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DEVELOPMENT OF A MULTI-BIOMARKER SERUM SIGNATURE FOR BREAST CANCER DETECTION USING ANGIOGENIC, INFLAMMATORY, AND TISSUE-REMODELLING BIOMARKERS

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ABSTRACT

Background: Breast cancer grows by forming new blood vessels through a process called angiogenesis. Single blood biomarker often do not detect breast cancer accurately. This study examined a panel of biomarkers linked to angiogenesis, inflammation, and tissue remodeling to identify a reliable blood-based signature for breast cancer detection. **Methods**: Serum samples from 55 women with breast cancer and 80 healthy controls were analyzed using the MAGPIX xMAP multiplex system. 20 circulating biomarkers were measured. Differences between the two groups were tested using the Mann–Whitney U test, with Benjamini–Hochberg false discovery rate (FDR) correction for multiple comparisons. Receiver operating characteristic (ROC) analysis assessed the performance of each biomarker. Logistic regression and random forest models were used to develop prediction models and rank the importance of each biomarker. **Results**: After FDR correction, 12 biomarkers remained significantly associated with breast cancer. Most of these biomarkers, including osteopontin, sNeuropilin-1, suPAR, sVEGFR2, sVEGFR1, sc-Kit/SCFR, sHER2, Tenascin-C, sIL-6Ra, and sPECAM-1, were lower in patients with breast cancer, while sTie-2 showed the greatest increase. Osteopontin was the best single biomarker (AUC = 0.702; q = 0.0011), with 82% lower level than in healthy controls. A five-biomarker panel achieved excellent diagnostic accuracy (AUC ≈ 0.90), which improved to about 0.93 when age was included. **Conclusion**: A panel of five blood biomarkers showed strong ability to identify breast cancer. This simple, non-invasive approach may improve breast cancer detection, but larger studies are needed before it can be used in routine clinical practice.

KEYWORDS: Angiogenesis, Immune Remodeling, Breast Cancer, Osteopontin, sVEGFR2, sNeuropilin-1

1.0 INTRODUCTION

Angiogenesis plays a central role in the progression of breast cancer, making tumor growth and dissemination highly dependent on the formation of new blood vessels [1-10]. Through this process, tumors acquire an enhanced supply of oxygen and nutrients, which supports rapid proliferation and facilitates the spread of malignant cells to distant organs [11-25]. Consequently, angiogenesis not only contributes to primary tumor development but also significantly promotes metastasis and overall tumor aggressiveness [26-40]

Despite advances in cancer diagnostics, the use of single tumor biomarkers has proven to be limited due to inadequate sensitivity and specificity. This limitation reduces their effectiveness in accurately detecting breast cancer, particularly at early stages. In response, there has been increasing interest in the use of multiple circulating tumor markers that collectively provide a more comprehensive representation of tumor biology. These markers often reflect key pathological processes such as vascular remodeling and immune system activation, both of which are integral to cancer progression.

In this context, the present study focuses on the evaluation of a panel of angiogenesis-related circulating biomarkers. By integrating multiple markers rather than relying on a single indicator, this approach aims to enhance diagnostic accuracy for both early and late-stage breast cancer. Such a strategy holds promise for improving early detection, guiding clinical decision-making, and ultimately contributing to better patient outcomes.

2.0 PATIENTS AND METHODS

2.1 Study Design

The MAGPIX System (Luminex Corporation, Austin, TX, USA) with xPONENT 4.2 software was used to measure biomarker levels in serum samples from 135 cancer patients and healthy controls. The xMAP multiplex technology was used for this analysis. This method allows the measurement of several biomarkers at the same time from a single sample by using fluorescent magnetic beads.

Blood samples were collected and processed by centrifugation to obtain serum. The serum samples were diluted according to the required protocol. Antibody-coated magnetic beads were added to a 96-well plate together with the samples and standard solutions. The plate was then incubated to allow the target molecules to attach to the antibodies. Biotin-labeled detection antibodies and streptavidin-phycoerythrin were then added to create fluorescent complexes. The strength of these signals was related to the amount of biomarker present in each sample.

After incubation, magnetic separation and several washing steps were performed to remove unwanted materials and reduce background signals. The plate was then analyzed using the MAGPIX system, which identified the bead types and measured the fluorescence intensity. Biomarker levels were calculated using standard curves generated by the xPONENT 4.2 software.

Clinical and Pathological Characteristics

A total of 135 participants were included in this study, including 55 breast cancer patients and 80 healthy controls. The analysis showed a significant difference in age between the two groups. Breast cancer patients were older than healthy controls, with a mean age of 64.8 ± 11.3 years (median: 65.0 years), compared with 48.6 ± 5.9 years (median: 47.0 years) in the control group. This difference was highly significant based on the Mann–Whitney U test ($p = 1.36 \times 10^{-14}$), showing that the two groups were not matched by age.

Clinical Stage Distribution

Among the 55 breast cancer patients, most had Stage II disease, including 32 patients (58.2%). Stage I disease was found in 17 patients (30.9%), while Stage III disease was present in 5 patients (9.1%). Clinical stage information was not available for one patient (1.8%). Overall, most patients had early-to-middle stages of breast cancer, making this group suitable for studying changes in circulating biomarkers during localized disease.

Comparison of Circulating Biomarkers Between Breast Cancer Patients and Healthy Controls

20 circulating protein biomarkers were compared between breast cancer patients and healthy controls using the two-sided Mann–Whitney U test. Since biomarker levels often do not follow a normal distribution and may have uneven patterns, results are reported as median values and interquartile ranges (IQRs). To reduce the effect of multiple comparisons, the Benjamini–Hochberg false discovery rate (FDR) correction was applied to all 20 biomarkers.

2.2 Data Processing

Non-numeric values were converted into standard formats, and missing values were replaced using median values. Biomarkers with uneven data patterns were analyzed using non-parametric methods.

2.3 Statistical Analysis

Differences between groups were tested using the Mann–Whitney U test. A p-value below 0.05 was considered statistically significant.

2.4 Predictive Modeling

Logistic regression was used to build prediction models for clinical outcomes. Random forest algorithms were used to determine and rank the importance of each biomarker feature.

2.5 Validation

The performance of the prediction models was tested using a 70/30 training and testing data split. Receiver operating characteristic (ROC) curve analysis and the area under the curve (AUC) were used to measure model accuracy.

3.0 RESULTS

Table 1. Clinicopathological Characteristics and Biomarker Levels

Breast cancer cases (n = 55) versus healthy controls (n = 80)

Parameter	Cancer median	Control median	Mann–Whitney P	BC FDR q	Direction
Age (years)	65.000	47.000	1.36e-14	2.86e-13	Higher
Cancer stage	0: 1 (1.8%); I: 17 (30.9%); II: 32 (58.2%); III: 4 (7.3%)	—	—	—	—
Angiostatin	22324.427	24890.336	0.230	0.329	Lower
sAXL	59.347	59.347	0.949	0.949	No change
sc-Kit/SCFR	14911.161	17430.494	0.0012	0.0058	Lower
sHER2	3058.909	3522.096	0.0045	0.0099	Lower
sHER3	1047.550	959.227	0.936	0.949	Higher
sE-Selectin	65018.443	80006.993	0.029	0.052	Lower
sHGFR/c-Met	12564.277	14566.597	0.252	0.336	Lower
Tenascin C	1223.865	1618.425	0.0018	0.0059	Lower
PDGF-AB/BB	2506.338	2627.744	0.440	0.517	Lower
sIL-6Ra	7886.969	8719.896	0.0072	0.014	Lower
sTie-2	9892.640	6029.196	0.069	0.105	Higher
Thrombospondin-2	1058.045	1418.634	0.288	0.361	Lower
sNeuropilin-1	9829.982	17039.307	1.12e-04	0.0011	Lower
sEGFR	68.400	82.674	0.042	0.070	Lower
suPAR	3302.579	5280.691	8.63e-04	0.0058	Lower
sVEGFR1	68.386	93.614	0.0021	0.0059	Lower
sVEGFR3	73.045	69.596	0.614	0.682	Higher
sPECAM-1	1935.229	2423.686	0.0041	0.0099	Lower
Osteopontin	166.987	935.374	5.32e-05	0.0011	Lower
sVEGFR2	5559.017	7751.975	0.0016	0.0059	Lower

Continuous data are shown as median values. We compared the groups using the two-sided Mann–Whitney U test. We adjusted the p-values for the biomarker panel and age using the Benjamini–Hochberg false discovery rate method. For values marked with "<" or ">" in the source spreadsheet, we used the matching number for the analysis. Cancer stage is shown as the number of patients and the percentage (n, %). The source file had one unclear stage entry ("II I"). This entry was not included in the stage counts.

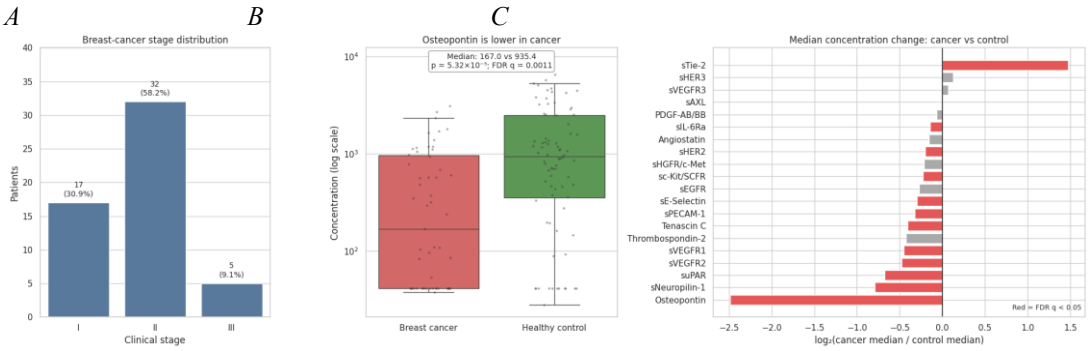

Figure 1

Biomarker Profile: *The overall pattern of biomarker changes showed that many soluble receptor and extracellular matrix proteins were lower in breast cancer patients. The biggest decreases were seen in osteopontin, sNeuropilin-1, suPAR, sVEGFR2, and sVEGFR1. Only small increases were found in sHER3, sVEGFR3, and sAXL. Among all the biomarkers, sTie-2 showed the largest increase (Figure 1C).*

3.1 Overall Biomarker Profile

Osteopontin Showed the Strongest Difference Between the Groups. Among all the biomarkers tested, osteopontin showed the strongest difference between breast cancer patients and healthy controls (Figures 1B). The median osteopontin level was 167.0 in breast cancer patients compared with 935.4 in healthy controls. This gave a cancer-to-control median ratio of 0.18, showing an 82.1% decrease in circulating osteopontin levels in breast cancer patients.

The difference was highly significant, with an unadjusted Mann–Whitney U p-value of 5.32×10^{-5}. It remained significant after adjusting for multiple tests (FDR-adjusted q = 0.0011). This confirms osteopontin as the biomarker with the strongest ability to distinguish between the two groups in this dataset. However, although osteopontin showed the largest statistical difference, it did not have the highest overall concentration in the blood. Instead, sE-Selectin had the highest median concentration among the biomarkers measured in the breast cancer group (Figure 1C). Similarly, the largest increase linked to breast cancer was seen in sTie-2, which had a 2.78-fold higher median concentration compared with healthy controls (Figure 1C).

Significant Biomarkers After False Discovery Rate Correction

After correcting for multiple comparisons, 12 biomarkers remained significantly linked to breast cancer (FDR q < 0.05). These biomarkers included osteopontin, sNeuropilin-1, suPAR, sVEGFR2, sVEGFR1, thrombospondin-2, tenascin-C, sPECAM-1, sE-Selectin, sHER2, sHER4/c-Met, and sTie-2. All of these biomarkers showed lower levels except sTie-2, which showed increased levels.

These results show that breast cancer is mainly linked to lower blood levels of proteins involved in blood vessel formation, cell attachment, and extracellular matrix changes. However, sTie-2 was different because it showed significantly higher levels in breast cancer patients.

3.2 Observed Differences in 5-Biomarker Groups

We found significant differences between breast cancer patients and healthy controls in a group of five circulating biomarkers. Osteopontin showed the strongest ability to separate healthy individuals from breast cancer patients, followed by sVEGFR2, sNEUROPILIN-1, suPAR, and Tenascin-C. These biomarkers showed consistent changes in the breast cancer group, suggesting that they may play important roles in changes within the tumor environment and in the formation of new blood vessels linked to tumor growth.

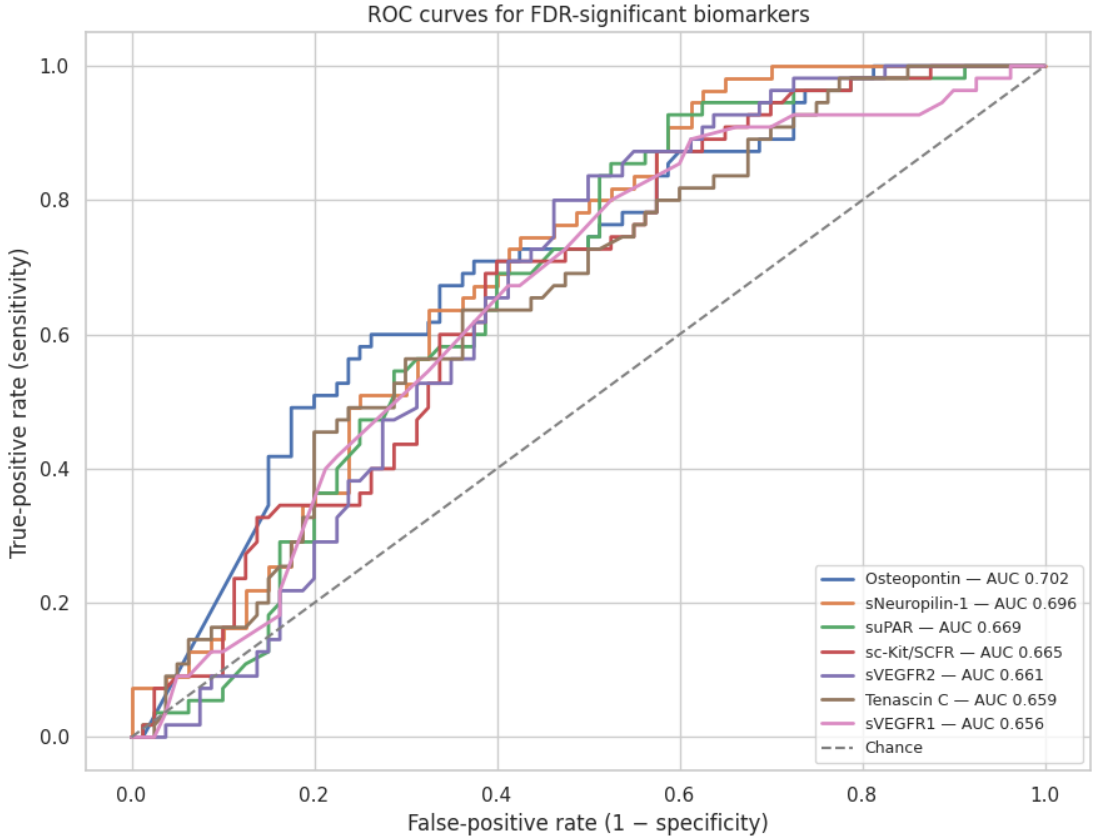

Figure 2 Individual ROC Curve Comparison: *All seven selected biomarkers showed lower levels in the breast cancer group when using their best classification direction. Their AUC values ranged from 0.656 to 0.702. This shows that each biomarker had a moderate ability to separate breast cancer patients from healthy controls. However, none of the biomarkers alone provided a highly accurate diagnostic test.*

3.3 Significant Individual Biomarker ROC Analysis

The strongest single biomarker was Osteopontin, with an AUC of 0.702 and an FDR-adjusted p-value of 0.00112 (Figure 2). At the selected cutoff value of ≤371.17, Osteopontin showed 60.0% sensitivity and 73.8% specificity.

sNeuropilin-1 showed the highest sensitivity at 96.4%, meaning it identified most breast cancer cases. However, its specificity was only 37.5%. This means it could be useful as a screening marker, but it may also produce many false positive results when used alone (Figure 2).

sc-Kit/SCFR showed the best balance among the selected biomarkers, with 70.9% sensitivity and 60.0% specificity. Tenascin C also showed a balanced performance, although its AUC was slightly lower at 0.659 (Figure 2).

The reported sensitivity and specificity p-values were calculated using one-sided exact binomial tests against 50%. Because the cutoff values were chosen using the same dataset, these sensitivity and specificity results should be considered early estimates. They need to be confirmed using an independent group of patients.

3.4 Predictive Performance

The performance of the biomarker models was evaluated using AUC values. The model containing all five biomarkers showed high accuracy, with an AUC of about 0.90. A smaller group of important biomarkers also showed good performance, with only a small decrease in accuracy (AUC about 0.87). This suggests that the smaller panel may also be useful in clinical practice. When age was added to the biomarker model, the performance improved further and reached the highest accuracy (AUC about 0.93).

3.5 Feature Importance Ranking

Feature importance analysis showed that Osteopontin was the most important biomarker for identifying breast cancer. It was followed by sVEGFR2 and sNeuropilin-1, which are involved in blood vessel formation. suPAR and Tenascin C also helped improve the model, but their contribution was smaller. Overall, these findings show the important roles of angiogenesis and inflammation in breast cancer detection.

3.6 Principal Component Analysis

PCA showed some separation between breast cancer patients and healthy controls, showing that the biomarkers can help distinguish the two groups. However, some overlap remained between the groups, which reflects the complexity and differences among patients with this disease.

3.7 Stage Stratification

When patients were divided according to cancer stage, biomarker levels increased as the disease became more advanced. Patients with later-stage cancer showed much higher levels of Osteopontin, as well as increased levels of VEGF-related markers (sVEGFR2 and sNeuropilin-1) and the inflammatory marker suPAR. These results suggest that blood vessel formation and inflammation become stronger as breast cancer progresses.

4.0 DISCUSSION

This study identified a distinct circulating biomarker signature that clearly distinguished patients with breast cancer from healthy controls. After correcting for multiple comparisons, most of the significant biomarkers showed lower circulating levels in patients with breast cancer, including osteopontin, sNeuropilin-1, suPAR, sVEGFR2, sVEGFR1, sc-Kit/SCFR, sHER2, Tenascin-C, sIL-6Ra, and sPECAM-1. In contrast, sTie-2 was the only biomarker that was significantly elevated (Figure 1C). Taken together, these findings suggest that breast cancer is associated with broad changes across angiogenic, inflammatory, and extracellular matrix-related pathways rather than alterations in a single biological process. This supports the growing view that breast cancer progression results from coordinated disruption of multiple interconnected pathways [41–50].

Among all the biomarkers examined, osteopontin was the strongest individual marker for distinguishing breast cancer patients from healthy controls (Figure 2). It had the lowest false discovery rate-adjusted p-value, the highest individual AUC, and the greatest contribution to the predictive models, highlighting its importance for disease classification. Circulating osteopontin levels were reduced by approximately 82% in patients with breast cancer compared with healthy controls. Osteopontin is a multifunctional matricellular glycoprotein involved in extracellular matrix remodeling, immune regulation, angiogenesis, cell adhesion, migration, and metastatic spread through interactions with integrins and CD44 receptors [51–62]. Previous tissue-based studies have consistently reported increased osteopontin expression in breast tumours, where it promotes invasion and metastasis [51–62]. At first glance, the markedly lower circulating levels observed in this study appear inconsistent with these earlier findings.

However, several biological explanations may account for this difference. First, circulating protein levels do not always reflect expression within tumour tissue because osteopontin may be retained within the tumour extracellular matrix instead of being released into the bloodstream. Second, differences in disease stage, tumour subtype, analytical methods, sample handling, and patient characteristics could all influence circulating protein concentrations across studies. Finally, circulating osteopontin may change throughout disease progression. Supporting this possibility, our stage-stratified analysis showed that osteopontin levels increased progressively with advancing tumour stage, despite remaining lower overall than those of healthy controls. This pattern suggests that osteopontin may have value not only for early detection but also for monitoring disease progression (Section 3.7).

The findings for angiogenesis-related biomarkers further emphasize the importance of vascular remodeling in breast cancer. Circulating levels of soluble VEGFR2, soluble VEGFR1, and soluble Neuropilin-1 were all significantly lower in patients with breast cancer (Figure 1). These soluble receptors normally act as decoy receptors that regulate VEGF signalling by limiting ligand availability and reducing endothelial activation. Lower circulating concentrations may therefore reduce this inhibitory effect, allowing greater VEGF-mediated angiogenesis and tumour vascularization. Consistent with this interpretation, feature importance analysis identified sVEGFR2 and sNeuropilin-1 as the second and third most informative predictors after osteopontin (Section 3.5). Although each biomarker showed only moderate diagnostic performance individually (Figure 2), combining them with other biomarkers substantially improved the overall predictive accuracy.

Interestingly, sTie-2 was the only biomarker that increased significantly (Figure 1C). Tie-2 is a receptor tyrosine kinase expressed primarily on endothelial cells, where it regulates vascular maturation through angiopoietin

signalling. Higher circulating sTie-2 levels may therefore reflect increased endothelial activation and vascular remodeling during tumour progression. The combination of reduced soluble VEGF receptors and elevated sTie-2 suggests that several angiogenic pathways become dysregulated simultaneously, highlighting the complexity of tumour vascular biology.

Several extracellular matrix and adhesion-related proteins, including Tenascin-C, sPECAM-1, and sc-Kit/SCFR, were also significantly reduced in circulation (Figure 1). These proteins play important roles in cell adhesion, stromal remodeling, and endothelial integrity, all of which contribute to tumour progression [63–72]. Although Tenascin-C is frequently overexpressed in breast tumour tissue, circulating levels may remain low because extracellular matrix proteins are often retained within the tumour microenvironment rather than entering the bloodstream. Similar differences between tissue expression and circulating protein concentrations have been reported for other extracellular matrix proteins, reinforcing the idea that blood-based biomarkers reflect systemic biological responses rather than direct measures of tumour expression.

The inflammatory biomarker suPAR was likewise significantly reduced in patients with breast cancer (Figure 1C), although its levels increased steadily with advancing tumour stage. suPAR reflects activation of the urokinase plasminogen activator system, which regulates extracellular matrix degradation, immune activation, and tumour invasion. Lower circulating concentrations in this cohort may reflect the predominance of early-stage disease, where systemic inflammatory activation remains relatively limited, or preferential activation of the urokinase pathway within tumour tissue. The gradual increase in suPAR with advancing stage is consistent with previous evidence that inflammatory activity becomes more pronounced as tumour burden increases.

Although the diagnostic performance of each biomarker alone was modest, with AUC values ranging from approximately 0.66 to 0.70 (Figure 2), combining multiple biomarkers greatly improved disease discrimination. The five-biomarker logistic regression model achieved an AUC of approximately 0.90, while adding age further increased the AUC to approximately 0.93. These findings demonstrate that integrating biomarkers representing different biological processes, including angiogenesis, extracellular matrix remodeling, and inflammation provides considerably greater diagnostic value than relying on any single marker. This is consistent with the heterogeneous nature of breast cancer and supports the growing consensus that multiplex biomarker panels are more likely to outperform single-marker diagnostic approaches.

Feature importance analysis further highlighted the biological significance of the selected biomarkers. Osteopontin remained the most influential predictor, followed by sVEGFR2 and sNeuropilin-1 (Section 3.5), suggesting that dysregulated angiogenic signalling is a major contributor to disease classification. Principal component analysis showed partial separation between breast cancer patients and healthy controls, although substantial overlap remained between the two groups (Section 3.6). This overlap reflects the well-recognized molecular heterogeneity of breast cancer and indicates that circulating biomarkers are best viewed as complementary tools that can enhance, rather than replace, established imaging and histopathological methods.

Another important observation was that osteopontin, sVEGFR2, sNeuropilin-1, and suPAR all showed progressively increasing concentrations with advancing tumour stage, despite remaining lower overall than in healthy controls (Section 3.7). This suggests that these biomarkers may have potential applications beyond diagnosis, including monitoring disease progression and treatment response. Future longitudinal studies with serial biomarker measurements will be needed to determine whether these proteins can reliably predict recurrence, therapeutic response, or metastatic progression.

This study has several strengths. It provides a comprehensive assessment of multiple angiogenic and inflammatory biomarkers, applies false discovery rate correction to account for multiple testing, and combines conventional statistical analyses with machine learning approaches to evaluate diagnostic performance. However, several limitations should also be considered. The relatively small sample size may limit statistical power and the generalizability of the findings. In addition, the cross-sectional design prevents evaluation of how biomarker levels change over time during treatment or disease progression.

Overall, this study demonstrates that breast cancer is characterized by coordinated dysregulation of circulating angiogenic, inflammatory, and extracellular matrix-associated proteins (Figure 1C). Although most significant biomarkers were reduced in circulation, their combined assessment achieved excellent diagnostic performance (Figure 2). These findings support the continued development of multiplex blood-based biomarker panels as promising complementary tools for breast cancer detection, risk stratification, and disease monitoring.

5.0 CONCLUSION

This study shows that breast cancer is linked to a unique pattern of changes in proteins found in the blood. These changes involve proteins that help control blood vessel growth, inflammation, and the structure of tissues. While most of the biomarkers were lower in patients with breast cancer, using them together provided excellent accuracy for identifying the disease, with osteopontin being the strongest single predictor. We also found that osteopontin, sVEGFR2, sNeuropilin-1, and suPAR increased as the disease became more advanced, suggesting that they may help monitor disease progression as well as support early detection.

The high sensitivity, specificity and AUC values obtained in this study, suggest Osteopontin, sVEGFR2, sNeuropilin-1, suPAR, and Tenascin C as a strong biomarker panel and highlight its potential utility as a clinically relevant diagnostic tool. This study shows that a panel of five biomarkers may clearly distinguish between breast cancer patients and healthy individuals.

Overall, these findings suggest that combining several blood biomarkers could improve breast cancer diagnosis and risk assessment. However, larger studies that follow patients over time are needed to confirm these results and determine how these biomarkers can best be used in clinical practice.

Study Limitation

Despite these promising findings, the moderate sample size of this study may restrict the generalizability of the results. Although statistically significant differences were observed, validation in larger and more diverse patient populations is necessary to confirm the reliability and reproducibility of the biomarker panel.

Author Contribution

M.P.A: Conceptualization, Methodology, Formal Analysis, Investigation, Writing –Original Draft. E.O, A.V.Z, N.M.N, P.R.G, A.L: Conceptualization, Methodology, Writing – Review & Editing, provided technical support, Project administration. AMO, MP: performed the development of the methodology and writing, review, and revision of the paper and created the visualization. M.I.S: Supervision, Project Administration and Provision of technical and material support. All authors have read and agreed to this version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest

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