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ORIGINAL RESEARCH

Novel Proteomic Insights into Hip Fractures in the Elderly: Unraveling Immunologic Biomarkers, Temporal Expression Patterns, and Clinical Correlations

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Background: Hip fractures in the elderly triggers a severe inflammatory immune response.

Methods: Peripheral blood samples from 16 elderly hip fracture patients and 16 healthy controls were analysed for 92 inflammatory biomarkers using proximity extension assay (PEA) at different stages after trauma.

Results: Dynamic trends in inflammatory proteins after surgery were assessed. Correlation analyses showed significant associations between inflammation-related proteins and clinical parameters. A prognostic risk score model was developed: on day 1, CCL19, FGF-19 and MCP-2 were significant; on day 3, TGF-α, FGF-5, CCL19, IL-22RA1 and IL-12B were included; and on day 7, IL-2RB, CCL19 and 4E-BP1 were significant. High-risk patients had a significantly lower rate of recovery compared with low-risk patients.

Conclusion: In this study, we have highlighted the complex inflammatory response during fracture healing and emphasised the importance of long-term monitoring of protein dynamics.

Keywords: geriatric hip fracture, immune, inflammation, prognosis, Olink

Introduction

Fragility fractures around the hip joint are common and serious injuries, and with an increasingly aging population, hip fractures have become a global public health problem.[1] Most hip fractures occur in falls and other related injuries, and osteoporosis is a major contributing factor; with aging, the body's bones become progressively more brittle and more susceptible to fracture.[2,3] In addition, the gradual decline in physical function and reduced mobility in the elderly is also an important factor in hip fractures in the elderly.[4] Hip fractures are associated with an increased mortality rate, which remains as high as 20–40% within 1 year after surgery.[1,5] The occurrence of hip fractures often leads to prolonged bedriddenness in the elderly, which poses a serious economic burden on patients and their families as well as on healthcare budgets; therefore, it is urgent to improve the cure rate of hip fractures in the elderly.

As research has progressed, the inflammatory response has been shown to be actively involved in multiple stages following hip fracture.[6] Inflammation is a key biological process for removing pathogens and maintaining tissue homeostasis, and the body releases a class of biologically active substances during the inflammatory response following

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trauma.[7] These mediators include cytokines, chemokines, and soluble mediators, which play regulatory and mediating roles in the inflammatory response.[8] In a previous study, our team revealed changes in the immune system at the cellular and molecular levels in elderly patients at different stages of hip fracture.[9] Fractured tissues release a large number of inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6).[10,11] These inflammatory mediators cause vasodilatation and increased permeability, which allows leukocytes and other immune cells to rapidly enter the wound site to remove dead tissue and defend against pathogens. Second, the inflammatory response also promotes healing and repair at the fracture site. Under the effect of inflammatory mediators, stem cells and osteoblasts will migrate to the wound site and begin to synthesize collagen and bone matrix, promoting the formation of new bone.[12] At the same time, the inflammatory response will also stimulate neovascularization and provide sufficient nutrients and oxygen, which will facilitate wound healing and repair.[13] In addition, it is worth noting that during the aging process, the body often exhibits a chronic low-grade inflammatory state known as "inflammaging".[14] This persistent inflammatory response not only increases the risk of chronic disease in older adults, but may also affect fracture healing. Studies have found that senescent skeletal stem cells produce a pro-inflammatory microenvironment, leading to poor fracture healing and accelerating the generalized aging process.[15] At the same time, aging-induced decreases in immune system function and increases in systemic pro-inflammatory states may slow the fracture healing process by affecting osteoclast numbers and activity.[16] However, there is no exact immunoassay indicator that can accurately reflect the disease development dynamics of hip fracture in the elderly, so exploring valuable disease dynamic indicators will become a major trend in the future development.

The Olink proteomics technology is a high-throughput proteomics technology that can detect hundreds of proteins simultaneously. By analyzing clinical data from a certain sample size, the expression levels of different inflammatory proteins in elderly hip fracture patients and their correlations with fracture healing and clinical indicators can be found. In this study, we studied the changes of 92 inflammatory proteins in the plasma of elderly hip fracture patients before and 1, 3, and 7 days after surgery by using Olink proteomics, and analyzed their correlation with the clinical indicators and prognosis of elderly hip fracture patients, in the hope of providing references to the early diagnosis and treatment of elderly hip fracture in the clinic and valuable proteins for prognosis, so as to detect the dynamic changes and regression of elderly hip fracture in a better way. We hope to provide valuable proteins for prognosis, so as to better detect the dynamic changes and regression of elderly hip fracture.

Method

Patient Characteristics

We included a total of 16 elderly healthy controls and 16 elderly hip fracture patients admitted to the Third Hospital of Hebei Medical University, baseline information for all subjects is shown in Table 1. Before inclusion in the study, all subjects gave informed consent and signed an informed consent form. We collected baseline data and clinical indicators including comorbidities and Harris Hip Score (HHS) from the patients[17] (Table 2). Exclusion criteria included (a)

Table 1 Baseline Characteristics of All Subjects

	Level	Control	Hip Fractures	p
n		16	16	
Gender (%)	Female	8(50.0)	11 (68.7)	0.28
	Male	8(50.0)	5 (31.3)	
Smoke (%)	No	10 (62.5)	12(75.0)	0.446
	Yes	6 (37.5)	4 (25.0)	
Alcohol (%)	No	12 (75.0)	11 (68.8)	0.694
	Yes	4 (25.0)	5 (31.2)	
Age (mean (SD))		75.13 (4.53)	79.50(6.59)	0.037
BMI (mean (SD))		20.85 (2.20)	22.42 (1.24)	0.115

Table 2 Baseline Characteristics of Elderly Hip Fracture Patients

	Level	Poor Prognosis	Good Prognosis	p
n		10	6	
Gender (%)	Female	6 (60.0)	5 (83.3)	0.676
	Male	4 (40.0)	1 (16.7)	
Smoke (%)	No	6 (60.0)	6 (100.0)	0.233
	Yes	4 (40.0)	0 (0.0)	
Alcohol (%)	No	6 (60.0)	5 (83.3)	0.676
	Yes	4 (40.0)	1 (16.7)	
Fracture type (%)	Femoral neck fracture	6 (60.0)	3 (50.0)	1
	Intertrochanteric fracture of the femur	4 (40.0)	3 (50.0)	
Diabetes (%)	No	6 (60.0)	5 (83.3)	0.676
	Yes	4 (40.0)	1 (16.7)	
HP (%)	No	2 (20.0)	2 (33.3)	1
	Yes	8 (80.0)	4 (66.7)	
CAD (%)	No	10 (100.0)	6 (100.0)	NA
Cerebral infarction (%)	No	6 (60.0)	4 (66.7)	1
	Yes	4 (40.0)	2 (33.3)	
Cause of fracture (%)	Fall	2 (20.0)	0 (0.0)	0.696
	Traffic Accident	8 (80.0)	6 (100.0)	
HHS (mean (SD))		65.10 (3.07)	73.33 (1.63)	<0.001
Age (mean (SD))		76.90 (6.44)	83.83 (4.45)	0.036
BMI (mean (SD))		21.52 (2.20)	23.92 (1.24)	0.029
DOH (mean (SD))		15.90 (1.66)	10.67 (2.16)	<0.001

Comorbidities with other types of fractures. (b) Those with malignant tumors. (c) Those with severe psychiatric disorders. (d) Combination of severe cardiac, hepatic, renal, and other organ dysfunction diseases.

To evaluate postoperative outcomes, we followed patients for 6 months. Treatment efficacy was initially assessed using the standard HHS criteria, where≥90 points is considered cured, 80–<90 markedly effective, 70–<80 effective, and <70 ineffective. However, to more closely align with real-world functional recovery and daily living demands in older adults, we further classified HHS≥75 as a "relatively good prognosis" and HHS <75 as a "relatively poor prognosis". This threshold reflects meaningful functional capacity needed for independent activity, thus offering a clinically pertinent basis for comparing patient outcomes. This study will be conducted in accordance with the ethical principles of the Declaration of Helsinki and approved by the Ethics Committee of the Third Hospital of Hebei Medical University (Ke-2023-051-1).

Serum Sample Collection

For 16 elderly patients with hip fractures, 4 mL of peripheral blood was drawn from the elbow vein outside the scope of normal treatment 24 hours after admission, 24 hours after surgery, and at 8 am on the 3rd and 7th days after surgery, and stored in an EDTA anticoagulation tube. The peripheral blood samples were centrifuged at 3000 rpm for 15 minutes to extract the plasma. The plasma was stored at −80°C for further analysis. We collected 4 mL of peripheral venous blood from 16 healthy subjects and extracted the plasma in the same way.

Analysis of Inflammation-Related Proteins

Peripheral blood plasma samples from elderly hip fracture patients and healthy elderly controls were analyzed using the Olink Inflammation Panel (Olink Proteomics, LC-Bio Technology Co., Ltd. Hangzhou, China), which is based on a highly sensitive and specific PEA technology that can simultaneously analyze 92 inflammation-related biomarkers.[18] In brief, each target protein was identified and bound by a pair of antibodies to its specific complementary DNA barcode, and then quantified using the high-throughput microfluidic real-time PCR instrument Biomark HD (Fluidigm, South San

Francisco, CA). The final test results were presented as normalized protein expression values and log2 conversion. For the Olink data, differentially expressed proteins were obtained using the limma package with a P value threshold of 0.05.[19] Heatmaps and volcano plots were visualized using the R package ggplot2. In addition, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed using ggplot2. In the enrichment analysis, all significantly differentially expressed proteins were mapped to each term or pathway in the GO or KEGG database, and then a hypergeometric test was used to determine the GO terms or KEGG pathways that were significantly enriched with differentially expressed proteins compared to a specific background. The results of the enrichment analysis were compared based on the background of all proteins and the 92 proteins in the Olink Inflammation Test Kit. The protein-protein interaction (PPI) network of differentially expressed proteins was constructed and visualized using Cytoscape (version 3.9.1).

Development of Prognostic Gene Profiles

As mentioned above, we defined elderly hip fracture patients with HHS scores less than 75 at 6 months postoperatively as having a poor prognosis. The research cohort consisted of 16 samples and univariate Cox regression analysis identified inflammatory proteins associated with prognosis. Feature selection was carried out via the least absolute shrinkage and selection operator (LASSO) algorithm in the glmnet package, with 1000 iterations to mitigate overfitting.[20] The final proteins were identified using a multivariate Cox regression model following the LASSO algorithm. Subsequently, the risk score was calculated using a linear combination of each selected gene as Risk score = $\sum$ (coef (β) * EXP(β)), where β denotes the regression coefficient. To further assess the stability of the model and reduce overfitting, we performed bootstrap resampling (n = 1000) for internal validation using the boot package due to our small sample size.[21] The survminer package was employed to generate Kaplan–Meier survival curves, and Log rank tests were applied to compare outcomes between groups. Model calibration was assessed by means of calibration curves created with the rms package, using a logistic regression model and 1000 bootstrap iterations. We conducted decision curve analysis (DCA) with the rmda package to estimate the net clinical benefit of the model across different threshold probabilities. Finally, the pROC package was used to compute receiver operating characteristic (ROC) curves and their corresponding area under the curve (AUC), providing an additional measure of predictive performance. All statistical analyses were conducted in R (version 4.2.2).

Result

Differences in Inflammatory Proteins in Elderly Hip Fractures and Healthy Controls

We used Olink's proximity extension assay (PEA) technology to measure 92 proteins in peripheral blood plasma samples from 16 elderly patients 24 hours after hip fracture, as well as from 16 control patients (Figure 1A). A total of 32 (34.78%) differentially expressed proteins (DEPs) were identified among the 92 proteins according to the adjusted $p < 0.05$, of which 12 DEPs were downregulated and 19 DEPs were upregulated (Figure 1B). We screened the top 5 proteins with significant differences; AXIN1, IL-6 and ST1A1 were significantly up-regulated in elderly hip bone fracture patients while TRAIL and TRANCE were significantly down-regulated compared to healthy controls (Supplementary Figure 1). Subsequently, KEGG analysis showed that the differentially expressed proteins were mainly enriched in cytokine-cytokine receptor interactions, TNF signaling pathway, NF-κB signaling pathway and PI3K-Akt signaling pathway, GO analysis showed that the differentially expressed proteins were mainly enriched in the extracellular space, cytokine activity, cytokine-mediated signaling pathway, extracellular region, and growth factor activity (Figure 1C and D). Correlation analysis of differentially expressed proteins between the two groups was performed and the protein-protein interaction network was constructed using the String database. A network of proteins centered on IL-6, which may play a specific role in the 24 hours after hip fracture, was obtained (Figure 1E).

Trends in Inflammatory Proteins at Different Time Points of Hip Fracture in the Elderly

We evaluated the dynamic trends of inflammatory proteins in elderly hip fracture patients at preoperative, 1, 3, and 7 days postoperatively. As shown in the Figure 2, we screened out the 10 most meaningful differential proteins, and we found

Figure 1 Comparison of inflammatory protein expression between preoperative and postoperative day one in elderly Hip fracture patients. (**A**) Heatmap of differential expression of inflammatory proteins. (**B**) Volcano plot of differentially expressed inflammatory proteins. (**C** and **D**) Enrichment analysis of differential expression of inflammatory proteins. (**E**) PPI network diagram of differentially expressed inflammatory proteins.

that TNFB, FIT3L, TRANCE, and CD244 showed a decreasing trend on postoperative day 1 compared with preoperative, and a gradual increasing trend on postoperative days 3 and 7. TNFB and TRANCE were able to return to preoperative levels on postoperative day 7, whereas the levels of FIT3L were higher than preoperative on postoperative day 7, and MCP-3, CCL23 and IL-6 showed an increasing trend at 1 day postoperatively and a decreasing trend at 3 and 7 days postoperatively compared with preoperative levels. IL-6 was able to return to preoperative levels at 7 days postoperatively, whereas MCP-3 was slightly higher than the preoperative level and CCL23 was slightly lower than the preoperative level.

In addition, we analyzed noteworthy differences in the levels of inflammatory proteins between each of the two different time points. IL-6, IL-8, and MCP-3 were significantly upregulated on postoperative day 1 compared to preoperative, whereas CD6 and TRANCE were significantly downregulated on postoperative day 1 (Figure 3A and Supplementary Figure 1). Enrichment analysis showed that these proteins were enriched in monocyte chemotaxis, cytokine activation, and immune response (Figure 3D and E). FIT3L was significantly upregulated on postoperative day 3 compared to postoperative day 1, while AXIN-1, CCL23, IL-8, and NT-3 showed a significant downregulation (Figure 3B and Supplementary Figure 2). GO enrichment analysis indicated that these proteins were enriched in chemokine activation, chemokine-mediated signaling pathways, cytokine activity, inflammatory response, and cell-cell signaling (Figure 3F and G). CCL11, EN-RAGE, TNFB, TRAIL, and TRANCE were significantly upregulated on postoperative day 7 compared to postoperative day 3 (Figure 3C and Supplementary Figure 3). We also performed GO and KEGG enrichment analyses to investigate the potential function of DEPs at 7 days postoperatively. In GO enrichment analysis, the results indicated that immune response and signaling were enriched (Figure 3H and I). Notably, differentially expressed proteins were significantly enriched in cytokine-cytokine receptor interactions, chemokine signaling pathway, IL-17 signaling pathway, and Toll-like receptor signaling pathway and NFκB signaling were enriched in KEGG analysis in all the above three time periods.

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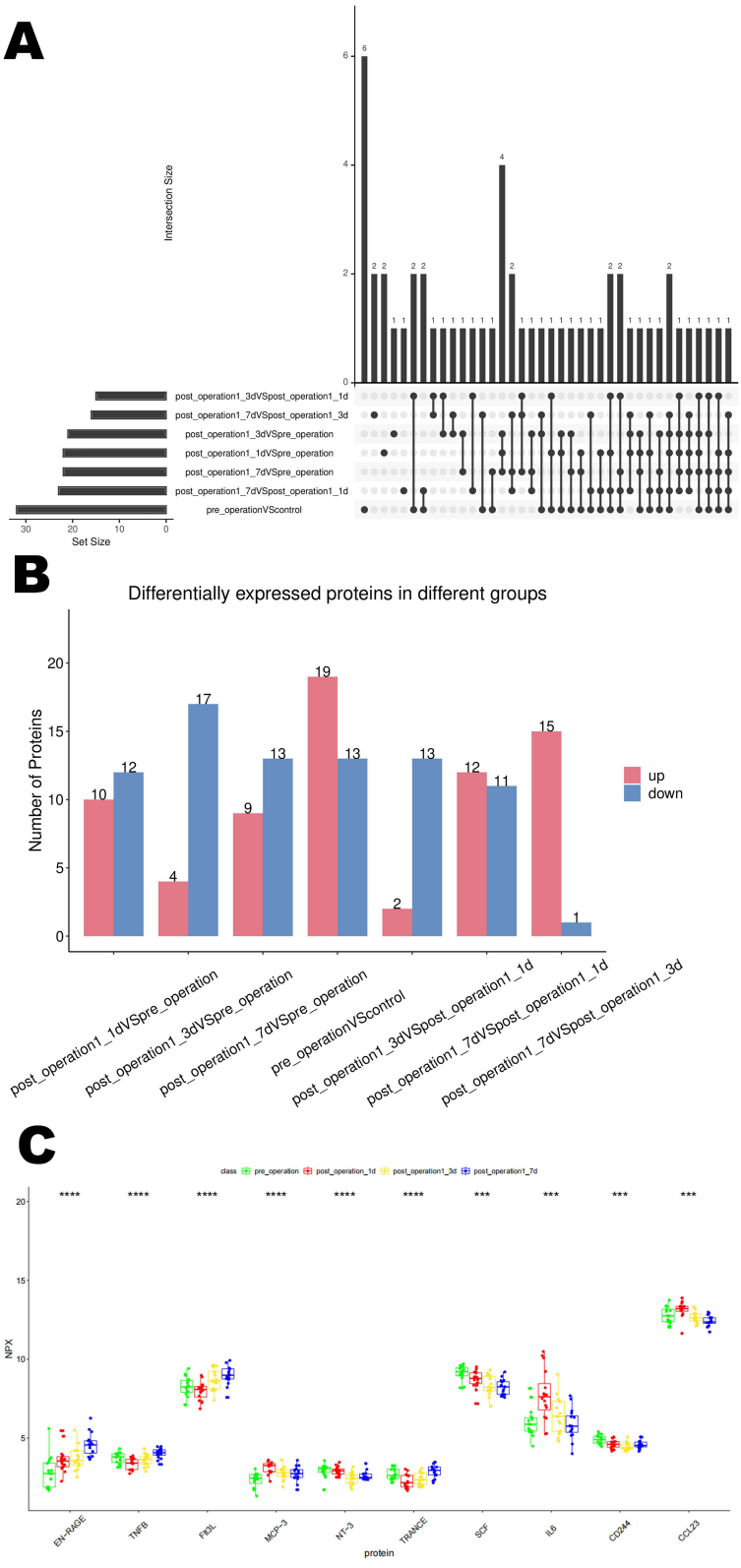

Figure 2 Differential expression of inflammatory proteins from preoperative to postoperative day 7. (**A**)Venn diagram showing the number of co-expressed proteins in healthy control, preoperative, postoperative day 1, postoperative day 3, and postoperative day 7 (**B**) Histogram of differential expression of inflammatory proteins from preoperative to postoperative day 7. (**C**) Significantly differentially expressed inflammatory proteins from preoperative to postoperative day 7.
Note: Statistical significance is denoted as follows: ***p<0.001, ****p<0.0001, two-tailed significance test.

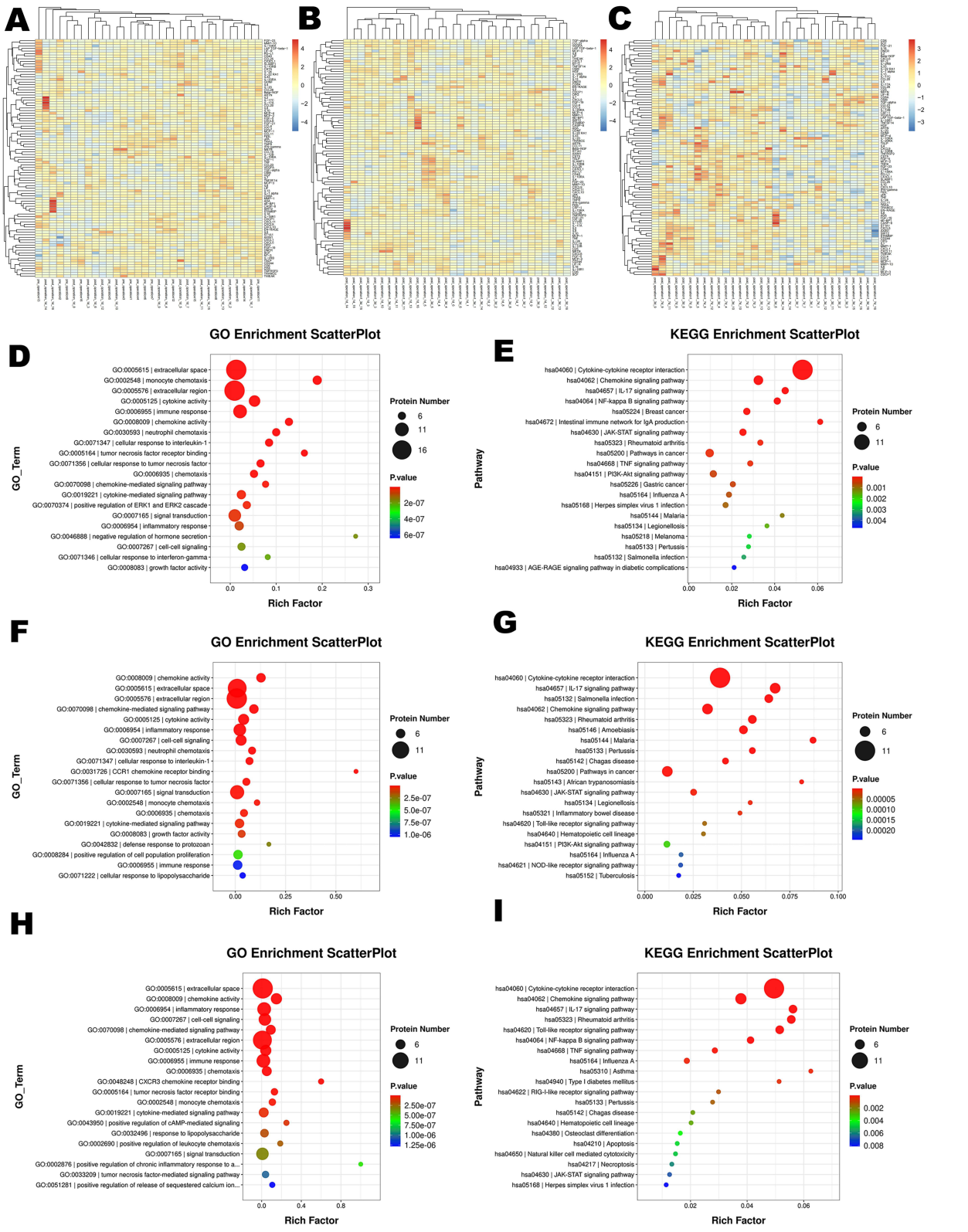

Figure 3 Analysis of differential expression and enrichment of inflammatory proteins across multiple time points. (**A**) Heatmap of differentially expressed inflammatory proteins from preoperative to postoperative day 1. (**B**) Heatmap of differentially expressed inflammatory proteins from postoperative day 1 to postoperative day 3. (**C**) Heatmap of differentially expressed inflammatory proteins from postoperative day 3 to postoperative day 7. (**D** and **E**) Enrichment analysis of inflammatory proteins expressed from preoperative to postoperative day 1. (**F** and **G**) Enrichment analysis of expressed inflammatory proteins from postoperative day 1 to postoperative day 3. (**H** and **I**) Enrichment analysis of inflammatory proteins expressed from postoperative day 3 to postoperative day 7.

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Correlation Between Inflammatory Protein Expression Levels and Clinical Indicators

We used linear mixed models to compare proteomic data at each time point with clinical laboratory measurements at that time in elderly hip fracture patients (Figure 4A–C). On the first postoperative day, factors positively correlated with platelet to lymphocyte ratio (PLR) levels were EN-RAGE, OSM, and TNFSF14, negatively correlated with TRANCE, TWEAK, and CD244, positively correlated with C-reactive protein (CRP) were IL-8, CCL23, MCP-3, SIRT2, and AXIN1, and negatively correlated with FGF-21, positively correlated with myoglobin (Mb) correlated factors were CCL23, EN-RAGE, IL-8, MCP-3, OSM, AXIN1, and IL-6, negatively correlated factors were TRAIL, TWEAK, TNF-β, CD6, and FGF-21, and positively correlated factors with monocyte to lymphocyte ratio (MLR) were IL-8, OSM, CCL23, and MCP-3, and negatively correlated factors were TNF-β, TRANCE, TRAIL, TWEAK and CCL11, positively correlated with neutrophil to lymphocyte ratio (NLR), CCL23, IL-8, negatively correlated with TRANCE, TRAIL, TWEAK, CCL11, TNF-β and CD6, positively correlated with hemoglobin (Hb), TRAIL, positively correlated with albumin to globulin ratio (A/G), CCL11 and FGF- 21, negatively correlated with CCL23, EN-RAGE, AXIN1, SIRT2, and TNFRSF14, and negatively correlated with cardiac troponin CTn), TRANCE, and TWEAK (Figure 4D). On postoperative day 3, the factor negatively correlated with CTn was IFN-γ. Negative correlation with CRP was FIT3L. Positive correlation with Hb was STAMBP. Positive correlation with Mb was AXIN1 and CCL23, and negative correlation with Mb was FIT3L and IFN-γ. Positive correlation with PLR was IL-6, NT-3, CCL23, and IL-7, and with MLR CXCL6, AXIN1, NT-3, IL-8, CCL23, and CXCL1, and NT-3, AXIN1, IL-8, and CCL23, and NLR, respectively (Figure 4E). Next, we correlated the levels of immunoinflammatory factors with the clinical indexes at 7 days post-operatively, and we found that the factors that were negatively correlated with Mb were EN-RAGE and TRAIL, the factor negatively correlated with NLR was EN-RAGE, the factor positively correlated with A/G was OSM, the factors negatively correlated were TRAIL, TNF-β, TRANCE, and TNFRSF9, the factors negatively correlated with CTn were

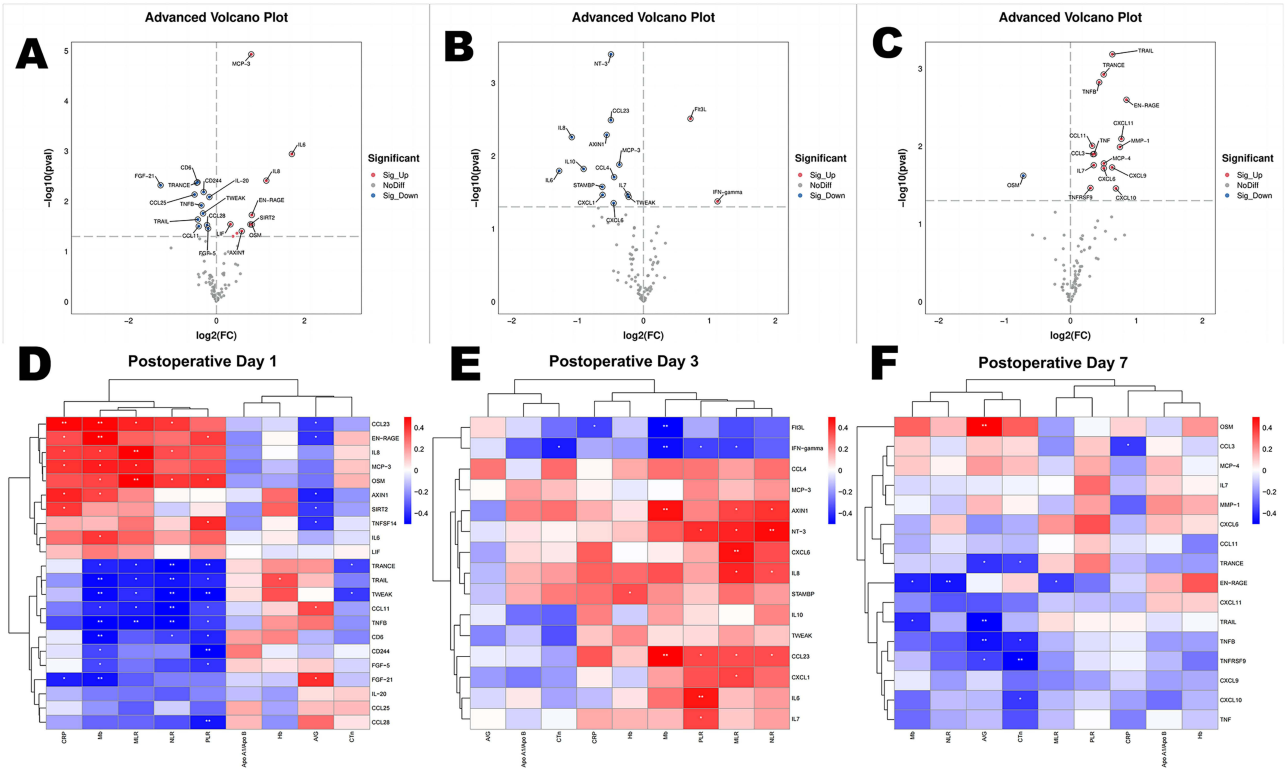

Figure 4 Volcano plots and correlation analysis of inflammatory proteins across multiple time points. (**A**) Volcano map of significantly differentially expressed inflammatory proteins from preoperative to postoperative day 1. (**B**) Volcano map of significantly differentially expressed inflammatory proteins from postoperative day 1 to postoperative day 3. (**C**) Volcano map of significantly differentially expressed inflammatory proteins from postoperative day 3 to postoperative day 7. (**D**) Heatmap showing the correlation between differential inflammatory proteins and clinical indicators in elderly Hip fracture patients at 1 day postoperatively, with the X-axis representing clinical indicators and the Y-axis representing inflammatory proteins. (**E**) Correlation between inflammatory proteins and clinical indicators at 3 days postoperatively versus 1 day postoperatively. (**F**) Correlation between inflammatory proteins and clinical indicators at 7 days postoperatively versus 3 days postoperatively.

TNFRSF9, TRANCE, TNF-β, and CXCL10, the factor negatively correlated with MLR was EN-RAGE, and the factor negatively correlated with CRP factor negatively correlated with MLR was EN-RAGE, and the factor negatively correlated with CRP was CCL3 (Figure 4F).

Correlation Between Inflammatory Protein Expression and Prognosis in Elderly Hip Fracture Patients

To further explore the potential link between the prognosis of elderly hip fracture patients and the immune-inflammatory response at different time points, we attempted to determine a risk score for each patient using inflammatory protein coefficients at different time points. We collected baseline data and clinical indicators, including comorbidities and Harris Hip Score (HHS), from 16 elderly hip fracture patients (Table 2). Prior to inclusion in the study, patients gave informed consent and signed an informed consent form. In the preoperative period, risk score = (0.047*IL-17C expression)+ (−0.070*IL-2RB expression)+ (0.001*FGF-21 expression)+ (0.031*IL10 expression)+ (0.249*TNF expression). On postoperative day 1, risk score = (0.001*CCL19 expression) + (0.003*FGF-19 expression) + (0.001 *MCP-2 expression). At postoperative day 3, risk score = (0.458*TGF-alpha expression) + (0.445*FGF-5 expression) + (0.001*CCL19 expression) + (0.197*IL-22RA1 expression) + (−0.009 *IL-12B expression). At postoperative day 7, risk score = (−4.972*IL-2RB expression) + (0.001*CCL19 expression) + (0.004*4E-BP1 expression). Subsequently, we predicted the prognosis of the patients using Kaplan-Meier. Based on the median risk score, participants were assigned to either a low-risk group or a high-risk group. In both groups, we found that the excellent recovery rate was lower in the high-risk group than in the low-risk group (Figure 5). To further validate our prognostic model, we performed bootstrap resampling (n = 1000) to assess its discriminatory power, measured by the area under the receiver operating characteristic curve (AUC). After bootstrap validation, the AUC values were 0.905 (95% CI: 0.673–1.000) for the preoperative stage, 0.778 (95% CI: 0.524–0.982) for the first postoperative day, 0.714 (95% CI: 0.491–0.950) for the third postoperative day, and 0.730 (95% CI: 0.500–0.964) for the seventh postoperative day, indicating that the model maintains robust predictive performance at each time point (Figure 5). In addition, calibration curves and decision curve analyses (DCA) further supported the stability and clinical utility of the model (Supplementary Figures 4–7).

Discussion

Hip fractures is a complex problem in elderly patients, and treatment and management require a combination of several aspects.[22] In addition to strengthening health management and preventive measures in the elderly, we need to continuously explore new treatments and techniques to improve cure rates and reduce mortality. In this study, we performed proteomic analysis of plasma from elderly hip fracture patients, we firstly horizontally compared the differential proteins between elderly hip fracture patients and healthy controls, secondly vertically compared the trend of inflammatory proteins in patients at different time points, and then correlated the expression levels of inflammatory proteins at each time point with the clinical indicators. Finally, we correlated the expression levels of inflammatory proteins with the prognosis of the patients. This study provides important clues for understanding the pathogenesis of hip fractures in the elderly and provides a basis for the development of personalized treatment and interventions.

The immune response in elderly patients after hip fracture is a dynamic process that usually enters the inflammatory response phase after the fracture and remains there for some time. Our study showed that MCP-3, IL-6, and CCL23 peaked on postoperative day 1, suggesting that these proteins may play a key role in the inflammatory phase of fracture repair. Consistent with our findings, Ishikawa et al observed high levels of MCP-1 and MCP-3 proteins in the periosteum and endosteum on day 1 of rib fracture, and another study also demonstrated elevated plasma IL-6 and MCP-3 levels after trauma, suggesting a role in inducing systemic inflammatory responses.[23,24] Shinohara et al found that MCP-3 expression could enhance osteoblast homing to the fracture repair site by recruiting MSCs.[25] Saribal et al found that IL-6 levels were significantly elevated in plasma 1 day after hip fracture surgery in the elderly.[26] CCL23 is a potential biomarker for acute compartment syndrome (ACS) in patients with tibial fracture, and IL6, CSF-1 and HGF Combined diagnosis is important in predicting ACS in tibial fracture patients.[27] While EN-RAGE, TNFβ, FIT3L and TRANCE peaked at 7 days postoperatively. Their trends suggest that they play important roles in the fracture healing process,

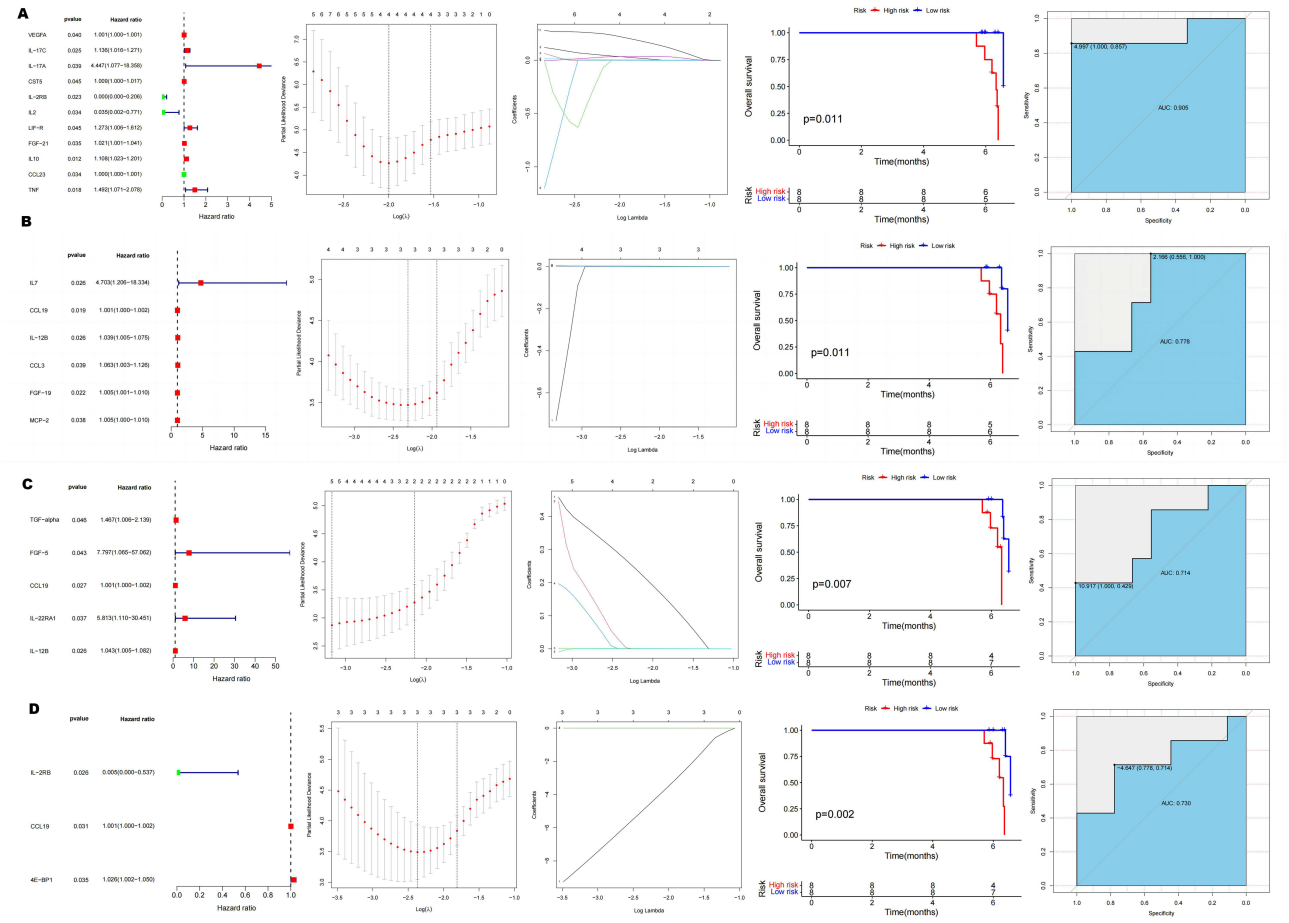

Figure 5 Construction and validation of risk scoring models at different time points. We performed univariate Cox regression and LASSO regression analyses to identify candidate prognostic inflammatory proteins. In the LASSO regression model, after selecting the best lambda values by cross-validation, the model gave the genes and their corresponding coefficients that had the greatest degree of influence on the survival data. Model equations were constructed to calculate risk scores. Patient prognosis was predicted using Kaplan-Meier survival analysis. In addition, the predictive effect of risk scores on patient prognosis was assessed using ROC curves. (**A**) Preoperative (**B**) Postoperative day 1 (**C**) Postoperative day 3 (**D**) Postoperative day 7.

which may involve cytokine regulation and activation of signaling pathways in fracture healing. EN-RAGE belongs to the toll-like receptor superfamily, which plays an important role in inflammation, either directly or through binding to advanced glycosylation end-products (AGEs) and advanced oxidized protein products (AOPPs).[28] As a pro-inflammatory cytokine, TNF-β plays an important role in skeletal diseases,[29] inhibiting the early stages of MSC osteoblast differentiation by down-regulating RUNX98 and activating NF-κB. FIT3L, a potent and specific DC growth factor, has been reported to expand and mature DCs in mice and humans.[30] The trends of these proteins may be closely related to the progression of disease dynamics in elderly hip fractures, and thus may serve as potential detectors of changes in the fracture course.

The comparative analysis between different time points unveiled significant upregulation of IL-6, IL-8, and MCP-3 on postoperative day 1, indicative of an early pro-inflammatory response.[31–33] In contrast, CD6 and TRANCE were downregulated on day 1, suggesting a possible anti-inflammatory response or immune modulation.[34,35] Enrichment analysis revealed their involvement in monocyte chemotaxis, cytokine activation, and immune response, emphasizing their crucial roles in the immediate postoperative phase. Postoperative day 3 saw a distinct profile, with FIT3L upregulated compared to day 1, while AXIN-1, CCL23, IL-8, and NT-3 exhibited significant downregulation. GO enrichment analysis highlighted the involvement of these proteins in chemokine activation, cytokine activity, and inflammatory response, providing insights into the evolving immune and inflammatory processes during the early recovery phase. On postoperative day 7, CCL11, EN-RAGE, TNFB, TRAIL, and TRANCE showed significant

upregulation compared to day 3. Enrichment analyses indicated the enrichment of immune response and signaling pathways. Notably, cytokine-cytokine receptor interactions, chemokine signaling pathway, IL-17 signaling pathway, Toll-like receptor signaling pathway, and NFκB signaling were significantly enriched, suggesting the persistence of inflammatory and immune responses at this later stage.

We also explored the correlation between the expression levels of inflammatory proteins and clinical indicators by comparing proteomic data from elderly hip fracture patients with concurrent clinical laboratory measurements. CRP is not only a risk predictor of hip fracture, but also closely related to the postoperative mortality of patients.[36,37] We observed a significant positive correlation with CCL23 at 7 days postoperatively compared to 1 day postoperatively, alongside a notable negative correlation with CCL11, MMP-1, and FTt3L. CRP, combined with these inflammatory factors, may collectively reflect the body's immune status during the recovery period following a fracture. Yao et al demonstrated that NLR, PLR, and systemic immune-inflammation indexes predicted postoperative pneumonitis in elderly hip fracture patients.[38] Our study indicates that TRANCE, TWEAK, CCL11, and TNF-βwere significantly negatively correlated with NLR at 1 day postoperatively compared with preoperatively, suggesting that these indices may promote lymphocyte proliferation and differentiation. By assessing the levels of these indicators and inflammatory factors, it's possible to preliminarily assess the presence of an inflammatory response and evaluate its severity and progression. Mb primarily facilitates oxygen storage and transport, and myoglobin is released when muscle cells are damaged or hypoxic to aid in oxygen metabolism.[39] In our study, we found that EN-RAGE showed a significant positive correlation with Mb, while TNF-β showed a significant negative correlation with Mb on postoperative day 1 compared to preoperative day 1. Furthermore, CCL23 showed a significant positive correlation with Mb, and FIT3L showed a significant negative correlation with Mb on postoperative day 3 compared to day 1. Interestingly, on postoperative day 7 compared to day 1, CCL23 continued to exhibit a significant positive correlation with Mb, potentially reflecting the impact of the inflammatory state of the disease on muscle tissue. Wang et al discovered that PLR predicted a low survival rate in elderly hip fractures.[40] Our results suggest a potential relationship between inflammatory factors and PLR, collectively contributing to the onset and progression of inflammatory responses.

In this study, for the prediction of recovery in elderly hip fracture patients, we designed a series of risk scoring models that utilise inflammatory protein expression at different time points pre- and post-operatively to determine the risk score for each patient. These models offer a novel approach to predicting patient outcomes and provide a dynamic tool for assessing recovery trajectories. Our longitudinal analysis of inflammatory proteins at various postoperative time points revealed significant trends. In the preoperative stage, we found that the expression levels of inflammatory proteins such as IL-17C, IL-2RB, and TNF had a significant effect on the prognosis of the patients. IL-17C is an important pro-inflammatory cytokine, which is correlated with the severity of several inflammation-related diseases.[41] IL-2RB plays a regulatory role in immune response, and its low expression may be related to the state of immunosuppression and thus affects patient recovery.[42] The high expression of TNF, a classical pro-inflammatory factor, is associated with increased inflammation and poor prognosis.[26] On postoperative day 1, our model incorporated CCL19, FGF-19, and MCP-2. The changes of these proteins reflected the dynamic process of inflammatory response in the early postoperative period. CCL19 plays an important role in the regulation of lymphocyte migration, and its high expression may be related to the enhancement of inflammatory response in the postoperative period.[43] FGF-19 is involved in cell metabolism and growth, and the changes in its expression level may be related to the body's stress response.[44] MCP-2 is a chemokine involved in the recruitment of monocytes and macrophages, and its high expression may be related to the body's stress response. MCP-2 is a chemokine involved in the recruitment of monocytes and macrophages, and its high expression may reflect increased inflammation and poor prognosis after surgery.[45] The high expression of MCP-2 may reflect the degree of postoperative inflammatory response. The mid course of the inflammatory response was further assessed on postoperative day 3 by our risk prediction model including TGF-α, FGF-5, CCL19, IL-22RA1 and IL-12B. TGF-α is a cell growth factor whose high expression may promote tissue repair but may also be associated with fibrosis and poor prognosis.[46] High expression of IL-22RA1, a receptor for IL-22, promotes tissue regeneration and repair, whereas low expression of IL-12B, a pro-inflammatory cytokine, may help to reduce the inflammatory response.[47,48] On postoperative day 7, our model incorporated IL-2RB, CCL19, and 4E-BP1 to assess patient recovery, reflecting the dynamic trends in the inflammatory profile and its impact on patient recovery over time. The low expression of IL-2RB

was consistent with the preoperative period and may reflect a state of persistent immunosuppression.4E-BP1 is a regulator of protein synthesis, and its high expression may be associated with cellular stress and recovery.[49]

Using Kaplan-Meier survival analysis, we found significant differences in prognosis between the high- and low-risk groups divided based on the median risk score. Patients in the high-risk group had a significantly lower rate of excellent recovery than those in the low-risk group, suggesting a key role for the inflammatory response in the prognosis of elderly hip fracture patients. The inflammatory response was more pronounced in patients in the high-risk group, which may lead to higher complication rates and poorer rehabilitation outcomes. These findings have important clinical applications. The inflammatory protein-based risk scoring system can be used as an early warning tool to help clinicians identify high-risk patients in a timely manner at various critical preoperative and postoperative time points. By identifying high-risk patients at an early stage, clinicians can take more proactive interventions, such as intensified anti-inflammatory therapy, personalised rehabilitation programmes and closer monitoring, to improve patient prognosis. In addition, this scoring system helps optimise the allocation of healthcare resources. High-risk patients require more medical resources and nursing attention, while low-risk patients can be treated with relatively simple measures. This rational allocation of resources helps to improve the overall efficiency and effectiveness of hospital treatment and reduce healthcare costs. However, the generalizability of these models may be affected by several factors. First, inflammatory responses may vary across age groups, gender, and ethnicity. For example, studies have found that C-reactive protein (CRP) levels are more sensitive in predicting metabolic syndrome in women than in men.[50] In addition, environmental factors, lifestyle, and genetic background may also influence the level of expression of inflammatory markers. Therefore, when applying the risk scoring model of this study to other environments or populations, further validation and adjustment are needed to ensure its applicability and accuracy. As it is more difficult to obtain samples, the current sample size is small and may yield inaccurate results, and we expect to increase the sample size and gradually correct the risk models in the future. Also, in-depth studies on the functions and mechanisms of action of these inflammatory proteins are needed to better understand their role in the fracture healing process.

To the best of our knowledge, this study is the first proteomic study of plasma from elderly hip fracture patients at different time intervals, and in addition, we simultaneously explored differences in the expression of inflammatory proteins between elderly people with hip fracture and healthy elderly people. By emphasizing immunological biomarkers can predict fracture risk and may delay the onset of osteoporosis and fragility fractures in older adults. However, some limitations remain in our study, an observational study of cytokine levels in peripheral plasma of elderly hip fracture patients at different time intervals. Although the development of hip fractures in elderly patients has been characterized at the level of inflammatory cytokines, no relevant conclusions can be drawn about the mechanisms of hip fracture. In addition, this study focused on the potential role of cytokines in fracture development and in the postoperative period, while ignoring genetics, environmental factors, and other relevant factors. To increase the clinical relevance of these biomarkers and small samples, validation and replication in longitudinal cohorts of different populations are needed.

In this study, we emphasize the complexity of the inflammatory response in the fracture healing process, and by observing the trends in protein dynamics at different pre- and postoperative time points, we can better understand the role of inflammatory proteins in the fracture healing process. Although some progress has been made, further studies are still needed to gain a deeper understanding of the role of the inflammatory response in the fracture healing process. Future studies can improve the accuracy and reliability of the study by expanding the sample size and increasing the time points. Also, in-depth studies on the functions and mechanisms of action of these inflammatory proteins are needed to better understand their role in the fracture healing process.

Data Sharing Statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

We obtained informed consent from all 32 subjects, the study will be conducted in accordance with the ethical principles of the Declaration of Helsinki, and our study was approved by the Ethics Committee of the Third Hospital of Hebei Medical University (Ke-2023-051-1).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas, have agreed on the journal to which the article has been submitted and agree to be accountable for all aspects of the work.

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Disclosure

The authors affirm that the research was carried out without any existing commercial or financial relationships that might be perceived as potential conflicts of interest.

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