# The terminal complement pathway is activated in septic but not in aseptic shoulder revision arthroplasties

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**Background:** The early diagnosis of suspected periprosthetic low-grade infections in shoulder arthroplasties is important for the outcome of the revision surgical procedures. The aim of this study was to investigate new biomarkers of infection in revision shoulder arthroplasties, taking into account the implant design, patient age, and comorbidities.
**Methods:** The study included 33 patients with shoulder arthroplasties undergoing revision surgical procedures. Microbiological diagnostic testing was performed in all cases. C-reactive protein serum levels and white blood cell counts were evaluated, and the periprosthetic tissue was stained immunohistologically for the terminal complement pathway components (C3, C5, and C9) and for CD68 and α-defensin.
**Results:** Microbiological diagnostic testing detected a periprosthetic infection in 10 reverse shoulder arthroplasties and in 4 anatomic shoulder arthroplasties, while the remaining 19 shoulder arthroplasties were classified as aseptic. We observed more *Staphylococcus epidermidis* infections in reverse shoulder arthroplasties and more *Staphylococcus aureus* infections in anatomic shoulder arthroplasties. The revision rate correlated with pre-existing comorbidities and number of previous surgical procedures. The C-reactive protein values and the incidence of specific periprosthetic radiolucent lines were significantly increased in septic revision cases. We found increased staining for all tested complement factors (C3, C5, and C9) but not for α-defensin and CD68 in septic tissue. The most interesting finding was that C9 separated septic from aseptic tissue with a predictive specificity of 100% and a sensitivity of 88.89%.
**Conclusion:** We observed a strong correlation between C9 expressions in septic revision tissue. We propose that the terminal complement pathway, especially C9 deposition, may be a potential biomarker to identify septic complications using tissue biopsy specimens.

Ethical approval for this study was given by the Institutional Review Board of the Otto-von-Guericke University Medical School, Magdeburg (IRB No. 150/12).

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The number of total shoulder arthroplasties is rapidly growing, with a 7-fold increase predicted during the next 15 years.[9] Today, various different types of shoulder prostheses for different medical and individual anatomic conditions exist. However, the design of these prostheses can be distinguished by 2 basic biomechanical principles: anatomic and reverse shoulder implants.

Because of the increasing number of failed primary shoulder arthroplasties, the number of septic and aseptic revision surgical procedures is a rising challenge in shoulder surgery. The rate of infection in shoulder endoprostheses is approximately 1%, comparable with the infection rate of other joints.[26] The most frequently detected pathogens in total shoulder joint arthroplasty are *Cutibacterium* (formerly *Propionibacterium*) *acnes*, *Staphylococcus epidermidis*, and *Staphylococcus aureus*.[30] The cause of infection can be a hematogenic infection (eg, pneumonia, urinary tract infection, or dental sinuses) or an intraoperative or perioperative infection, which can appear as an early or late infection. Clinically, an early periprosthetic joint infection (PJI) is accompanied by the onset of pain, loss of function, and other signs of inflammation such as fever, wound-healing disorders, or the development of local erythema. Furthermore, the presence of a systemic reaction is indicated by increased systemic inflammation parameters such as the white blood cell (WBC) count and C-reactive protein (CRP) level.[27] A late infection, however, is often more difficult to diagnose because of the lack of systemic inflammation owing to the formation of a biofilm. Sometimes, radiologically detectable periprosthetic radiolucent lines (RLLs) indicate the failure of secure fixation of the implant in the case of low-grade septic complications. Microbiological analysis of the synovial fluid, however, detects low-grade infection cases only at very late stages, when the biofilm is already producing planktonic bacteria. A second surgical intervention using a spacer implant, consisting of antibiotics and bone cement, is inevitable in most cases. Because low-grade infections are difficult to diagnose at early stages and a late diagnosis makes appropriate treatment impossible without explantation of the prosthesis, there is a need for biomarkers to diagnose the infection at an early time point. To develop biomarkers for the early diagnosis of a low-grade infection, the understanding of different pathways activated during the infection is of utmost importance.

The detection of α-defensin has been proposed to be a marker for PJI.[4,13,46] The α-defensin protein is a 2- to 6-kDa antimicrobial peptide, which is predominantly activated by gram-negative and -positive bacteria. It is secreted by neutrophils and macrophages and is able to bind pathogens in the synovial fluid and impede cell wall synthesis.[13,46] However, there have been reports of false-positive test results in the case of adverse tissue reactions.[11]

Another important component of the immune response to bacterial infection is the complement system.[41] The main purpose of the complement system is the destruction of foreign or dead cells, activation of immune defense cells, and opsonization of pathogens.[22] Therefore, the activation of the complement pathway predominantly occurs during the early infection phase.[17,20] The system recognizes foreign structures activating 3 different pathways, which converge to the common component C3; the terminal common pathway is initiated with C5 being cleaved into C5a and C5b. C5b starts the formation of the membrane attack complex by recruiting C6, C7, C8, and C9. The membrane attack complex is the cytolytic end product of the terminal complement cascade resulting in osmotic lysis and thereby cell death.[10]

The presence of macrophages in tissue biopsy specimens has been proposed to be an indicator for septic complications, as they are part of the nonspecific immune response by removing pathogens via phagocytosis and also part of the adaptive immune response by recruiting other immune cells. Immunostaining for CD68 shows the presence of monocytes and macrophages, as a first hint of the inflammatory tissue response.[28,37]

The hypothesis of this study was that the terminal complement pathway in combination with α-defensin would provide better evidence of discrimination between aseptic loosening and PJI in total shoulder arthroplasties. To test this hypothesis, we investigated aseptic and septic tissues of shoulder endoprosthesis revision surgical procedures regarding the design of the shoulder implant, patient characteristics, bacterial diagnostic testing, and proposed biomarkers.

## Materials and methods

### Patients

In this retrospective basic research study, 33 consecutive shoulder revision surgical procedures performed for aseptic and septic revision reasons between February 2011 and April 2016 were included. The demographic data of all patients (age at surgery, implantation time, radiologically detected RLLs, number of previous surgical procedures, and comorbidities) were recorded (Table I). Before surgery, serum levels of CRP (in milligrams per liter) and WBC count (in giga-particle [Gpt] per liter) were determined. Infections were identified according to Musculoskeletal Infection Society criteria and Infectious Diseases Society of America criteria.[25,27]

**Table I** Biometric characteristics of study population

| Total shoulder arthroplasty group | % of cohort | Age, yr | Implantation time, yr | No. of previous revision surgical procedures |
|---|---|---|---|---|
| Reverse | | | | |
| Septic | 30.3 | 64.2 ± 6.57 | 1.8 ± 2.36 | 1 (5 of 10) or 2 (4 of 10) |
| Aseptic | 6.06 | 84.5 ± 16.26 | 1.87 ± 1.59 | 1 (1 of 2) |
| Anatomic | | | | |
| Septic | 12.12 | 71.94 ± 8.13 | 2.87 ± 2.95 | 3 (1 of 4) |
| Aseptic | 51.51 | 73.0 ± 9.21 | 3.62 ± 5.08 | 1 (3 of 17) or 2 (1 of 17) |

### Histologic analysis and immunohistochemical staining

The periprosthetic tissues were fixed overnight in 4% paraformaldehyde. The tissue was embedded in paraffin and cut into 4-μm sections. Immunohistochemical staining was performed using the following antibodies: α-defensin (Acris; OriGene Technologies, Rockville, MD, USA); CD68 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and C3, C5, and C9 (Quidel, San Diego, CA, USA). Corresponding IgG antibodies were used as isotype control for the respective staining. The area of red immunofluorescence was calculated as the percentage of the picture and was analyzed using ImageJ (version 1.5; National Institutes of Health, Bethesda, MA, USA) in comparison with the isotype control staining.

### Microbiological diagnostic testing

Periprosthetic tissue samples were minced and mechanically homogenized on an Ultra-Turrax Drive Control Dispergierer system (IKA-Werke, Staufen im Breisgau, Germany) at 6000 rpm for 2 minutes in intervals with direction change. In brief, the homogenized samples were inoculated on agar plates: Columbia agar with 5% sheep blood (Becton Dickinson, Heidelberg, Germany) and chocolate agar and Schaedler agar (Oxoid, Munich, Germany) under aerobic conditions with 5% carbon dioxide and anaerobically at 35°C ± 1°C. In addition, the samples were inoculated in thioglycolate and Schaedler broth (bioMérieux, Marcy-l'Etoile, France) at 35°C ± 1°C for 14 days. The identification of pathogens was performed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (VITEK MS; bioMérieux).

### Radiographic analysis

For the evaluation of periprosthetic bone resorption, the radiographs were analyzed for the location and extent of RLLs around the implant (Sanchez-Sotelo et al[38]). To determine the degree of implant loosening, the humeral component of the shoulder was subdivided into 8 radiologic zones.[24] The glenoid was not investigated because of the small number of aseptic reverse implants.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 7; GraphPad Software, San Diego, CA, USA) and SPSS (IBM, Armonk, NY, USA). MedCalc (MedCalc Software, Ostend, Belgium) was used for calculation of C9 staining predictive sensitivity and specificity. The plots and bar charts show medians with standard deviations. To test statistical significance, we used the Mann-Whitney *U* test, with thresholds of $P < .01$, $P < .05$, and $P < .1$.

## Results

### Demographic data and patient characteristics

Thirty-three patients undergoing shoulder revision procedures were included. Microbiological diagnostic testing indicated PJI in 14 patients as the reason for revision. Nineteen patients were considered to have aseptic revisions (Fig. 1, *A*), owing to rotator cuff insufficiency and instability (9 of 19), glenoid component loosening (1 of 19), glenoid erosion (2 of 19), prosthesis dislocation (3 of 19), periprosthetic fracture (2 of 19), or humeral osteolysis (2 of 19).

The septic group of 14 patients was subdivided into 10 patients with reverse and 4 with anatomic shoulder implants, whereas the aseptic group included 2 reverse and 17 anatomic implants (Table I; Fig. 1, *A*). Altogether, 19 patients (57.6%) underwent aseptic surgery, and a bacterial infection was diagnosed in 14 patients (42.4%). It is interesting to note that 71.4% of the septic cases received a revision of a reverse shoulder prosthesis while only 28.6% of the patients with anatomic implants showed a septic complication. The average age of the patients at the time of surgery was similar in both groups (73.3 years in aseptic group and 72.1 years in septic group). The implantation time before revision surgery was approximately 3 years (3.44 ± 4.84 years in aseptic group and 2.19 ± 2.28 years in septic group) (Table I).

The number of previous surgical procedures before revision surgery on the respective joint is presented in detail in Table I. The number of previous surgical procedures in the septic total shoulder arthroplasty group was greater than that in the aseptic total shoulder arthroplasty group, with 9 of 11 patients having at least 1 or 2 previous shoulder surgical procedures and 1 patient having 3 previous surgical procedures on the shoulder. In the aseptic group, 5 patients had at least 1 or 2 prior operations. The remaining 14 patients had no previous operation before revision surgery.

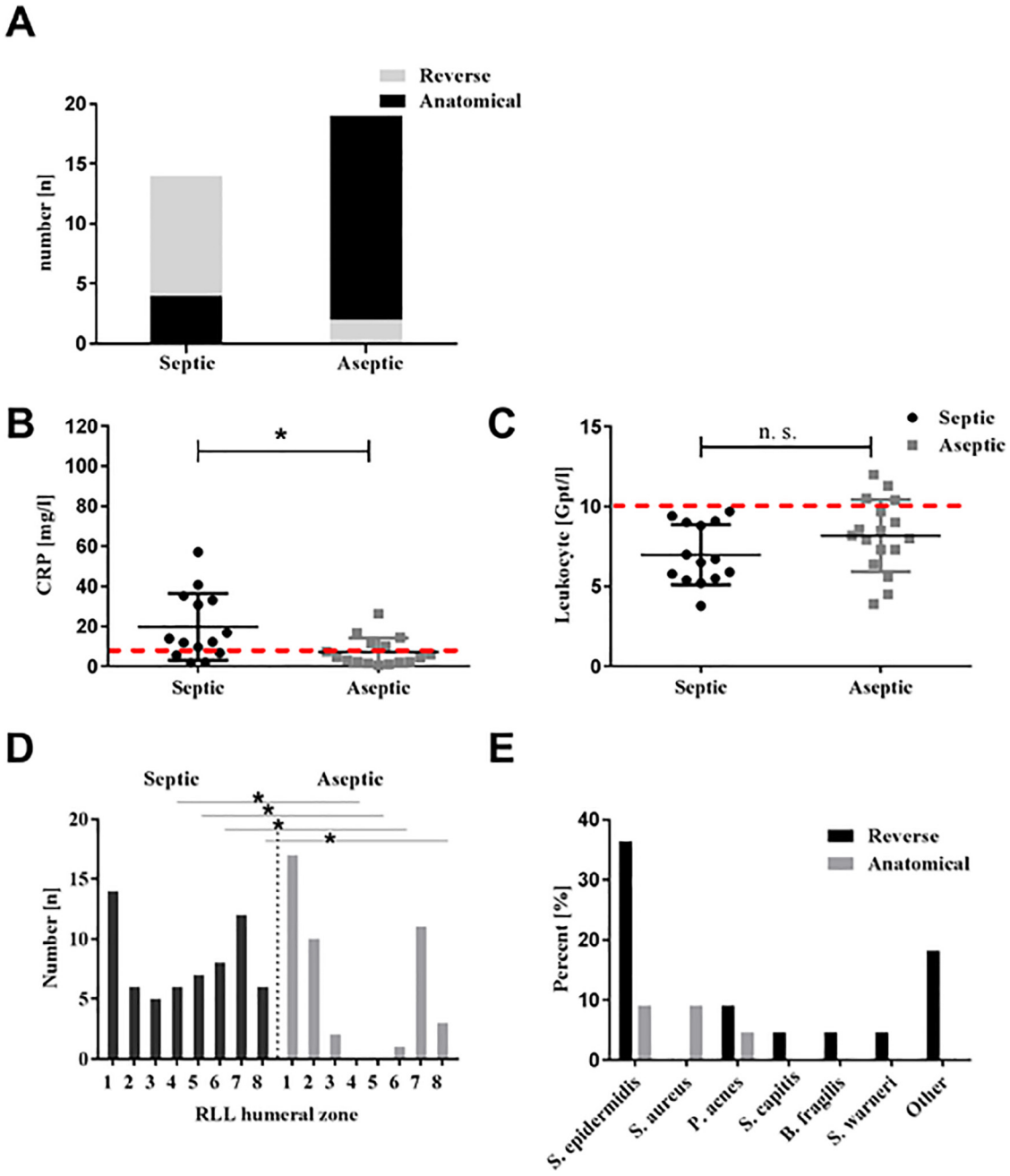


**Figure 1** Characterization of septic and aseptic shoulder prostheses. (**A**) The study included 33 implants (14 septic and 19 aseptic). (**B**) C-reactive protein (*CRP*) values (in milligrams per liter) in septic (*black circles*) and aseptic (*gray squares*) groups. The CRP value was significantly increased in septic samples. The pathologic threshold of 5 mg/L is indicated (*red dashed line*). (**C**) The white blood cell count (in giga-particles per liter) was not changed between the septic and aseptic groups. The normal range of 10 Gpt/L is indicated (*red dashed line*). (**D**) The radiolucent lines (*RLLs*) of the humeral zones were analyzed using the Neer zones.[24] The septic group exhibited increased RLLs compared with the aseptic group. (**E**) Prevalence of most frequent pathogens in reverse and anatomic shoulder prostheses. $*P < 0.05$. *ns*, not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The comorbidities of the patients are presented in Table II. Patients with septic complications more often had diabetes and renal insufficiency, whereas patients with aseptic revision showed more neurologic comorbidities such as Parkinson disease or polyneuropathy.

To evaluate the presence of systemic inflammation, the CRP value and WBC count were analyzed in the blood of all patients. When the CRP value was compared between the aseptic (4.55 mg/L) and septic (13.10 mg/L) groups, the septic group showed a higher variability in CRP values than the aseptic

**Table II** Comorbidities of patients

| | Reverse | Anatomic |
|---|---|---|
| Aseptic | Renal insufficiency, 1 of 2<br>Heart failure, 1 of 2 | Parkinson disease, 3 of 17<br>Polyneuropathy, 1 of 17 |
| Septic | Diabetes, 4 of 10<br>Renal insufficiency, 3 of 10<br>Metal allergy, 1 of 10 | Heart failure, 1 of 4<br>Renal insufficiency, 1 of 4<br>Pneumonia, 1 of 4 |

group ($P \leq .0138$) (Fig. 1, *B*). No difference, however, was observed regarding the WBC count in septic and aseptic patients (Fig. 1, *C*). The aseptic group showed an average WBC count of 8.2 Gpt/L, while the average WBC count in the septic group was 6.6 Gpt/L. As the normal maximum value of the WBC count is 10 Gpt/L, no pathologic elevation of the WBC count was observed in either group ($P = .157$).

The evaluation of RLLs on the radiographic images showed clear differences in the bone resorption pattern between septic and aseptic cases (Fig. 1, *D*). The radiographs of the septic group exhibited RLLs in all 8 Neer[25] zones of the humerus. An interesting finding was that the aseptic group mainly exhibited loosening in zones 1 and 7. The other RLL zones were mainly not affected in the aseptic group, as indicated by the fact that zones 4, 5, 6, and 8 were more frequently found in the septic group than in the aseptic group (zone 4, $P = .03$; zone 5, $P = .01$; zone 6, $P = .02$; and zone 8, $P = .04$). Because of the low number of aseptic reverse cases (n = 2), we did not further investigate glenoid loosening.

The identification of bacterial strains is summarized in Figure 1, *E*. An interesting finding was that *S epidermidis* showed the highest frequency in the reverse shoulder implants (36.4%) while *S aureus* (9.1%) was only found in the anatomic prostheses. The reverse shoulder implants showed a higher variety of *Staphylococcus* strains compared with the anatomic prostheses. Other bacteria, such as *Staphylococcus capitis* (4.5%), *Bacteroides fragilis* (4.5%), and *Staphylococcus warneri* (4.5%), were found in small numbers only in the reverse group but not in the anatomic group. In contrast, *C acnes* was found in the anatomic (4.5%) and reverse (9.1%) shoulder implants.

## Distribution of septic marker proteins in periprosthetic revision tissue

As α-defensin staining has been proposed to be a marker for infected tissue, we stained septic and aseptic periprosthetic revision tissue to assess the tissue presence and distribution of this marker protein (Fig. 2, *A*). We found both tissue types to be positive for α-defensin, with no significant difference between the septic and aseptic groups (0.5171% ± 0.4187% and 0.1307% ± 0.1631%, respectively; $P = .2224$).

To further analyze the tissue response in septic revision tissue, we assessed the presence of macrophages as inflammatory marker cells using CD68 staining (Fig. 2, *B*). Again, we found macrophages to be present in septic tissue, as well as aseptic tissue, and the quantification of the fluorescence showed no difference between septic and aseptic tissue (0.097% ± 0.02% and 0.014% ± 0.03%, respectively; $P = .1135$).

The terminal pathway of the complement system is a key component of the host defense against bacteria. We investigated this pathway using antibodies for C3, C5, and C9 to evaluate their tissue presence (Fig. 2, *C-E*). We found increased deposition of all studied complement components in septic tissue. C3, as the first common activated component, was significantly increased in the septic tissue (0.23% ± 0.2175% in septic tissue and 0.029% ± 0.027% in aseptic tissue, $P = .031$) (Fig. 2, *C*). The statistical difference increased with C5, as the following component starting the terminal pathway (0.16% ± 0.08% in septic tissue and 0.022% ± 0.013% in aseptic tissue, $P = .005$) (Fig. 2, *D*). An interesting finding was that C9, as the furthest downstream component of this pathway, distinguished between septic and aseptic tissue with a specificity of 100% and a sensitivity of 88.89% (0.662% ± 0.161% in septic tissue and 0.025% ± 0.073% in aseptic tissue, $P = .0008$) (Fig. 2, *E*).

## Discussion

In this study, we analyzed 33 consecutive total shoulder revisions with respect to reasons for revision, radiologically detectable RLLs, patient characteristics, and microbiological diagnostic testing. The aim of the study was to determine whether biomarkers such as α-defensin and the terminal complement pathway have the potential to discriminate between septic and aseptic total shoulder arthroplasty loosening. Therefore, aseptic (57.6%) or septic (42.4%) periprosthetic tissues obtained at the time of revision surgery were tested. The findings of our study suggest that particularly the deposition of the terminal complement component C9 discriminates between PJI and aseptic loosening in shoulder endoprostheses with an extremely high sensitivity of 100%.

Comorbidities, including diabetes, renal disease, or heart failure, are known to increase the risk of periprosthetic infection.[2,5,6,29,32] We likewise observed an increased number of patients with diabetes and renal insufficiency in our septic cohort; however, we also found patients with these comorbidities in the aseptic cohort and found septic patients without these comorbidities. Furthermore, the risk of PJI increases with each revision operation by about 10%.[12,15,43] In our cohort, there was also an increase in the number of previous surgical procedures in the septic subgroup, which may partially explain the increased number of PJIs. Again, there were also patients with PJI who did not undergo a prior revision surgical procedure and some of these patients did not have any relevant comorbidities. However, these factors clearly contributed to the finding of an increased number of

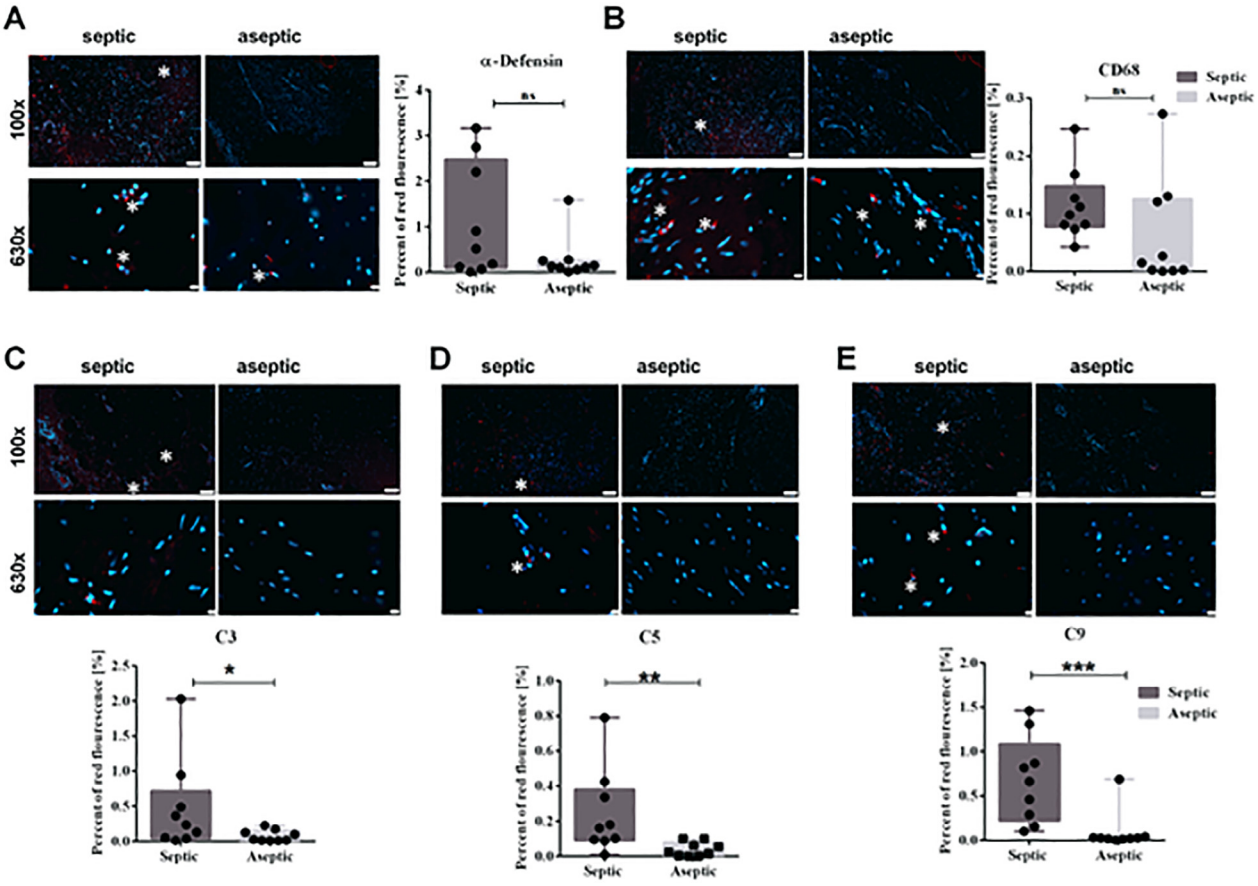


**Figure 2** Local inflammatory response in septic and aseptic periprosthetic tissue. Representative immunohistochemical staining of periprosthetic tissue of septic and aseptic patients is shown: α-defensin (**A**), macrophages (CD68) (**B**), and terminal complement pathway (**C-E**) (N = 9; n = 27). The values indicate the percentage of positive staining given as the median of 3 images per patient. Two different magnifications (100× and 630×) are given. The scale bars (*white bars*) are 100 µm for 100× magnification and 2 µm for 630× magnification (n = 3; N = 9). **P* < 0.05; ***P* < 0.01; ****P* < 0.001. *ns*, not significant; *n*, total number of measurements; *N*, number of samples.

septic complications with a reverse design but did not explain the increased number completely.

One difference between the anatomic and reverse prostheses, according to their biomechanical principles, is that screws are used for fixation of the glenoid component in the reverse shoulder prosthesis. It has been shown that in the wide surface grooves of the screws, the contact area of bacteria to the material is increased and the adhesion energy is enhanced, allowing better bacterial adherence on the surface.[35,36,42,47] In line with these observations, it has been shown that a type of stemless shoulder prosthesis, which was fixed with a porous coated hollow screw, was more prone to infection than other anatomic shoulder prosthetic designs.[19] This accounts for the increased infection rate of screw-fixed prostheses.

We also observed a difference in the bacterial spectrum between anatomic and reverse shoulder implants. This difference might be explained by the fact that some strains prefer to adhere to smooth surfaces while other bacteria would rather colonize on rough surfaces.[48] It is interesting to note that it has been reported that *S epidermidis* shows increased adhesion on rougher surfaces,[44,45] suggesting an explanation for the higher frequency of *S epidermidis* infections in reverse prostheses. Furthermore, *S epidermidis* was mostly found in a polymicrobial colonization, whereas *S aureus* was mostly found as a monoculture. Previous studies showed that *S aureus* exhibits a higher virulence than *S epidermidis*, which supports a polymicrobial biofilm.[14,21]

One key problem of low-grade PJI is the late diagnosis. To test whether an increase in markers of systemic inflammation could be observed in the septic patients, we analyzed the serum CRP value and WBC count. We observed an increase in the CRP level to 19.84 mg/L, which was statistically significant. However, the predictive value of CRP measurement to identify infections of endoprostheses has been described to be low,[31,39] indicating that especially the low-grade infection is a local phenomenon with a very low systemic involvement. Therefore, the gold standard for the diagnosis of low-grade PJI is, at the moment, the examination of an arthroscopic or open tissue biopsy specimen.[31]

We stained for the presence of macrophages in the septic and aseptic tissue using CD68 as a marker. There was no significant difference in macrophage presence in septic

periprosthetic tissue compared with aseptic revision tissue. The presence of macrophages in aseptic tissue might be attributed to the presence of wear particles phagocytosed by macrophages.[18,21,33,40]

Another proposed biomarker for bacterial infection is α-defensin.[8] Using immunohistologic staining, we also found α-defensin in the periprosthetic tissue of septic as well as aseptic samples. Quantitative analysis of the immunofluorescence showed no significant increase in α-defensin in septic tissue compared with aseptic tissue. Therefore, the predictive value of α-defensin as a biomarker for infection in our shoulder cohort was low. This finding might be explained by other studies showing that the presence of metal wear particles can also induce the expression of α-defensin.[4,7] This might be a reason for the positive α-defensin staining in the aseptic tissue.

One of the key parts of the immune system involved in the host defense against bacteria is the complement system.[1,23,34] To investigate its activation, we stained for 3 different components (C3, C5, and C9) of the terminal part of the complement pathway in septic and aseptic tissue. C3 is the pivotal component of all 3 pathways of activation, while C5 and, in particular, C9 are members of the common late pathway and their deposition indicates a terminal part of the cascade. Quantitative analysis showed a significant increase in all complement factors in the septic group, with C9 being able to detect septic tissue with a high specificity and sensitivity. In contrast to our findings regarding α-defensin, we found almost no presence of complement factors in aseptic cases, making the activation of the complement system a highly specific marker for infection.[3,16,49]

We are well aware of some limitations of our study, which mainly are the low number of included revision cases and the quite diverse reasons for revision; moreover, the location of the periprosthetic tissue for sampling was not clearly defined. Larger cohort studies, as well as studies including other joints, are currently being performed to further substantiate the findings of our study; however, our results clearly provide evidence that the analysis of complement activation, in particular C9 deposition, can allow early discrimination of PJI.

## Conclusion

We found a marked increase in terminal complement pathway expression in septic revision tissue in our cohort of shoulder arthroplasty revisions, suggesting its function as a new biomarker to diagnose PJI early. The very high specificity and sensitivity of C9 staining make it a useful biomarker for the detection of PJI in tissue biopsy specimens.


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