Elevated Plasma ACE2 and Shifted Ang 1-7/Ang II Ratios: A Novel Multi-Analyte Signature for Distinguishing Breast Cancer Patients from Healthy Controls—A Pilot Case-Control Study

Biwash Ghimire , Pradeep Giri , Susan Tavernier , Sarah E. Hobdey , Ali Aghazadeh-Habashi *

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Article

Elevated Plasma ACE2 and Shifted Ang 1-7/Ang II Ratios: A Novel Multi-Analyte Signature for Distinguishing Breast Cancer Patients from Healthy Controls—A Pilot Case-Control Study

Biwash Ghimire [1], Pradeep Giri [1], Susan Tavernier [2], Sarah E. Hobdey [3,4,5] and Ali Aghazadeh-Habashi [1*]

[1] Idaho State University, Pocatello, ID, USA
[2] School of Nursing, University of Alaska Anchorage, Anchorage, AK
[3] Idaho State University, Meridian, Idaho, USA
[4] Veterans Affairs Medical Center, Boise, Idaho, USA
[5] Idaho Veterans Research and Education Foundation, Boise, Idaho, USA
* Correspondence: habaali@isu.edu

Abstract

Background: The renin-angiotensin system (RAS), traditionally known for its role in cardiovascular regulation, has also emerged as a key regulator of tumor progression and metastasis. Dysregulation of the RAS components has been implicated in breast cancer due to the significant presence of the RAS-related proteins in the breast tissue. This study aims to identify the dysregulated RAS components and investigate their potential as prognostic biomarkers. **Methods**: A pilot case-control study was carried out with 21 treatment-naïve breast cancer patients and 17 healthy controls. Plasma levels of Ang 1-7, Ang II, ACE2 and some cytokines were measured using LC-MS/MS and ELISA. ROC curves were used to assess changes in biomarker levels across the RAS components. **Results**: Breast cancer patients show significant dysregulation of the RAS components and Interleukin-10. The ratio of Ang 1-7/Ang II was reduced by over two-fold in breast cancer patients (p = 0.0442). While plasma ACE2 was significantly elevated in breast cancer patients (p = 0.0005), IL-10 was significantly suppressed (p = 0.0420). In exploratory logistic regression analysis, ACE2 showed potential as a classifier with improved discrimination when combined with Ang 1-7 and Ang II (AUC = 0.9396, accuracy = 92.59%). However, due to the small sample size and methodological limitations, these findings require further validation. **Conclusions**: Our hypothesis-generating study highlights the potential of RAS components as circulatory biomarkers, given their high accuracy in distinguishing breast cancer patients from healthy individuals. Despite promising results, external validation of this data in a larger, more diverse study cohort is recommended to generalize the findings.

Keywords: ACE2, angiotensin peptides, cytokines, LC-MS/MS, multi-analyte model, breast cancer biomarkers

1. Introduction

Breast cancer is the most prevalent cancer among women, affecting millions of people around the world, characterized by its heterogeneous nature and variable outcomes. Due to the complex nature of the pathophysiology of breast cancer, it is essential to understand the molecular mechanisms responsible for tumor development and advancement. Among these mechanisms, the Renin-Angiotensin System (RAS) has received significant attention for its role in cancer biology. RAS is primarily known for its functions in cardiovascular regulation and fluid balance. However, recent studies have also explored its role in various cancer processes, including cell proliferation, migration, and angiogenesis [1–3].

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The RAS is a complex cascade of peptides, enzymes, and receptor proteins composed of two distinct and opposing arms (Figure 1). The precursor protein of the RAS, Angiotensinogen, is cleaved by Renin to release the decapeptide Angiotensin I (Ang I)[4]. Ang I is then cleaved by the angiotensin-converting enzyme (ACE) to form the biologically active peptide Angiotensin II (Ang II). Ang II modulates a diverse range of physiological effects, including vasoconstriction, vascular smooth muscle cell proliferation, and hypertrophy of the heart vessel wall, through its interaction with the Ang II type 1 receptor (AT1R) [5]. The axis of Ang II/ACE/AT1R is known as the classical arm of RAS. A homolog of ACE, ACE2, cleaves Ang II to Angiotensin (1-7) (Ang 1-7). Ang 1-7 is a heptapeptide with affinity to the Mas receptor (MasR), to produce effects that directly oppose the actions of Ang II [6–8]. This axis of Ang 1-7/ACE2/MasR is known as a protective axis of the RAS, which acts as a counter-regulatory to the classical arm[9].

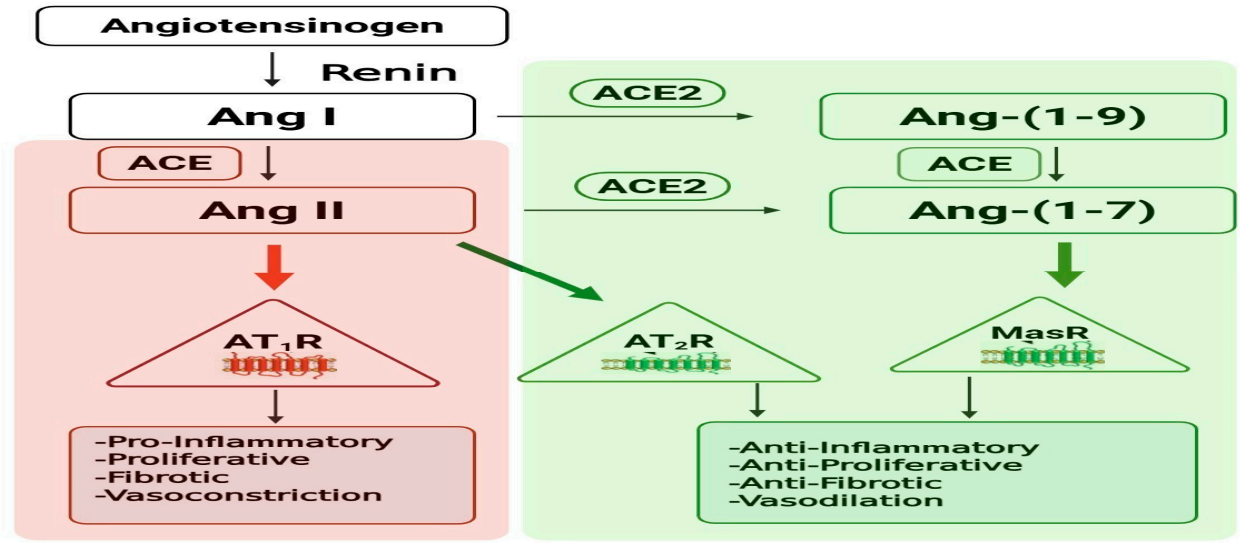

Figure 1. The schematic representation of the RAS pathways: the classical axis (red) and the protective axis (green). Ang = Angiotensin, ACE= Angiotensin-converting enzyme, AT1R= Angiotensin II type I receptor, AT2R= Angiotensin II type 2 receptor, MasR= Mas receptor

The discovery of ACE2 has revealed its role in maintaining the balance between the two RAS axes. Circulating levels of soluble ACE2 in plasma and urine have been linked to various diseases, including cancer [10–14]. The increase in plasma circulating levels of ACE2 may be considered a part of the body's defense mechanism to neutralize higher Ang II levels. Higher urinary or plasma levels of ACE2 were reported in patients suffering from hypertension, heart failure, microalbuminuria, and overt vascular disease[15,16].

In breast tissue, the RAS promotes tumorigenesis through various mechanisms, including cell proliferation, angiogenesis, metastasis, and immune modulation. Classical RAS activates the PI3K/AKT signaling pathway via AT1R activation, thereby suppressing apoptosis by downregulating caspase-9 expression. Activation of MAPK/ERK signaling pathways, together with PI3K/AKT signaling, induces increased expression of Bcl2, a potent anti-apoptotic regulator that promotes cell survival [17,18]. The classical axis also induces epithelial-mesenchymal transition (EMT) through the phosphorylation of AKT and activation of TGF-β/Smad cascade. One of the products of this cascade, the Snail1-Smad3/4 complex, in advanced stages, reduces E-cadherin expression, allowing cells to detach and metastasize [1,2,19]. The lymph node metastasis is guided by focal adhesion kinase (FAK)/Ras homolog gene family member A (RhoA) signaling. This cascade is activated through CXCR4/Sdf-1α signaling, which is stimulated by the Ang II/AT1R axis, suggesting an important role of RAS in cancer metastasis[1,20].

The RAS is a central factor in breast cancer angiogenesis, acting through the classical pathway. Ang II is a key stimulator of vascular endothelial growth factor (VEGF), which is a driver of

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angiogenesis [21]. Angiogenesis is a crucial hallmark of cancer that supports supplying nutrients and oxygen to the rapidly growing tumor mass [1,14,22].

The immunomodulatory function of the RAS is well-studied and well-known. The infamous cytokine storm in COVID-19 patients has been directly linked to the overactivation of the RAS system, driven by reduced lung ACE2 levels [23]. This overactivation activates nuclear factor kappa B (NF-κB) and MAPK signaling, which drive the secretion of inflammatory cytokines such as IL-1, IL-6, and TNF-α, further augmenting the inflammatory process [24,25]. IL-10 is a potent anti-inflammatory cytokine; however, its pleiotropic effects make it a paradoxical, context-dependent regulator, acting as both a pro- and anti-cancer cytokine [26]. While activation of the Ang 1-7/MasR axis suppresses these actions, it is essential to understand the complex interplay between RAS and the inflammatory cascade in breast cancer. Because of the extensive role of RAS in breast cancer, our study attempts to identify any of its components as disease biomarkers to predict and potentially suggest therapeutic interventions for breast cancer.

2. Materials and Methods

2.1. Materials

Ang 1–7 (Anaspec, AS-61039) and Ang II (Anaspec, AS-20633) were acquired from Anaspec Inc. (Fremont, CA, USA). (Asn1, Val5)-Ang II (Sigma-Aldrich A6402-1MG) was used as an internal standard (IS) and was obtained from Sigma Aldrich (St. Louis, MO, USA). cOmplete™ Mini Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO) was added to plasma samples to prohibit further protein and peptide degradation. Waters C18 SPE cartridges (Sep-Pak WAT020805) were purchased from Waters (Milford, MA, USA). LC-MS grade water, acetonitrile, and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). A positive-pressure manifold for solid-phase extraction was purchased from Agilent Technologies (Agilent Technologies Inc., Santa Clara, CA, USA). Human ACE2 ELISA kit was purchased from RayBiotech (GA, USA), and custom Procartaplex 7-plex plate was ordered from Life Technologies Corporation (NY, USA).

2.2. LC-MS/MS System

LC-MS/MS system included liquid chromatography (Shimadzu, MD, USA) with a binary pump (LC-30AD), an autosampler (SIL-30AC), a controller (CBM-20A), a degasser (DGU-20A5R), a column oven (CTO-20A), and an ABSciex QTRAP 5500 mass spectrometer (SCIEX, Foster City, CA, USA) with electron spray ionization (ESI) source. The chromatograms were monitored and analyzed using Analyst 1.7 software (SCIEX, Foster City, CA, USA). The separation was performed on a Synergi™ Fusion-RP column (2.5 µm, 100 × 2 mm) from Phenomenex (Torrance, CA, USA). All analyses were carried out in positive-ion mode.

2.3. Human Subjects

The institutional review board approved the study through the protocol number IRB-FY2020-273. Pre-collected plasma samples from breast cancer patients from St. Luke's Hospital (Boise, ID, USA) and healthy control individuals' samples from SpeciCare (Gainesville, GA, USA) were purchased for analysis. The samples were venous blood plasma collected with EDTA coagulant. The samples were preserved by freezing them at -80°C and transported frozen on dry ice. Samples underwent one freeze-thaw cycle after receiving and before analysis. A protease inhibitor cocktail was added to all aliquots prior to peptide analysis, according to the manufacturer's instructions. All breast cancer patients were treatment-naïve, defined as none of the patients being subjected to chemotherapy or radiotherapy prior to sample collection.

The inclusion criteria for the breast cancer patients were treatment-naïve female cancer patients aged over 18 years. Inclusion criteria for control groups were similar-aged female volunteers with no

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history of cancer. No exclusion criteria were applied to either cohort. No stratification by tumor stage or receptor status was performed a priori.

2.4. *Sample Preparation Procedures*

Ang 1-7 and Ang II were extracted from plasma samples using a Solid Phase Extraction (SPE) procedure based on a previously established method by Cui et al., with minor changes[27,28]. 200 µL of the plasma samples were mixed with 100 µL of 25 ng/mL IS. Then, 2 µL of Formic acid was added to make the final concentration of 1%. Waters C18 SPE cartridges were simultaneously preconditioned with 3 mL of ethanol, followed by 3 mL of deionized water. Previously mixed samples were loaded onto the cartridge and allowed to interact with the stationary phase by applying positive nitrogen flow via a positive-pressure manifold. The column was then washed with 3 mL of deionized water, followed by elution with 3 mL of methanol containing 5% formic acid. The eluent was collected and dried using a Savant 200 SpeedVac system (ThermoFisher Scientific, Waltham, MA, USA). The dried samples were reconstituted in 100 µL of acidified water containing 0.1% formic acid, from which 30 µL was injected into the LC-MS/MS system to quantify angiotensin peptide concentrations.

2.5. *LC-MS/MS Method*

Ang 1-7, Ang II and IS were separated using a Synergi RP column (2 × 100 mm) with a particle size of 2.5 µm from Phenomenex (Torrance, CA, USA). The column temperature was maintained at 40°C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) pumped as a gradient elution. The gradient program was set as 5% ACN to 30% ACN for 4 minutes. The gradient was held constant for 4-8 minutes, then reduced to 5% ACN at 9 minutes and maintained for another 1 minute. The flow rate was set at 0.3 mL/min, and the injection volume was 30 µL.

The analytes were detected in positive ion mode using multiple reaction monitoring (MRM) in an Electrospray ionization (ESI) source. Previously optimized source gas parameters were used [28]. The set parameters are as follows: curtain gas, 30 psi; collision gas, medium; ion-spray voltage, 5500V; temperature, 300°C; nebulizer gas (ion source gas 1), 20 psi; and turbo gas (ion source gas 2), 25 psi. The specific MRM transition of m/z values used for quantification of Ang 1-7 were 450.4 → 647.0 and 450.4 → 110.1. Ang II was analyzed using m/z of 523.9 → 263.3 and 523.9 → 784.5. (Asn1, Val5)-Ang II used as internal standard was analyzed at m/z of 516.5 → 769.4 and 516.6 → 110.1. Collision energy (CE) and declustering potential (DP) used for Ang 1-7 were 20 and 90 V, for Ang II were 30 and 25 V, and for IS were 25 and 25 V, respectively. An entrance potential of 10 V and a cell exit potential (CXP) of 20 V were used for all of the analytes.

2.6. *Measurement of ACE2 Levels*

Plasma levels of ACE2 were measured by Enzyme-linked immunosorbent assay (ELISA) using the Human ACE2 ELISA kit in duplicates according to the manufacturer's recommendations. Briefly, 100 µL of the standards and samples were applied to the commercial ELISA plate pre-coated with capture antibodies and incubated for 2.5 hrs at room temperature. The plate was washed with the washing solution, and 100 µL of biotin antibody was added to each well, which was incubated for 1 hour. The plate was washed again, then the Streptavidin solution was added and incubated for 45 minutes. For colorimetric detection, 100 µL of TMB substrate was added, and the reaction was incubated for 30 minutes; the reaction was then stopped by adding 50 µL of stop solution. The absorbance was measured immediately at 450 nm.

2.7. *Measurement of Cytokine Levels*

An Immune-bead array assay was performed using bead-based technology with a custom Procartaplex 7-plex and analyzed on the Luminex platform. 25 µL plasma samples were diluted 4-

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fold, then aliquoted into the 96-well preconfigured plate and analyzed for interleukins (IL)-1, IL-6, IL-8, IL-10, INF-γ, and TNF-α according to the manufacturer's instructions. Standards were run alongside the samples, prepared in duplicate.

2.8. Statistical Analysis

The data were presented as means ± standard error of mean (SEM) unless stated otherwise. They were tested for normality using the Shapiro-Wilk test. A group comparison of normally distributed data was performed using Student's t-test with Welch correction assuming unequal variances. In contrast, non-normal data were analyzed using the Mann-Whitney U test. The correlation of continuous variables was quantified using Spearman's correlation coefficient. The predictive capacity of the variables was initially assessed using univariate logistic regression for each predictor, followed by multivariable models incorporating additional variables. In these analyses, the response variable was binary, representing the presence or absence of breast cancer. The strength of association was determined using the odds ratio (OR), and the discriminatory performance of the models was assessed using receiver operating characteristic (ROC) analysis. The confidence interval level was set at 95%, and $p \leq 0.05$ was considered statistically significant.

Internal validation was performed using bootstrap resampling with 1,000 iterations. For each bootstrap sample, data were resampled with replacement, and the logistic regression model (ACE2 + Ang 1-7 + Ang II) was refitted. Model performance was assessed by calculating the optimism-corrected AUC. Optimism was defined as the difference between the apparent AUC (from the original dataset) and the mean bootstrap AUC. The optimism-corrected AUC represents the expected performance in new data. Bootstrap 95% confidence intervals were calculated using the percentile method (2.5th and 97.5th percentiles of the bootstrap distribution).

Given the small sample size and ACE2's strong discriminatory performance, quasi-complete separation was anticipated in the standard logistic regression model. To obtain stable, bias-corrected estimates, Firth's penalized logistic regression was applied as a supplementary analysis [29]. The univariate Firth model was fitted on all participants with available ACE2 measurements (n = 35), while the full multivariable Firth model was fitted on the complete-case subset with all three predictors available (n = 27; 13 controls, 14 patients). AUC was calculated from predicted probabilities of each Firth model using ROC analysis.

Statistical analysis for group comparisons and correlations along with their visualization were carried out in GraphPad Prism 10. Logistic regression, stepwise selection method for identification of optimal set of variables, and bootstrap resampling were carried out in Jamovi 2.6.26. Post hoc power analysis was performed in Gpower 3.1.9.7 to provide insight into the required sample sizes for future studies. Data visualization for logistic regression and logit identification was performed in R version 4.5.0, RStudio 2025.05.0 build 496. The list of libraries used in R analyses is as follows: tidyverse, readxl, ggplot2, ggpubr, dplyr, pROC, forestplot, broom, pROC and gridExtra [30–34].

3. Results

3.1. Characteristics of Control and Patient Cohort

Our study analyzed de-identified plasma samples from 17 healthy subjects and 21 breast cancer patients having different tumor grades at different clinical stages. The variables such as age, body mass index (BMI), medication history, comorbidities and various clinical stages of breast cancer were considered for further analysis with respect to their association with the RAS peptides.

The study cohort consisted exclusively of women in both the control and patient groups. The average ages of the healthy and patient populations were 60.90 ± 12.70 years and 65.14 ± 12.98 years, respectively (p = 0.3208), suggesting the groups were comparable. The mean BMI of the patients was in the class I obesity range at 33.44 ± 14.60 kg/m^2.

The clinical stages of these patients were based on the standard pTNM classification, which uses TNM (tumor, node, and metastasis) staging systems based on the excised or physically examined

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tumor to define the extent of cancer. In this classification, pT1 refers to tumors measuring 1-2 cm, whereas pT2 refers to tumors measuring 2-5 cm across. pT1 is subdivided into pT1mi (< 0.1 cm), pT1a (0.1 - 0.5 cm), pT1b (0.5 - 1 cm), and pT1c (1-2 cm). Most tumors (n = 17, 80.95%) were pT1, indicating a tumor size less than 2 cm at its greatest dimension. This group was subdivided into pT1a (n = 4, 19.05%), pT1b (n = 4, 19.05%), and pT1c (n = 9, 42.85%). The remaining patients' tumors were pT2 (n = 3, 14.29%), suggesting that the largest number of participants had tumors measuring 1-2 cm (Table 1).

Table 1. Clinical characteristics, comorbidities, disease state, and medication history of breast cancer patients.

Clinical Characteristics	
Age (years)	65.14 ± 12.98
BMI (kg/m^2)	33.44 ± 14.60
Clinical stage	n (%)
-pT1a	4 (19.05)
-pT1b	4 (19.05)
-pT1c	9 (42.85)
-pT2	3 (14.29)
-pN0	16 (76.19)
-pN1a	2 (9.52)
-pN1c	1 (4.76)
-pN1mi	1(4.76)
Tumor grade	n (%)
-G1	16 (76.19)
-G2	3 (14.29)
-G3	1 (4.76)
ER+, n (%)	18 (85.71)
PgR+, n (%)	17 (80.95)
HER2+, n (%)	4 (19.05)
Comorbidities	n (%)
-Diabetes	2 (9.52)
-Cardiac arrhythmia	4 (19.05)
-Hypertension	10 (47.62)
-Peripheral Vascular Disease	1 (4.76)
-Congestive Heart Failure	1 (4.76)
Tobacco use, n (%)	10 (47.62)
Medication History	n (%)
-Aspirin (any dose)	3 (14.29)
-NSAIDs	4 (19.05)
-Statins	4 (19.05)
Platelets (per μL)	(255.74 ± 55.4) x 10^3
Total Bilirubin (mg/dL)	0.65 ± 0.40
Creatinine (mg/dL)	0.79 ± 0.18
WBC (per μL)	(7.32 ± 2.20) x 10^3

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Hemoglobin (g/dL)	13.02 ± 1.66

Data presented as Mean ± SD unless mentioned otherwise. BMI, Body Mass Index; HER2+, Human Epidermal Growth Factor Receptor 2 positive; NSAID, non-steroidal Anti-inflammatory drugs; WBC, white blood cells. N = 20 for all variables except Age, where N = 21. Clinical stage is based on the standard pTNM classification, where T = tumor, N = nodes, and M = metastasis. Tumor grades are classified based on differentiation and aggressiveness, where G1 = well differentiated and least aggressive; G2 = moderately differentiated and moderately aggressive, and G3 = poorly differentiated and most aggressive.

Pathological N categories (pN classification) describe the extent of lymph node involvement by cancer. Here, pN0 means no regional lymph node metastasis was identified (n = 16, 76.19%), pN1 (n = 4, 19.05%) suggests the cancer has spread to 1 -3 axillary lymph nodes. pN1 is further divided into pN1mi (n = 1, 4.76%) referring to micro-metastasis of cancer cells between the size of 0.2 - 2 mm, pN1a (n = 2, 9.52%) in which cancer has spread to 1-3 axillary lymph nodes with at least one metastasis exceeding 2 mm. pN1b category suggests cancer is present in internal mammary lymph nodes on the same side as the primary tumor. When both pN1a and pN1b criteria are met, it is designated as pN1c (n = 1, 4.76%) [35,36].

Tumor grades are based on how closely the cancer cells resemble normal cells of the tissue. Most participants had a grade 1 tumor (G1; n = 16, 76.19%), indicating that the cancer cells are slow-growing and well-differentiated, with a strong resemblance to healthy cells. Grade 2 (G2; n = 3, 14.29%) suggests moderately differentiated, faster-growing cells, whereas grade 3 (G3; n = 1, 4.76%) consists of very aggressive, poorly differentiated cancer cells (Table 1) [37]. Depending on the level of human epidermal growth factor receptor 2 (HER2) expression in breast cancer cells, they are classified as HER2-positive ($HER2^+$) or HER2-negative ($HER2^-$) [38]. $HER2^+$ cancers are considered more aggressive as the HER2 protein is involved in mitotic signaling, leading to faster growth [39]. Our study cohort included 2 patients (9.52%) with $HER2^+$ cancer. Similarly, based on estrogen receptor (ER) and progesterone receptor (PgR) status, breast cancer is divided into ER^+/ER^- and PgR^+/PgR^- groups. Our study population included n = 18 (85.71%) ER^+ and n = 17 (80.95%) PgR^+ (Table 1).

Study participants reported cardiovascular and cardiometabolic comorbidities, including Diabetes (n = 2, 9.52%), Cardiac arrhythmia (n = 4, 19.05%), hypertension (n = 10, 47.62%), peripheral vascular disease (n = 1, 4.76%), and congestive heart failure (n = 1, 4.76%) (Table 1). The participants also reported use of Aspirin at any dose (n = 3, 15%), non-steroidal anti-inflammatory drugs (NSAIDs) (n = 4, 19.05%), and statins (n = 4, 19.05%). 47.62% (n = 10) of the patients reported using tobacco in any form. Specifics regarding the status, duration and frequency of tobacco use were not available. The average value of serological markers was within the normal range (Table 1).

3.2. Effect of Breast Cancer on Ang 1-7 and Ang II Levels

The Ang 1-7 and Ang II concentrations were calculated by comparing the areas of the peaks of the analytes obtained from the plasma sample to the standard curve drawn from spiked blank plasma in the LC-MS/MS chromatograms. Among the samples analyzed, the resulting Ang 1-7 and Ang II data for two patient samples and one control sample were omitted because the plasma samples were hemolyzed. They were, however, considered for the ratio as they were indicative of RAS balance. Two samples, one from each of the Ang II control and patient groups, were excluded from analysis because they were below the limit of quantification. Two samples from the control group were omitted due to persistent column blockage during the analysis. The final sample sizes in the Ang 1-7 groups were 16 and 17; the Ang II groups were 14 and 15; and the ratios were 15 and 17 for the control and patient groups, respectively.

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We did not see a significant difference (r = -0.1177, p = 0.5814) between plasma levels of Ang 1-7 between the control (43.40 ± 13.04 ng/mL) and the breast cancer patients (24.39 ± 2.23 ng/mL) (Figure 2a). Plasma levels of Ang II were elevated in breast cancer patients (0.20 ± 0.04 ng/mL) compared to the controls (0.12 ± 0.01 ng/mL), but not significantly different (r = 0.2048, p = 0.3591) (Figure 2b). To assess the balance of the RAS effector peptides between the two groups, Ang 1-7 levels were normalized to Ang II levels. The resulting ratio was significantly lower (r = -0.4196, p = 0.0442) in breast cancer patients (169.47 ± 30.33) than in controls (473.20 ± 129.07) (Figure 2c), suggesting a significant RAS pathway dysregulation in breast cancer patients.

Post hoc power analysis indicated that the group comparisons for Ang 1-7, Ang II, and the Ang 1-7/Ang II ratio were underpowered, with calculated powers of 0.400, 0.546, and 0.726, respectively. We calculated required sample sizes of 89, 49 and 34 per group for Ang 1-7, Ang II and the ratio, respectively, to achieve a statistical power of 0.95 at a 95% CI.

3.3. Effect of Breast Cancer on ACE2 Levels

The concentrations of ACE2 in the plasma samples were determined by comparing the average absorbance values of duplicate samples to a standard curve generated from the standards. A standard curve was drawn over a concentration range of 0.027 ng/mL to 20 ng/mL. Two patients' samples with a coefficient of variance (CV) greater than 20% were omitted for analysis. One sample from the patient group was considered a technical failure because its value was over 200 times the average of the remaining samples.

ACE2 levels in the breast cancer patients (1.33 ± 0.17 ng/mL) were significantly higher (r = 0.6678, p = 0.0005) than the levels in the control population (0.74 ± 0.04 ng/mL) (Figure 2d). Post hoc power analysis showed that the group comparison for ACE2 was reasonably powered for a pilot study, with a computed power of 0.939 at 95% CI.

3.4. Effect of Breast Cancer on Cytokine Levels

There was a significant reduction (p = 0.0420, D = 0.7302, 95% CI = 0.0096, 0.4896, n = 15,19) in plasma IL-10 levels in patients (1.28 ± 0.08 pg/mL) compared to controls (1.53 ± 0.09 pg/mL) (Fig. 2a). Data below the limit of quantification (LOQ) were omitted from analysis, which included two samples each from control and patients' groups for IL-10 (LOQ < 0.8 pg/mL).

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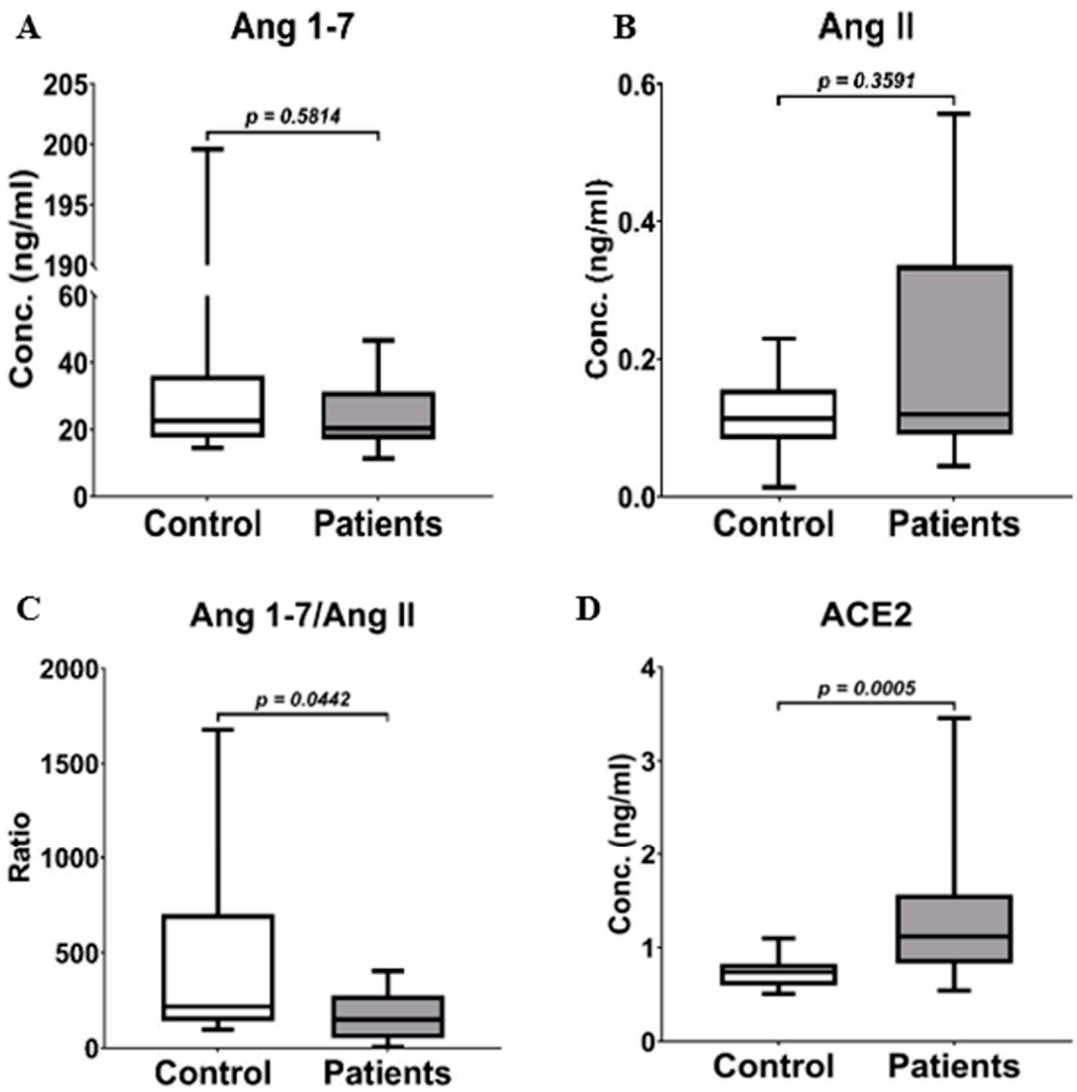

Figure 2. The boxplots of plasma concentrations of Ang 1-7 (A), Ang II (B), and the Ang 1-7/Ang II ratio (C) in the control group (n = 16) and breast cancer patients (n = 17). The boxplot of plasma ACE2 concentration (D) in the control group (n = 16) and breast cancer patients (n = 19). Statistical comparison was performed using the Mann-Whitney U test, and statistical significance was set at.

While tests for IL-6 (LOQ < 2.0 pg/mL), IL-8 (LOQ < 0.3 pg/mL), and IFN-γ (LOQ < 7 pg/mL) were carried out, more than 50% samples of at least one group were below the level of quantification. Therefore, no significant differences were found, and the data are presented here as descriptive statistics. The level of IL-6 was found to be 8.58 ± 2.42 pg/mL in the control group (n = 10), and 30.45 ± 18.68 pg/mL in the patient group (n = 7) (Fig. 3b). IL-8 was lower in the patient group (1.73 ± 0.57 pg/mL, n = 7) compared to that of the control group (3.75 ± 1.48, n = 12) (Fig. 3c). IFN-γ level in the patient group (n = 6) was 38.87 ± 25.33 pg/mL. In contrast, in the control group (n = 5), it was 12.51 ± 2.23 pg/mL (Fig. 3d). The plasma concentrations of TNF-α, IL-1α, and IL-1β were below the detection limit for all samples (Data not shown). Post hoc power analysis indicated an underpowered group comparison for IL-10, with a computed power of 0.501. The required sample size to achieve a power of 0.95 with a 95% CI would be 66 per group. Power analyses for other cytokines were not performed due to the limited data above the detection limit obtained from the analyses.

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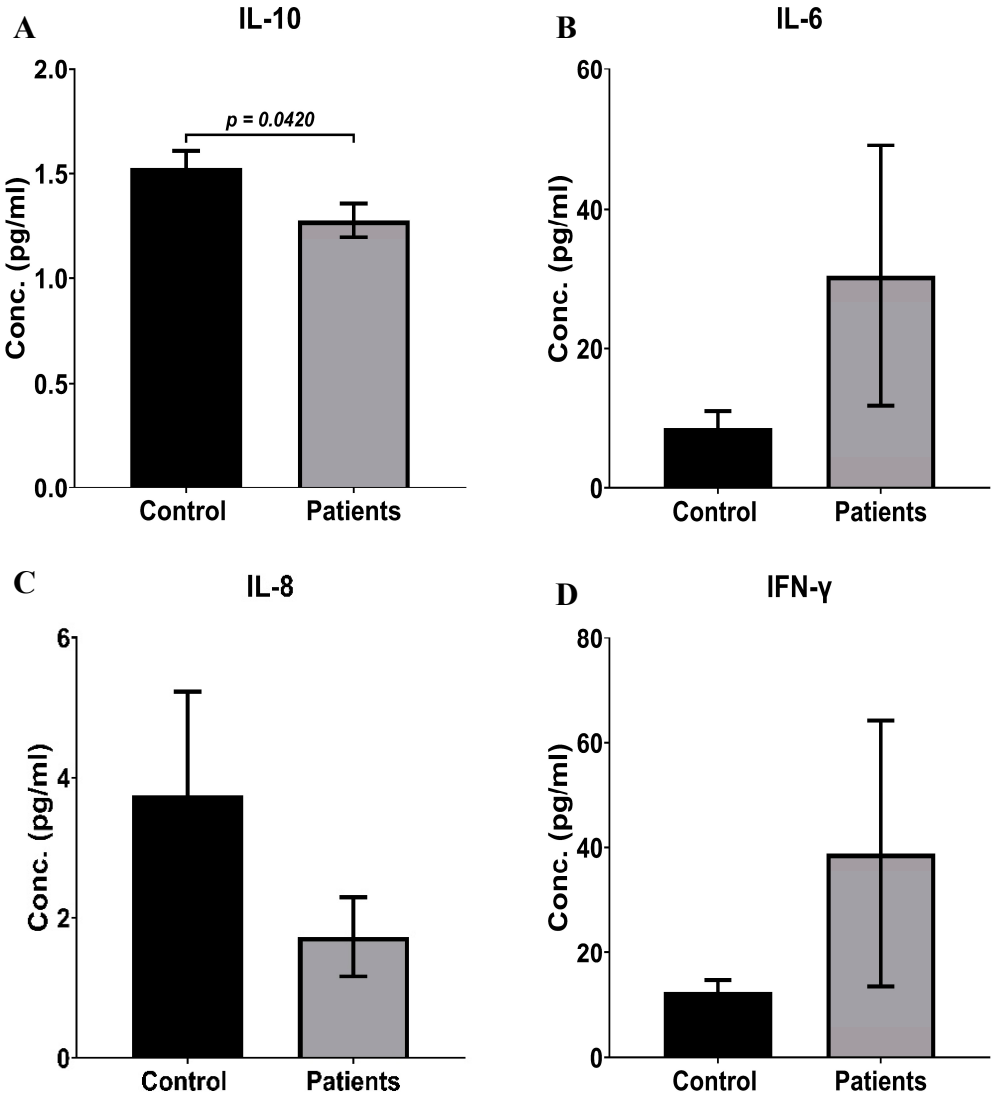

Figure 3. Bar graphs of plasma concentrations of IL-10 (a), IL-6 (b), IL-8 (c), and IFN-γ (d) in the control group and in breast cancer patients. Data are presented as mean ± SEM. Statistical comparison between the groups (Controls, n = 15 and Patients, n = 19) was performed using Student's t-test for IL-10, and statistical significance was set at $p < 0.05$ (*). No significant differences were found for IL-6 (n = 10, 7), IL-8 (n = 12, 7), and INF-γ (n = 5, 6), likely due to small sample sizes; data are presented as mean ± SEM.

3.5. Effect of Hypertension and Tobacco Use on the RAS System in Breast Cancer Patients

As there was a significant difference in IL-10 levels between groups, we analyzed the effects of hypertension on components of the RAS and IL-10. We did not observe a significant difference (r = 0.0204, p = 1.0000) in Ang 1-7 levels between the non-hypertensive (26.09 ± 4.50, n = 7) and hypertensive groups (23.92 ± 2.42, n = 7). Similarly, no differences were observed in Ang II levels (r = -0.0952, p = 0.8357, n = 6, 7) or in Ang 1-7/Ang II levels (r = -0.1111, p = 0.7756, n = 6, 9) between hypertensive and non-hypertensive groups. ACE2 (r = -0.7778, p = 0.0120, n = 6, 9) was significantly reduced in the hypertensive group, whereas IL-10 (D = -1.0578, p = 0.0488, n = 6, 10) was significantly elevated (Fig. 4). Similarly, the use of tobacco did not have significant effect in the Ang 1-7 (r = 0.3016, p = 0.3511), Ang II (r = 0.2143, p = 0.5358), Ang 1-7/Ang II (r = -0.0833, p = 0.8148), and ACE2 (r = -0.2099, p = 0.4894) levels (Fig. 4b). IL-10 was significantly elevated (D = -1.1345, p = 0.0270) among tobacco users (Fig. 4d).

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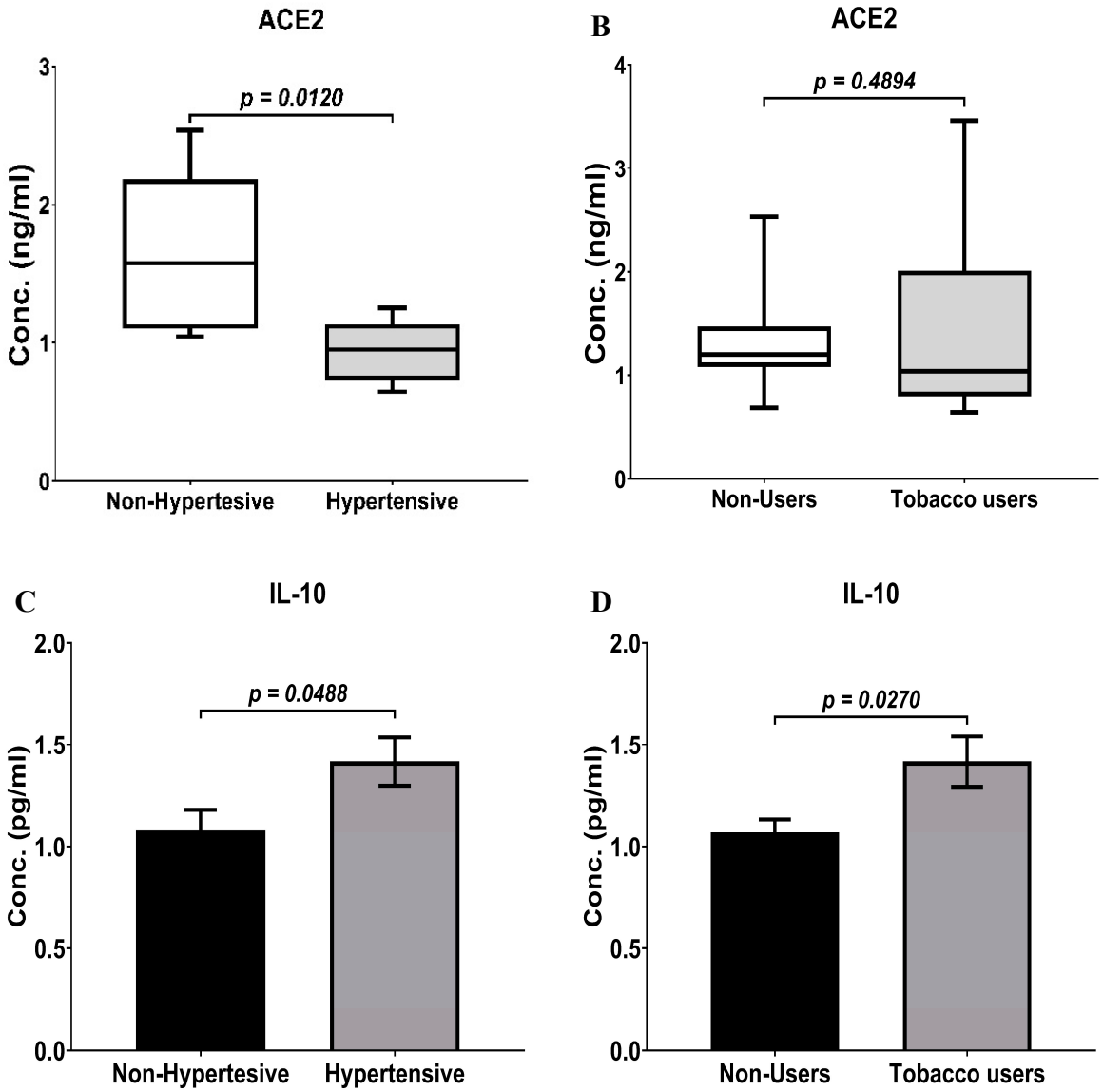

Figure 4. The boxplot shows the effects of hypertension (a) and tobacco use (b) on the plasma concentration of ACE2 in breast cancer patients. The bar diagrams show the effects of hypertension (c) and tobacco use (d) in breast cancer patients. The group comparison for ACE2 was done using the Mann-Whitney U test, and IL-10 was done with the student's t-test with Welch correction assuming unequal variances. Data are presented as mean ± SEM for the bar diagram.

To further assess the potential confounding effect of hypertension on primary study outcomes, a sensitivity analysis was performed by restricting the cancer group to non-hypertensive patients and comparing them against the full control cohort. ACE2 remained significantly elevated in non-hypertensive cancer patients (1.65 ± 0.62 ng/mL) compared to controls (0.74 ± 0.04 ng/mL) ($p < 0.0001$), reinforcing that the ACE2 elevation observed in the primary analysis reflects cancer-associated biology rather than hypertension related confounding. In contrast, the Ang 1-7/Ang II ratio did not reach statistical significance in this restricted sub-group ($p = 0.3403$), likely attributable to the reduced statistical power resulting from the smaller cancer sub-group (n = 6). The directional trend remained consistent with the primary analysis (188.40 ± 38.48 in cancer vs. 473.20 ± 129.07 in controls), suggesting that the loss of significance reflects insufficient power rather than the absence of effect.

3.6. Correlation of RAS Components and IL-10

Plasma concentration of Ang 1-7 did not show any correlation with the plasma concentration of Ang II (r = 0.1641, $p = 0.3950$), suggesting the possibility that Ang II may not be a primary substrate

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for the formation of Ang 1-7 (Supplementary Material 1 Table 2). A similar finding was reported in a previous study of hypertensive subjects, in which these two effector peptides were negatively correlated in males but showed no correlation in females [40]. Ang 1-7 did not show significant correlations with ACE2 (r = -0.3322, p = 0.0729) or IL-10 (r = 0.3236, p = 0.0811) (Supplementary Material 1 Table 2). Similarly, Ang II was not correlated with ACE2 (r = 0.1811, p = 0.3659) or IL-10 (r = -0.1092, p = 0.5954) (Supplementary Material 1 Table 2). ACE2 also showed a moderately negative association with IL-10 (r = -0.4123, p = 0.0212).

3.7. Correlation of RAS Components and IL-10 with Biological Variables

The biological variables used in this study are age and BMI. The age data were available for both the control and patient groups, but the BMI data was available only for the patient group. Therefore, the correlation between BMI and analytes was not performed in the control group. There was no significant correlation of age with Ang 1-7 (r = 0.0982, p = 0.5865), Ang II (r = 0.2076, p = 0.2798), Ang 1-7/Ang II (r = -0.2611, p = 0.1498), ACE2 (r = 0.0960, p = 0.5833), or IL-10 (r = 0.0449, p = 0.8009) (Supplementary Material 1 Table 2). While BMI showed strong correlation with Ang II (r = -0.5516, p = 0.0438) in the breast cancer patients (Fig 5A, Supplementary Material 1 Table 2), it did not show any correlation with Ang 1-7 (r = -0.0676, p = 0.8051), Ang 1-7/Ang II (r = 0.2426, p = 0.3467), ACE2 (r = 0.1084, p = 0.6687), or IL-10 (r = 0.2043, p = 0.4161) (Supplementary Material 1 Table 2).

3.8. Correlation of RAS Components and IL-10 with Hematological and Biochemical Markers

Hematological markers, comprising quantified hemoglobin, white blood cells (WBC), and Platelets, along with biochemical markers including serum bilirubin and creatinine, were correlated with the RAS components and IL-10 of breast cancer patients to understand how dysregulation of the RAS impacts these variables. Ang 1-7 showed a strong negative correlation with platelets (r = -0.5147, p = 0.0436) (Fig. 5B, Supplementary Material 1 Table 2). Hemoglobin was positively correlated with Ang II (r = 0.5827, p = 0.0248), whereas it was negatively correlated with Ang 1-7/Ang II ratio (r = -0.6270, p = 0.0083) (Fig. 5C, D). Similarly, ACE2 showed a strong correlation with WBC (r = 0.5515, p = 0.0237). IL-10 did not show a significant correlation with any of the above-mentioned serological markers. We did not see any correlation between serum bilirubin and creatinine and the RAS peptides or IL-10 levels. The complete correlation matrix between the groups is presented in Supplementary Material 1, Tables 2 and 3.

3.9. Logistic Regression

A binomial logistic regression was performed to identify variables associated with the likelihood that individuals in the study population would be in the Control or Patient group. We developed a model via bidirectional stepwise regression. Initially, we used a univariate baseline, creating a separate model for each variable (Ang 1-7, Ang II, Ang 1-7/Ang II, ACE2, and IL-10) with our groups (Controls and Patients) as the dependent variables. The performance of the model was assessed using Akaike Information Criterion (AIC), deviance, McFadden's pseudo R^2 (R^2_{McF}), and the p-value of the predictor. In the univariate model, ACE2 was determined as the best predictor (AIC = 36.49, deviance = 32.49, R^2_{McF} = 0.3269, and $p < 0.0001$).

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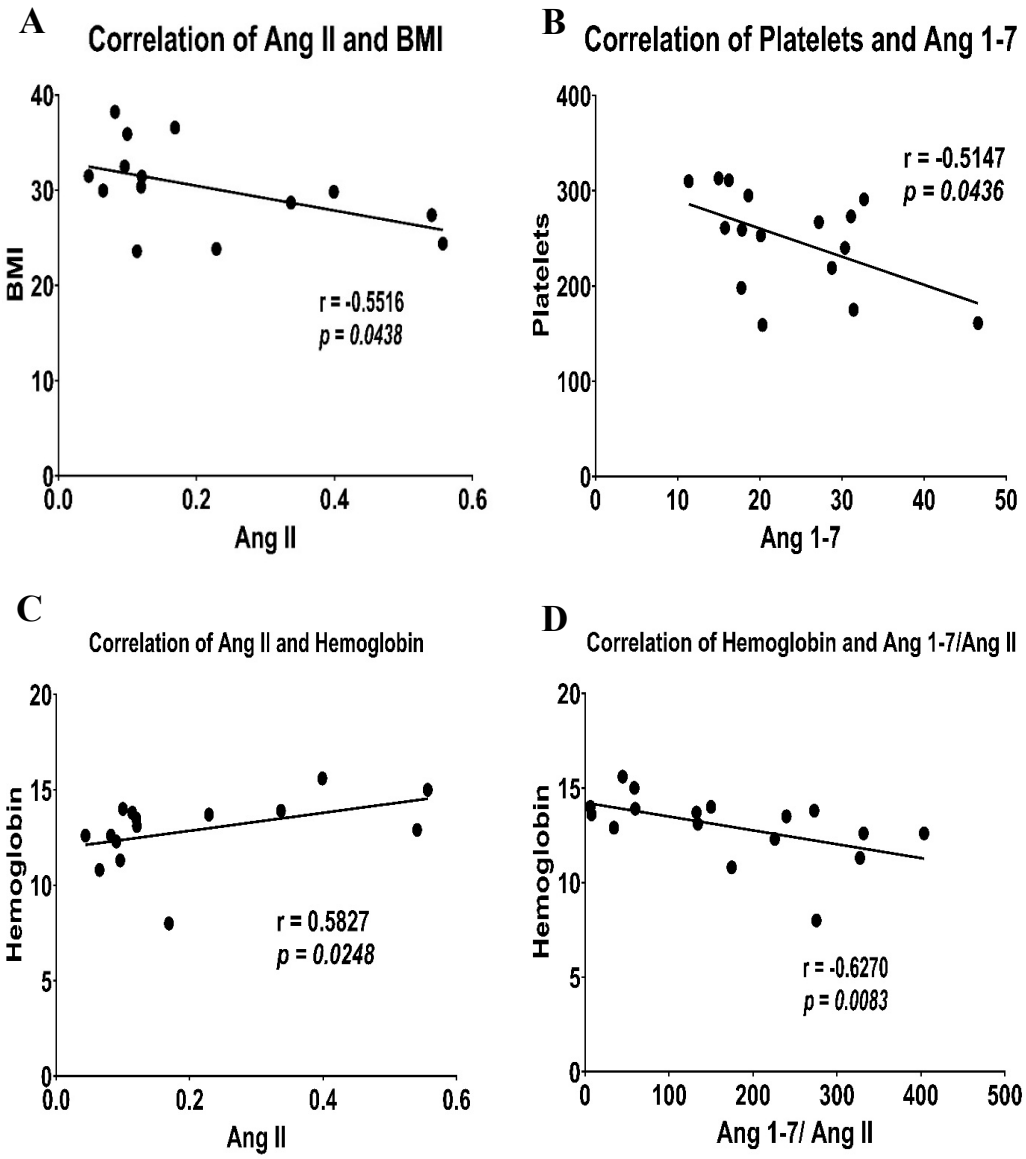

Figure 5. The correlation graphs of plasma concentrations (ng/ml) of Ang II against BMI (kg/m^2) (a), Ang 1-7 (ng/ml) against platelets (counts per µl) (b), Ang II (ng/ml) against Hemoglobin (g/dl) (c), and Ang 1-7/Ang II ratio against hemoglobin (g/dl) (d). A statistical test was performed using Spearman's correlation coefficient (r), with $p < 0.05$ considered statistically significant.

After determining ACE2 as a baseline predictor, we added each of the remaining variables (Ang 1-7, Ang II, Ang 1-7/Ang II, and IL-10) to generate bivariate models. The model assessment was conducted using the same metrics as before, with the addition of the variance inflation factor (VIF) to account for multicollinearity-induced inflation of regression coefficients. The combination of ACE2 and Ang II was the best predictor of the disease outcome (AIC = 23.48, deviance = 17.47, $R^2_{McF} = 0.5326$, and VIF = 1.05). The model consisting of ACE2 only as a predictor was defined using the following equation:

$$\boldsymbol{Logit\,(P) = -4.95 + 5.68\,x\,[ACE2]}$$

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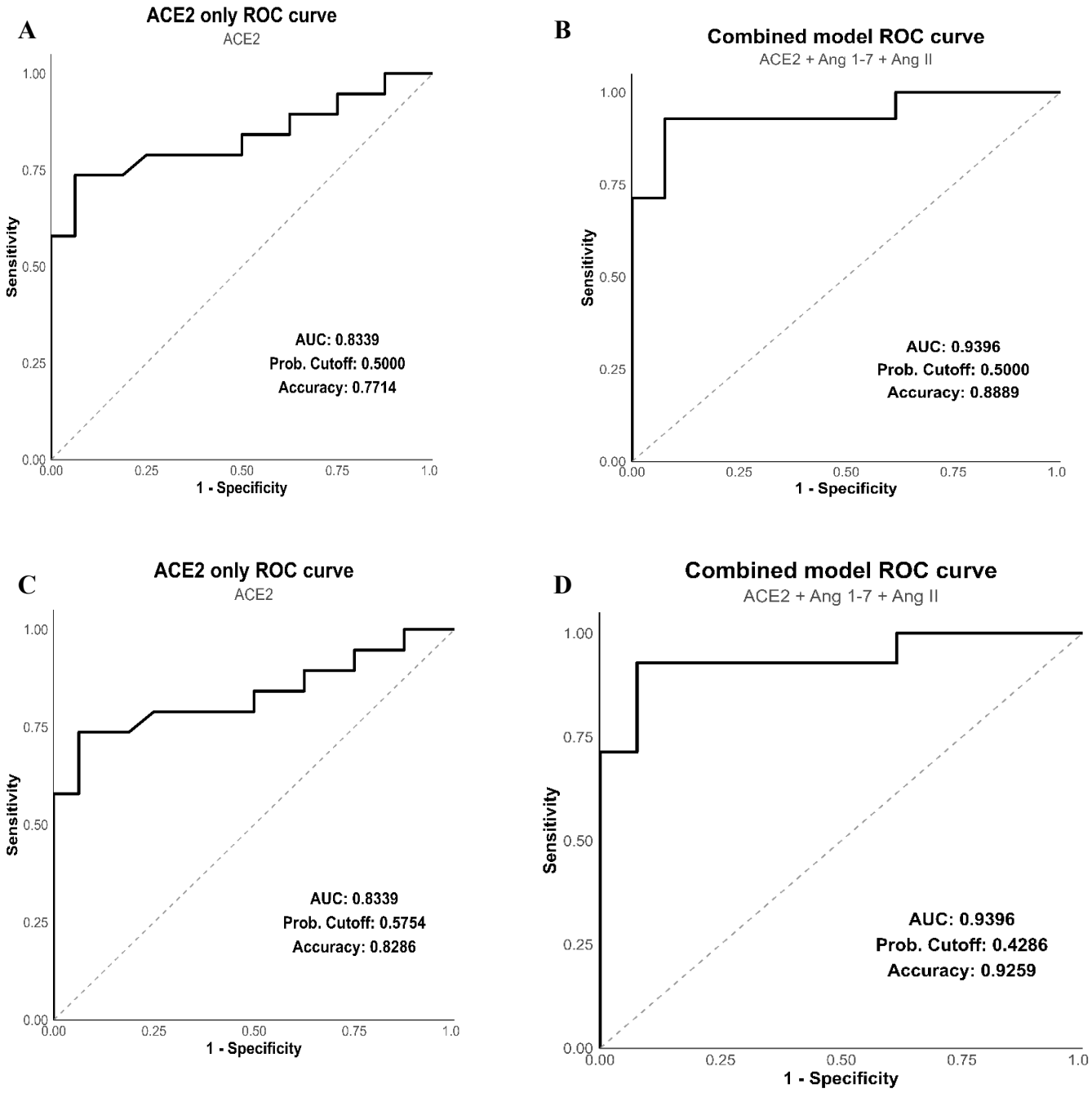

Figure 6. Receiver operating characteristic (ROC) curves showing the performance of the ACE2 univariate model (A) and the multivariate model using ACE2, Ang 1-7, and Ang II together (B) at an arbitrary threshold of 0.5. The ROC curves with calculated Youden's index (J) for the ACE2 univariate model (C) and the multivariate model using ACE2, Ang 1-7 and Ang II together (D). ACE2, Angiotensin Converting Enzyme; Ang, Angiotensin; AUC, Area under curve; Youden's index (J) represented as cutoff.

This forward stepwise selection process continued by adding the remaining variables sequentially based on model performance. Our optimal model was determined to be a combination of ACE2, Ang 1-7, and Ang II, with a strong model fit (R^2_{McF} = 0.5366), AIC = 25.33, deviance = 17.33, no multicollinearity (max VIF = 1.08), and ACE2 as the significant predictor ($p < 0.0001$). Validation of this model was done by calculating statistical performance metrics based on receiver operating characteristic (ROC) analysis using a cutoff value of 0.5. The area under the ROC curve (AUC) was 0.9396, with an accuracy of 0.8889. This shows that our model achieved outstanding discrimination between the outcomes, with an overall accuracy of 88.89%.

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Our next step was to identify the optimal threshold to maximize diagnostic accuracy. This was done by calculating Youden's index, which maximizes the balance between sensitivity and specificity. We calculated the cutoff value (J) for our biomarker panel to be 0.4286, which was lower than the threshold for ACE2 alone (J = 0.5754) (Fig. 6C, D). However, the combined panel of RAS biomarkers showed improved accuracy of 92.59% with a lower cutoff than 88.89% at an arbitrary cutoff of 0.5 (Fig. 7). The combined model was defined using the following equation:

$$\boldsymbol{Logit\,(P) = -9.64 + 9.57\,x\,[ACE2] - 0.007\,x\,[Ang\,1-7] + 6.3\,x\,[Ang\,II]}$$

The ROC curve showed a sharp rise to high sensitivity, suggesting the model was likely to identify true positives. The high AUC value indicates excellent separation between the groups, enabling clinically relevant analysis. Our panel of biomarkers (ACE2, Ang 1-7, and Ang II) represents a robust diagnostic tool for breast cancer patients.

We further calculated the odds of the outcome for each predictor variable cluster in our model. The odds ratio (OR) of ACE2 was calculated to be 14282.76 (p = 0.0168, 95% CI = 5.59 – 3.65 x 10^7), showing that a one-unit increase in ACE2 increased the odds of breast cancer by several thousand-fold (Fig. 7). Similarly, we observed a large OR (568.57) for Ang II which was not statistically significant (p = 0.2561, 95% CI = 0.01 – 3.23 x 10^7). Although Ang 1-7 showed minimal association with the outcome (OR = 0.99, p = 0.7312, 95% CI = 0.95 - 1.03) independently, it was an integral part of the overall multivariable model, improving model accuracy when used with ACE2 and Ang II. The extremely wide confidence intervals observed for ACE2 and Ang II indicate high variability in biomarker concentrations. The point estimates obtained from the model suggest potential for clinically meaningful associations.

3.9.1. Bootstrap Validation

Internal validation using 1,000 bootstrap resamples demonstrated minimal model optimism (optimism = -0.0103), indicating the model is not overfit to the training data. The optimism-corrected AUC was 0.9498 (95% bootstrap CI: 0.84 - 1.000), confirming robust discriminatory performance comparable to the apparent AUC of 0.9396. The bootstrap distribution showed consistent model performance across resamples (mean AUC = 0.9498, median = 0.9560, SD = 0.05), supporting the stability of our biomarker panel's discriminatory ability. Some bootstrap samples exhibited quasi-complete separation due to a small sample size (n = 27) and strong predictor effects, leading to unstable coefficient estimates. However, AUC-based discrimination remained consistent across resamples, confirming robust model performance.

3.9. 2 Firth's Penalized Logistic Regression

To address the quasi-complete separation produced by ACE2 in the standard logistic regression model, evidenced by the unstable odds ratio estimate (OR = 14,282.76, 95% CI: 5.59 – 3.65 × 10^7), Firth's penalized logistic regression was applied to obtain bias-corrected estimates. The bias-corrected estimates are tabulated in Supplementary Material 1, Table 4. In the univariate Firth model (n = 35), ACE2 remained a highly significant predictor of breast cancer status (OR = 138.05, 95% CI: 4.80 – 14,817.63, p = 0.0004, AUC = 0.8339, 95% CI: 0.693 – 0.975). In the full multivariable Firth model (n = 27), ACE2 remained the sole significant predictor (OR = 1,644.86, 95% CI: 10.29 – 7,910,616, p = 0.0004), with Ang II showing a large but non-significant positive association (OR = 49.63, 95% CI: 0.044 – 4,281,882, p = 0.271) and Ang 1-7 showing a near-neutral effect (OR = 1.00, CI: 0.9674 – 1.0190, p = 0.9902), consistent with the standard model results. The full model was statistically significant (Likelihood ratio test [LRT], p = 0.0018) with an AUC of 0.9339 (95% CI: 0.835 – 1.000), demonstrating that discriminatory performance was preserved under penalized estimation and was not attributable to separation artifact. Confidence intervals remain wide due to the small sample size and should be interpreted accordingly.

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3.9.3. Decision Curve Analysis

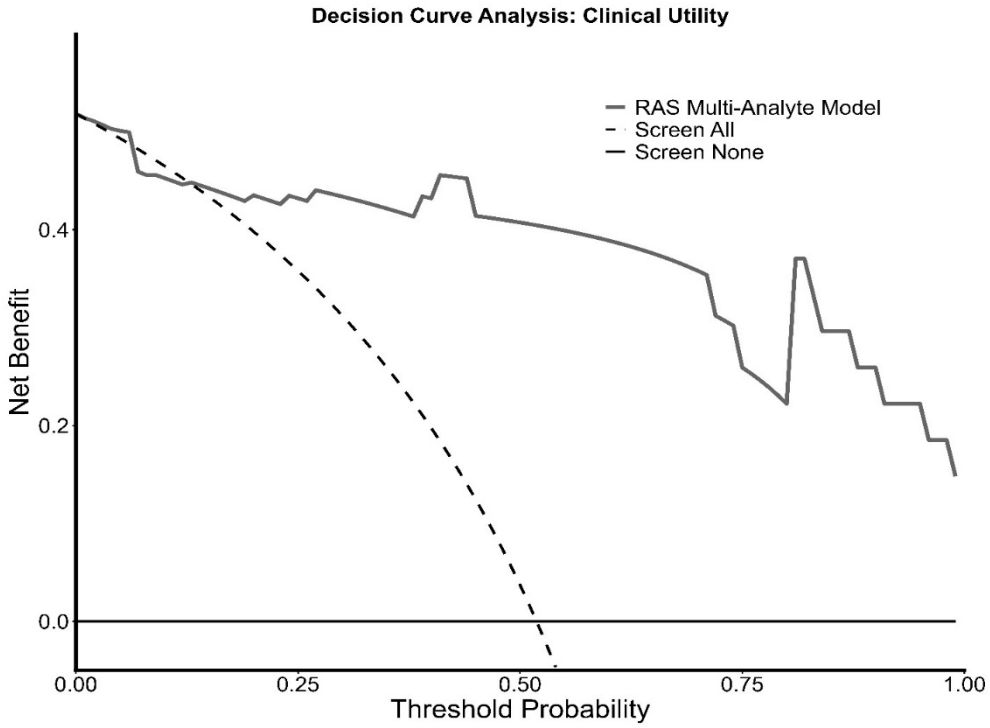

Figure 8. The Decision Curve shows that the RAS Multi-Analyte Model (bold grey line) maintains a superior net benefit over the 'Screen All' strategy (dashed line) across most threshold probabilities, particularly in the 20% to 50% risk range.

To assess the clinical net benefit of the RAS biomarkers, decision curve analysis (DCA) was employed. The model consistently outperformed the 'screen all' strategy across the majority of the threshold probability range. At a clinical threshold of 30%, the model yielded a net benefit of 0.43, compared to 0.31 for the 'screen all' approach. At a higher risk threshold of 50%, the model maintained a substantial net benefit (0.41) while the 'screen all' strategy offered no benefit (0.04) (Fig 8, Supplementary material 1 Table 5). This demonstrates the model's potential to reduce unnecessary clinical interventions without sacrificing sensitivity.

4. Discussion

In this study, we determined the concentrations of the RAS components (Ang 1-7, Ang II, Ang 1-7/Ang II, and ACE2) and a panel of cytokines (IL-6, IL-8, IL-10, and IFN-γ). We compared them between healthy controls and breast cancer patients to determine the effect of the disease on these biomarkers. Our results show that although Ang 1-7 and Ang II were not significantly affected by breast cancer, their ratio was significantly lowered. This finding indicates that breast cancer shifts the balance of RAS towards the pro-tumorigenic classical axis.

While the suppression of Ang 1-7 in breast cancer tissues is well-reported [22,41]. There was a significant gap in the circulating Ang 1-7 levels in the breast cancer patients. In another study conducted in 102 breast cancer patients, serum Ang II level was identified as a strong predictor of breast cancer mortality [42]. Our results showed no alteration in circulating Ang 1-7 levels, and a small change in Ang II levels in breast cancer patients suggests that the absolute quantities of Ang 1-7 and Ang II alone may not be as informative in a small study population. Therefore, we normalized the concentration of Ang 1-7 with Ang II to obtain a ratio that could represent the RAS balance. We observed a significant reduction in Ang 1-7/Ang II, suggesting that the RAS balance shifted towards the classical axis. This finding is consistent with previous studies demonstrating dysregulation of the RAS in breast cancer tissues and cell lines [1,43–45].

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Contrary to our finding, Zuo et al found that plasma ACE2 was significantly lowered in breast cancer patients when compared to healthy controls. However, ACE2 concentrations were elevated after treatment with chemotherapeutic agents and were associated with a poor prognosis [46]. We saw a significant increase in the ACE2 levels of breast cancer patients. Additionally, temporal and spatial variation in ACE2 expression may play a role in different stages of cancer. A critical strength of our study is the treatment-naïve status of all breast cancer patients, allowing attribution of elevated ACE2 levels to disease pathophysiology rather than treatment effects. ACE2 is also variable across racial and ethnic groups. A previous study on COVID-19 showed that ACE2 expression across different tissues was significantly higher in East Asian females than in both sexes of the Caucasian and African study populations [47]. A combination of these factors may explain the discrepancies we see in the two studies. Increased ACE2 levels may be due to the compensatory response to inflammation or an immune response related to cancer. This is evident from increased plasma ACE2 levels across various RAS-related diseases, including cardiovascular disease and COVID-19 [48–50]. Plasma and urinary ACE2 concentrations have also been associated with different metabolic and cardio-renal disorders [9,10,16]. Despite higher plasma ACE2 levels, patients with connective tissue diseases who presented with reduced ACE2 enzyme activity had considerable circulating ACE2 autoantibodies[15]. A similar effect was observed with the prevalence of circulating ACE2 autoantibodies in rheumatoid arthritis[51]. Interestingly, we saw a significant reduction of ACE2 levels in patients with hypertension as a comorbidity. One consideration for this analysis is that 4 (67%) of the non-hypertensive cohort had other comorbidities, of which 3 were cardiovascular-related. The sample size was too small to conduct a multivariate analysis to account for confounding variables. We did not see any significant difference in ACE2 levels due to tobacco consumption. While studies suggest smoking cigarettes upregulates ACE2 expression, they have only been investigated in the lung and the respiratory tract tissues, not in systemic circulation [52,53].

The role of IL-10 in breast cancer is not fully understood, although it is well accepted that IL-10 plays an important role. It acts as both a pro- and an anti-inflammatory cytokine, promoting and suppressing tumor progression [54,55]. IL-10 promotes tumor progression by activating STAT3, thereby inhibiting apoptosis and evading immune surveillance. Furthermore, it inhibits dendritic cell function, downregulates the human leukocyte antigen class I molecule on the tumor cell surface, and suppresses the cytotoxicity of natural killer cells [56–58]. On the other hand, it inhibits tumor progression by downregulating the synthesis of pro-angiogenic factors and suppressing local pro-inflammatory and pro-tumorigenic cytokine release [26,59–61]. We observed lower IL-10 levels in breast cancer patients than in controls (Fig 3a). While IL-10 levels are generally shown to be elevated in cancer patients, studies have shown that plasma IL-10 levels can be lower in ER^+ and PR^+ breast cancer subtypes [62]. In addition, IL-10 levels were lowered after chemotherapy in ovarian cancer patients [63]. We also saw IL-10 levels significantly elevated in the hypertensive group. While the dysregulation of the IL-10 response is complex, IL-10 levels are expected to be lowered in chronic cardiovascular disease, such as hypertension [64]. Studies have shown that IL-10 levels can increase with the use of antihypertensive medications [65–67]. We also saw an increase in IL-10 levels of patients who used tobacco, which is consistent with previous studies [68–70]. While our findings are consistent with some previous findings, a study with a larger sample size considering a greater number of variables such as use of antihypertensive drugs, duration of use, type of tobacco use (smoking, chewing, patch, etc.), status and duration of tobacco use, etc. is required to get a better picture of dysregulation of ACE2 and IL-10.

We tested the association among the RAS components in all participants by calculating correlation coefficients. We did not see any correlation between Ang 1-7 and Ang II levels. This is consistent with a previous study showing there was no correlation between Ang 1-7 and Ang II in females [33], as our study cohort was exclusively composed of female subjects. Similarly, we did not observe a significant correlation between Ang 1-7 and Ang II individually, with ACE2, but we observed a moderate negative correlation between the Ang 1-7/Ang II ratio and ACE2. Although no direct correlations between ACE2 and the effector peptides have been previously evaluated, evidence

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indicates that plasma ACE2 is derived from ACE2 shedding from tissue-bound protein [71]. The soluble ACE2 retains its catalytic activity to degrade Ang II [72]. Therefore, with increased Ang II, plasma ACE2 may increase as a compensatory mechanism, leading to a negative correlation between ACE2 and the Ang 1-7/Ang II ratio [9].

We also saw that Ang II was negatively correlated with BMI. Under normal physiological conditions, adipose tissue expresses components of the RAS, such as angiotensinogen. Higher BMI, or more adipose tissue, means high angiotensinogen and subsequently high Ang II levels, leading to increased cancer risk. Many studies report that this holds across various pathological conditions, such as diabetes and cancer [18,73]. Interestingly, in a large study carried out in 2020, this trend was not true for females with breast cancer and cervical cancer. Inverse association for breast cancer incidence was seen when assessed against an increase in BMI [74]. Our findings support this notion, as we observe an inverse relationship between Ang II and BMI. This could be due to lower concentrations of estradiol and progesterone in the elderly females, low insulin-like growth factor-1 (IGF-1) levels and early differentiation of breast cancer tissues [74,75]. Additionally, the use of cytotoxic chemotherapy is often linked to cachexia or adipose tissue atrophy. This factor, combined with inflammation-induced high Ang II levels, may allow low BMI to coexist with high Ang II [76].

We saw a significant negative correlation between Ang 1-7 and platelets. Platelets express the Mas receptor and, upon activation, release nitric oxide, contributing to the antithrombotic effect [77]. Ang 1-7 has also been shown to stimulate platelet recovery in thrombocytopenia, suggesting a supportive role in platelet regeneration and production [78]. Hemoglobin did not show a significant correlation with Ang 1-7; however, it was positively correlated with Ang II and negatively correlated with the Ang 1-7/Ang II ratio. Ang II, via AT1R activation, stimulates erythropoietin secretion and acts as a growth factor for erythrocyte precursors in the bone marrow [79,80]. Treatments such as angiotensin receptor blockers (ARBs) and ACE inhibitors reduce hemoglobin and erythropoietin levels [80,81]. This shows that our findings are consistent with the previous studies.

We determined an optimal model with a panel of biomarkers involving ACE2, Ang II and Ang 1-7 to predict the occurrence of breast cancer with excellent predictive performance using the Youden index, which offers the maximum potential effectiveness of a biomarker and is a common summary measure of the ROC curve. Although ACE2 alone showed strong discrimination between the groups, adding Ang 1-7 and Ang II to the model improved both the AUC and the model's accuracy. The model, initially built with a standard cutoff of 0.5, also showed strong discrimination, with a Youden's index of 0.86 and optimal cutoff of 0.4203 for our combined model, indicating higher accuracy. The lower cutoff value may be due to the wide confidence intervals for the variables in the forest plot, suggesting high variability in the data. Although the model fared well at higher cutoffs, we optimized it to avoid misclassifications of patients as controls. The improvement in accuracy demonstrates meaningful clinical benefit from a combination of biomarkers, consistent with established findings that multiple biomarkers are better predictors than any single biomarker alone [82–84].

The forest plot (Fig 8) shows a detailed insight into the individual contributions of the biomarkers in the model. ACE2 was identified as the strongest independent predictor, with statistical significance, highlighting its central role in disease progression, consistent with previous studies showing that ACE2 may be altered during tumorigenesis and serve as a biomarker for multiple diseases [85–89]. Although statistically insignificant, Ang 1-7 showed a near-neutral effect and improved the model's performance. This finding reflects the complex counter-regulatory balance of the protective RAS towards its negative impact. Ang II showed a substantially large positive association, highlighting its important role as an independent risk factor of breast cancer. This supports the fact that Ang II plays a critical role in cancer progression [45,90,91]. The overall model performance supports the clinical relevance of this comprehensive biomarker panel for cancer prediction.

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Limitations

While the findings of this pilot study strongly predict the presence of breast cancer, external validation in an independent cohort is warranted. The model was validated internally on a small sample size with high accuracy. However, breast cancer is a complex disease; therefore, external validation with a large study population is required to generalize this finding. In addition, RAS is dysregulated in many diseases; thus, this model may be limited in accurately diagnosing a single disease. Consequently, similar model development with ACE2 and other RAS components may be required across different disease models to differentiate between them. Due to these limitations, our model should be used as a proof-of-concept for general breast cancer screening, not as a diagnostic tool.

5. Conclusions

This proof-of-concept study provides compelling evidence of significant RAS dysregulation in treatment-naïve breast cancer patients. Our results show that elevated plasma ACE2 levels and a markedly reduced Ang 1-7/Ang II ratio serve as distinct indicators of disease state, suggesting a shift towards a pro-tumorigenic classical axis.

The most significant contribution of this work is the identification of a novel multi-analyte signature comprising ACE2, Ang 1-7, and Ang II in distinguishing breast cancer patients from healthy controls with remarkable accuracy (AUC = 0.9396). This model outperformed individual RAS components, underscoring the importance of assessing the balance between the classical and protective RAS axes rather than focusing on isolated peptides or proteins. The observed correlation between RAS components and IL-10 suggests a complex interplay between RAS dysregulation and the immune microenvironment in breast cancer.

While the findings are promising, we acknowledge the limitations inherent in a pilot study, including a temporal gap in sample collection and a small sample size. However, internal stability analysis and the robustness of the bootstrap-validated model support the biological validity of these biomarkers.

In conclusion, plasma ACE2 and Ang 1-7/Ang II ratio represent potential circulatory biomarkers. If validated in larger, multicenter, prospective cohorts, this model could provide a minimally invasive tool for early breast cancer detection, potentially guiding more personalized therapeutic strategies targeting the RAS pathway.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org., Figures and tables: Supplementary Material 1.

Author Contributions: Conceptualization, A. A.-H.; methodology, A.A.-H., B.G., P.G. and S.E.H.; software, A.A.-H. and B.G.; validation, A.A.-H. and B.G.; formal analysis, A.A.-H., B.G., P.G. and S.E.H.; investigation, A.A.-H. and B.G.; resources, A.A.-H. and S.T.; data curation, A.A.-H., B.G., P.G. and S.E.H.; writing—original draft preparation, A.A.-H. and B.G.; writing—review and editing, A.A.-H., B.G., P.G. S.T. and S.E.H.; visualization, A.A.-H., B.G. and P.G.; supervision, A.A.-H.; project administration, A.A.-H.; funding acquisition, A.A.-H. and S.T. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Idaho State University (protocol code IRB-FY2020-273, February 29, 2024).

Informed Consent Statement: Patient consent was waived due to eligibility of this study for Certificate of Exemption under the Category 4: Secondary Research for which Consent is Not Required, (ii) Information, which may include information about biospecimens, is recorded by the investigator in such a manner that the identity

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of the human subjects cannot readily be ascertained directly or through identifiers linked to the subjects, the investigator does not contact the subjects, and the investigator will not re-identify subjects.

Data Availability Statement: The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Abbreviations

The following abbreviations are used in this manuscript:

RAS	Renin Angiotensin System
ACE	Angiotensin-Converting Enzyme
Ang I	Angiotensin I
Ang II	Angiotensin II
Ang 1-7	Angiotensin (1-7)
AT1R	Angiotensin II Type 1 Receptor
AT2R	Angiotensin II Type 2 Receptor
MasR	Mas Receptor
NF-κB	Nuclear factor kappa B
FAK	Focal adhesion kinase
EMT	Epithelial-mesenchymal transition
SPE	Solid Phase Extraction
MRM	Multiple reaction monitoring
ESI	Electrospray ionization
CE	Collision energy
DP	Declustering potential
CXP	Cell exit potential
BMI	Body mass index
$HER2^+$	Human Epidermal Growth Factor Receptor 2 positive
NSAIDs	Non-steroidal anti-inflammatory drugs
T	Tumor
N	Nodes
M	Metastasis
pN	Pathological N categories
ER^+	Estrogen receptor positive
PgR^+	Progesterone receptor positive
AIC	Akaike Information Criterion
R^2_{McF}	McFadden's pseudo R^2
VIF	Variance inflation factor
ROC	Receiver operating characteristic

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