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ORIGINAL ARTICLE

High-precision immune-related plasma proteomics profiling predicts response to immunotherapy in patients with triple-negative breast cancer

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ABSTRACT

Objective: While immunotherapy holds great potential for triple-negative breast cancer (TNBC), the lack of non-invasive biomarkers to identify beneficiaries limits the application.

Methods: Paired baseline, on-treatment, and post-treatment plasma samples were collected from 195 TNBC patients receiving anti-PD-1 immunotherapy in this retrospective study conducted at the Fudan University Shanghai Cancer Center (FUSCC) for sequential high-precision proteomic profiling.

Results: ARG1, NOS3, and CD28 were identified as plasma proteins significantly associated with the response to immunotherapy in neoadjuvant settings or in advanced stages of TNBC. Matched single-cell RNA sequencing data were incorporated to correlate peripheral plasma with the tumor microenvironment. Furthermore, the Plasma Immuno Prediction Score was developed to demonstrate significant predictive power for evaluating the efficacy and prognosis of patients undergoing neoadjuvant immunotherapy.

Conclusions: The results underscore the importance of systemic immunity in the immunotherapy response and support the use of plasma protein profiles as a feasible tool for enhancing personalized management of immunotherapy in breast cancer.

KEYWORDS Triple-negative breast cancer; immunotherapy; non-invasive biomarkers; efficacy prediction; plasma proteomic profiling

Introduction

Triple-negative breast cancer (TNBC) stands out as a specific subtype of breast cancer lacking the expression of three pivotal receptors: estrogen receptor (ER); progesterone receptor (PR); and human epidermal growth factor receptor 2 (HER2)[1]. TNBC is associated with a high risk of early recurrence and unfavorable patient outcomes, accounting for approximately 15% of invasive breast cancers[2]. Unlike other breast cancer types, TNBC presents unique challenges due to the absence of targets for existing therapeutic agents. This absence of receptors, coupled with molecular heterogeneity, leads to differential patient responses to conventional treatments, rendering TNBC management more challenging[3].

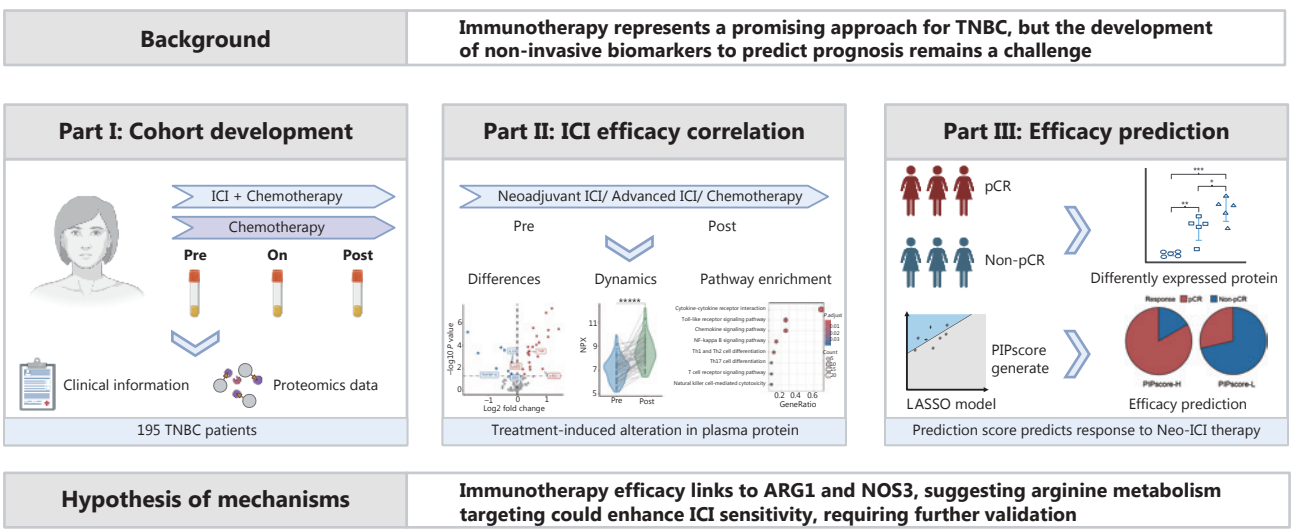
Blocking the programmed death-1 (PD-1) protein or the PD-1 ligand (PD-L1) *via* immune checkpoint inhibitors (ICIs) has drastically altered the systemic treatment landscape for TNBC[4]. ICIs, when combined with chemotherapy, significantly improve patient outcomes in early and metastatic settings, achieving durable responses in a substantial fraction of patients[5]. However, this therapeutic evolution underscores a pressing need for reliable predictive biomarkers. Existing markers, such as tumor mutational burden (TMB), microsatellite

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instability (MSI), and PD-L1 expression, have been shown to correlate with ICI responsiveness[6]. However, these markers often fail to fully anticipate therapeutic responses, highlighting the complex and multifactorial nature of immune responses[7]. This complexity suggests that a focus on the tumor microenvironment (TME) alone is insufficient for predicting treatment outcomes. A more comprehensive treatment approach that includes consideration of systemic immunity elements, such as circulating immune cells and cytokine profiles, is crucial[8]. Given the significant heterogeneity of treatment responses, the quest for effective therapeutic biomarkers that encompass local and systemic immune factors is pivotal.

The proximity extension assay (PEA), a next-generation plasma proteomics technology, allows for more sensitive and precise quantification of plasma proteins[9,10]. Plasma proteomics has greatly advanced cancer diagnosis and treatment by enabling the non-invasive and frequent detection of plasma proteins[11,12]. This technology can identify protein signatures that correlate with treatment outcomes and aid the development of personalized cancer treatment strategies. For example, it was reported that elevated baseline serum leukemia inhibitory factor (LIF) levels are related to poor clinical outcomes in patients treated with immune checkpoint blockade therapies (ICBs)[13]. Moreover, pre-treatment serum immune proteomics have been pivotal in decoding the dynamic systemic immune responses to preoperative chemotherapy in patients with gastric adenocarcinoma[14]. The detection of unique protein signatures related to immune responses has expedited the identification of new biomarkers essential for early disease detection and monitoring the effectiveness of treatments, such as chemotherapy and immunotherapy. Despite these advances, the application of plasma proteomics to TNBC immunotherapy has not been fully explored. Specifically, the changes in plasma proteins before and after immunotherapy for TNBC, as well as the role of these proteins in predicting the efficacy of immunotherapy, have not been thoroughly investigated.

In this study an immunotherapy cohort of TNBC with plasma immune proteomics, single-cell RNA sequencing (scRNA-seq), and clinicopathologic data was developed. Correlations between plasma immune proteomics and the localized tumor environment were established and key plasma markers significantly associated with immunotherapy efficacy were established (**Study flowchart**).



Study flowchart Part I profiled 92 plasma proteins (Olink Target 96 Immuno-Oncology panel) across pre-, on-, and post-treatment phases in a triple-negative breast cancer (TNBC) cohort predominantly receiving immunotherapy (n = 169) with a minority undergoing chemotherapy (n = 26). Part II performed differential expression analysis of plasma proteins between pre- and post-treatment timepoints, revealing dynamic changes in protein levels accompanied by pathway enrichment that demonstrated significant immune activation signatures. Part III identified key biomarkers through differential expression analysis between responders and non-responders and developed the Plasma Immuno Prediction Score (PIPscore; AUC = 0.858) for stratifying responders to neoadjuvant immunotherapy. This study established a comprehensive plasma proteomic signature for predicting immunotherapy response in TNBC, integrating dynamic treatment monitoring with robust clinical validation through a novel PIPscore model. *P < 0.05, **P < 0.01, ***P < 0.001.

Materials and methods

Patient cohorts

In this retrospective study plasma samples were obtained from patients with TNBC who were treated with immunotherapy at the Fudan University Shanghai Cancer Center (FUSCC) (Shanghai, China) between July 2021 and October 2022. Patients enrolled in this study met the following criteria: pathologically confirmed TNBC; age ≥18 years; Eastern Cooperative Oncology Group (ECOG) performance status score of 0–2; and received immunotherapy or chemotherapy. The participants were divided into three distinct groups based on the treatment approach: group A, neoadjuvant therapy; group B, adjuvant therapy; and group C, advanced disease stage. Group A consisted of 101 patients who received neoadjuvant immunotherapy under the camrelizumab + chemotherapy regimen and 26 patients who received neoadjuvant chemotherapy as determined by their physicians. Group B consisted of 26 patients who received adjuvant immunotherapy under the atezolizumab + chemotherapy regimen. Group C consisted of 42 patients who received immunotherapy for advanced TNBC under the camrelizumab + chemotherapy regimen.

Clinical information, including age, group, treatment, response, and tumor node metastasis (TNM) staging, is listed in **Table S1**. All samples were stored at −80°C until processing. The study complied with the ethical principles of the Declaration of Helsinki and was approved by the Ethics Committee of FUSCC (050432-4-1911D) in 2019 for biological specimen collection and by the SIBPT Ethics Committee (PJ2022-47) in 2022 for project establishment. Written informed consent was obtained from each patient before any study-specific investigation was performed.

Plasma immune proteomics

Proteomic profiling was performed on blood plasma from the patient cohorts using the Olink Target 96 Immuno-Oncology Panel (**Table S2**). The Olink platform is based upon in-solution binding of two polyclonal antibody pools to a target protein and subsequent hybridization and enrichment of two unique single-stranded DNA probes to create a double-stranded barcode unique for the antigen[15].

All blood plasma samples and randomly selected duplicate samples were randomized and plated in four 96-well plates. Each plasma sample was processed and analyzed individually. Samples were processed according to the manufacturer's instructions. Briefly, the 92 antibody pairs labeled with DNA oligonucleotides bound to their respective proteins in the samples. Then, oligonucleotides that were brought into proximity were hybridized and extended using a DNA polymerase. This newly created piece of DNA barcode was amplified by PCR. Finally, the amount of each DNA barcode was quantified by microfluidic qPCR. Microfluidic qPCR was quantified by an Olink Signature Q100. Normalized protein expression (NPX) values for each protein per patient were produced by Olink NPX Signature software. NPX is the Olink relative protein quantification unit on a log2 scale. A high NPX value equals a high protein concentration.

Olink implemented a robust quality control (QC) system with multi-level normalization to ensure assay reliability. Four predefined controls were spiked into each sample to monitor procedural steps. Triplicate inter-plate controls (IPC) were embedded on each plate and the median of the IPC triplicates was used to normalize assays, which compensated for technical variability between runs and plates.

Plasma ARG1, NOS3, and CD28 assays

The ARG1, NOS3, and CD28 plasma concentrations in TNBC patients prior to neoadjuvant immunotherapy were measured by enzyme-linked immunosorbent assay (ELISA) using reagent kits for human ARG1 (Multi Sciences, Hangzhou, China), NOS3 (BOSTER, Wuhan, China), and sCD28 (BOSTER) according to the manufacturer's instructions.

Functional enrichment analyses

The differential plasma protein expression before and after treatment was tested using the Wilcoxon rank-sum test. Proteins with a P value < 0.05 were selected for enrichment analyses. The enrichment analysis was performed on the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology Biological Process (GO) to categorize the biological functions of genes identified in these three modules using the "clusterProfiler" package in R.

Tissue sample collection and single-cell RNA library preparation and sequencing

All sample collection procedures complied with the regular clinical practices. Fresh tumor biopsies obtained by needle

puncture were initially stored in tissue storage solution (Miltenyi Biotec, China) on ice. The samples were then placed in RPMI-1640 medium and enzymatically digested with a gentle MACS Tumor Dissociation kit (Miltenyi Biotec) for 60 min at 37°C following the manufacturer's protocol. The dissociated cells were passed through a 70-mm cell strainer in RPMI-1640 medium with 10% FBS until uniform cell suspensions were obtained. The cells were subsequently passed through additional cell strainers and centrifuged at 400 × g for 10 min and red blood cells were removed. The Single Cell 3′ Library and Gel Bead kit V3 (10× Genomics, China) and Chromium Single Cell A Chip kit (10× Genomics) were used for scRNA-seq. The cell suspension was loaded onto the Chromium Single-cell Controller (10× Genomics) to generate single-cell gel beads in the emulsion (GEMs) per the manufacturer's protocol. Briefly, single cells suspended in PBS with 0.04% bovine serum albumin were added to each channel and the captured cells were lysed. The released RNA was barcoded *via* reverse transcription in individual GEMs. Reverse transcription was performed at 53°C for 45 min, followed by 85°C for 5 min, then held at 4°C. Complementary DNA was generated and amplified, and the quality was assessed using an Agilent 4200 (CapitalBio Technology, Beijing, China) following the manufacturer's instructions. Sequencing libraries were constructed using the Single Cell 3′ Library Gel Bead kit V3. The libraries were sequenced using an Illumina NovaSeq 6000 with a paired-end 150-base pair (PE150) reading strategy (Oebiotech, Shanghai, China).

scRNA-seq data preprocessing and cellular clustering

The Cell Ranger software pipeline (version 3.1.0) provided by 10× Genomics was utilized for the analysis. This pipeline was used to demultiplex cellular barcodes, map reads to the genome and transcriptome using the STAR aligner, and down-sample reads as needed to generate normalized aggregate data across samples. The resulting output was a matrix of gene counts *vs.* cells. The unique molecular identifier (UMI) count matrix was then obtained. To identify and exclude doublet droplets, the R package DropletUtils (version 1.12.3) was used. Then, raw gene expression matrices were analyzed using Seurat. Briefly, all cells expressing < 200 or > 8000 genes were excluded, as well as cells that contained <500 UMIs or > 20% mitochondrial counts. The default parameters were utilized unless otherwise indicated. For the clustering of all cell types, 2000 variable genes were identified and principal component analysis (PCA) was applied to the dataset to reduce dimensionality after regressing for the number of UMIs, percentage mitochondrial genes, and cell cycle. The top 20 informative principal components were used for clustering and uniform manifold approximation and projection (UMAP). Clusters in the resulting two-dimensional UMAP representation consisted of distinct cell types, which were identified based on the expression of marker genes.

K-means clustering and least absolute shrinkage and selection operator (LASSO) regression

An unsupervised learning algorithm (K-means clustering) was used to demonstrate the pretreatment plasma protein expression patterns. LASSO regression was used to generate the plasma immuno prediction score (PIPscore), which predicted the immunotherapy response. The lambda value (λ = 0.0785) was calculated by 3-fold cross-validation *via* the minimum mean cross-validated error. Non-zero coefficients generated by LASSO regression using the above lambda value are indicated in **Figure S8A**. The glmnet R package[16] was used to perform the LASSO regression.

Statistical analysis

The normality of the variables was tested using the Shapiro–Wilk normality test. Statistical significance for normally distributed variables was estimated using paired/unpaired Student's t tests for comparisons of two groups; variables not normally distributed were analyzed using the Wilcoxon rank-sum test. One-way ANOVA or Kruskal-Wallis tests were applied for comparisons among multiple groups. Correlation coefficients were computed by Spearman or Kendall rank correlation, as indicated. The R package (pROC) was used to plot and visualize receiver operating characteristic (ROC) curves and calculate the area under the curve (AUC) to evaluate the treatment response accuracy of different indices. A two-sided P value < 0.05 was considered statistically significant for all analyses and P values were designated as * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001, and ***** < 0.00001, or not significant (ns). All data were analyzed using R Statistical Software (version 4.1.0).

Results

Overall synopsis of the plasma proteome profiling of patients with TNBC

A total of 195 patients diagnosed with TNBC were included in this study (**Figure 1A**). These patients were divided into three distinct groups (groups A-C), representing neoadjuvant, adjuvant, and advanced therapeutic interventions, respectively. Most patients were enrolled in treatment regimens that incorporated immunotherapy. Detailed information about the specific treatment protocols and number of patients assigned to each group are shown in **Table S1**. The cohort under study was also marked by the inclusion of comprehensive clinical pathologic information and paired scRNA-seq (n = 11), which provide granular insight into intratumoral heterogeneity and cellular functionality.

The plasma collection was strategically studied at three critical treatment periods (pre-treatment, on-treatment, and post-treatment), which provided a timeline to determine potential therapy-induced proteomic changes (**Table 1**). High-sensitivity and -specificity proteomic analysis of the plasma samples was performed using a proximity extension assay (PEA) with the Olink Target 96 Immuno-Oncology panel, as illustrated in **Figure 1B**. Importantly, the robustness of the analysis was underscored by the lack of significant batch effects, as evidenced in **Figure S1**. After successful gathering of the plasma proteomic data and the corresponding clinical pathology patient information, a comprehensive bioinformatics analysis was undertaken to explore potential correlations between the plasma proteome and clinical outcomes.

Plasma proteome profiles differed between diverse TNBC subgroups

A comprehensive analysis of plasma proteome profiles in untreated TNBC patients revealed interesting associations with tumor (T) stage and node (N) stage, which provided valuable insight into disease biology and potential therapeutic strategies.

Distinct protein expression patterns were associated with different T and N stages (**Figure S2A, B**). Specifically, IL-33

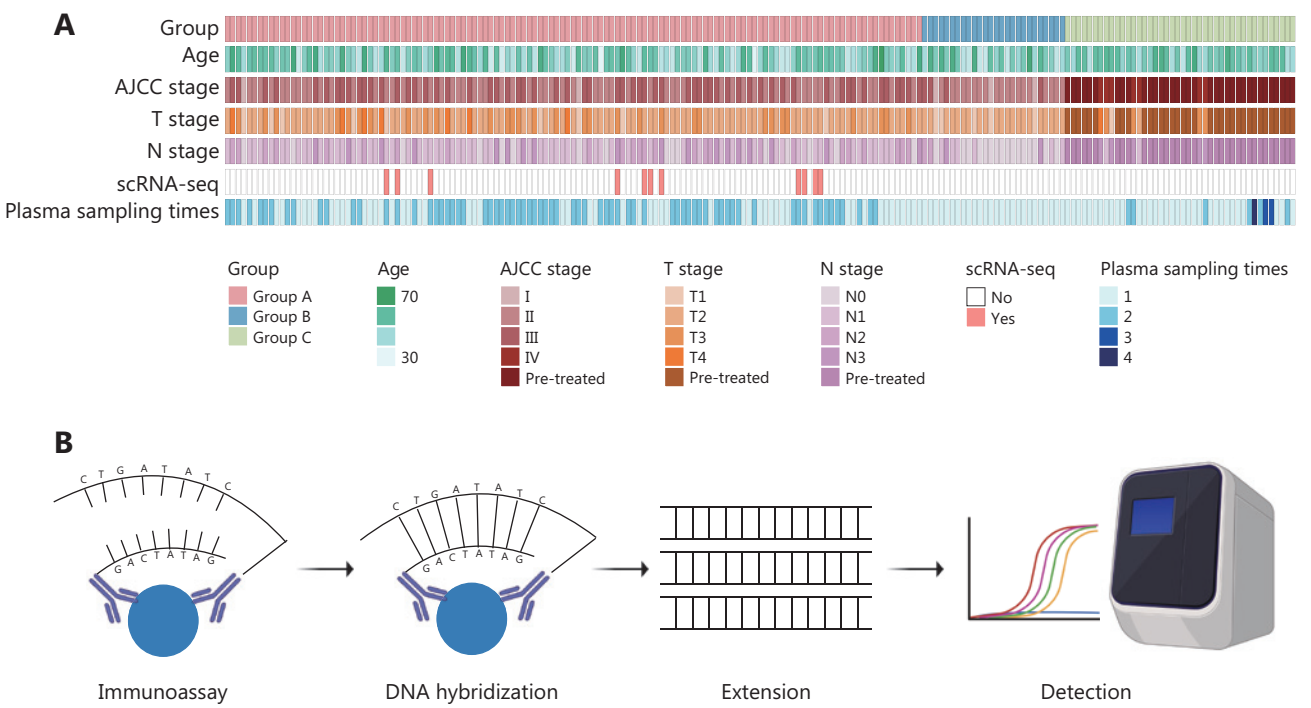


Figure 1 Patient cohort characteristics and plasma proteomics workflow. (A) Heatmap representing the availability of clinicopathologic data, plasma sampling, and single-cell sequencing for patients; (B) Core steps of the proximity extension assay (PEA) technology.

Table 1 Patient and plasma sample distribution

Group	Cohort	Total patients, *n*	Patients with plasma samples, *n*			Total plasma samples, *n*
			Pre-treatment only	Post-treatment only	Both pre- and post-treatment	
A	Neoadjuvant immunotherapy	101	16	25	60	161
	Neoadjuvant chemotherapy	26	9	5	12	38
B	Adjuvant immunotherapy	26	26	0	0	26
C	Advanced immunotherapy	42	12	21	9[a]	55

[a]In Cohort C (advanced immunotherapy), the "Both pre- and post-treatment" category (defined as patients with at least one pre-treatment and one post-treatment sample) includes 9 patients with varying sampling strategies: 6 patients collected 1 pre-treatment + 1 post-treatment sample (total 12 samples), 2 patients collected 1 pre-treatment + 1 on-treatment + 1 post-treatment samples (total 6 samples), and 1 patient collected 1 pre-treatment + 2 on-treatment + 1 post-treatment samples (total 4 samples). The total number of samples in this category is 22.

and FGF2 were elevated in T3 and 4 stages, suggesting roles in inflammation and angiogenesis, respectively[17,18]. The levels of MUC-16 and MMP-7, proteins known for roles in tumor invasion and metastasis[19,20], were increased with T stage progression (**Figure S2C**). Similarly, an increase in the IL-8 level, which is involved in immune responses and lymph node metastasis[21], and a decrease in the CRTAM level, indicating a potential inverse relationship with lymph node involvement, were noted with N stage progression (**Figure S2D**).

This intricate interplay of protein expression continued when comparing early- (AJCC stage I-II) and late-stage (AJCC stage III-IV) TNBC patients (**Figure S2E**). Proteins, such as CRTAM, IL15, and PD-L2, known for their roles in immune response regulation, were prominently expressed in early-stage TNBC[22-24]. In contrast, late-stage TNBC exhibited an increase in proteins, such as MUC-16, MMP7, MMP12, IL-8, and FGF2, suggesting involvement in disease progression, angiogenesis, and immune evasion[20,25-27].

Immunotherapy-induced alterations in plasma protein expression in TNBC patients

First, the changes in plasma protein profiles in response to immunotherapy were determined. The analysis revealed that one-half of the 92 proteins had significant changes in paired and unpaired tests following neoadjuvant immunotherapy (group A; **Figures 2A** and **S3A**), suggesting complex systemic immune responses. A substantial number of immune-related factors, including CXCL9, CXCL10, CCL3, IFN-gamma, and LAMP3, had increased levels, suggesting the potential enhancement of immune activity against cancer cells due to therapy (**Figure 2B**)[28-30]. In contrast, the post-treatment reduction in MUC-16 levels, a biomarker frequently overexpressed in various cancers, suggested a significant alteration in the immune response following immunotherapy[31]. Function enrichment and signaling pathway analyses were performed based on the KEGG and GO databases to further explore the biological functions of the alternating proteins (**Figure 2C, D**). Some signaling pathways were significantly upregulated after neoadjuvant immunotherapy, including the cytokine-mediated signaling pathway, toll-like receptor signaling pathway, and leukocyte migration.

Differential responses were observed in plasma protein profiles between patients who achieved a pathologic complete response (pCR) and patients who did not achieve a pCR (non-pCR) following neoadjuvant immunotherapy (**Figure 2E**). An elevation in 12 specific proteins in pCR patients post-treatment, including stress-induced HO-1[32], pro-inflammatory IL-6[33], and immune suppressive PD-L1[34], could indicate an enhanced immune response against cancer (**Figure 2F**). Conversely, non-pCR patients exhibited a post-treatment rise in 3 proteins, including the pro-inflammatory cytokine, TNF[35], and the immune checkpoint, LAG3[36], potentially indicating heightened immune activity or cancer resistance mechanisms (**Figure 2G**).

The analysis of plasma proteome profiles in advanced TNBC patients undergoing immunotherapy (group C) revealed significantly different protein expression pre- and post-treatment, like the observations in neoadjuvant

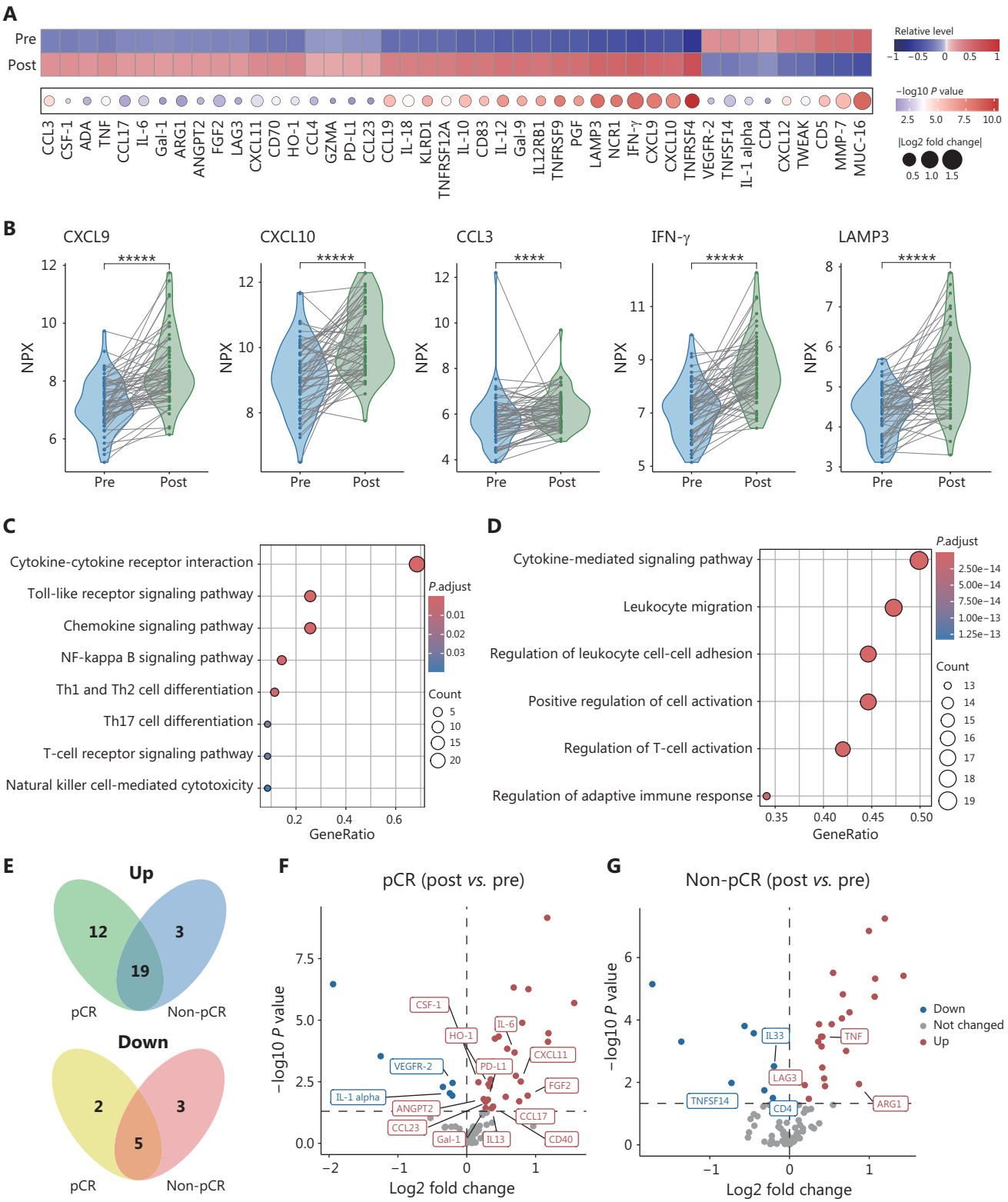


Figure 2 Changes in plasma proteins in patients undergoing neoadjuvant immunotherapy (group A). (A) Significantly altered plasma proteins ($P < 0.05$) in patients before and after neoadjuvant immunotherapy by paired tests ($n = 60$ paired); (B) Changes in plasma protein levels of CXCL9, CXCL10, CCL3, IFN-γ, and LAMP3 in patients before and after neoadjuvant immunotherapy; (C) KEGG analysis of changes in plasma

protein levels during neoadjuvant immunotherapy; (D) GO analysis of changes in plasma protein levels during neoadjuvant immunotherapy; (E) Venn diagram showing the overlap of differentially expressed plasma proteins between pCR and non-pCR groups after neoadjuvant immunotherapy; (F) Volcano plot showing differentially expressed plasma proteins unique to pCR patients after neoadjuvant immunotherapy (n = 33 paired samples). Colored and labeled proteins indicate significant changes (P < 0.05) only observed in the pCR group; (G) Volcano plot showing differentially expressed plasma proteins unique to non-pCR patients after neoadjuvant immunotherapy (n = 26 paired samples). Colored and labeled proteins indicate significant changes (P < 0.05) only observed in the non-pCR group. ****P < 0.0001, *****P < 0.00001.

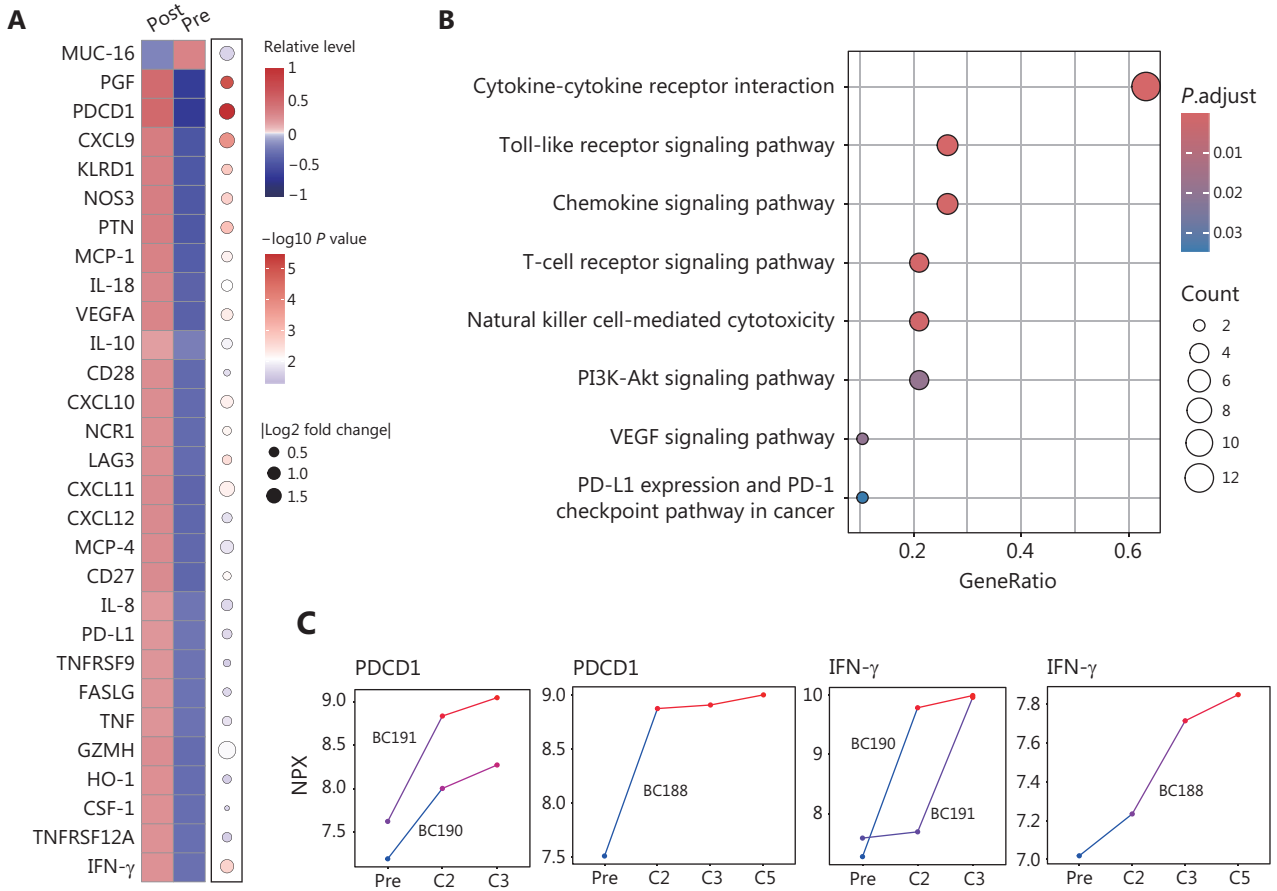


Figure 3 Changes in plasma proteins in advanced patients undergoing immunotherapy (group C). (A) Significantly altered plasma proteins (P < 0.05) in advanced TNBC patients before and after immunotherapy by unpaired tests (before n = 21, after n = 30); (B) KEGG analysis of changes in plasma protein levels in advanced patients undergoing immunotherapy; (C) Line graphs showing the progressive increase in PDCD1 and IFN-γ levels in advanced TNBC patients over the course of immunotherapy.

immunotherapy (**Figure 3A**). KEGG pathway analysis revealed distinct enrichment patterns between advanced and neoadjuvant immunotherapy settings. Patients with advanced TNBC showed unique enrichment in PI3K-Akt, VEGF, and PD-1/PD-L1 checkpoint pathways (**Figure 3B**). Conversely, neoadjuvant immunotherapy was distinctively characterized by enrichment in T helper cell differentiation pathways, including Th1, Th2, and Th17 (**Figure 2C, D**). Moreover, PDCD1 and IFN-γ showed dynamic upregulation during treatment (**Figure 3C**). The observed PDCD1 upregulation may signify an intensified immune response, corroborated by the optimal therapeutic response [partial response (PR)] observed in patients BC190 and BC191 and a complete response (CR) in patient BC188[37]. An increased IFN-γ level, which is crucial for the immune response and anti-tumor activity, may suggest an effective anti-tumor response[30]. The

analysis encompassed a broader array of proteins, which are represented in additional line graphs showing the dynamic changes throughout treatment (**Figure S3B**), while focusing on these key proteins. Although preliminary, these findings hint at the potential use of these plasma proteins as dynamic biomarkers for monitoring therapeutic response in late-stage disease.

Chemotherapy-induced alterations in plasma protein expression in TNBC patients

A similar analysis was also performed on patients receiving neoadjuvant chemotherapy (group A). Significant changes in plasma protein levels before and after treatment were noted (**Figures 4A and S4**). Divergent patterns of plasma protein

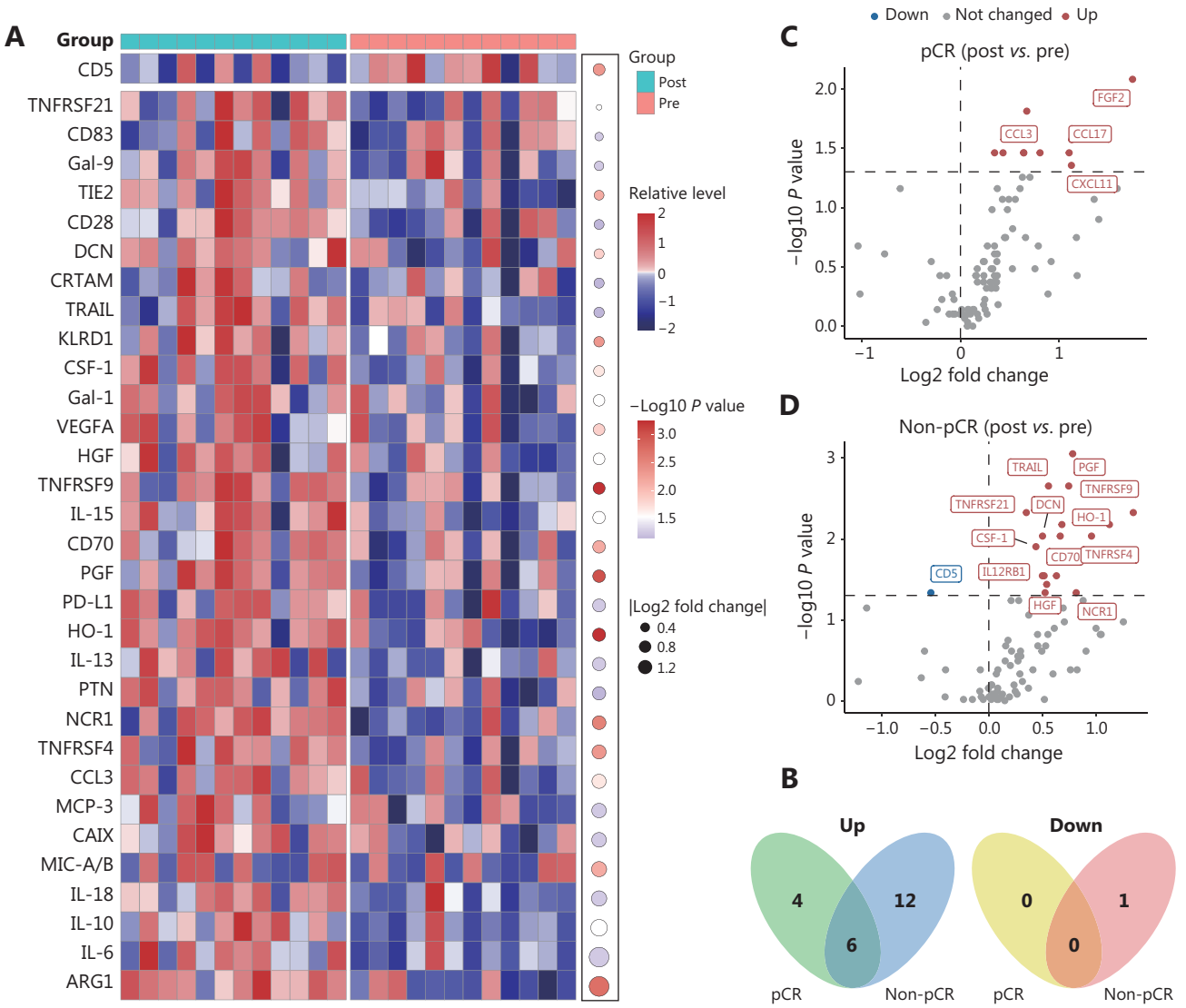


Figure 4 Changes in plasma proteins in patients undergoing neoadjuvant chemotherapy (group A). (A) Significantly altered plasma proteins (P < 0.05) in patients before and after neoadjuvant chemotherapy by paired tests (n = 12 paired); (B) Venn diagram showing the overlap of differentially expressed plasma proteins between pCR and non-pCR groups after neoadjuvant chemotherapy; (C) Volcano plot illustrating shifts in plasma protein levels in patients before and after neoadjuvant chemotherapy by unpaired tests (before n = 7, after n = 11). Colored and labeled proteins indicate significant changes (P < 0.05) observed only in pCR group; (D) Volcano plot illustrating shifts in plasma protein levels in patients before and after neoadjuvant chemotherapy by unpaired tests (before n = 13, after n = 6). Colored and labeled proteins indicate significant changes (P < 0.05) only observed in the non-pCR group.

alterations were also demonstrated between patients who achieved a pCR and patients who did not (non-pCR), suggesting a potential correlation between plasma protein changes and the chemotherapy response (**Figure 4B-D**).

Correlations between plasma protein levels and the TME in TNBC patients

The correlations between systemic immunity and the TME were evaluated, given the focus of most existing cancer immunology studies on the TME. A further step was undertaken to explore the interplay between plasma protein levels and the TME building on our previous plasma proteomics analysis. Specifically, pre-treatment research biopsies were collected from 11 untreated TNBC patients. scRNA-seq was performed on these biopsy samples utilizing established protocols. The results showed that cells were predominantly clustered based on cell type, forming nine transcriptionally distinct clusters (**Figure S5A**). All annotated cell types were detected in each patient with varying proportions of cell types between cases, indicating no patient-specific subpopulations in the integrated dataset (**Figure S5B**). The markers that distinguished the main cell types are shown in **Figures S5C and D**. Furthermore, subsequent analyses subdivided the T cells and myeloid cells into additional subclusters, ultimately revealing 15 distinct populations (**Figure S5E-H**).

Indeed, the analysis uncovered intriguing relationships between plasma protein levels and cell type presence within the TME that hint at specific biological roles (**Figure S6A**). Specifically, higher levels of CRTAM, a protein crucial for T-cell activation[38], were positively correlated with an increased proportion of $CD4^+$ T cells in the TME (**Figure S6B**), suggesting that CRTAM may modulate the recruitment and/or retention of $CD4^+$ T cells and influence immunologic dynamics within the tumor. Concurrently, an elevated level of FGF2 protein, a potent angiogenic and wound-healing growth factor[39], had a positive association with the presence of epithelial cells within the TME (**Figure S6C**), indicating that FGF2 might enhance an environment conducive to epithelial cell survival and proliferation, thereby impacting tumor growth and cellular heterogeneity. In contrast, an inverse relationship was noted between the level of IL-10 protein, an anti-inflammatory cytokine[40], and the proportion of $CD8^+$ T cells in the TME (**Figure S6D**), suggesting that an elevated IL-10 level may foster an immunosuppressive environment within the tumor, potentially limiting the infiltration or survival of cytotoxic $CD8^+$ T cells and aiding in immune evasion.

Taken together, these findings shed light on the intricate interplay between systemic immune markers and cellular dynamics within the TME, underscoring the importance of interpreting these interactions within the larger framework of the immune landscape and systemic host response.

Pre-treatment plasma protein levels predict the immunotherapy response and prognosis in patients with advanced TNBC

Advanced TNBC patients undergoing immunotherapy (group C) were categorized into two groups [complete or partial response (CRPR) as good responders and stable or progressive disease (SDPD) as poor responders], according to RECIST version 1.1. Comparative analysis of plasma protein expression between these groups revealed that IL-6, NOS3, VEGFA, KLRD1, and CSF-1 were significantly associated with poor prognosis in poor responders (SDPD; **Figure 5A, B**). Furthermore, univariate Cox analysis demonstrated that IL-6, NOS3, VEGFA, KLRD1, and CSF-1 were significantly associated with shorter progression-free survival (PFS) in advanced TNBC patients undergoing immunotherapy (**Figure 5C**).

Pre-treatment plasma protein levels predict the neoadjuvant immunotherapy response and patient prognosis

Plasma protein expression was compared between the different response groups to identify key proteins with predictive value for the efficacy of neoadjuvant immunotherapy (group A; **Figure 6A, B**). Three proteins were identified with a $P < 0.05$ level of significance, including ARG1 and CD28, which were highly expressed in pCR patients, while NOS3 had low expression. The plasma levels of ARG1, NOS3, and CD28 were determined to validate these findings using ELISA in different response groups (**Figure 6C**). The ELISA results corroborated the previous observations from Olink plasma proteomics sequencing, demonstrating concordant differential expression patterns of these proteins across response groups. This orthogonal validation not only confirmed the reliability of the proteomic findings but also substantiated the robustness of the Olink plasma proteomics platform. Furthermore, ARG1, NOS3, and CD28 demonstrated predictive capabilities for the neoadjuvant immunotherapy response

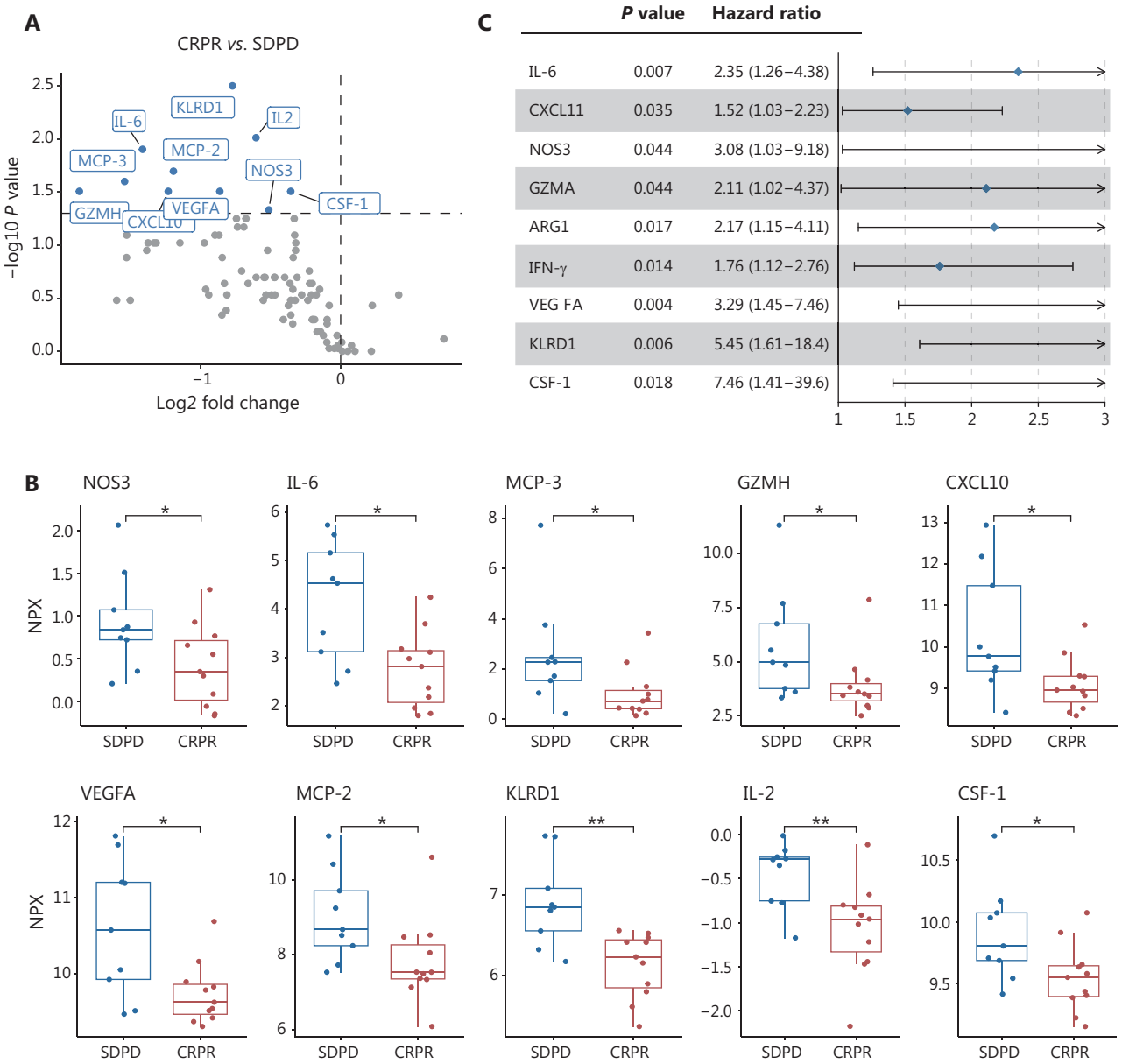


Figure 5 Plasma proteome predicts immunotherapy response in advanced TNBC patients (group C). (A) Volcano plot of differential plasma protein expression in advanced TNBC patients prior to immunotherapy, categorized by RECIST scores. Patients who achieved a complete or partial response (CRPR) are represented on the right, while those with stable disease or progressive disease (SDPD) are shown on the left; (B) Normalized plasma protein expression in different response groups of advanced TNBC patients prior to immunotherapy; (C) Univariate Cox proportional hazards analysis of pre-treatmernt plasma proteins associated with progression-free survival (PFS) in advanced TNBC patients following immunotherapy. *P < 0.05, **P < 0.01.

with AUCs of 0.653, 0.655, and 0.686, respectively (**Figure S7**). Pre-treatment plasma proteins identified as predictors of PFS are listed in forest plots with hazard ratios (**Figure 6D**). The univariate Cox analysis revealed that some pre-treatment plasma proteins, including MUC-16, NOS3, GZMH, and IL-5, were significantly associated with PFS following neoadjuvant immunotherapy. NOS3 and IL-5 remained significant predictors in a subsequent multivariate Cox analysis. Specifically, NOS3 had a 12-month AUC of 0.95 and an 18-month AUC of 0.81, indicating the potential as a robust

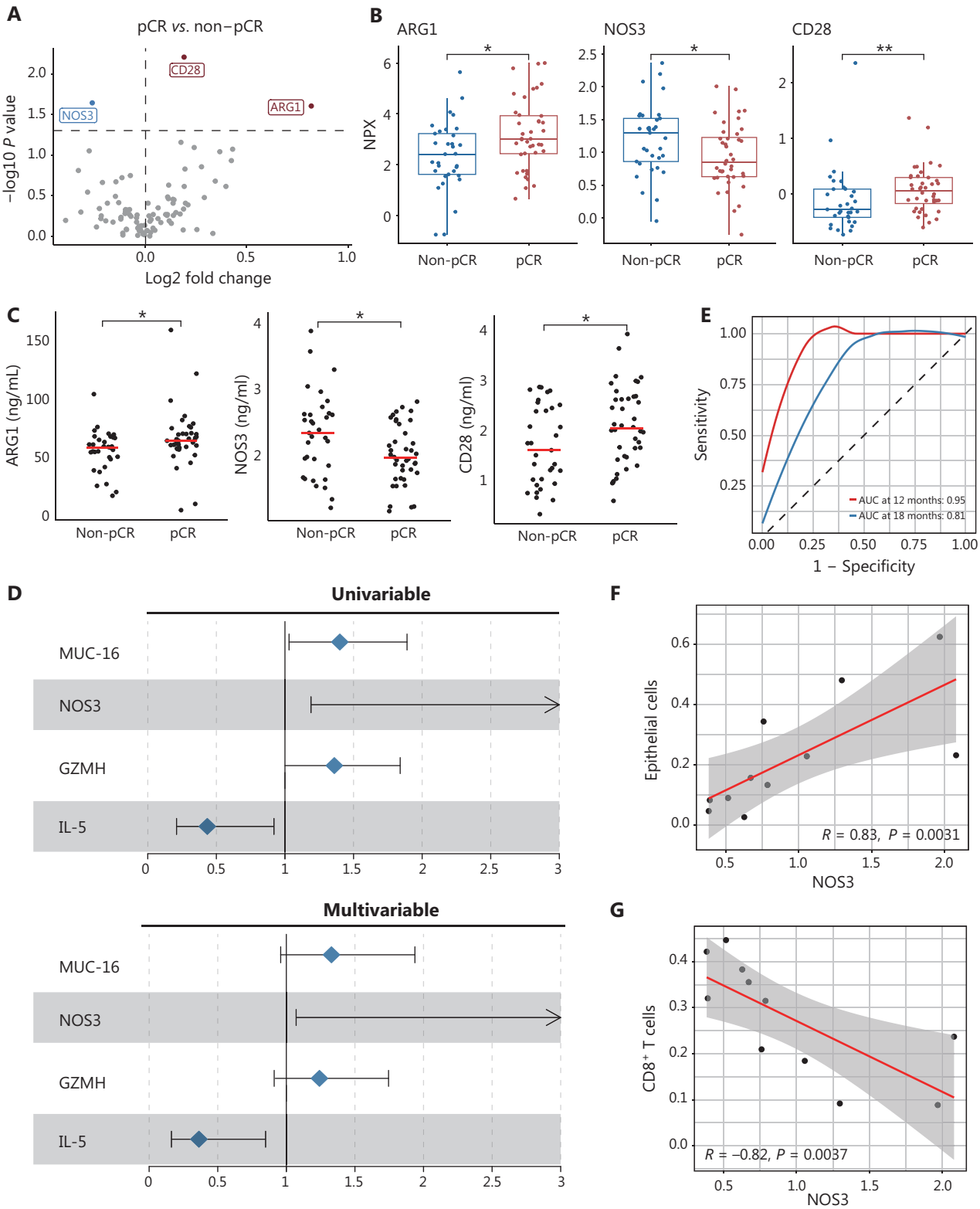


Figure 6 Plasma proteome predicts neoadjuvant immunotherapy (group A) response in patients with TNBC. (A) Differential plasma protein expression collected before neoadjuvant immunotherapy for patients in the pCR (right) and non-pCR groups (left); (B) Normalized plasma protein expression in different response groups of patients prior to neoadjuvant immunotherapy; (C) Plasma ARG1, NOS3, and CD28 proteins

were determined by ELISA in different response groups of patients prior to neoadjuvant immunotherapy; (D) Univariate and multivariate Cox proportional hazards analysis of pre-treatment plasma proteins associated with progression-free survival (PFS) following neoadjuvant immunotherapy; (E) Receiver operating characteristic (ROC) curves for predicting progression-free survival (PFS) using NOS3 levels following neoadjuvant immunotherapy at 12 and 18 months; (F) Correlation between pre-treatment plasma protein NOS3 levels and epithelial cell abundance in the tumor microenvironment (TME); (G) Correlation between pre-treatment plasma protein NOS3 levels and CD8+ T cell abundance in the TME.

biomarker for neoadjuvant immunotherapy efficacy and prognosis (**Figure 6E**).

Additional analysis revealed that among untreated TNBC patients, higher plasma NOS3 levels were positively correlated with increased epithelial cell abundance and inversely correlated with $CD8^+$ T cell abundance in tumor tissues (**Figure 6F, G**). These correlations suggested that elevated NOS3 levels might contribute to creating a TME that is less favorable for effective immune responses.

Prediction scores predict the response to neoadjuvant immunotherapy

To enhance clinical predictability the LASSO model was used to refine the prediction of neoadjuvant immunotherapy (group A) outcomes. The optimal lambda value was determined through 3-fold cross-validation, minimizing the mean cross-validated error (**Figure S8A**). Predictive performance was rigorously evaluated. Specifically, the model achieved an area under the precision-recall curve (PR-AUC = 0.903; **Figure S8B**) and high accuracy in the confusion matrix (**Figure S8C**), which confirmed robust classification capability.

Using this approach, the Plasma Immuno Prediction Score (PIPscore) model was developed, which incorporates ARG1, NOS3, CD28, IL-18, CXCL12, and PTN, achieving an AUC of 0.858 (**Figure 7A** and **7B**). Patients were stratified into high (PIPscore-H) and low (PIPscore-L) score groups based on the ROC curve. Notably, a higher PIPscore was associated with a favorable response to neoadjuvant immunotherapy, whereas a lower score predicted a poorer treatment response (**Figure 7C**). Importantly, this model also demonstrated a strong predictive effect on the prognosis of breast cancer patients undergoing neoadjuvant immunotherapy with a 12-month AUC of 0.96 and an 18-month AUC of 0.77 (**Figure 7D**).

A comprehensive analysis was performed on untreated patients to determine the correlation between the PIPscore and plasma protein levels and to further elucidate the clinical significance of the PIPscore (**Figure 7E**). The PIPscore was positively correlated with several immune-related plasma proteins, such as IFN-γ and LAMP3. GO analysis was performed on the plasma proteins correlated with the PIPscore to further understand the biological significance of these correlations (**Figure 7F**). Notably, the GO analysis revealed enrichment in immune-related pathways, such as regulation of T cell activation and leukocyte migration. These findings provide a more detailed understanding of the functional roles of these proteins, highlighting the potential mechanisms by which the PIPscore may predict patient prognosis and response to neoadjuvant immunotherapy.

Discussion

A comprehensive analysis of plasma proteome changes in response to immunotherapy in TNBC patients was performed in this study. Systemic immune responses were mapped and critical plasma protein biomarkers correlated with therapeutic outcomes were identified utilizing high-sensitivity proteomic techniques and integrating clinical data with scRNA-seq. Importantly, these findings underscored the value of non-invasive approaches in monitoring and predicting immunotherapy efficacy. Additionally, the PIPscore, a non-invasive predictive model that demonstrated strong clinical utility in both forecasting neoadjuvant immunotherapy responses and stratifying patient prognosis, was developed.

The observed alterations in plasma protein levels following immunotherapy highlight the complex interplay between the immune system and the TME in TNBC patients. These changes suggested that plasma protein alterations might be valuable indicators of the immune response and potentially provided new targets for future therapeutic interventions. The findings herein underscored the importance of immune-related proteins, such as CXCL9 and IFN-γ, which were elevated post-immunotherapy and indicative of robust immune activation. Indeed, this heightened immune response is crucial for the efficacy of immunotherapy, suggesting that these proteins could be leveraged as biomarkers to optimize treatment timing

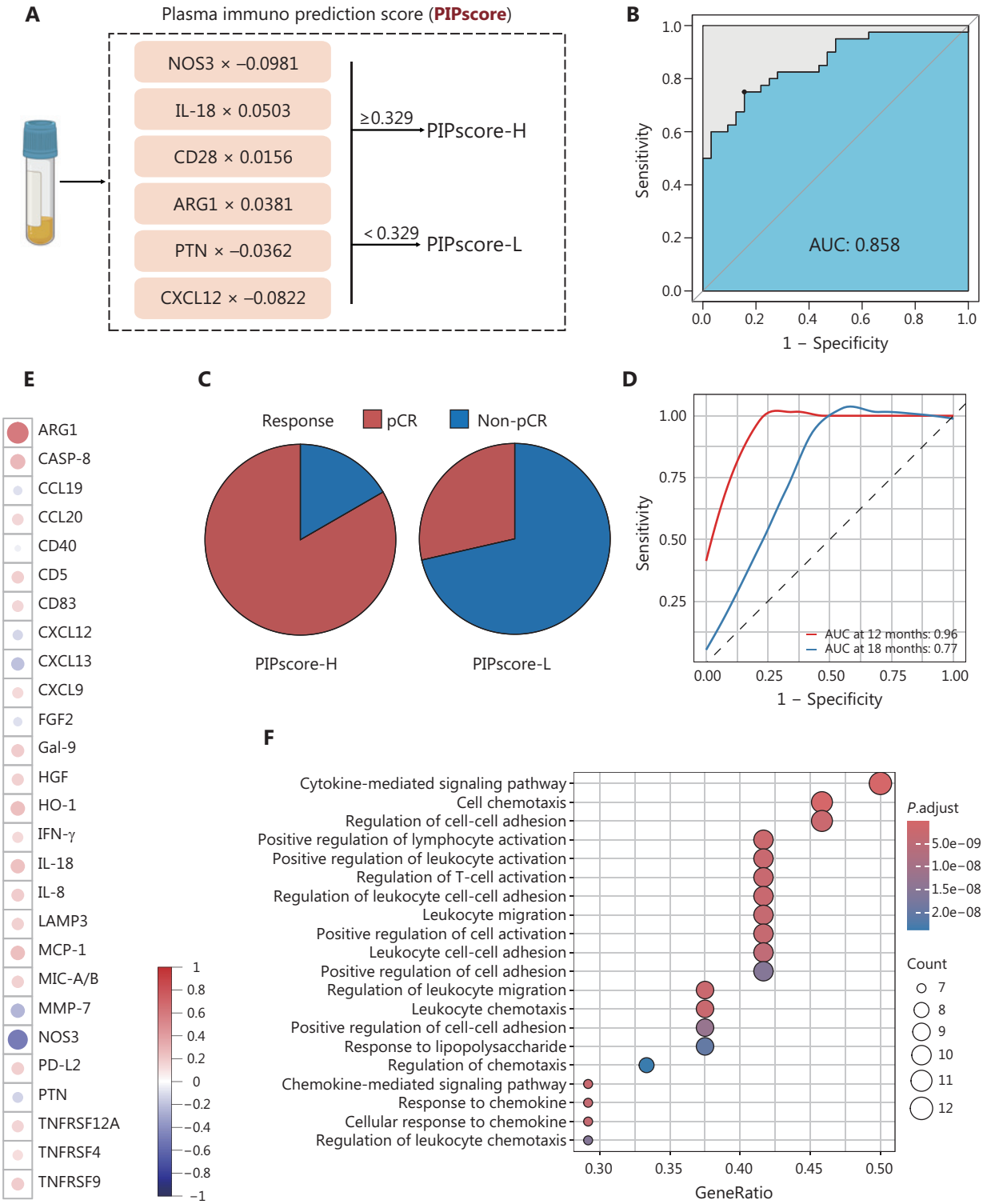


Figure 7 Prediction scores predict the response to neoadjuvant immunotherapy (group A). (A) Plasma immune prediction score (PIPscore) formula generated by using the least absolute shrinkage and selection operator (LASSO) model for plasma proteins. Patients with a PIPscore ≥ 0.329 are considered the high PIPscore population, while patients with a PIPscore < 0.329 are considered the low PIPscore population; (B) ROC curve demonstrating the predictive accuracy of the PIPscore for neoadjuvant immunotherapy response; (C) Rates of pCR in patients with a high PIPscore (≥ 0.329) compared with patients with a low PIPscore (< 0.329); (D) Receiver operating characteristic (ROC) curves for

predicting progression-free survival (PFS) using the PIPscore following neoadjuvant immunotherapy at 12 and 18 months; (E) Heatmap of correlation coefficients between the PIPscore and plasma protein levels in untreated patients; (F) GO analysis of plasma proteins correlated with the PIPscore.

and dosage, thereby enhancing patient outcomes. Conversely, a reduction in MUC-16 implied a decrease in tumor burden, highlighting the impact of therapy on tumor dynamics.

Notably, ARG1, CD28, and NOS3 were identified as key proteins that can predict the response to neoadjuvant immunotherapy with high accuracy, as demonstrated by the generated prediction scores. In this study, we focused on the predictive value of NOS3 as a plasma protein biomarker in the context of immunotherapy for TNBC patients. Patients achieving a pCR had lower NOS3 levels than non-responders, suggesting that NOS3 is a reliable biomarker for predicting benefit from neoadjuvant immunotherapy. Lower NOS3 levels also correlated with better immunotherapy responses in advanced TNBC patients, indicating the utility of NOS3 as a biomarker across different disease stages. The ability of NOS3 to reflect real-time changes in the TME and systemic immune response makes NOS3 valuable for monitoring treatment efficacy and adjusting strategies accordingly.

In previous studies ARG1 was shown to be an important component of the urea cycle[41] because ARG1 can catalyze the hydrolysis of arginine to ornithine and urea. NOS3, which is known as endothelial NOS, utilizes L-arginine as a substrate to synthesize nitric oxide[42]. The increase in ARG1 expression can deprive the substrate of NOS3 and even cause NOS uncoupling[43]. Elevated arginase in plasma promotes arginine catabolism and leads to arginine deprivation in tumor cells. This mechanism suggests that targeting arginine metabolism with therapeutic arginase could be a salvage strategy for patients who fail immunotherapy, although further experimental validation is required[44]. CD28, a co-stimulatory molecule essential for T-cell activation, has a critical role in the immune response by enhancing T-cell proliferation and cytokine production[45]. CD28 was included in the PIPscore in the current study because CD28 reflects systemic T-cell activity. Higher levels of CD28 in plasma may indicate a more robust T-cell response, which could contribute to better immunotherapy outcomes. In future studies, the biological mechanisms of these proteins will be explored through *in vitro* experiments (e.g., L-arginine deprivation on T-cell function) and *in vivo* murine models to validate the roles in the immunotherapy response.

A PIPscore based on key proteins (ARG1, NOS3, CD28, IL-18, CXCL12, and PTN) was also constructed. The PIPscore had a strong correlation with the response to neoadjuvant immunotherapy, suggesting the potential clinical utility of the PIPscore as a predictive tool. High PIPscores were associated with positive responses to neoadjuvant immunotherapy, whereas low PIPscores were linked to poor responses. The positive correlation between the PIPscore and multiple immune-related plasma proteins further validates the biological relevance of the PIPscore. Pathway analysis revealed enrichment in immune-related pathways, such as T cell activation and leukocyte migration, providing a mechanistic basis for the predictive capability of the PIPscore. The combination of these six proteins in the PIPscore allows for a comprehensive assessment of metabolic suppression, TME immunosuppression, and systemic T-cell activation, and highlights the importance of integrating systemic and local immune factors in immunotherapy response prediction.

Despite providing valuable insights, the study had a few limitations. First, the study population was relatively small, which may limit the generalizability of the findings. Specifically, the number of patients receiving late-stage immunotherapy was limited, preventing the development of a predictive model. However, rigorous statistical measures were applied to ensure the robustness of the results. Moreover, the study did not include detailed mechanistic investigations to elucidate the underlying biological processes driving the observed associations. Future corollary studies should focus on mechanistic studies to provide a deeper understanding of how these plasma proteins influence immunotherapy responses.

Conclusions

In conclusion, the current study underscored the predictive value of NOS3 with ARG1 and CD28 as non-invasive plasma protein biomarkers for neoadjuvant immunotherapy in TNBC patients. The PIPscore, a non-invasive model based on these six plasma proteins, was developed. The PIPscore correlated strongly with immunotherapy responses, highlighting the potential clinical utility of the PIPscore. Demonstrating

excellent predictive performance, the PIPscore effectively evaluated the efficacy and prognosis of TNBC patients receiving immunotherapy. This non-invasive approach might facilitate personalized management of immunotherapy in patients with breast cancer and offer a practical tool for clinicians to tailor treatment strategies.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

Author contributions

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Performed the analysis: Yuling Xiao, Hang Zhang.
Wrote the paper: Yuling Xiao, Hang Zhang, Yi Xiao.

Data availability statement

All plasma immune proteomics data generated by this study have been deposited in **Table S2**. All scRNA-seq data can be viewed in the National Omics Data Encyclopedia (NODE) (http://www.biosino.org/node): OEP003394. Additional data related to this paper may be requested from the authors.

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