: Quantitative real-time polymerase chain reaction; n: Number of samples

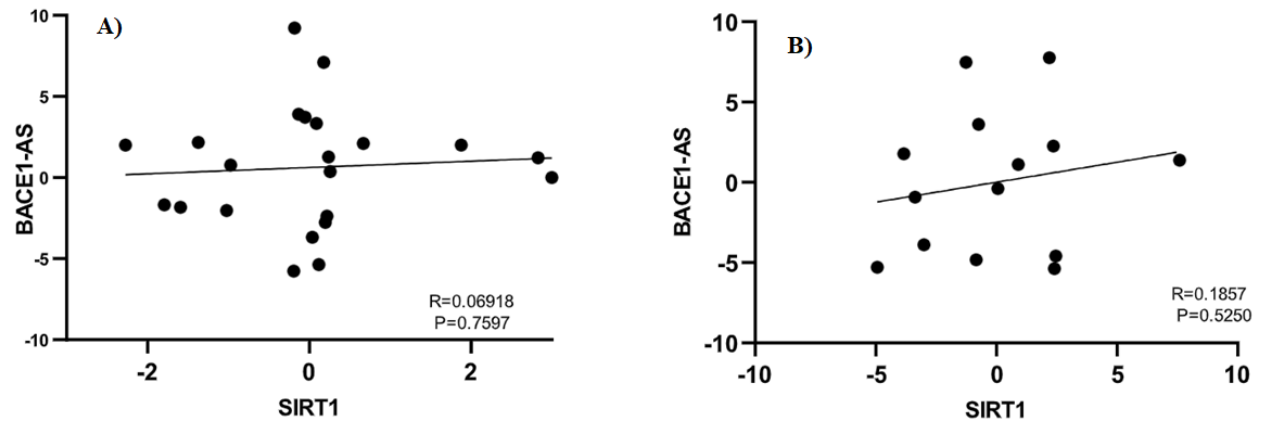


Figure 7. This figure shows the correlation between BACE1-AS and SIRT1 expression in peripheral blood leukocytes. (A) Correlation between BACE1-AS and SIRT1 expression in peripheral blood leukocytes from healthy controls. (B) Correlation between BACE1-AS and SIRT1 expression in peripheral blood leukocytes from breast cancer patients. Each point represents an individual participant.

BACE1-AS: β -site amyloid precursor protein cleaving enzyme 1 antisense RNA; SIRT1: Sirtuin 1

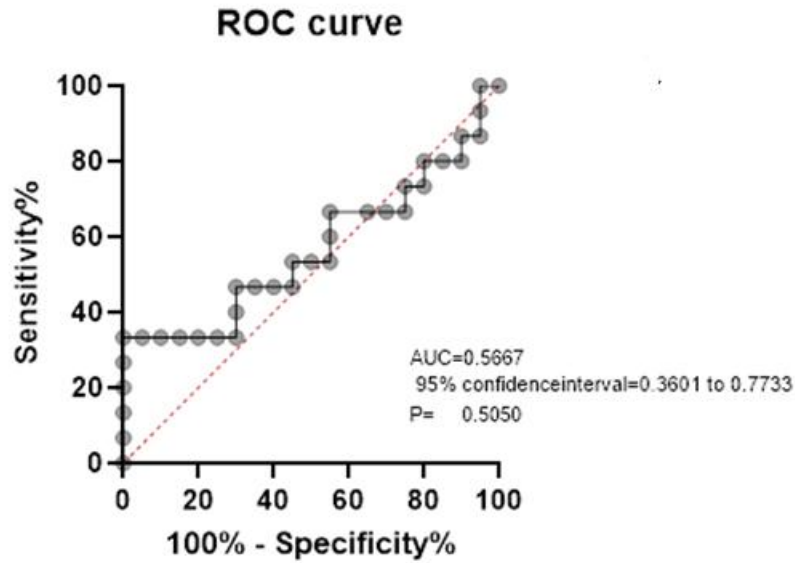


Figure 8. This figure shows the ROC curve of leukocyte BACE1-AS expression for discriminating breast cancer patients from healthy controls. The AUC was 0.5667, with a 95% confidence interval of 0.3601–0.7733 and $P = 0.5050$, indicating that the diagnostic performance was not statistically significant.

BACE1-AS: β -site amyloid precursor protein cleaving enzyme 1 antisense RNA; ROC: Receiver operating characteristic; AUC: Area under the curve; CI: Confidence interval