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Serum MicroRNAs and Tissue EGFR in Triple-Negative Breast Cancer: An Exploratory Case-Control Study

Sahar M. Hussein*[a], Ruaa Maan Attallah[b], Noora A. Hadi[c], Roya Hadi Al-Haddad[d]

[a] Department of Molecular and Medical Biotechnology, College of Biotechnology, Al-Nahrain University, Baghdad, Iraq
[b] Department of Forensic Chemistry, Higher Institute of Forensic Sciences, Al-Nahrain University, Baghdad, Iraq
[c] Department of Applied Biological Science, College of Biotechnology, Al-Nahrain University, Baghdad, Iraq
[d] Scientific Research Commission, Baghdad, Iraq

* **Corresponding Author:** Sahar M. Hussein. Email: saharhadad84@gmail.com

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ABSTRACT

Background: Triple-negative breast cancer (TNBC) is an aggressive and heterogeneous subtype for which reliable molecular biomarkers remain an important research need. We aimed to compare serum miR-21, miR-30, and miR-125 expression and tissue *EGFR* mRNA expression between TNBC cases and their respective control groups, and to explore biomarker correlations and apparent diagnostic discrimination.

Methods: This hospital-based case-control study was conducted from April 2024 to November 2025. Among 105 women with histopathologically confirmed HER2-negative breast cancer, 32 patients with TNBC underwent molecular analysis. Serum miRNAs were compared with healthy female controls, whereas tumor-tissue *EGFR* mRNA was compared with histopathologically confirmed benign breast tissue because healthy breast tissue could not be obtained ethically. Expression was measured by reverse-transcription quantitative polymerase chain reaction. Group comparisons used ΔCt values; receiver operating characteristic (ROC) analyses used $2^{-\Delta\Delta Ct}$ values and were exploratory and unvalidated.

Results: Relative to their respective control groups, TNBC cases had higher miR-21 (4.30-fold) and tissue EGFR (6.69-fold) expression and lower miR-30 (0.02-fold) and miR-125 (0.13-fold) expression (all $P < .001$). In exploratory analyses restricted to TNBC participants, serum miR-30 and miR-125 were positively correlated ($r = 0.601$, $P < .001$, $n = 30$), meeting the Bonferroni-adjusted threshold; all other correlations were weak and nonsignificant. Apparent AUCs were 0.876 for miR-21, 1.000 for miR-30, 1.000 for miR-125, and 0.865 for EGFR; assay-specific ROC sample sizes ranged from 44 to 47 participants.

Conclusion: Serum miRNA and tissue EGFR expression differed between TNBC cases and their distinct comparator groups. The markers were measured in different biological compartments, the cohort was small, reference genes were not validated for stability, and the ROC results were not validated internally or externally. Therefore, these results are exploratory and do not establish a combined clinical biomarker panel.

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INTRODUCTION

Breast cancer is the most frequently diagnosed malignancy among women worldwide and remains a major cause of cancer mortality.[1] It comprises biologically heterogeneous diseases with distinct clinical behavior and treatment response. Triple-negative breast cancer (TNBC), defined by absent estrogen receptor (ER) and progesterone receptor (PR) expression and lack of human epidermal growth factor receptor 2 (HER2) overexpression or amplification, is characterized by substantial molecular heterogeneity, relatively aggressive behavior, and limited receptor-directed treatment options.[2,3]

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression posttranscriptionally and may function as oncogenic or tumor-suppressive regulators.[4,5] miR-21 is widely described as an oncogenic miRNA and has been linked to proliferation, invasion, apoptosis resistance, and treatment resis-

tance in breast cancer.[6] By contrast, members of the miR-125 and miR-30 families have tumor-suppressive functions in several experimental contexts. miR-125 can regulate ERBB-family signaling, whereas reduced miR-30 expression has been associated with epithelial–mesenchymal transition and invasive behavior.[7–11]

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase involved in cell proliferation and survival. EGFR overexpression is observed in a substantial subset of TNBC and has been associated with aggressive clinicopathological features and treatment resistance.[12–14]

Although serum miRNAs and tissue EGFR may each provide information relevant to TNBC biology, they represent different biological compartments and should not be treated as interchangeable components of a single circulating test. The diagnostic potential of individual miRNAs also requires rigorous validation because small case-control studies may yield optimistic estimates.[15–17]

The primary objective of this study was to compare serum miR-21, miR-30, and miR-125 expression between women with TNBC and healthy female serum controls and to compare tumor-tissue *EGFR* mRNA expression between TNBC tissue and benign breast tissue. Secondary exploratory objectives were to describe pairwise correlations among the measured markers in paired TNBC specimens and to estimate the apparent, in-sample discriminatory performance of each marker separately.

METHODS

Study design, setting, participants, and ethics

This hospital-based case-control study was conducted at Al-Amal Hospital from April 2024 to November 2025. Reporting was prepared in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement for case-control studies.[18] The study protocol was reviewed and approved by the Scientific Committee of the Ministry of Health (approval no. 41113; 11 December 2023). Written informed consent was obtained from all participants before enrollment, and the study was conducted in accordance with the Declaration of Helsinki.

A total of 105 women with histopathologically confirmed HER2-negative breast cancer were consecutively recruited following clinical and pathological assessment by the specialist medical team. Thirty-two met the local immunohistochemical criteria for TNBC and were included in the molecular analyses; the remaining 73 HER2-negative non-TNBC cases contributed only to the descriptive clinicopathological characterization of the overall breast cancer cohort. For every TNBC participant, the serum sample and corresponding tumor-tissue specimen were obtained from the same individual.

Eligibility for the breast cancer group required female sex and histopathological confirmation of breast cancer. Comorbidities were not used as exclusion criteria for breast cancer cases. Healthy female serum controls were reported to be aged 30 to 40 years and were eligible if they had no family history of breast cancer and no history of autoimmune disease, diabetes mellitus, or hypertension. Information on menopausal status, body mass index, reproductive history, parity, smoking, hormonal therapy, medication use, breast imaging, clinical breast examination, and benign breast disease was not available for complete case-control characterization. For the EGFR analysis, control RNA was obtained from breast tissue specimens that were histopathologically confirmed as benign, because obtaining breast tissue from healthy women solely for research purposes was not ethically feasible. Accordingly, the comparator group for tissue EGFR differed from the healthy serum-control group used for circulating miRNAs.

Treatment status at sampling and detailed exclusion criteria related to other malignancies, inflammatory or infectious conditions, pregnancy, corticosteroid exposure, chemotherapy, radiotherapy, and surgery were not consistently available in the study records. These variables were therefore not used to define the analytic cohort and are acknowledged as potential sources of residual confounding.

Clinical and pathological assessment

Tumors were classified using local immunohistochemical assessment of ER, PR, and HER2 and were grouped as Luminal A-like, Luminal B-like (HER2-negative), or TNBC. The exact ER, PR, and Ki-67 cutoffs applied during routine clinical classification were not available for retrospective verification. The original dataset used the combined label “Stage 0–I”; however, no separate Stage 0 diagnosis could be verified from the available records. To avoid implying an unconfirmed in situ category, the descriptive tables below report this group as stage I. Disease stage was otherwise summarized as stages I–IV.

Outcomes

The prespecified primary outcome for this report was the difference in expression of each individual biomarker between TNBC cases and its appropriate comparator group: serum miRNAs versus healthy serum controls and tumor-tissue EGFR versus benign breast-tissue controls. Pairwise correlations and individual-marker ROC analyses were treated as secondary exploratory outcomes. No combined multivariable biomarker model was developed or validated.

Sample collection and RNA extraction

For serum RNA extraction, 400 µL of serum was mixed with 600 µL of TRIzol reagent in a sterile RNase-free microcentrifuge tube. Quantitative assessment of hemolysis was not performed, and further details on blood collection tubes, clotting time, centrifugation, aliquoting, storage duration, and freeze–thaw cycles were not available. These omissions limit evaluation of preanalytical variability in circulating miRNA measurements.

Tumor tissue was obtained by ultrasound-guided

needle biopsy. Each biopsy was divided into two portions: one was submitted to the medical team for histopathological confirmation of malignant cells, and the second was immediately transferred to an RNase-free microcentrifuge tube containing 1 mL TRIzol reagent and stored at −20 °C until total RNA extraction and cDNA synthesis. The corresponding EGFR control specimens were histopathologically confirmed benign breast tissue. Tumor-cell percentage, microdissection, stromal or immune-cell contamination, RNA integrity number, the interval from biopsy to stabilization, and EGFR protein expression by immunohistochemistry were not assessed.

RNA concentration and purity were evaluated with a Quantus fluorometer (Promega, USA). Complementary DNA was synthesized from purified RNA using stem-loop reverse-transcription primers for miRNAs and gene-specific primers for EGFR.

Reverse-transcription quantitative PCR

Reverse-transcription quantitative PCR (RT-qPCR) was performed with SYBR Green chemistry on a StepOnePlus Real-Time PCR System (Applied Biosystems/Thermo Fisher Scientific, USA). Primer sequences are presented in Table 1. U6 small nuclear RNA served as the endogenous reference for serum miRNAs, and *GAPDH* served as the reference gene for tissue EGFR. U6 was used in the original protocol because of its historical use in miRNA studies; however, its stability was not experimentally validated in this cohort. Evidence from circulating-miRNA research indicates that U6 may vary between individuals and disease states, so results normalized to U6 require cautious interpretation.[19–21]

Following initial denaturation at 95°C for 10 minutes, amplification comprised 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. Melting-curve analysis was performed after amplification. A single sharp melting peak was observed for each target, with no apparent primer-dimer or nonspecific amplification. A randomly selected subset of samples was analyzed in duplicate or triplicate and produced consistent amplification profiles. The number of technical replicates for each assay was not recorded in a form permitting a complete MIQE table.[22]

Relative expression calculation

Relative expression was calculated by the comparative threshold-cycle method.[23,24] For each sample, ΔCt was calculated as $Ct_{target} - Ct_{reference}$. ΔΔCt was calculated as ΔCt_{sample} − mean $\Delta Ct_{control}$, using the appropriate serum or tissue control group. Relative expression was then calculated as $2^{-\Delta\Delta Ct}$. Values greater than 1 indicate higher expression and values less than 1 indicate lower expression relative to the corresponding control calibrator.

Statistical analysis

Analyses were performed using SPSS version 24. All tests were two-sided, and $P < .05$ was considered nominally significant. Between-group expression comparisons were performed on ΔCt values with the Mann–Whitney U test; $2^{-\Delta\Delta Ct}$ values were used for descriptive fold-change presentation. Pairwise associations among miR-21, miR-30, miR-125, and EGFR were assessed with Pearson correlation coefficients on ΔCt values in TNBC participants only (pairwise deletion); controls were not included. Because six pairwise correlations were examined, a Bonferroni-adjusted significance threshold of $\alpha = .05/6 = .0083$ was also considered as a multiplicity sensitivity analysis.

ROC analyses evaluated each marker separately using $2^{-\Delta\Delta Ct}$ values. Cutoffs therefore represent fold-change values. For upregulated markers (miR-21 and EGFR), larger test values were treated as more indicative of TNBC; for downregulated markers (miR-30 and miR-125), test direction was inverted so that lower expression indicated TNBC. Optimal cutoffs were selected with the Youden index. Because of limited RNA availability, assay-specific ROC sample sizes were miR-21, 28 TNBC cases and 17 controls; miR-30, 30 and 17; miR-125, 30 and 17; and EGFR, 25 TNBC tissues and 19 benign controls. No bootstrap correction, cross-validation, or external validation was performed; therefore, AUC, sensitivity, and specificity are apparent in-sample estimates.[16]

RESULTS

Participant flow and clinicopathological distribution

The hospital-based cohort comprised 105 women with histopathologically confirmed HER2-negative breast cancer recruited from April 2024 to November 2025. Thirty-two women met the local immunohistochemical definition of TNBC and entered the molecular analyses, whereas the remaining 73 women with non-TNBC HER2-negative breast cancer contributed only to descriptive clinicopathological characterization. Paired serum and tumor-tissue specimens were obtained from all 32 TNBC participants. Because RNA was unavailable in selected specimens, analyzable counts varied by assay: miR-21 included 28 TNBC cases and 17 healthy serum controls (45 participants); miR-30 included 30 cases and 17 controls (47 participants); miR-125 included 30 cases and 17 controls (47 participants); and tissue EGFR included 25 TNBC tumor specimens and 19 histopathologically confirmed benign breast-tissue controls (44 participants). Thus, healthy female serum controls were used only for the circulating-miRNA comparisons, whereas benign breast tissue was the comparator for the EGFR analysis.

Luminal A-like tumors were the most frequent local IHC-based subtype (46.7%, $n = 49$), followed by TNBC (30.5%, $n = 32$) and Luminal B-like (HER2-negative) tumors (22.9%, $n = 24$) (Table 2). Because the exact local ER, PR, and Ki-67 thresholds were not available, Table 2 presents the recorded surrogate definitions without adding unverified cutoffs.

Stage I accounted for 47.6% ($n = 50$) of the overall cohort, followed by stage II (32.4%, $n = 34$), stage III

Table 1. Primer Sequences

Target	Primer type	Sequence (5′→3′)
miR-21	Stem-loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTAT TCGCACTGGATACGACCAACA
miR-21	Forward	TAGCTTATCAGACTGATGTTGA
miR-21	Universal reverse	GTGCAGGGTCCGAGGT
miR-30a	Stem-loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTAT TCGCACTGGATACGACCTTCCA
miR-30a	Forward	TGTAAACATCCTCGACTGGAAG
miR-30a	Universal reverse	GTGCAGGGTCCGAGGT
miR-125b	Stem-loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTAT TCGCACTGGATACGACACAA
miR-125b	Forward	TCCCTGAGACCCTAACTTGTGA
miR-125b	Universal reverse	GTGCAGGGTCCGAGGT
U6	Forward	CTCGCTTCGGCAGCACA
U6	Reverse	AACGCTTCACGAATTTGCGT
EGFR	Forward	AGGCACGAGTAACAAGCTCAC
EGFR	Reverse	ATGAGGACATAACCAGCCACC
GAPDH	Forward	AGAAGGCTGGGGCTCATTTG
GAPDH	Reverse	AGGGGCCATCCACAGTCTTC

Table 2. Distribution of Locally Assigned IHC-Based Molecular Subtypes (HER2-Negative, *N* = 105)

Subtype	Recorded IHC definition	*n*	%
Luminal A-like	ER-positive and/or PR-positive; HER2-negative; lower-proliferative local category	49	46.7
Luminal B-like (HER2-negative)	ER-positive and/or PR-positive; HER2-negative; PR-low and/or higher-proliferative local category	24	22.9
TNBC	ER-negative; PR-negative; HER2-negative	32	30.5

Abbreviations: ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; PR, progesterone receptor; TNBC, triple-negative breast cancer. Exact ER, PR, and Ki-67 cutoffs were not available for retrospective verification.

Table 3. Tumor-Stage Distribution According to Locally Assigned Molecular Subtype

Subtype	Stage I	Stage II	Stage III	Stage IV
Luminal A-like (*n* = 49)	30 (61.2%)	13 (26.5%)	5 (10.2%)	1 (2.1%)
Luminal B-like, HER2-negative (*n* = 24)	11 (45.8%)	9 (37.5%)	3 (12.5%)	1 (4.2%)
TNBC (*n* = 32)	9 (28.1%)	12 (37.5%)	7 (21.9%)	4 (12.5%)

Table 4. Available Characteristics of the TNBC Molecular Cohort (*n* = 32)

Domain	Characteristic	*n* (%)
Disease stage	Stage I	9 (28.1)
Disease stage	Stage II	12 (37.5)
Disease stage	Stage III	7 (21.9)
Disease stage	Stage IV	4 (12.5)
Specimen availability	Paired serum and tumor tissue from the same participant	32 (100)

(14.3%, *n* = 15), and stage IV (5.7%, *n* = 6). TNBC had a larger proportion of stage III–IV disease than the two hormone-receptor-positive local categories (Table 3).

The available characteristics of the 32-case TNBC molecular cohort are summarized in Table 4. The table is restricted to variables that could be verified from the supplied study records.

Age, menopausal status, tumor size, nodal status, histological grade and subtype, lymphovascular invasion, treatment status at sampling, metastatic-site details, and BRCA status were not available for complete reporting and were not imputed.

Serum miRNA expression

Serum miR-21, miR-30, and miR-125 expression levels differed between TNBC cases and healthy serum controls on ΔCt-based Mann–Whitney testing (all $P < .001$). Relative to the healthy-control calibrator, miR-21 was 4.30-fold higher (ΔΔCt = −2.11), whereas miR-30 and miR-125 were lower at 0.02-fold (ΔΔCt = 5.61) and 0.13-fold (ΔΔCt = 3.00), respectively (Table 5).

Table 5. Relative Expression of Serum miRNAs in TNBC Cases and Healthy Serum Controls

Biomarker	Group	*n*	Mean ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$	*P* value
miR-21	TNBC	28	14.90	−2.11	4.30	<.001
miR-21	Healthy serum control	17	17.01	Reference	1.00	
miR-30	TNBC	30	5.45	5.61	0.020	<.001
miR-30	Healthy serum control	17	−0.17	Reference	1.00	
miR-125	TNBC	30	6.17	3.00	0.125	<.001
miR-125	Healthy serum control	17	3.17	Reference	1.00	

Statistical comparisons used ΔCt values and the Mann–Whitney *U* test. Relative expression was calculated with the healthy serum-control group as calibrator.

Table 6. Relative Tissue *EGFR* mRNA Expression in TNBC and Benign Breast-Tissue Controls

Biomarker	Group	*n*	Mean ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$	*P* value
EGFR	TNBC tumor tissue	25	12.50	−2.74	6.69	<.001
EGFR	Benign breast tissue	19	15.25	Reference	1.00	

Statistical comparison used ΔCt values and the Mann–Whitney *U* test. Relative expression was calculated with benign breast tissue as the calibrator.

Tissue *EGFR* mRNA expression

Tumor-tissue *EGFR* mRNA expression was higher in TNBC than in benign breast-tissue controls on ΔCt-based Mann–Whitney testing ($P < .001$). Relative expression was 6.69-fold higher in TNBC tissue (ΔΔCt = −2.74) (Table 6). This comparison does not involve healthy serum controls.

Exploratory pairwise correlations

In TNBC participants only ($n = 23$–30 pairs; controls not included), serum miR-30 and miR-125 were positively correlated ($r = 0.601$, $P < .001$), remaining significant at the Bonferroni-adjusted threshold of $P < .0083$. All other correlations were weak and nonsignificant ($|r| \leq 0.317$, all $P \geq .12$) (Figure 1).

Exploratory ROC analysis

The ROC analyses used relative expression ($2^{-\Delta\Delta Ct}$) values and assay-specific samples (Figure 2). For miR-21, the apparent AUC was 0.876 (95% CI, 0.760-0.992; $P < .001$). A cutoff of 3.568 yielded 89.3% sensitivity (25 of 28 TNBC cases) and 88.2% specificity (15 of 17 controls).

For miR-30, the apparent AUC was 1.000 ($P < .001$), with lower expression indicating TNBC and complete separation between patients and controls; the 95% CI was 1.000–1.000 as reported by the software and was not informative because of complete separation. A Youden-optimal cutoff of 0.064 or less yielded 100% sensitivity (30 of 30) and 100% specificity (17 of 17) (Figure 2B).

For miR-125, the apparent AUC was 1.000 (95% CI, 1.000–1.000 as reported by the software; not informative because of complete separation; $P < .001$); a cutoff of 0.178 or less yielded 100% sensitivity (30 of 30) and 100% specificity (17 of 17).

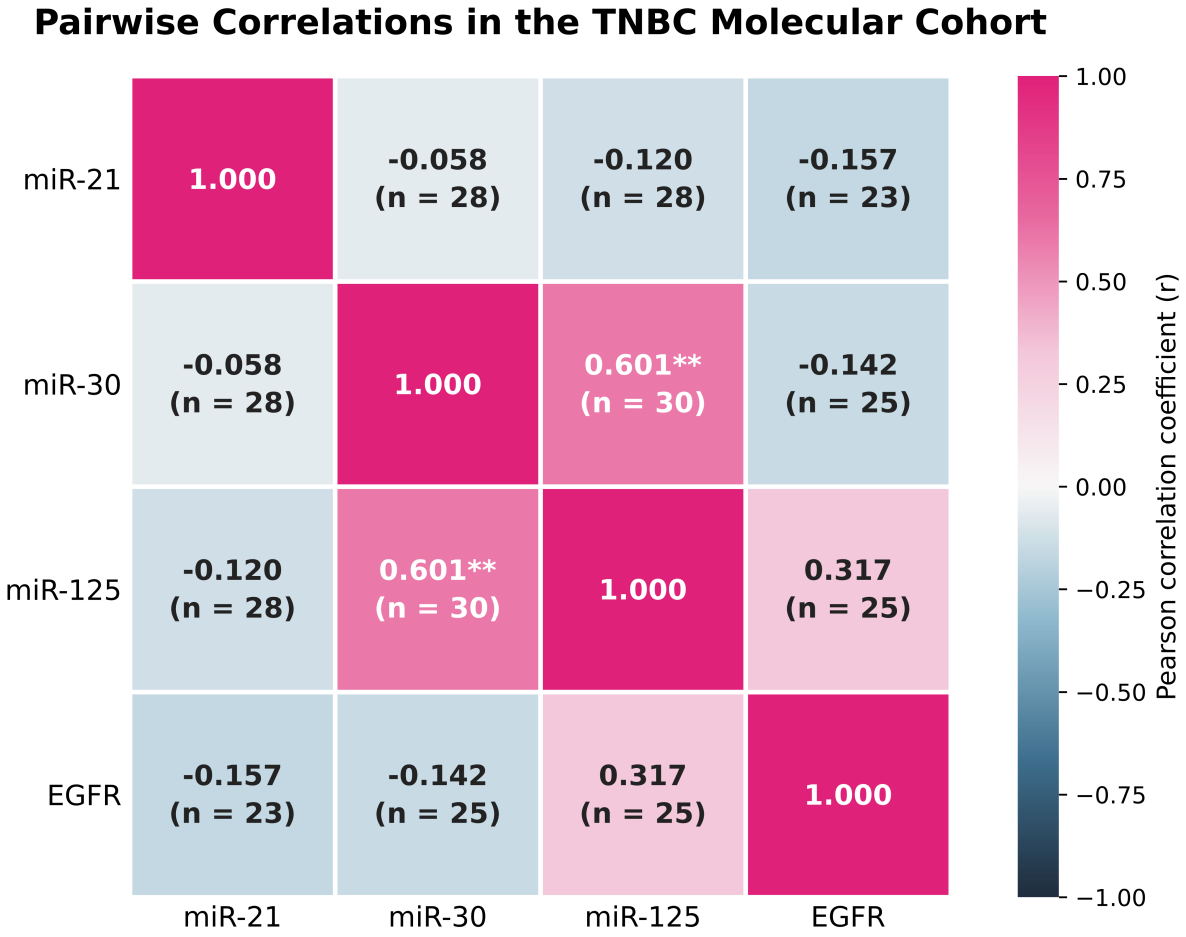
For tissue EGFR, the apparent AUC was 0.865 (95% CI, 0.751-0.980; $P < .001$), with higher expression indicating TNBC. A Youden-optimal cutoff of 2.704 or higher yielded 72.0% sensitivity (18 of 25) and 100% specificity (19 of 19) (Figure 2D).

The two AUC values of 1.000 represent complete separation in these small, selected, in-sample datasets. Because no resampling or independent validation was performed, they should not be interpreted as expected clinical performance. Moreover, the EGFR ROC analysis used benign tissue rather than healthy serum controls, so its AUC is not directly comparable with the serum-miRNA AUCs.

DISCUSSION

In this hospital-based case-control study, the descriptive cohort of 105 HER2-negative breast cancers comprised Luminal A-like, Luminal B-like (HER2-negative), and TNBC categories. The TNBC subgroup had a larger proportion of stage III–IV disease than the locally assigned luminal categories, consistent with the aggressive clinical behavior frequently reported for TNBC.[2,25,26] The original Stage 0–I label could not be verified as including a separate Stage 0 diagnosis and was conservatively presented as stage I. Similarly, exact local ER, PR, and Ki-67 cutoffs were unavailable; subtype comparisons should therefore be interpreted as descriptive classifications recorded in routine practice.

The present data showed higher serum miR-21 and lower serum miR-30 and miR-125 expression in TNBC than in healthy serum controls. These findings are directionally consistent with literature describing miR-21 as an oncogenic miRNA and miR-30 and miR-125 family members as context-dependent tumor-suppressive regulators.[4–11,17] Mechanisms involving PTEN, PDCD4, epithelial–mesenchymal transition, ERBB2, and ERBB3 have been reported in previous experimental studies; none of these pathways or targets was measured directly here. They therefore provide



** P < .001; significant after Bonferroni correction for six comparisons (α = .0083).
TNBC participants only; controls not included. Correlations computed on ΔCt values.

Figure 1. Pairwise Pearson Correlations Among Serum miRNAs and Tissue EGFR in the TNBC Molecular Cohort Pearson correlations were computed on ΔCt values using pairwise deletion. Analyses were restricted to TNBC participants; controls were not included. The miR-30/miR-125 correlation ($r = 0.601$, $P < .001$) remained significant after Bonferroni correction for six comparisons ($\alpha = .0083$). Correlations involving EGFR are cross-compartment associations and do not demonstrate a direct regulatory relationship.

literature-based hypotheses rather than mechanistic results of the present dataset.

Tissue *EGFR* mRNA was higher in TNBC than in benign breast tissue, which is compatible with previous reports of EGFR dysregulation in TNBC.[12–14] However, serum miRNAs and tissue EGFR were measured in different biological compartments and normalized to different controls. Although serum and tumor tissue samples were paired within each TNBC participant, the weak, nonsignificant correlations between tissue EGFR expression and each serum miRNA do not support a coordinated expression pattern in this cohort. Cross-compartment correlations cannot establish direct biological regulation, especially without EGFR protein assessment or functional experiments. The positive miR-30/miR-125 correlation in TNBC may reflect coordinated down-regulation but may also partly reflect shared U6 normalization; it should be regarded as hypothesis-generating.

The individual-marker ROC estimates were exploratory. The complete apparent separation observed for miR-30 and miR-125 is unusual in heterogeneous clinical disease and is particularly vulnerable to spectrum bias, selection bias, small-sample optimism, and overfitting.[15,16] Assay-specific case and control counts were reported to clarify the attainable sensitivity and specificity fractions. Because no cross-validation, bootstrap correction, or external cohort was available, the observed AUCs should not be interpreted as validated diagnostic accuracy. In addition, no multivariable equation, calibration analysis, or validation was performed; consequently, this study does not demonstrate a combined miRNA–EGFR diagnostic panel.

Normalization is another important consideration. U6 was used as the endogenous control for serum miRNAs in the original protocol, but its stability was not evaluated in this cohort. Published studies show that U6 can vary in serum, and contemporary recommendations favor study-specific validation of stable endogenous controls, often combined with exogenous spike-ins and careful preanalytical

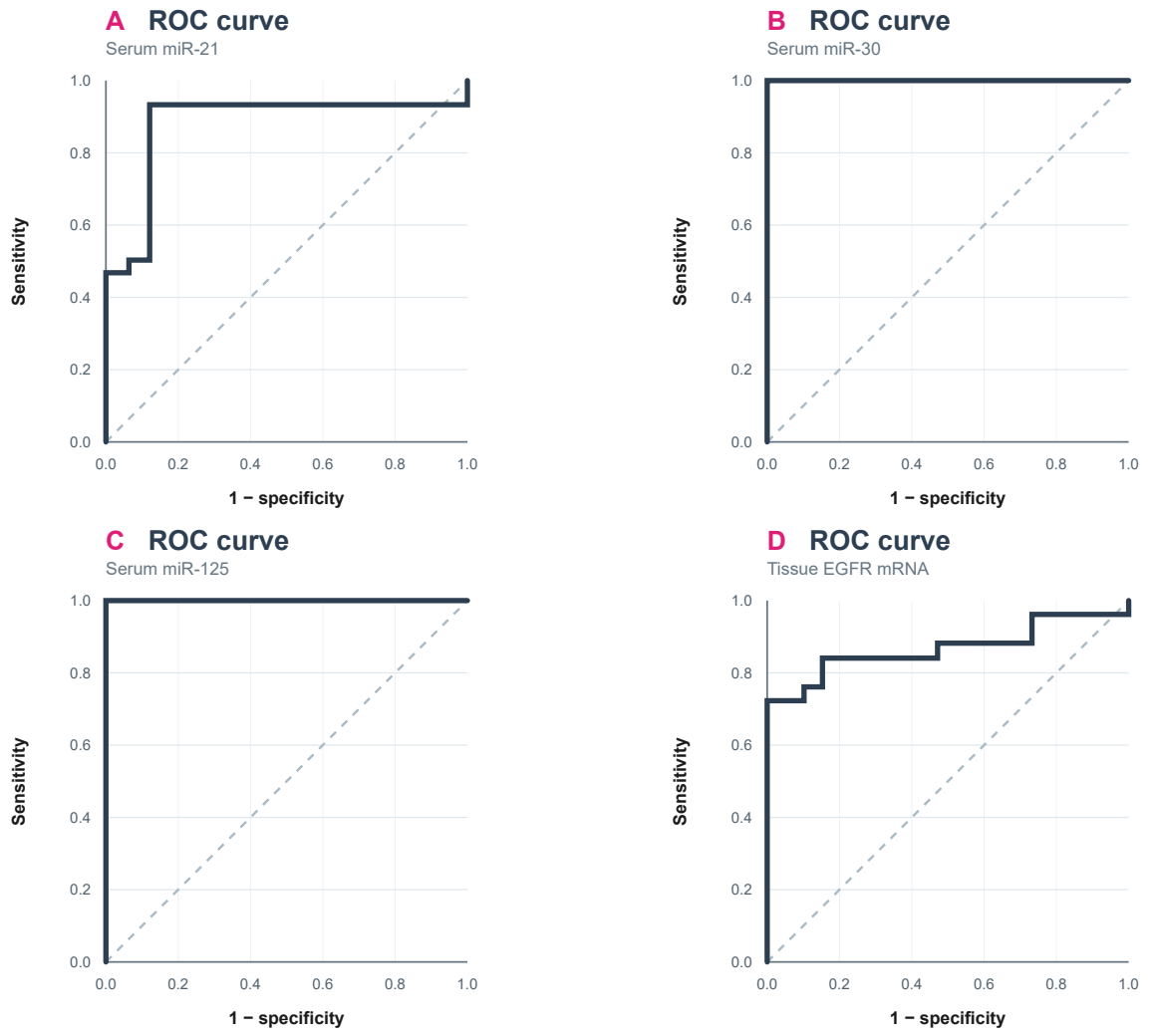


Figure 2. Receiver Operating Characteristic Curves for Individual Markers
A, Serum miR-21; B, serum miR-30; C, serum miR-125; and D, tissue *EGFR* mRNA. Curves show apparent in-sample discrimination and were not cross-validated or externally validated. Serum miRNAs were compared with healthy serum controls; tissue EGFR was compared with benign breast tissue.

quality control.[19–21] The absence of quantitative hemolysis assessment and incomplete documentation of serum processing may also influence circulating-miRNA estimates. These limitations do not prove that the observed group differences are spurious, but they reduce confidence in their magnitude and reproducibility.

Future work should use prospectively standardized collection, quantitative hemolysis assessment, validated normalization strategies, detailed clinical covariates, and independent multicenter cohorts. Emerging platforms such as microneedle-based diagnostics, two-dimensional hybrid nanomaterials, and cell-mediated delivery systems illustrate broader directions in cancer detection and therapeutic delivery.[27–29] These technologies were not assessed in this study; they are discussed solely as potential future translational applications and should not be construed as direct evidence for the biomarkers identified here.

Limitations and future directions

This study has several limitations. First, the molecular cohort was small and hospital-based, and the exclusion of 73 non-TNBC breast cancers from molecular analysis limits generalizability and prevents evaluation of TNBC specificity relative to other breast cancer subtypes. Second, serum miRNAs and tissue EGFR were measured in different biological compartments and against different control sources. Third, important case and control characteristics—including menopausal status, body mass index, reproductive history, smoking, medications, inflammatory conditions, treatment status at sampling, tumor size, nodal status, grade, histological subtype, lymphovascular invasion, metastatic status, and BRCA status—were unavailable or incompletely recorded. The newly supplied control age range of 30–40 years could not be reconciled with the age values reported in the original manuscript; the previous claim of age matching and

the unsupported age-by-stage ANOVA were therefore removed. Residual confounding, including by age, cannot be excluded.

Fourth, quantitative hemolysis assessment, complete serum-handling details, RNA integrity number, tumor-cell percentage, microdissection, stromal or immune-cell contamination, and time-to-stabilization data were unavailable. Fifth, U6 and GAPDH stability was not formally evaluated using tools such as geNorm or NormFinder. Sixth, the correlation analyses were exploratory, based on 23 to 30 paired TNBC observations, and the miR-30/miR-125 association may partly reflect shared U6 normalization. Finally, ROC estimates were calculated on small assay-specific datasets without cross-validation, bootstrap optimism correction, calibration analysis, or external validation. These limitations preclude clinical implementation and require prospective confirmation with transparent reporting and prespecified analysis plans.

CONCLUSION

TNBC cases showed higher serum miR-21, lower serum miR-30 and miR-125, and higher tumor-tissue *EGFR* mRNA relative to their respective control groups. These parallel findings may inform future TNBC biomarker research, but they do not establish a unified circulating miRNA–EGFR profile or a validated combined diagnostic panel. Larger prospective studies with appropriate control selection, standardized preanalytics, validated reference genes, complete clinical covariates, and independent validation are required before clinical utility can be assessed.

ETHICAL CONSIDERATIONS

The study was approved by the Scientific Committee of the Ministry of Health (approval no. 41113; 11 December 2023). Written informed consent was obtained from all participants before enrollment. All procedures were conducted in accordance with the Declaration of Helsinki.

DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request, subject to applicable ethical and institutional restrictions.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

FUNDING

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AI DISCLOSURE

AI-assisted language tools were used to support language editing, revision drafting, formatting, and organization of responses to reviewers. The authors remain responsible for the scientific content, data accuracy, analysis, interpretation, and final approval of the manuscript.

AUTHOR CONTRIBUTIONS

SMH: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization, Project administration, Funding acquisition. RMA: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Project administration, Funding acquisition. NAH: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Supervision, Project administration, Funding acquisition. RHA: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

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