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POSTOPERATIVE EVALUATION OF CD MARKER EXPRESSION ON MONOCYTES AND NEUTROPHILS IN FRACTURE-RELATED INFECTIONS

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ABSTRACT

Abstract: Background: Fracture-related infection (FRI) represents a significant post-traumatic complication, with diagnosis often hindered by its heterogeneous clinical presentation, and differentiating FRI from non-infectious conditions can be difficult. **Aim**: This study aimed to investigate the relationship between the expression of immune markers **CD64** and **CD66b** on monocytes and neutrophils in patients with FRI, focusing on postoperative changes. **Methods**: Blood samples were collected from 72 patients suspected of having FRI in closed fractures, including 11 positive and 63 negative cases. Flow cytometry was used to measure the fluorescent intensities of CD64 and CD66b on neutrophils and monocytes. The ratio of **CD64/CD66b** was calculated to further explore the relationship between these markers. **Results**: The analysis revealed that the **CD64/CD66b ratio** differed significantly between preoperative and postoperative measurements in FRI-positive patients. This change in immune marker expression was more pronounced in patients with confirmed infections, suggesting that these markers may be indicative of infection dynamics after fracture healing. Additionally, delayed fracture healing was found to influence immune response, affecting marker expression. The study also identified that the expression ratios of **NCD64/NC66b** (neutrophil CD64 to CD66b) and **MCD64/MCD66b** (monocyte CD64 to CD66b) were valuable in predicting FRI presence. **Conclusion**: The study provides evidence that **NCD64/NC66b** and **MCD64/MCD66b** ratios are potential suggestive diagnostic biomarkers for FRI. Changes in the expression of these markers, particularly after surgery, reflect infection status, and may aid in the diagnosis and management of FRI, offering insights into infection progression and fracture healing in adult patients.

INTRODUCTION

Fracture-related infection (FRI) is a significant complication that may develop after a bone injury, and its varied clinical features can make diagnosis challenging. Distinguishing FRI from non-infectious conditions is often complex. [1] Despite significant progress in the diagnosis and management of FRI, it continues to pose considerable challenges for orthopedic surgeons. Diagnosis of FRI relies on a combination of clinical assessment, imaging findings, serum inflammatory biomarkers, microbiological culture results, and therapeutic response, which can vary considerably across different patients. Traditional serum markers such as white blood cell (WBC) count, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) are commonly used in diagnosing FRI. However, their diagnostic utility is affected by several factors, including variations in their half-life and the influence of other conditions. [2]

Fracture-related infections (FRIs) primarily result from bacterial contamination at the fracture site. In open fractures, contamination may occur at the time of injury, whereas in closed fractures, it can be introduced during surgical intervention. The likelihood of developing FRI is significantly influenced by the extent of soft tissue damage; for instance, while closed tibial shaft fractures have an estimated infection rate of 1–2%, this can escalate to approximately 42.9% in open fractures with severe soft tissue involvement. A standardized definition of FRI, incorporating both confirmatory and suggestive diagnostic criteria, was established in 2018. [3]Expression of the CD64 antigen on neutrophils has been under investigation for some years as a biomarker of infection and sepsis. It has several

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characteristics that make is well suited for clinical application on resting neutrophils CD64 expression is low and after activation it is significantly up regulated within few hours. Once the activation stimulus disappears, CD64 expression returns to its basal level in few days. [4]

Cluster of differentiation 64 (CD64), also known as the FcγR1 receptor, is a monoclonal antibody that recognizes the FcγR1 neutrophil receptor. While CD64 expression is normally low on resting neutrophils, it is significantly upregulated upon activation by pro-inflammatory cytokines such as interferon-γ (IFN-γ), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and granulocyte colony-stimulating factor (G-CSF), typically within 4 to 6 hours of infection. This upregulation allows for the clear differentiation between resting and activated neutrophils.[5]

CD64 plays a key role in phagocytosis, which underscores it/s functional relevance during infection. Upon the onset of infection, CD64 expression on neutrophils is induced by cytokines and other mediators released by macrophages in response to pathogen-associated molecular patterns (PAMPs). Under normal conditions, CD64 is expressed on only a small fraction of circulating polymorphonuclear leukocytes (PMNs); however, in the presence of infection, neutrophil CD64 (nCD64) expression increases rapidly in response to microbial wall components, complement split products, and various pro-inflammatory cytokines.[6] Additionally, CD66b is a granulocyte-specific activation marker, with its expression being upregulated during immune activation. Overexpression of CD66b has been observed in cases of neutrophil dysfunction, particularly in infections caused by *Staphylococcus aureus*. Markers such as CD11b, CD64, and CD66b are indicative of neutrophil activation, exhibiting low baseline expression on resting neutrophils, but significantly elevated levels following pro-inflammatory stimuli [7]

The objective of this study was to investigate the expression levels of CD64 and CD66b as diagnostic markers for fracture-related infections (FRI) in adult patients. Specifically, the study aimed to evaluate how the expression of these immune markers can distinguish between patients with positive and negative FRI, providing a more reliable method for identifying infection. Also the combined values of CD64 and CD66b could be reliable tool in early detection of infection and distinguish it from positive and Negative.

MATERIALS AND METHODS

A total of 72 patients presenting with suspected Fracture-Related Infection (FRI) were enrolled in the study. After obtaining confirmatory results from microbial culture, 11 patients were diagnosed with a positive FRI, while 63 patients were found to be free of infection. Detailed baseline data were systematically collected, including demographic variables such as age, gender, and the duration of symptoms. Additionally, the expression levels of immune markers CD64 and CD66b were recorded to assess their potential role in the pathogenesis of FRI. FRI was defined based on clinical criteria, including signs of infection such as redness, swelling, and purulent discharge, in conjunction with positive microbial culture results [Table 1] Postoperatively, all patients were closely monitored for the development of any confirmatory signs of infection over a follow-up period of 10 weeks, allowing for a standardized timeframe to assess infection status and to limit the duration of the study. Both preoperative and postoperative expressions of CD64 and CD66b were meticulously measured and documented at designated intervals. These immune markers were specifically chosen due to their potential involvement in the inflammatory response associated with fracture healing and infection. The collected data were subsequently analyzed using statistical methods to compare and assess the differences in the expression of these markers before and after surgery, with a focus on their relevance to the diagnosis and prognosis of FRI. Patients included in the study were above 18 years of age, with no history of prior antibiotic use and no known infectious diseases, while those below 18, with recent antibiotic use, or with existing infections were excluded.

Table 1. Confirmatory and Suggestive criteria of a Fracture-Related Infection according to Metsemakers et al. [3]

Confirmatory Criteria	Suggestive Criteria
Presence of a fistula, sinus tract, or wound dehiscence that communicates with the bone or implant	• Persistent or progressively worsening pain (especially without weight-bearing) • New-onset pain at the fracture site • Localized erythema (redness) • Swelling around the affected area • Increased skin temperature over the fracture site • Fever ≥ 38.3°
Visible presence or discharge of purulent material from the wound site.	• Bone resorption or lysis near the fracture or implant site • Signs of implant loosening • Presence of sequestra (segments of devitalized bone) • Delayed or absent fracture healing (e.g., nonunion) • Periosteal new bone formation, especially in areas distant from the original fracture line or in already united fractures.

Isolation of phenotypically identical pathogens from at least two distinct deep tissue or implant samples obtained during surgery	Isolation of a pathogenic microorganism from a single deep tissue or implant culture may support the diagnosis of fracture-related infection, especially when correlated with clinical and radiological findings.
Microorganisms identified via histopathological examination using targeted bacterial or fungal staining methods, or detection of more than five polymorphonuclear leukocytes per high-power field.	Continuous, worsening, or newly appearing wound drainage extending past the early postoperative phase, in the absence of a clear alternative cause, may suggest a fracture-related infection. New-onset joint effusion

Table 2. Baseline data of the Enrolled patients

Variables	FRI Pos	FRI Neg	Chi-Value	P- Values
Gender: Male :Female	6 5	31 30	0.05	0.54
Age	38.81(20.06)	44.57(19.32)	18.66	0.68
Clinical Symptoms:			57.57	0.000
Fever	0	13		
Fistula	2	0		
Local Warmth	0	3		
Pain	0	16		
Pus	4	0		
Redness	1	17		
Swelling	1	12		
Wound Drainage	3	0		
Duration from fracture to admission	16.98(16.31)	8.09(12.04)	7.76	0.000
Duration from admission to surgery	94.54(163.84)	27.03(57.54)	3.64	0.001
Duration of surgery	5.36(1.68)	4.82(1.60)	20.44	0.000
ESR	6(4-7)	6(5-10)	1.16	0.118
TLC	6987(6784-8798)	7500(6700-8826)	2.13	0.969
CRP	2.5(1.67-2.87)	2.13(1.85-2.56)	1.34	0.486
CD64	4243.09(2456-7656)	6000(2554-7876)	8.21	0.03
CD66b	460.73(324-675)	495.97(319-676)	1.98	0.69
CD64/CD66b	12.16±6.81	12.29±6.76	15.47	0.001
NCD64/NCD66b	10.04±4.95	10.81±4.23	20.98	0.001
MCD64/MCD66b	10.68±9.32	12.76±8.85	11.82	0.001

Quantitative measurements of CD64 and CD66b

Blood samples were collected in both stained and unstained tubes. In each tube, 10 µl of CD45 was added. To the stained tubes, 10 µl of CD64 FITC was added, along with 5 µl of CD66b PE Mouse anti-human antibody. The tubes were gently shaken to ensure thorough mixing and incubated in the dark for 15 minutes. Following incubation, 920 µl of lysing buffer was added to each tube, and the samples were incubated for an additional 10 minutes in the dark.

Subsequently, the tubes were centrifuged, and the supernatant was discarded. To each tube, 920 µl of sheath fluid was added, and the samples were centrifuged again. The supernatant was discarded, and 500 µl of sheath fluid was added. The tubes were mixed well, and the resulting mixture was transferred to Falcon tubes for analysis.

The samples were analyzed using a BD FACS Canto flow cytometer, and data were collected through BD FACS Diva Software. The analysis focused on the expression of CD64 and CD66b, specifically measuring the following parameters: **NCD64**: The average fluorescence intensity of CD64 on neutrophils, **NCD64/CD66b**: The ratio of the average fluorescence intensity of CD64 to CD66b on neutrophils, **MCD66b**: The average fluorescence intensity of CD66b on monocytes, **MCD64**: The average fluorescence intensity of CD64 on monocytes, **MCD64/MCD66b**: The ratio of the average fluorescence intensity of CD64 to CD66b on monocytes.

Statistical Analysis

The significance of the differences between the groups before and after surgery was analyzed using the **Wilcoxon signed-rank test**. This non-parametric test was applied to compare paired data, specifically evaluating the changes in marker expression before and after surgery. A p-value of less than 0.05 was considered statistically significant, indicating a meaningful difference between the pre-operative and post-operative measurements.

RESULTS

Patients were categorized into Fracture-Related Infection Positive (FRI POS) and Negative (FRI NEG) groups based on culture reports. The mean age was 38.81 (±20.06) years in the FRI POS group and 44.57 (±19.32) years in the FRI NEG group, with no significant difference. The mean duration from fracture to admission was significantly longer in the FRI POS group (16.98 ±16.31 days) compared to the FRI NEG group (8.09 ±12.04 days, p=0.03). Time from admission to surgery was also significantly higher in the FRI POS group (94.54 ±163.84 days, p<0.0001). Although the mean surgery duration was similar in both groups, the difference was statistically significant.FRI POS patients showed clinical signs such as pus (n=4), wound drainage (n=3), fistula (n=2), and redness/swelling (n=1), with a highly significant association (p<0.0001). Biomarker analysis revealed significant differences in immune markers between groups. The CD64/CD66b ratio was significantly lower in the FRI POS group, both in neutrophils (NCD64/NCD66b) and monocytes (MCD64/MCD66b), indicating a potential role in FRI diagnosis. No significant differences were observed in gender or age distribution, suggesting these factors do not influence FRI occurrence in this cohort. [Table 2]

When the biomarkers where tested post-surgery mostly at the 10th day or around it was observed that the FRI Positive Patients significant increase were observed in the expression of CD64, NCD64, and NCD66b post-surgery, indicating a shift toward reduced immune cell activation or inflammation post-surgery. There was an **increase in MCD64** and **MCD66b** expression, indicating active involvement of macrophages and neutrophils in the healing process. The **CD64/CD66b**, **NCD64/NCD66b**, and **MCD64/MCD66b** ratios significantly shifted, suggesting changes in the balance of immune cell populations post-surgery. In **FRI Negative Patients** Although some increases in **CD64** and **MCD64** were observed, **CD66b expression** did not show a significant change, indicating a less pronounced immune response post-surgery in this group. **Immune marker ratios**, such as **CD64/CD66b** and **NCD64/NCD66b**, reflected a shift in immune activity, though these changes were less marked compared to the FRI-positive group. [Table 3]

The findings highlight the distinct immune responses before and after surgery in both FRI-positive and FRI-negative patients, with more pronounced changes observed in the FRI-positive group, suggesting a stronger inflammatory response linked to infection and healing processes. This indicates that NCD64/NCD66b and MCD64/MCD66b are good biomarker for Fracture Related Infection. The graph illustrates the mean and standard deviation of expressions of the biomarkers which indicates that CD64 expression is higher in FRI Pos group with statistical significant difference (0.03). NCD64 and MCD64 also showed significant difference (p<0.000). This demonstrated that CD64, NCD64 and MCD64 markers are significantly upregulated in FRI pos cases suggesting potential diagnostic role in identifying FRI. [Figure 1] however, CD66b doesn't show any significance between FRI Pos and FRI Neg, but NCD66b and MCD66b increased in positive group (p<0.000) suggesting as a potential biomarkers. [Figure 2] In case of CD64 and CD66b ratios, CD64/CD66b and NCD64/NCD66b appears to be a suggestive biomarker for distinguishing from positive and Negative. The lack of significance MCD64.MCD66b Suggest it may have limited diagnostic utility for FRI. [Figure 3]

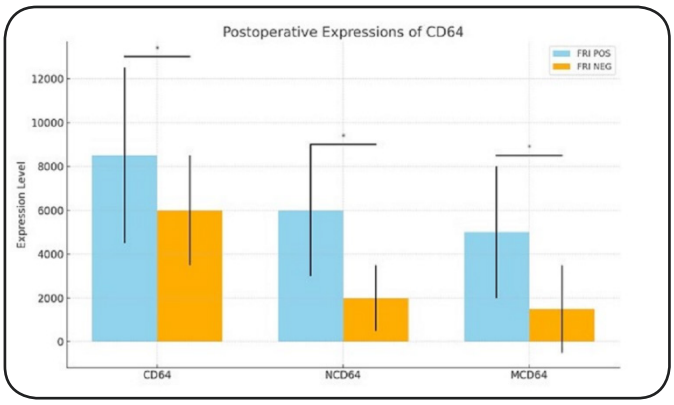

Figure 1. Comparison of Postoperative CD64 expressions in both groups.

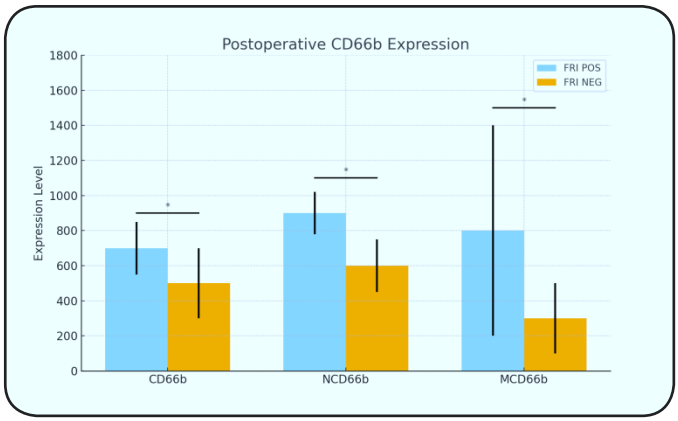

Figure 2. Comparison of Postoperative CD66b expressions in both groups.

Table 3. Post Surgery expressions of Biomarkers in both the Groups

Biomarker	FRI POS	FRI NEG	Z- Value	P-Value
CD64	8541.02±3874.23	6000±2937.17	4.76	0.03
CD66b	656.75±123.30	495.97±212.00	0.67	0.69
CD64/CD66b	19.66±10.01	12.29±6.76	4.83	0.000
NCD64	5840.59±1631.79	2211.54±1125.45	7.36	0.000
NCD66b	8028.80±101.01	584.49±172.24	7.36	0.000

NCD64/NCD66b	10.81±4.23	5.12±8.72	6.20	0.000
MCD64	5364.02±2438.23	2570.54±2274.26	5.39	0.000
MCD66b	694.41±1108.97	280.34±186.86	5.69	0.000
MCD64/MCD66b	12.70±8.85	12.96±16.64	1.62	0.10

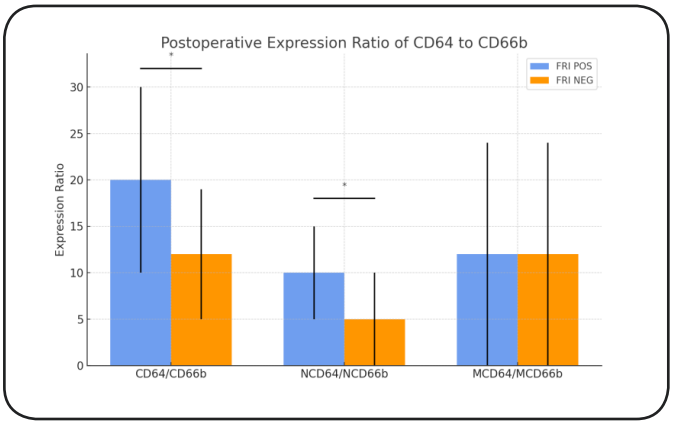

Figure 3. Comparison of Postoperative Ratio of CD64/ CD66b expressions in both groups.

Correlation of Immune markers

A correlation analysis was performed between various immune markers in fracture-related infection (FRI) positive patients. The results indicated several significant relationships:

CD64 and CD66b were positively correlated ($r = 0.65$, $p = 0.02$), suggesting that an increase in CD64 expression is associated with a corresponding increase in CD66b expression in these patients. **MCD66b** was positively correlated with the ratio of **CD64/CD66b** ($r = 0.72$, $p = 0.01$), indicating a strong association between the expression of MCD66b and the combined expression of CD64 and CD66b. **MCD64** was positively correlated with the ratio of **MCD64/CD66b** ($r = 0.81$, $p = 0.02$), showing a significant relationship between MCD64 expression and its ratio with CD66b. On the other hand, **CD66b** was negatively correlated with **MCD66b** ($r = -0.65$, $p < 0.05$), suggesting an inverse relationship between CD66b and MCD66b expression. Additionally, **CD66b** was negatively correlated with the ratio of **CD64/CD66b** ($r = -0.79$, $p = 0.003$), further supporting the idea that higher CD66b expression might be inversely related to the combined expression of CD64 and CD66b in these patients. These findings highlight the complex interplay between these immune markers and suggest that the ratios of certain markers may provide valuable insights into the immune response during FRI.

DISCUSSION

The diagnosis of fracture-related infections (FRIs) has traditionally relied on clinical signs, imaging, and nonspecific serum inflammatory markers, with limited studies focusing on biomarkers. This study investigated the diagnostic accuracy of CD markers CD64 and CD66b in patients suspected of having FRI. While serum inflammatory markers such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are commonly used to aid in FRI diagnosis, they often lack specificity, especially in the early phases of infection.

Most of the existing literature on CD64 and CD66b markers has focused on their role in diagnosing infections in contexts such as prosthetic joint infections (PJI) and sepsis, but their application in FRIs has not been well studied. CD64 and CD66b are surface markers expressed on neutrophils and are upregulated in response to bacterial infections. This makes them potentially useful as specific biomarkers for identifying infections in orthopedic trauma settings.

In this study the fluorescent Intensity of CD64 and CD66b on neutrophils and Monocytes was detected by Flow Cytometry. The Wilcoxon Ranked Test revealed that NCD64/NCD66b and MCD64/CD66b and CD64/CD66b was found significant among the FRI positive and Negative group. Previous studies have used the same biomarkers but in different diseases like Rheumatoid arthritis sepsis. Previous studies have used a single CD35 or CD64 as the research object, and its correlation with bacterial or viral infection has been reported to be high and low respectively, and the sensitivity and specificity were also quite different [8]. Another study on the ratio of CD35/ CD64 was reported to have 100% sensitivity and 86% specific for patients with rheumatoid arthritis. The sensitivity of diagnosis of bacterial infection was 67% and the specificity was 80%.[9], and the results are in consistent with the present study. **Raikwar A et al.,** in their previous study tested for the same biomarkers at different postoperative and compared them in both the groups the results were found to be statistically significant and further proceeding their research the ratios of the fluorescent study was analysed The current study is the extended version of this study.[10]

Toru Doi et al., in their findings, suggest that **CD64** serves as an effective marker for detecting postoperative infections, particularly in high-risk patient groups such as those undergoing orthopedic surgeries or dealing with fracture-related infections (FRI). The study emphasizes the utility of **CD64** in improving **early diagnosis** and facilitating **timely intervention**, which is critical for preventing complications in patients who are vulnerable to infections post-surgery. By serving as a reliable biomarker, **CD64** helps clinicians identify infection early, leading to better management of patient care and potentially improving outcomes by initiating appropriate treatments before infections progress. This insight contributes to advancing clinical practices and reinforcing the role of immune markers in enhancing diagnostic precision for postoperative infections. As in our study CD64 also showed the significant observations.[11]

Iris Streimish in their study these findings suggest that neutrophil CD64 measurement may enable neonatal clinicians to safely discontinue antimicrobial therapy at 24 hours in clinically stable neonates with CD64 levels below the established cutoff, without awaiting definitive microbiological culture results.[12]

Due to the scarcity of original data and comprehensive studies on the specific role of CD64 in fracture-related infections

(FRI), it has become challenging to directly compare findings across different research efforts. While CD64 has been widely studied and its usefulness demonstrated in the context of various bacterial infections and sepsis, its application to FRI remains underexplored. In many infectious diseases, CD64 expression on neutrophils has been shown to serve as an early and reliable biomarker of infection, particularly bacterial in nature. Its upregulation in response to pro-inflammatory cytokines has been consistently observed in conditions such as sepsis, where CD64 expression correlates with disease severity and prognosis. However, due to the lack of dedicated studies on fracture-related infections, drawing firm conclusions on its diagnostic value in this specific context remains difficult, underscoring the need for further research in this area.

Another study on CD64 expression on neutrophils demonstrates equivalent efficacy to traditional infection markers in the diagnosis of various musculoskeletal infections. This biomarker provides a reliable and efficient method for identifying infections, with the added advantage of reflecting the activation of neutrophils, which are key players in the immune response to infection. By offering a more sensitive and specific approach compared to conventional markers such as C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR), CD64 can potentially lead to faster diagnosis and more targeted interventions in musculoskeletal infection cases, improving patient outcomes. Furthermore, it can serve as an important tool for monitoring the progress of infection and response to treatment, helping clinicians make more informed decisions regarding management.[13]

CD64 expression is a reliable biomarker for diagnosing localized musculoskeletal infections, offering a sensitive and specific method for detecting infection. As an activation marker on neutrophils, CD64 reflects the immune response, making it particularly valuable in identifying infections in musculoskeletal tissues. However, its diagnostic performance may be influenced by prior antibiotic use, as antibiotics can modulate immune responses, potentially leading to reduced neutrophil activation and altered CD64 expression. Therefore, while **CD64** remains a robust marker for infection, its interpretation should consider the potential effects of antibiotic therapy, which may affect its sensitivity and accuracy in certain cases.[14]

The discrepancy in CD64 expression levels between our study and some previous studies may be attributed to differences in study design, patient populations, timing of sample collection, or the presence of other underlying conditions. Nevertheless, the consistent finding across studies is the potential role of CD64, and possibly the CD64/CD66b ratio, as biomarkers for FRI. Incorporating these biomarkers into routine clinical practice could enhance the early detection and management of FRI, leading to improved patient outcomes.

CONCLUSION

This is the first study on Fracture-Related Infections (FRI) to evaluate biomarker ratios, expanding on previous work that focused on biomarker expression. The analysis reveals significant differences in key variables namely, fracture-to-admission time, admission-to-surgery time, and CD64/CD66b ratios between FRI-positive and FRI-negative patients. FRI-positive patients presented earlier than FRI-negative ones, possibly due to symptomatic infection prompting timely care. The significantly longer delay from admission to surgery in FRI-positive cases may reflect preoperative infection control efforts. Notably, ratios of CD64/CD66b (including neutrophil and monocyte subsets) differed significantly between groups, indicating potential utility as diagnostic markers. Some biomarkers showed positive or negative correlations, suggesting interdependence that may impact diagnostic accuracy. The study is ongoing with a larger cohort to establish cut-off values via ROC analysis, aiming to enhance early FRI detection and treatment. Future research should prioritize standardizing and validating CD64/CD66b biomarkers, assessing their prognostic value, and exploring targeted therapies. Development of cost-effective, point-of-care diagnostics, integration of molecular and genetic approaches, and use of AI for early diagnosis and personalized care could significantly improve FRI outcomes and reduce healthcare burden.

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