



Immunohistochemical examination in arthrofibrosis of the knee joint

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Abstract

Introduction Arthrofibrosis (AF) is the result of increased cell proliferation and synthesis of matrix proteins (collagen I, III, and VI). Especially after invasive knee surgery, e.g., ligament reconstruction or knee replacement, abnormal fibroblast proliferation with pathological periarticular fibrosis can be observed leading to severely limited joint motion. The pathogenesis of AF is currently not fully understood. The present work aims to determine pathogenic factors.

Materials and methods A descriptive, histological and immunohistochemical comparative study was performed on tissue samples of 14 consecutive patients undergoing arthrolysis for joint stiffness due to AF. Seven human autopsy specimens served as control. Samples were stained for expression of relevant markers such as CD68, α -smooth muscle actin (ASMA), beta-catenin, BMP-2 and examined for the histological grade of AF (cell-rich versus cell-poor) and compared to a control. Furthermore, a microscopic evaluation of the samples for cell differentiation and number was performed.

Results Tissue sections of cell-rich fibrosis showed a significantly higher expression of CD68 compared to the control with less than 10% of CD68 positive cells ($p=0.002$). In cell-poor fibrosis no statistically significant difference was obvious ($p=0.228$). Expression of ASMA in synovia, vessels, cell-rich and cell-poor fibrosis showed median values of 2.00 in the AF group and 1.75 in the control. Both groups differed significantly ($p=0.003$). AF tissue showed a significantly difference in expression of β -catenin ($p<0.001$) compared to the control. The overall difference between AF and control group in expression of BMP-2 was also statistically significant ($p=0.002$).

Conclusions Expression of CD68, ASMA, beta-catenin and BMP-2 is significantly increased in AF tissue samples. Based on presented findings, histological evaluation and immunohistochemical assessment of CD68, ASMA, β -catenin and BMP-2 expression may prove useful to diagnose AF and to analyze AF activity.

Keywords Arthrofibrosis · Knee · Immunohistochemical examination · CD68 · ASMA · Beta-catenin · BMP-2

Introduction

Arthrofibrosis (AF) is a severe complication that arises after joint trauma or surgery on the knee [1]. It is characterized by a persistent and sometimes painful limitation in the range of motion (ROM) of the affected knee [1–3] which is caused by a massive intraarticular proliferation of fibroblasts and increased synthesis of extracellular matrix proteins [1]. After anterior cruciate ligament (ACL) reconstruction knee stiffness can occur in up to 11% of the patients [2] and leads to a significantly poorer outcome compared to knee ligament surgery without postoperative complications [4].

The high risk of recurrence after arthroscopic treatment and the development of knee osteoarthritis in 78% of AF patients indicate the high socio-economic impact of AF. Therefore, a better understanding of the pathomechanism is of great importance [5, 6].

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With the detection of active myofibroblasts in AF, it was suggested that these cells contribute to the development of AF through their role in tissue repair and scar formation. Ruppert et al. [7] set up histopathological diagnostic criteria for AF based on the expression of β -catenin, as they found a direct correlation between the amount of β -catenin positive fibroblasts and the degree of AF. Together with E-cadherin, β -catenin forms the cell adhesion complex and regulates differentiation, proliferation and embryonic development of cells via the Wnt signalling pathway [8]. Beta-catenin was shown to be a central mediator of pro-fibrotic activity in patients with systemic sclerosis [9].

Beside the excessive formation of fibrinous connective tissue, recent studies also observed increased enchondral ossification in AF [7, 10]. Pfitzner et al. [11] found elevated concentrations of Bone morphogenetic protein 2 (BMP-2) in the synovial fluid of patients with AF after total knee arthroplasty. There was a linear correlation between the concentration of BMP-2 in the synovial fluid and the soft tissue density of the anterior joint capsule [12]. This led to the conclusion that BMP-2, which induces heterotopic osteogenesis through enchondral ossification [13] also plays a crucial role in the pathogenesis of AF. However, there has not been any immunohistochemical detection of BMP-2 in arthrofibrotic tissue so far.

Therefore, the aim of the current study was to give further insight into the pathogenesis of AF by evaluating the expression of β -catenin, BMP-2, α -smooth muscle protein and CD68 immunohistochemically. Special attention was thereby drawn to the histological grades of AF, the cell numbers and differentiation. A deeper understanding of the molecular biological and cellular alterations in AF tissue may contribute to improved diagnostic and therapeutic strategies.

Materials and methods

A descriptive, histological and immunohistochemical comparative study was performed on tissue samples of 14 consecutive patients undergoing arthrolysis due to AF in the

hospital of the work group. The AF had developed secondary to injuries and surgical interventions of the knee joint (Table 1). The AF was diagnosed on the basis of clinical criteria. Patients with knee stiffness due to mechanical reasons, osteoarthritis of the knee and rheumatic disorders were excluded. In all cases arthroscopic arthrolysis with scar tissue removal was carried out. The tissue samples were taken during the therapeutic intervention. Among the patients were 3 women and 11 men. The mean age was 38 years. 7 human autopsy specimens with physiological knee status served as control group. Among these donors were 3 women and 3 men. There was no information about person number seven. Mean age was 86 years. The age difference is due to the limited availability of young autopsy specimens. Body donors with pre-existing knee diseases and knee osteoarthritis were excluded.

The samples were transferred to four percent formalin and fixed therein for at least 24 h. The paraffin embedding was done by machine. Five μ m thick sections of the preparations were made. For the light microscopic examinations, a hematoxylin eosin (HE) staining and a Giemsa staining were performed.

Immunohistochemical procedures

In the present work, the immunohistochemical representations were obtained by indirect methods.

Immunohistochemical presentation of CD68

The primary antibody used was anti-CD68 from DakoCytomation (San Francisco, California, USA) and diluted 1:20 in low cross buffer. The buffer serves to minimize unspecific binding, cross-reactivity and matrix effects in immunohistochemical staining. The paraffin sections were then incubated for 16 h at 4 °C with the primary antibody. The antibody was then washed off with phosphate buffer solution (PBS) and the post block of the horse radish peroxidase (HRP) polymer kit (Zytomed Systems, Berlin, Germany) was applied for 20 min thereafter for immunohistochemistry by indirect method. After washing the substrate with PBS, the AEC

Table 1 Interventions that led to the development of AF

Type of intervention	Number of patients
ACL (anterior cruciate ligament) graft after rupture of the ACL	6
ACL graft and meniscal refixation after rupture of the ACL and meniscal tear	2
Status after ACL rupture without intervention	2
ACL graft with knee joint infection after rupture of the ACL	1
Osteosynthesis and PCL (posterior cruciate ligament) fixation after tibial fracture with PCL tear	1
Implantation of a total knee replacement	2

(3-Amino-9-ethylcarbazole) chromogen consisting of 20 μ l of AEC Chromogen solution per 1 ml of AEC Substrate Buffer was added and the sections incubated therewith until a clearly visible color development occurred. The reaction was then stopped with distilled water and the sections then counterstained with hematoxylin QS for 5 min. A positive and negative control of the liver of a sheep were performed.

Immunohistochemical presentation of α -smooth muscle actin (ASMA)

The anti-actin antibodies, α -smooth muscle Cy3 (Sigma-Aldrich, St. Louis, Missouri, USA) that was diluted 1:200 in Low Cross Buffer and the antibody anti-alpha smooth muscle actin from abcam with a dilution of 1:62.5 used. The incubation was also carried out for 16 h at 4 °C. The excess of the antibody was washed off with PBS and the post block applied for 40 min. After washing again with PBS, the sections were incubated for 30 min with the HRP polymer consisting of secondary antibody and horseradish peroxidase. There was a washing step with PBS. Thereafter, the AEC chromogen consisting of 20 μ l of AEC chromogen in 1 ml of AEC Substrate Buffer was applied to the paraffin sections. When a sufficient color reaction was seen, the chromogen was rinsed with distilled water. Subsequently, the sections were counterstained with hematoxylin for 5 min and blued in tap water. Finally, the sections were covered in water in Dako Faramount Aqueous Mounting Medium (Dako, Santa Clara, California, USA). As positive and negative controls, two preparations with rawhide were used.

Immunohistochemical presentation of β -Catenin

The paraffin sections were washed off with PBS. Subsequently, the blocking solution was applied for 5 min. A washing step with PBS followed. Now, the primary anti- β -catenin antibody was diluted 1:250 in low cross buffer and the sections were incubated at 4 °C in the refrigerator for 16 h. The next day, the sections were washed again with PBS and then applied the post block as a reinforcing reagent for 20 min. After washing again, the incubation with the HRP polymer consisting of secondary antibody and horseradish peroxidase was carried out for 30 min. This was then washed off again with PBS. To visualize the binding of the secondary antibody to the primary antibody, 20 μ l of AEC chromogen were diluted to 1 ml of AEC Substrate Buffer, applied to the sections and left on the sections for about 10 min until a visible color reaction occurred. The reaction was stopped with distilled water. The sections were then counterstained with hematoxylin QS and rinsed with distilled water after 5 min. At the end, the paraffin sections were blued for about 5 min in tap

water and finally watered in Dako Faramount Aqueous Mounting Medium. The positive and negative controls were two preparations of breast cancer.

Immunohistochemical presentation of BMP-2

An antigen unmasking was performed by HIER method. To block non-specific binding, the post-block was applied for 5 min. Antibody anti-BMP-2 was diluted at a ratio of 1:250 in low cross buffer and the sections incubated therewith for 16 h at 4 °C. The next day, the excess of the antibody was washed off with PBS. In the next step, a 30-min incubation with the HRP polymer was performed. This was followed by a wash with PBS. This was followed by the application of the AEC chromogen diluted in AEC substrate buffer until a visible color reaction occurred. The reaction was stopped with distilled water, the sections counterstained with hematoxylin QS, bleached in tap water for about 5 min, and watered in Dako Faramount Aqueous Mounting Medium. The positive and negative controls were preparations of the femur and brain of a neonatal mouse.

Histological evaluation

The histological evaluation of the preparations was carried out on the Olympus (Olympus Europe, Hamburg, Germany) microscope, model BX53F. The sections were analyzed at 4 \times 50 magnification and all stained cells were counted out. They were classified according to a distinction between four different tissue types. These were synovium, vessels, cell-rich fibrosis and cell-poor fibrosis. For this cellularity grading all stained cells were counted and divided into four different grades. Grade 1 featured < 10% stained cells in this tissue, grade 2 was 10–25% positive cells, grade 3 included tissues with 25–50% stained cells, and in grade 4 > 50% of the cells had to be positive. The entire specimen was always assessed and evaluated instead of defining individual fields of tissue sections as the “region of interest”. Grade 1 and 2 were classified as cell-poor AF. Grade 3 and 4 were classified as cell-rich AF in the recent study.

To determine the ratio of fibroblasts to fibrocytes, a different grading of AF according to the criteria of Ruppert et al. [7] was used, measuring the distance between fibroblasts. Low fibroblast cellularity meant AF grade 1, for grade 2 the spacing between the fibroblasts had to be slightly less than two cell lengths and tissue with a high fibroblast cellularity with less than a cell length distance between the fibroblasts was assigned to AF grade 3. By means of the HE staining, fibroblasts and fibrocytes were counted and the ratio of these was recorded to be able to make statements about the activity of the tissue.

Statistical analysis

Due to the small sample size and lack of normal data distribution, non-parametric tests were used to further evaluate data. The Mann–Whitney U test was used to compare the metric data of two independent samples. Thus, the expression of the four proteins (CD68, ASMA, β -catenin, BMP-2) between AF collective and control group was compared. Correlations between two variables were determined by means of Spearman rank correlation analysis. It was examined whether an undirected linear relationship exists between two variables. A p value < 0.05 was considered statistically significant. The statistical analysis of the data was done with the program SPSS Statistics, Version 22. IRB admittance for the study has been obtained from the ethics committee of University Medical Center Freiburg (will be completed after acceptance) (# 305/10).

Results

The mean time between index operation and arthroscopic arthrolysis, when tissue harvest was performed, amounted to 22.79 months (2–81 months). Of the included 14 AF patients, three (21.4%) were classified according to Ruppert et al. [7] as AF grade 1, seven (50%) as AF grade 2 and four (28.6%) as grade 3. The HE staining was used to determine the ratio of fibroblasts to fibrocytes in fibrotic tissue sections. The proportion of fibroblasts in the tissue sections analyzed,

was between 60 and 95%. On average, arthrofibrotic tissue showed a fibroblast content of about 80%.

Presentation of immunohistochemical markers

Immunohistochemical presentation of CD68

The tissue sections of cell-rich fibrosis showed a statistically significant high expression of CD68 in the AF group, compared to the control group with less than 10% of CD68 positive cells ($p = 0.002$). In cell-poor fibrosis no statistically significant difference to the control group could be determined ($p = 0.228$). Evaluation of CD68 antibody staining revealed cytoplasmic staining of cells in form of granules with particular perinuclear accentuation (Fig. 1).

Expression of α -smooth muscle actin (ASMA)

After staining, the ASMA antibody presented a very clear expression of the protein. Especially endothelial cells of vessels showed a strong cytoplasmic staining. In areas of cell-rich and cell-poor fibrosis, fibrous, wavy course of ASMA staining were observed (Fig. 2). In the synovium ASMA was expressed only very rarely. The highest expression of ASMA was found in vessels of AF and control group. On average, over 50% of endothelial cells (grade 4) expressed the protein here. But no significant difference between the groups could

Fig. 1 Immunohistochemistry of CD68 of an AF patient. **a** Strong staining of synovial cells, magnification $\times 40$. **b** No staining of endothelial cells, magnification $\times 40$. **c** Strong staining in areas of cell-rich fibrosis, magnification $\times 20$. **d** Weak staining in areas of low-cell fibrosis, magnification $\times 40$

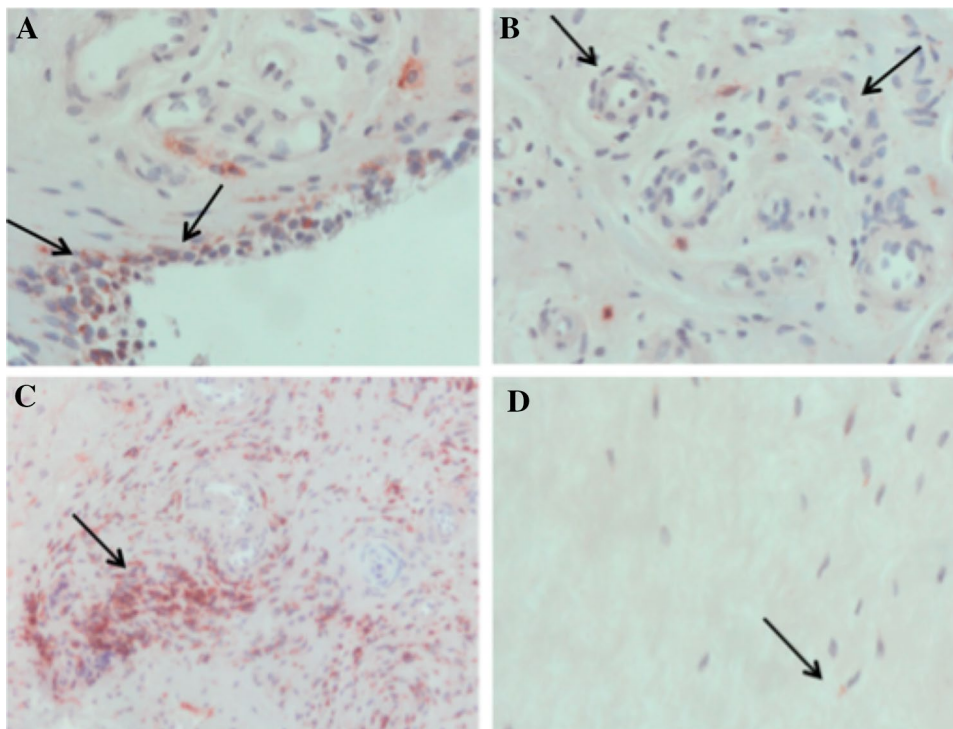
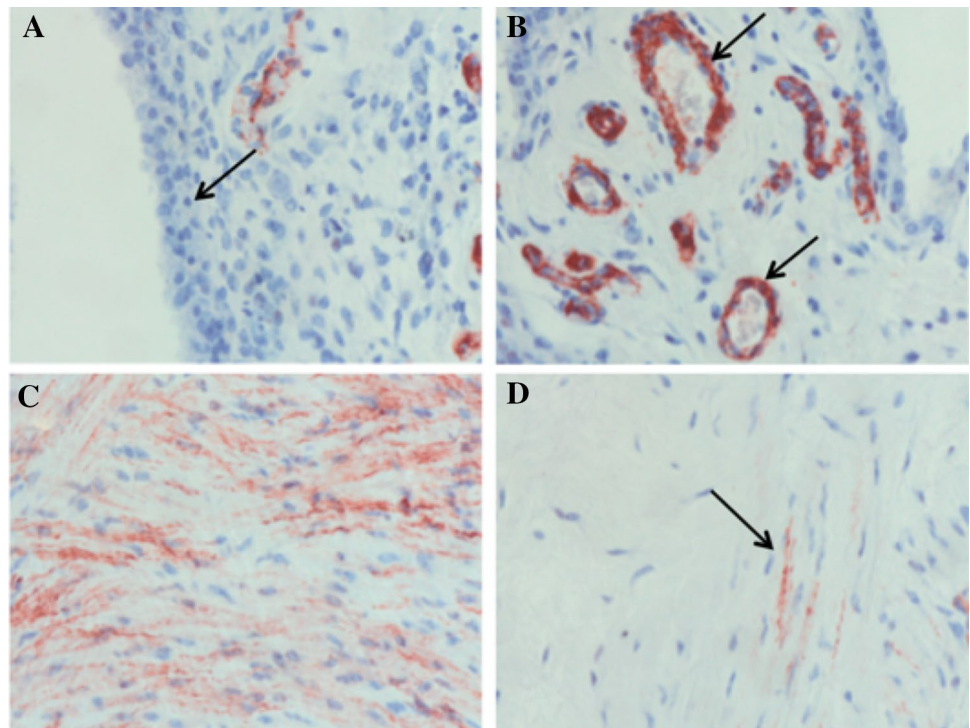


Fig. 2 Immunohistochemistry of ASMA of an AF patient. **a** No staining of the synovium, magnification $\times 40$. **b** Strong staining of endothelial cells, magnification $\times 40$. **c** Expression in the area of cell-rich fibrosis, magnification $\times 40$. **d** Weak staining in the area of cell-poor fibrosis, magnification $\times 40$



be seen ($p = 1.000$). Expression of ASMA in synovium, vessels, cell-rich fibrosis and cell-poor fibrosis showed a median of 2.00 in the AF group and 1.75 in the control group. The two groups differed significantly ($p = 0.003$).

Expression of β -catenin

Staining with β -catenin antibody was weak compared to immunohistochemistry of CD68 and ASMA, which made evaluation on the microscope more difficult. Cytoplasmic expression of β -catenin was observed, cell nucleus and cell membrane showed no staining (Fig. 3). In the synovium of the AF group, expression of β -catenin averaged 2.00, which corresponds to staining of 10–25% of synovial cells compared to a value of 1.00 in the control group. Based on the expression of β -catenin in synovium, a statistically significant difference between AF group and control group was found ($p = 0.003$). The strongest expression of β -catenin was found in endothelial cells of AF patients, with a median of 4.00 ($p < 0.001$). An equally high level of β -catenin was seen in fibroblasts of cell-rich fibrosis. With a median expression of 4.00 in the AF group, the difference to the control group was statistically significant ($p < 0.001$). In areas of low-cell fibrosis of the AF group, the expression of β -catenin averaged 1.00, which was also significantly different ($p = 0.047$) to the control group. In terms of total tissue, the median expression of β -catenin in tissues of AF patients was 2.63 and 1.00 in the control group. Overall, both groups showed a statistically significant difference in expression of β -catenin

($p < 0.001$). Overall, β -catenin showed the strongest expression compared to CD68, ASMA and BMP-2.

Expression of BMP-2

Immunohistochemical presentation of BMP-2 was weak. Cytoplasmic staining revealed mainly fibroblasts near vessels (Fig. 4). The strongest expression of BMP-2, with a significant difference compared to the control group, was observed in tissue sections of AF patients with cell-poor fibrosis ($p = 0.006$). The median here was 2.00. Very weak staining was seen in the control group, averaging 1.00. On average, expression of BMP-2 in synovium, vessels, cell-rich fibrosis, and cell-poor fibrosis presented a median of 1.50 in the AF group and 1.00 in the control group, respectively. The overall difference between AF group and control group in the expression of BMP-2 was thus statistically significant ($p = 0.002$).

Time dependent influence on protein expression

In the AF group, the relationship between time elapsed between index surgery and arthroscopic arthrolysis and the level of expression of CD68, ASMA, β -catenin and BMP-2 was determined by mean expression in synovial fluid, vessels, cell-rich and cell-poor fibrosis. A rank correlation analysis was performed. None of the four proteins revealed a relationship between staining intensity and time elapsed between index surgery and tissue harvesting.

Fig. 3 Immunohistochemistry of β -catenin in an AF patient. **a** Slight staining of the synovial cells, magnification $\times 40$. **b** Marked staining of endothelial cells, magnification $\times 40$. **c** Staining in the area of cell-rich fibrosis, magnification $\times 40$. **d** Weak staining in the area of low-cell fibrosis, magnification $\times 40$

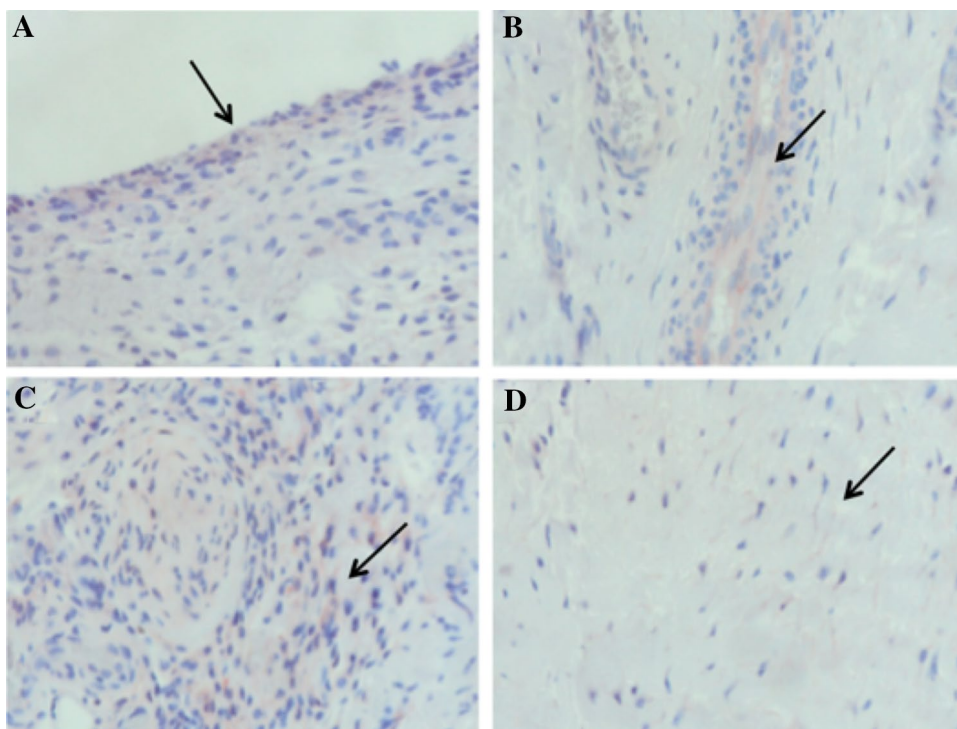
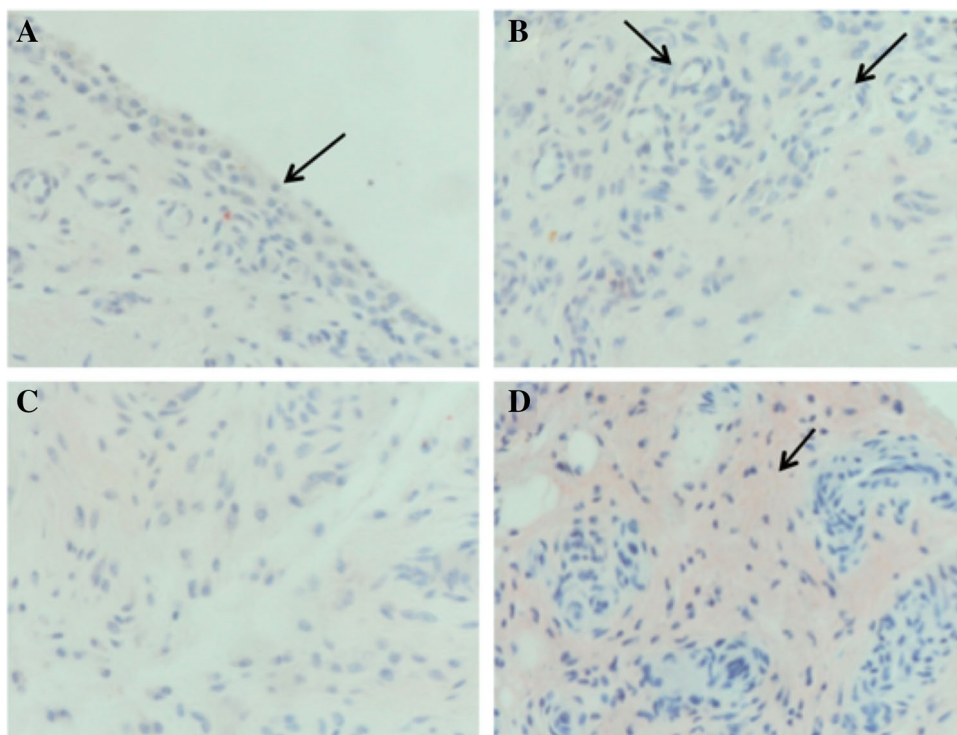


Fig. 4 Immunohistochemical presentation of BMP-2 of an AF patient. **a** No staining of synovial cells, magnification $\times 40$. **b** No staining of endothelial cells, magnification $\times 40$. **c** Hardly staining in areas of cell-rich fibrosis, magnification $\times 40$. **d** Staining in areas of low-cell fibrosis, especially perivascular, magnification $\times 40$



Discussion

The present study reveals the increased expression of CD68, ASMA, β -catenin and BMP-2 in tissue samples

derived from patients suffering from AF of the knee. The histological and immunohistochemical findings contribute to a better understanding of the pathophysiology of AF and may provide a new approach for diagnosing and classifying the disease.

Expression of CD68

CD68 is a transmembrane glycoprotein that is highly expressed by human monocytes and tissue macrophages. These immune cells have been shown to be inhomogeneously distributed within the synovial sublining layer in inflammatory diseases such as osteoarthritis and rheumatoid arthritis [14]. In this context, the synovial tissue sublining CD68 expression can even serve as a biomarker indicating the inflammatory activity [15]. Our analyses revealed a strong CD68 expression in the synovial cover cell layer of AF patients, which is well in line with observations described by Ruppert et al. [7]. Furthermore, we detected a significantly stronger expression of CD68 in the synovial sublining layers of cell-rich AF, whereas only weak expression was found in low-cell AF (Fig. 1). Taken together, cell-rich tissue sections presumably feature a higher inflammatory activity compared to tissue sections with less fibroblasts.

Expression of α -smooth muscle actin (ASMA)

Regarding the pathogenesis of AF, the increased secretion of pro-fibrotic molecules such as transforming growth factor- β 1 (TGF- β 1) seems to account for the transformation of resident fibroblasts into activated myofibroblasts [16]. These myofibroblasts are mechano-responsive and highly contractile extracellular matrix producing cells [17]. Immunohistochemically, they are characterized by the expression of ASMA. During the current investigations, a strong cytoplasmic ASMA staining of endothelial cells was detected in the AF and the control group, as ASMA is widely expressed within vascular smooth-muscle cells. Furthermore, sublining areas of cell-rich fibrosis featured a significantly stronger expression of ASMA compared to controls (Fig. 2). Areas of cell-poor fibrosis showed a less pronounced, but still increased expression of ASMA. These findings suggest, that cell-rich areas within the fibrotic tissue demonstrate a high rate of fibroblast to myofibroblast transformation. This rate seems to be lower in areas of cell-poor fibrosis. Faust et al. conducted an *in vitro* study on synovial fibroblasts isolated from arthrofibrotic and control synovial biopsies [16]. In response to TGF- β 1, ASMA expression revealed a significantly higher fibroblast to myofibroblast transition rate in arthrofibrotic fibroblasts compared to controls. Ruppert et al. as well as Unterhauser et al. described a significant increase of myofibroblast differentiation in AF as revealed by immunohistochemical staining of ASMA in ten and nine patient tissue samples, respectively [7, 17]. However, a potential correlation between ASMA expression and cellularity was not investigated. Nevertheless, it was hypothesized, that these activated myofibroblasts do not appropriately initiate apoptosis at a given time, but contribute to fibrotic tissue

formation by excessively synthesizing matrix components into the intercellular space [18].

Expression of β -catenin

Membranous and cytoplasmic β -catenin contributes to forming the so-called adherens junctions, which are involved in regulation and coordination of cell–cell adhesion [19]. Intracellular β -catenin plays a key role in activating the canonical Wnt signaling transduction, which is of paramount importance in the regulation differentiation and proliferation of cells [8]. In the literature, a connection between an increased β -catenin expression and the development of fibrosis has repeatedly been described [20–23]. The results of the presented study demonstrate predominately cytoplasmic expression of β -catenin, which was significantly increased in synovial cell, in endothelial cells as well as in fibroblast of cell-rich and low-cell areas of AF samples (Fig. 3). The current findings go well in line with observations described by Ruppert et al. also demonstrating a strong cytoplasmic expression of β -catenin in endothelial cells and fibroblasts in AF [7]. In the same way, there was no β -catenin expression detected in the nucleus or the membrane.

Expression of BMP-2

TGF- β has been known to play a key role in the pathogenesis of AF due to an increased inflammatory reaction [6]. Pfitzner et al. discovered the concentration of BMP-2 in the synovial fluid to be significantly higher in arthrofibrotic knees compared to controls [11]. BMP-2 belongs to the TGF- β superfamily, has versatile functions in osteoinduction and regeneration [13], but is also known to modulate inflammatory tissue reactions [24–26]. For the first time, the current study reveals an increased cytoplasmic expression of BMP-2 in fibroblasts of AF patient samples (Fig. 4). Fibroblasts expression of BMP-2 was predominately found perivascular in tissue sections with cell-poor fibrosis. In a less pronounced manner, the increased expression was also evident in tissue sections with cell-rich fibrosis. It remains elusive whether fibroblast actively synthesize BMP-2 or upregulate its expression in response to circulating proinflammatory cytokines. The latter may be supported by another observation made in the above cited study by Pfitzner et al., showing a significant upregulation of BMP-2 expression in a fibroblast cell line after being exposed to the synovial fluid of AF patients [11]. BMP-2 features a strong osteoinductive stimulus on mesenchymal stem cells (MSC) and osteoblasts. Nevertheless, investigating MSC and osteoblasts in the context of AF was not subject of the present study. In literature, conflicting data exist whether AF is associated with heterotopic ossification. Even though Pfitzner et al. found an increased density of the anterior knee capsule (grey scale

values) on plane X-rays of AF patients and revealed a linear correlation between the BMP-2 concentration in the synovial fluid and the radiological density, the grey values did not reach the density of bone and no radiologically visible ossifications were present in any of the cases [11]. Accordingly, Ruppert et al. did not find signs of ossification on any of their AF tissue sections [7]. On the other hand, other studies detected heterotopic ossification in AF after total knee arthroplasty [10, 27, 28]. As a matter of fact, unravelling the specific impact of BMP-2 in the context of AF requires further investigation.

Correlation analysis

Rank correlation analysis did not show a correlation of time elapsed between the index surgery and the arthroscopic arthrolysis and the expression levels of CD68, ASMA, β -catenin or BMP-2. This may be due to the fact that the activity of the AF and the course of disease usually do not follow a straight unidimensional (or linear) pattern.

Distribution of cellularity

Classifying the cellularity of fibroblasts in the AF group revealed a distribution with AF grade 1 in 21.4%, AF grade 2 in 50% and AF grade 3 in 28.6% of the cases. This distribution pattern is comparable to the one described by Ruppert et al. in a larger population [7].

Limitations and strengths

Limitations of the present study are the small sample size, a heterogenic population and the lacking assessment of osteoblast activity. Strengths of the study are the revealed association of cell density and CD68 as well as ASMA expression in AF, the provided evidence of a highly significant upregulation of β -catenin and BMP-2 in AF tissue samples and the indicated relationship between cell density and activity of AF. Further studies with larger population should be initiated to confirm the results.

Conclusion

The expression of CD68, ASMA, β -catenin and BMP-2 is significantly increased in AF tissue samples. Based on the presented findings, histological evaluation and immunohistochemical assessment of CD68, ASMA, β -catenin and BMP-2 expression may prove useful to diagnose AF and to analyze AF activity.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest with regard to the current study.

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