

Macroscopical, Histological, and *In Vitro* Characterization of Nonosteoarthritic Versus Osteoarthritic Hip Joint Cartilage



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ABSTRACT: Osteoarthritis (OA) might affect chondrocyte culture characteristics and complement expression. Therefore, this study addressed the interrelation between macroscopical and microscopical structure, complement expression, and chondrocyte culture characteristics in non-OA and OA cartilage. Femoral head cartilage samples harvested from patients with femoral neck fractures (FNFs) and OA were analyzed for macroscopical alterations using an in-house scoring system, graded histologically (Mankin score), and immunolabeled for complement regulatory proteins (CRPs) and receptors. Morphology of monolayer cultured chondrocytes isolated from a subset of samples was assessed. The macroscopical score distinguished the FNF and OA cartilage samples and correlated significantly with the histological results. Chondrocyte phenotype from FNF or OA cartilage differed. Complement receptor C5aR, CRPs CD55 and CD59, and weakly receptor C3aR were detected in the investigated FNF and OA cartilage, except for CD46, which was detected in only two of the five investigated donors. The in-house score also allows inexperienced observers to distinguish non-OA and OA cartilage for experimental purposes.

KEYWORDS: osteoarthritis, chondrocyte, hip joint cartilage, complement regulatory proteins, complement receptors, Mankin score

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Introduction

Osteoarthritis (OA) is a chronic disease, which affects, depending on age, a large number of patients. More than 60% of the German population suffers from OA by the time of their 65th birthday. In combination with demographic changes, OA has led to an enormous rise of health-care costs over the past two decades.¹ Various nonmodifiable entities such as age, genetic, metabolic, biochemical, biomechanical, and modifiable factors, such as obesity, are all suspected to support this degenerative change.^{2,3} It has been observed that OA is initiated by multiple microtraumas to the joint or one single traumatic event.⁴ However, the exact etiology remains unclear. Unfortunately, there is currently no effective biological and/or pharmacological therapy available for OA, despite some efforts in OA-related research. Within OA, there is a shift of the tightly regulated anabolic and catabolic process toward the catabolic state resulting in the upregulation of inflammatory markers, most notably interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α).³ Numerous animal model studies have shown the role of IL-1 β in the upregulation of catabolic enzymes matrix

metalloproteinases (MMP)-1, -3, and -13, while also identifying its ability to suppress the anabolic process, within the extracellular matrix (ECM), by inhibiting type II collagen and proteoglycan expression in chondrocytes.⁵ Furthermore, IL-1 β may initiate apoptosis via production of nitric oxide or reactive oxygen species within the chondrocyte.⁶ Similarly, TNF- α exhibits enhanced expression not only in traumatic articular cartilage but also in OA synovium, inducing the same MMP enzymes as IL-1 β , which suggests a synergistic catabolic effect during OA.⁷ This loss of homeostasis between anabolic and catabolic factors might contribute to activation of the complement cascade as recently demonstrated for OA pathogenesis.⁸ Complement activation results in the secretion of chemotaxins arising from cleaved complement factors, such as anaphylatoxins C3a and C5a. In addition, it induces opsonization of pathogenic particles, and finally, pathogen or cell lysis by membrane attack complexes (MACs).⁸ The complement split fragments C3a and C5a bind to anaphylatoxin receptors, C3aR and C5aR, and induce a multiple inflammatory response, including cytokine release, vasoconstriction and permeability, histamine release,



and leukotaxis, as well as platelet aggregation.⁹⁻¹¹ Complement regulatory proteins (CRPs) protect cells from lysis induced by complement activation. The anaphylatoxin receptors are expressed on various cell types.¹² Interestingly, C5aR expression was observed as normal in OA and rheumatoid cartilage, while its upregulation was noted in normal, rheumatoid arthritis samples, but not in OA cartilage, when chondrocytes were stimulated by IL-1 β .¹³ In addition to C5aR, we wanted to demonstrate the expression of C3aR and crucial CRPs in OA and non-OA cartilage. We hypothesized in 2007 that complement expression could be involved in OA¹⁴ and demonstrated the complement regulation by cytokines in cultured chondrocytes, in cartilage *in situ* as well as in another joint-associated musculoskeletal connective tissue cell type, tenocytes, and altered/healthy tendon tissue.¹⁵⁻¹⁷ An impaired expression of particular CRPs could contribute to OA⁸ and was, therefore, investigated here.

Femoral neck fracture (FNF) is a typical feature of older patients suffering from sarcopenia and osteoporosis.^{18,19} An FNF usually cannot be treated by conservative strategies due to loss of femoral head vascularization. Therefore, endoprothetic joint replacement of the hip is usually performed. OA diagnosis is based mainly on clinical mobility and pain scores, eg, Western Ontario and McMaster Universities Arthritis Index, visual analog scale, and radiologic measurements, such as joint space narrowing (eg, Kellgren score). Histology provides more detailed information about OA progression in cartilage, but it cannot be applied in the clinical routine. When human articular cartilage is recruited for *in vitro* studies, latent OA might markedly influence chondrocyte culture characteristics. For this reason, we developed an in-house macroscopic scoring system to categorize cartilage samples in a first look manner with respect to OA-related changes. Other scoring systems such as ICRS, O'Driscoll, MOCART, OAS scores, or scores adapted and proposed by Goebel et al, 2012, are specialized to assess the degree of cartilage repair of local defects and might not be suitable for less experienced persons.²⁰⁻²³

Therefore, the aim of this study was to compare joint cartilage derived from FNF and hip OA patients using both microscopic and, a novel in-house, macroscopic scoring system while further analyzing chondrocyte culture characteristics. In addition, the expression of specific cell surface-located complement pathway-associated glycoproteins and complement anaphylatoxin receptors was assessed.

Materials and Methods

Samples. Samples of 37 patients (17 males, 20 females; 18 FNF, 19 hip OA; Table 1) were harvested during joint replacement surgery in accordance with the institutional ethics committee of the Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin. The research was conducted in accordance with the principles of the Declaration of Helsinki, and patients gave their written, informed consent to participate. The average age of all donors included in the study was 68.72 years. The age

Table 1. Donor characteristics of samples included.

Total number of donors	37			
Gender	Male	Female		
	17 (45.9%)	20 (54.1%)		
Age	≤60	≤75	≤90	≤100
	6 (16.2%)	21 (56.8%)	9 (24.3%)	1 (2.7%)
	OA		FNF	
	19 Donors (51.3%)		18 Donors (48.7%)	

range was between 30 and 91 years. In a subset of samples (15 donors), primary human chondrocytes were isolated from the femoral heads of patients who underwent joint replacement due to OA (4 donors) and FNF (11 donors).

Macroscopical scoring. All femoral heads included in this study were scored macroscopically using the self-designed score before the joint cartilage was scratched off for chondrocyte isolation. Observers (one or two observers per time point, but different observers at different time points) were blinded for the diagnosis (FNF/OA). If two observers were included, the results were averaged. The topics such as color, extent of lesions (quadrants affected), consistency, and cartilage thickness were addressed during the scoring process as summarized in Table 2.

Isolation and culturing of articular chondrocytes. Apart from a 1-cm² area reserved for histology, cartilage chips were harvested from macroscopically, mostly unaffected cartilage areas of human femoral head articular cartilage with a macroscopical score of ≤9, since at higher scores, the amount and quality of the remaining cartilage appeared too low for successful cell isolation. The rationale to also include OA-affected cartilage was to study the effect of the OA milieu in the joint on chondrocyte phenotype. The harvested cartilage was weighed and cut into small slices (~1 mm²) followed by digestion with pronase derived from *Streptomyces griseus* at 20 mg/mL (7 U/mg, Roche Diagnostics) in Ham's F-12/Dulbecco's modified Eagle's medium (50/50, Biochrom-Seromed) for 30 minutes at 37 °C. Subsequently, the samples were treated with collagenase NB5 derived from *Clostridium histolyticum* at 1 mg/mL (Serva) in chondrogenic growth medium and slowly stirred in a spinner flask overnight. Isolated chondrocytes were resuspended in growth medium (Ham's F-12/Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum (Biochrom AG), ascorbic acid (25 μg/mL), 1% essential amino acids, partricin (0.5 μg/mL), and penicillin/streptomycin (both: 50 IU/mL). Cell vitality after cell isolation was determined using trypan blue exclusion assay. Cells were counted and then seeded in culture flasks (Cell plus culture flask; Sarstedt AG) at 42,857.142 cells/cm² for cell expansion and subsequent storage in liquid nitrogen at passage 1.

Table 2. Self-designed macroscopical scoring system.

	GRADE
Osteoarthritis macroscopically	
<i>Color</i>	
White, Beige	0
Yellowish	1
Gray-brown/Other discolorations	2
Expansion of osteoarthritic changes	
<i>How many quadrants of the femoral head contain recognizable arthritic tissue?</i>	
No osteoarthritis	0
Up to 25%	1
More than 25% up to 50%	2
Up to 75%	3
More than 75% up to 100%	4
<i>Degree of severity of osteoarthritic changes?</i>	
Healthy	0
Little	1
Moderate	2
Severe	3
Cartilage consistency (when cutting)	
Normal	0
Soft	1
Mushy	2
Cartilage thickness	
Normal (ca. >1 mm)	0
Focally thin (clearly <1 mm)	1
Mostly thin	2
Special	
Cartilage–Bone–Osteophytes (chondro-/osteophytes), defects	
complete cartilage loss (no points)	
Maximal: 13 score points	

Culture morphology. For the measurement of cell morphology and the number of adherent cells, freshly isolated cells (passage 0) of 15 donors were seeded into Petri dishes (35 mm diameter) at 52,083.33 cells/cm² and cultured for 72 hours. Measurement was taken using cell^D software at ×100 magnification under a light microscope (Axioskop 40, Zeiss), while cellular morphology, including length (μm), width (μm), and overall cell area (μm²), was assessed at ×200 magnification. Images were taken at ×100 using a XC30 camera system (Olympus, Europa Holding).

Decalcification and embedding of cartilage bone cylinders. A 1-cm² cartilage–subchondral bone sample was fixed using 4% paraformaldehyde (PFA) solution (USB, USA) for three days. Twenty-two samples were recruited for histology (derived from 11 female and 11 male donors, 16 of them suffering from FNF and 6 from OA). The samples were placed in a volume 20 times their own volume of formic acid

to ensure satisfactory decalcification. The acid solution was changed every 24 hours, while a chemical end point test was also carried out on the supernatant to assess the rate of decalcification. When chemical testing exhibited no significant residual calcium, the samples were rinsed in water and incubated in 5% ammonia solution (Merck KGaA) for 30 minutes to ensure neutralization. Samples were then rinsed under running tap water for 24 hours to remove any residual acid. Dehydration involving a series of ascending ethanol solutions was completed over a 48-hour period. Following this, each sample was embedded in paraffin at 60 °C with subsequent sectioning using a microtome Microm HM 325 (Thermo Fisher Scientific).

Histological staining. The histological staining was conducted on paraffin sections (thickness between 3 and 5 μm). Paraffin was removed using a series of descending alcohol solutions. For hematoxylin and eosin (H&E) staining, the sections were incubated for 4 minutes in Harris hematoxylin solution (Sigma-Aldrich), briefly rinsed in tap water and then counterstained for 1.5 minutes in eosin Y (Sigma-Aldrich). For the safranin-O staining, the slides were incubated for 10 minutes in Weigert's iron hematoxylin (Carl Roth GmbH) and afterward rinsed under running tap water for 10 minutes. Subsequently, each section was stained in 0.001% fast green solution (Sigma-Aldrich) for five minutes. After rinsing the slides briefly in 1% acetic acid, the slices were stained in 0.1% safranin-O solution (Merck KGaA) for up to five minutes. For alcian blue (AB) staining, the sections were incubated for 3 minutes in 1% acetic acid followed by 1% AB (Carl Roth GmbH) for 30 minutes. Then, they were rinsed in 3% acetic acid and washed in deionized water (A. dest.) (two minutes). Additionally, cell nuclei were counterstained in nuclear fast red aluminum sulfate solution (Carl Roth GmbH) for five minutes.

Finally, all the slides were dehydrated using an ascending alcohol series and mounted with Entellan mounting medium (Merck KGaA). Images of the slides were taken using the Axioskop 40 light microscope and the Olympus digital camera XC30.

Microscopical scoring for OA. The stained sections were further analyzed by applying the Mankin score^{24,25} to them by one observer. The observers were blinded for the diagnosis (FNF, OA) of the cartilage donors. Both cartilage structure and chondrocyte distribution were scored, while safranin-O and AB staining allowed the grading according to the amounts of glycosaminoglycans detectable in the sample. Finally, tide-mark integrity was assessed for blood vessel penetration.

Immunofluorescence labeling. Paraffin sections of five donors (two with OA, two with FNF, and one with OA and FNF) were immunolabeled for complement receptors (C5aR and C3aR) and CRPs (CD46, CD55, and CD59) using specific antibodies combined with a counterstaining of the cell nuclei by 4',6-diamidino-2-phenylindole (DAPI). The sections were deparaffinized and fixed in 4% PFA before being rinsed for two minutes in tris-buffered saline (TBS; 0.05M Tris, 0.015M NaCl, pH 7.6). The sections were overlaid for 20 minutes with 0.1% pronase solution at 37 °C for demasking and rinsed two times

with TBS (2 minutes). Then, a 20-minute incubation period at room temperature (RT) with 100 μ L protease-free blocking buffer (5% donkey serum [Merck Millipore] diluted in TBS) followed. Negative controls included omitting the primary antibody or using mouse IgG₁ as primary antibody during the staining procedure. The primary antibodies (rabbit anti-human C3aR [1:30, Assay Biotechnology], mouse anti-human C5aR [1:100, GeneTex, Biozol], mouse anti-human CD46, CD59 [1:20 and 1:40, both AbD Serotec], and goat anti-human CD55 [1:40, R&D Systems]) were diluted in blocking buffer containing 0.1% Triton X-100 (Sigma-Aldrich) and incubated overnight at 4 °C in a humidified chamber. After rinsing three times with TBS, the labeled secondary antibody (donkey anti-rabbit or donkey anti-mouse-Alexa488 or -Alexa555, 10 mg/mL; Life Technologies Corporation) was diluted 1:200 in blocking buffer containing 0.1% Triton X-100 and 0.1 μ g/ μ L DAPI and incubated for one hour at RT in a dark room. Labeled sections were rinsed with TBS three times and covered with Fluoromount G (Biozol Diagnostica). The fluorescence images were acquired using a confocal laser scanning microscope (SPEII; Wetzlar Leica).

Statistical analysis. Data were expressed as an average value with standard deviation or median and analyzed using GraphPad Prism 5. The ROUT test was performed to identify outliers. If applicable, the Kolmogorov–Smirnov test was used to analyze the data for the presence of a Gaussian distribution. In the presence of a Gaussian distribution, unpaired *t*-test was applied to the interval scaled data (Fig. 4: femoral head diameter, Fig. 5B: cell numbers) to compare the respective data with the control. The Mann–Whitney test was used for ordinally scaled values, for which a Gaussian cannot be determined (Fig. 3: score values). Statistical significance was set at a *P* value of ≤ 0.05 . The Pearson coefficient was determined to assess correlations.

Results

Patients. Patients were sorted according to their age following the WHO classification as follows: less than 60 or 60 years (aging), between 61 and 75 years (old), between 76 and 90

Table 3. Additional donor characteristics of FNF and OA samples.

FNF: Donor number		18			
Gender	Male 50% (9)	Female 50% (9)			
Age ^a	≤ 60 5.6% (1)	61–75 50% (9)	76–90 38.8% (7)	91–100 5.6% (1)	
BMI (Average)	24 \pm 2.84				
Comorbidities	Diabetes 16.7% (3)	Cardiovascular disease 72.2% (13)	Infection 27.8% (5)	Kidney disease 55.6% (10)	
OA: Donor number		19			
Gender	Male (8) 42.1%	Female (11) 57.9%			
Age	≤ 60 26.3% (5)	61–75 63.2% (12)	76–90 10.5% (2)	91–100 0% (0)	
BMI^b (Average)	27 \pm 5				
Comorbidities	Diabetes 5.3% (1)	Cardiovascular disease 47.4% (9)	Infection 10.5% (2)	Kidney disease 5.3% (1)	

Note: ^aThe FNF donors were significantly older than the OA donors. ^bThe FNF donors had a significantly lower BMI than the OA donors.

years (older), and between 91 and 100 years (very old). Most of them were between 61 and 75 years (21, 56.76%) and females (20, 54.05%). The average age of the OA cartilage donors included was 63.68 years, whereas that of the FNF cartilage donors was 74 years. The body mass index (BMI) was significantly higher in OA compared with FNF patients (Table 3). The percentage of each comorbidity (diabetes, cardiovascular diseases, infection, and kidney disease) was generally higher in the FNF patients (Table 3).

Grading of OA using the self-designed macroscopical scoring system and the Mankin score for histological scoring. A self-designed score system was used to determine

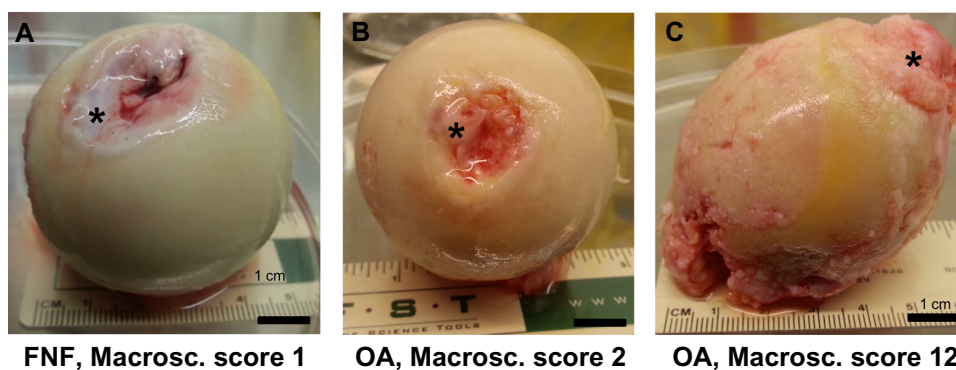


Figure 1. Exemplary macroscopical images of explanted femoral heads and scoring results. Depicted are femoral heads of three representative donors. (A) FNF, macroscopical score 1, (B) OA, macroscopical score 2, and (C) OA, macroscopical score 12.

Notes: *Insertion of the femoral head ligament. Scale bar: 1 cm.

Abbreviation: Macrosc., macroscopical.

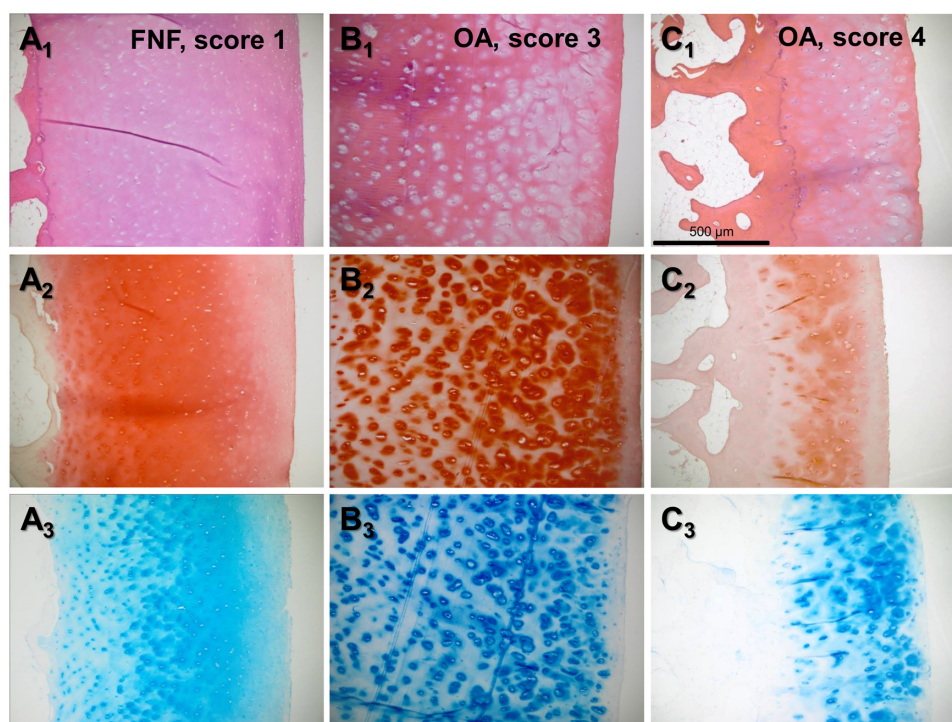


Figure 2. Exemplary histological images of cartilage samples. H&E (A₁–C₁), Safanin O (A₂–C₂), and AB (A₃–C₃) stained paraffin sections of femoral head articular cartilage of three representative donors are shown. (A) FNF, Mankin score 1, (B) OA, Mankin score 3, and (C) OA, Mankin score 4.

Note: Scale bar: 500 µm.

the macroscopical grade of cartilage degradation (Table 2 and Fig. 1A–C). Using this scoring system, the same samples were evaluated by inexperienced versus experienced observers. The differences of the scoring results between both observer groups were not significant (data not shown). For histological evaluation of OA-associated changes (Fig. 2), the Mankin score was used. The macroscopical score was significantly higher in patients with hip OA compared with those suffering from FNF (Fig. 3A). The median of the macroscopical score for OA was 11, whereas that for FNF was 3.5 ($P = 0.0002$). The histological score did not significantly differ between FNF and OA ($P = 0.0241$). The median of FNF samples was 3 and that of OA samples was 5.5 (data not shown). The correlation between the macroscopical and the Mankin scoring results including all the patients was significant ($P = 0.0001$) and also that of the OA samples separately ($P = 0.0357$), but not that of the FNF samples separately ($P = 0.25$; Fig. 3B_{1–3}).

Femoral head diameters. When the femoral head diameters were measured using the X-ray images, the male femoral heads were, as expected, significantly larger compared with the female ($P = 0.0001$), but no significant differences between FNF- and OA-derived femoral heads could be detected ($P = 0.313$; Fig. 4A and B).

Chondrocyte morphology, adherence, and vitality. Cells were isolated enzymatically, and the percentage of adherent cells as well as cell morphology (overall cell area, length, and width) was determined after 72 hours of culturing (Fig. 5A_{1–2}).

No significant correlation could be detected between the cell density of cultured chondrocytes and the macroscopical score ($P = 0.673$; data not shown). The cell density in OA-derived chondrocyte cultures was lower than that in FNF samples ($P = 0.0264$, Fig. 5A and B and Table 4). The percentage of dead cells (trypan blue positive) after isolation was around 3%. The number of isolated cells showed high variance (between 0.86 and 7.1 mio isolated chondrocytes/g cartilage were counted [data not shown]). The average number of isolated cells was lower in FNF cartilage (2.21 mio isolated chondrocytes/g) than in OA cartilage (3.84 mio isolated chondrocytes/g; Table 4, not significant). It did not correlate with the donor age ($P = 0.582$; data not shown) or the macroscopical score ($P = 0.382$, Fig. 5C). However, the histological score correlated significantly with the cell number per gram ($P = 0.031$, Fig. 5D), so that a higher score went along with a lower cell number. Nevertheless, the number of OA samples ($n = 4$) versus FNF ($n = 11$) used for cell isolation was too low to calculate a valid correlation. As determined for FNF and OA samples separately, after 72 hours of culturing, an average 67.18% versus 73.25% of the cells adhered, detectable by an elongated shape with focal adhesion sites formation (Fig. 5A₁, Table 4). The average cell density of FNF and OA samples together was 366 cells/mm² (data not shown). The average cytoplasmic area of all samples (FNF and OA) was 630 µm² with a length of 62.27 µm and a width of 17.93 µm (data not shown). FNF samples had a smaller cell area (559.45 versus 824 µm², not significant, $P = 0.104$), and the cells were shorter (59.27 versus 70.5 µm) than the cells of OA samples

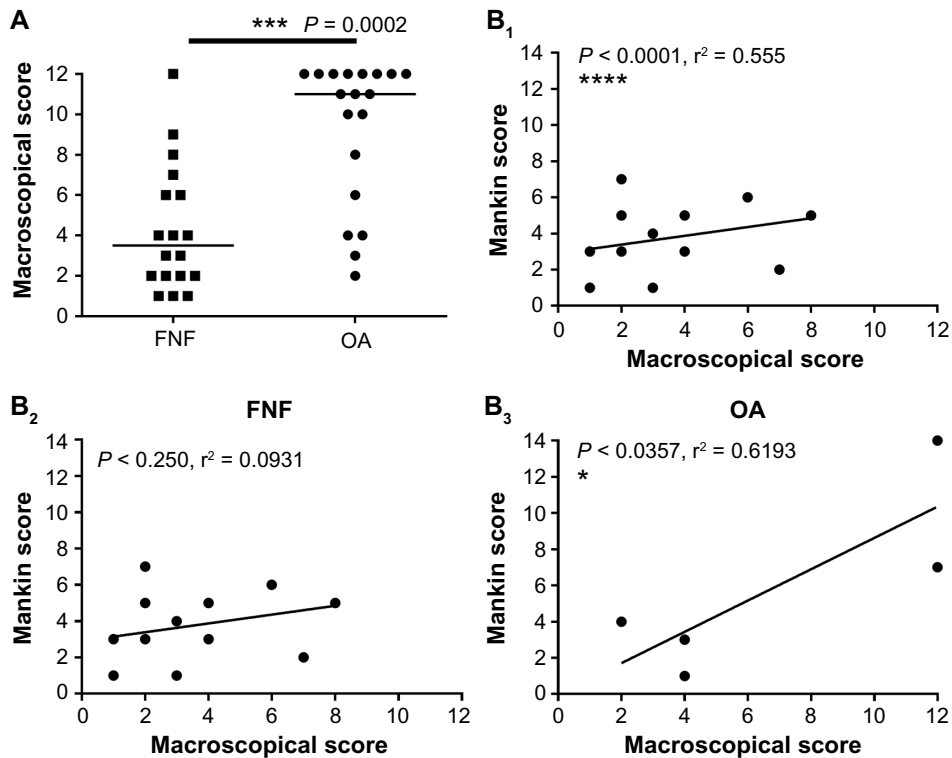


Figure 3. Macroscopical and Mankin scoring results of OA and FNF cartilage samples. (A) Macroscopical scoring results of FNF and OA samples. (B₁) Correlation between all macroscopical and Mankin scoring data (FNF and OA) and separated for FNF (B₂) and OA (B₃). **Note:** *Represent significant differences.

(not significant, $P = 0.206$). Cells derived from FNF samples grew at a significantly higher cell density in culture (426.36 versus 200 cells/mm, $P = 0.0264$), but presented a lower number of adhering cells (67.18% versus 73.25%, not significant, $P = 0.147$) than OA samples.

Detection of regulatory complement proteins *in situ*. C3aR receptor protein revealed only a weak immunoreactivity in the investigated samples. C5aR was expressed by the cells of the surface cartilage layer. The CRP CD46 could be found in two of the five donors analyzed. CD55 was synthesized in the samples of all investigated donors. Signals were not only confined to the cells but also observable in the whole lacunae. Nevertheless, it could not be found in the interterritorial ECM.

Protein expression of CD59 was shown in all investigated donors with divergent distribution (Fig. 6).

Discussion

In this study, a simple in-house macroscopical score was established, and we approved it to distinguish FNF and OA cartilage samples. Macroscopical scoring results correlated significantly with the histological analysis. It is advisable to categorize the quality of cartilage used for chondrocyte isolation since chondrocyte phenotype differed between FNF or OA cartilage. The complement receptor C5aR and the weakly expressed receptor C3aR, as well as CRPs CD55 and CD59, were detected in the investigated FNF and OA cartilage

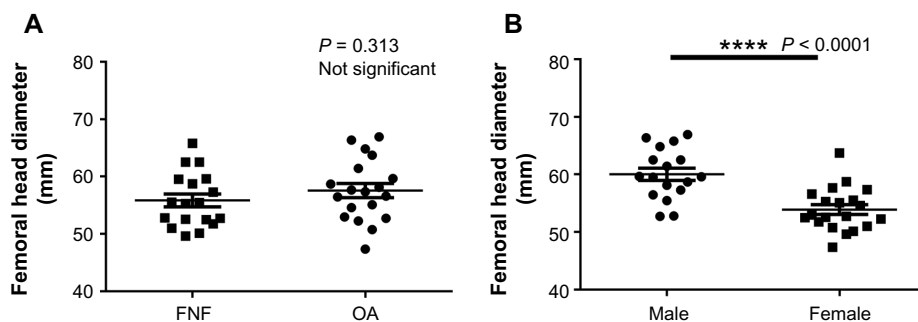


Figure 4. Femoral head diameters with respect to OA and gender. (A) Femoral head diameters of FNF and OA donors. (B) Femoral head diameters of male and female donors. **Note:** *Represent significant differences.

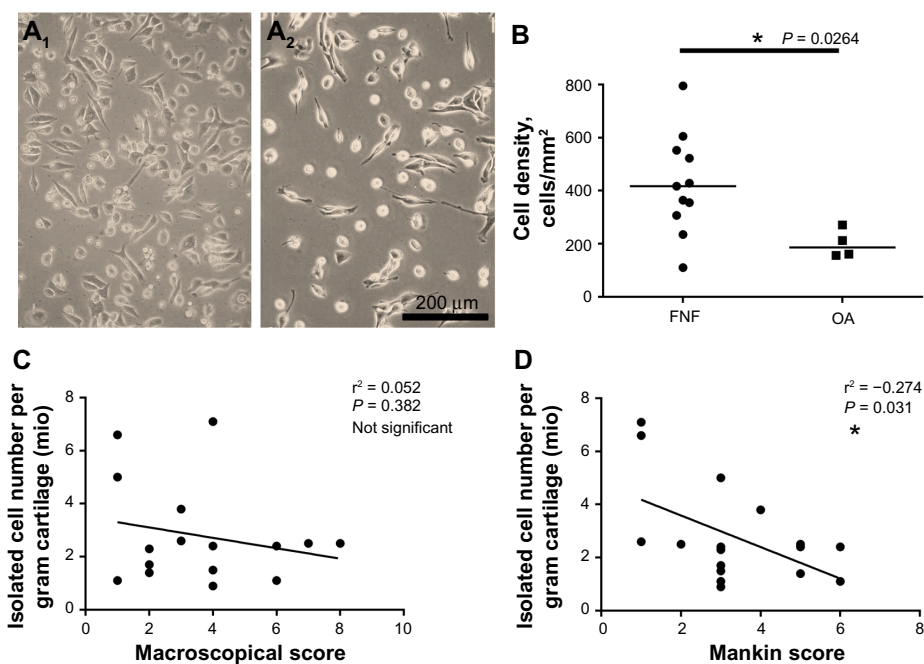


Figure 5. Vitality, adherence, and cytoplasmic area of cultured femoral head chondrocytes isolated from FNF or OA samples. Images of cultured chondrocytes derived from femoral head cartilage prone to FNF (A₁) or OA (A₂). (B) Cell density in FNF and OA samples. (C) Isolated cell numbers per gram versus macroscopical scoring results. (D) Isolated cell numbers per gram versus Mankin scoring results. Images and calculations derived from chondrocytes cultured for 72 hours.

Note: Scale bar: 200 μm .

samples. However, CD46 could only be found in two of the five investigated donors.

The macroscore was established for a rapid cartilage grading before cell isolation as simple as possible to allow even inexperienced laboratory staff to estimate OA in the recruited samples. Indeed, the results deduced from this easy-to-handle macroscopical score did not show major differences between experienced and inexperienced observers. This score could help to spare the time-consuming histology.

The total donor cohort analyzed macroscopically reflected a higher number of female individuals than male individuals as usually reported for OA.²⁶ On average, hip OA led to total joint replacement with 63.7 years, whereas the FNF (FNF) occurred around 10 years later with 74 years. A higher number of these FNF patients suffered simultaneously from other diseases such as diabetes, infections, kidney disease, and cardiovascular diseases than the OA patients. This fact might be age dependent. However, it was reported that women who have hip OA also attain a higher rate of comorbidities.²⁷ Furthermore, the BMI in the OA group was significantly higher.

Comparison of the OA and FNF patient-derived cartilage samples indicated a highly significant difference between both groups concerning the macroscopical score (macroscopical scoring values of OA samples varied between 2 and 12 [median: 11]), and that of the FNF group in a similar range (also between 1 and 12), but the median was lower (median: 3.5). However, only samples with a scoring value lower than 9 were used for cell isolation, since samples with higher scores presented insufficient amounts of cartilage. From these samples, only the macroscopically barely changed cartilage was recruited. Also, the FNF group presented only three samples with a low macroscopical score (one score point), indicating macroscopically nearly unaffected cartilage. For this observation, age-associated and early OA changes might be responsible since the average age in the FNF group was around 74 years.

Histological scoring can be done using several scoring systems.^{28,29} The Mankin scoring system, which was selected in this study, is well-established one.³⁰ The Mankin and the recently recommended Osteoarthritis Research Society

Table 4. Characteristics of FNF- and OA-derived chondrocytes cultured for 72 hours.

DONOR	ISOLATED CELL NUMBER PER G CARTILAGE	CELL DENSITY CELLS/mm ²	CELL ADHERENCE [%]	CELL AREA [μm^2]	CELL LENGTH [μm]	CELL WIDTH [μm]
Average value OA ($n = 4$)	2.6 ± 1.7	$200 \pm 53.7^*$	73.3 ± 4.99	824 ± 267.6	70.5 ± 12.8	19.5 ± 3.1
Average value FNF ($n = 11$)	2.2 ± 1.1	426.4 ± 187.8	67.2 ± 10.3	559.5 ± 128.9	59.3 ± 4.8	17.4 ± 3.3

Note: *Significant difference, $P = 0.0264$.

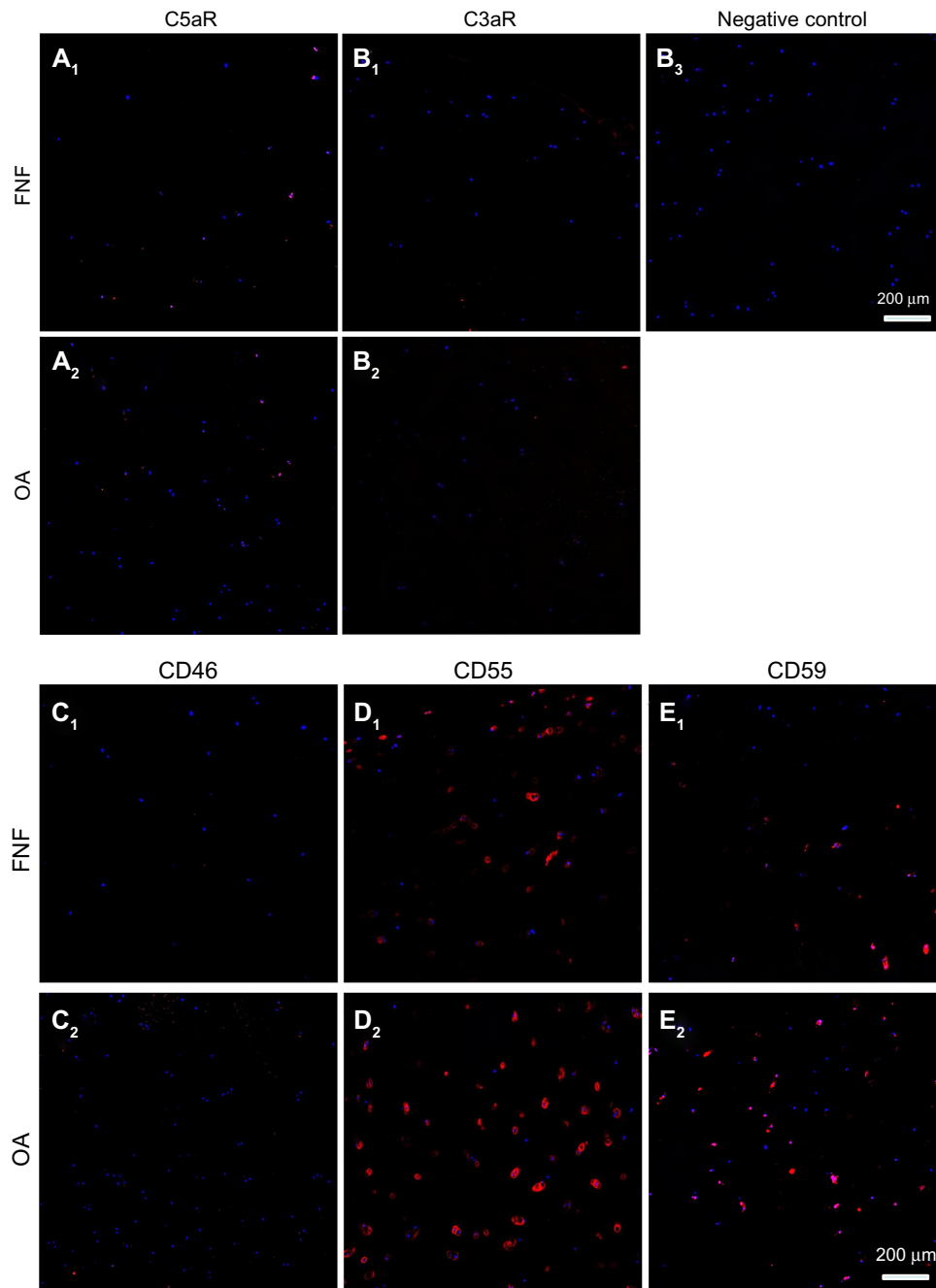


Figure 6. Expression of complement receptors (C5aR and C3aR) and CRPs (CD46, CD55, and CD59) in cartilage in situ. FNF and OA cartilage sections immunolabeled for (A₁₋₂) C5aR, (B₁₋₂) C3aR, (C₁₋₂) CD46, (D₁₋₂) CD55, and (E₁₋₂) CD59.

Notes: Cell nuclei were counterstained using DAPI (blue). Scale bar: 200 μm. Negative controls (B₃) performed by using only the secondary antibody revealed no unspecific staining.

International (OARSI) cartilage histopathology assessment system revealed a high reliability.³¹ The histological score and the macroscopical score correlated significantly. Many samples revealed focal OA due to incongruity of the femoral head and acetabulum. However, the Mankin score revealed differences between OA and FNF group (not significant). One has to consider the fact that the macroscopical score allows an assessment of the whole femoral head, whereas for histology, only one exemplary sample was selected, which represented an area of the best cartilage quality of the whole head. This cartilage area

was selected to be representative for the cartilage subsequently harvested for cell isolation. Therefore, the histology was performed to get information concerning the chondrocytes isolated later from the same samples.

It has been reported that chondrocyte morphology changes in hip OA.³² Holloway et al, detected a subpopulation of chondrocytes with multiple elongated cytoplasmic extensions.³² We found the tendency of a larger cell area in OA samples compared with FNF cartilage, but the length and width of the cells did not significantly differ. The reason remains unclear. However, we



evaluated only cell culture characteristics, but we did not measure the chondrocyte diameter in the histological sections. This was not necessary, since we wanted to elucidate chondrocyte differences in culture, which might influence future *in vitro* experiments.

OA is known to be associated with a higher rate of cell death *in situ*.³³ It is also known that aging cartilage reveals increased rates of apoptosis.³⁴ The cell vitality of cultured chondrocytes was slightly lower in OA than in the FNF group, but the difference was not significant. In agreement with the larger cell dimensions, the cell density of cultured chondrocytes was significantly lower in the OA samples compared with the FNF-derived chondrocytes. Against expectation, the cell numbers isolated were higher in OA than in FNF cartilage. This observation could be explained by the higher donor age in the FNF group and the lower ECM stability in the OA cartilage, which allows a more complete and easier digestion by the enzymes used for cell isolation. The cell adherence was lower in the FNF group, which could be explained by remnants of pericellular ECM after digestion. This might impair the rate of adherent cells. Nevertheless, we found no significant correlation between the donor age and the isolated cell numbers (data not shown).

Complement has recently been implicated in OA pathogenesis.^{8,14,15} We previously observed gene expression and regulation of complement receptors and cell surface-associated regulatory proteins in cultured chondrocytes.¹⁵ Now, we studied the expression of selected complement receptors and regulatory proteins *in situ*. In agreement with the study by Onuma et al.¹³, we detected in all samples, irrespective of either prone to OA or not, an expression of C5aR, but only a focal and faint immunoreactivity for C3aR. C5aR seems to play a superior role in articular cartilage, in addition to its role in traumatic cartilage injury.³⁵ In addition, we demonstrated the CRPs CD55 and CD59 in all the analyzed samples, but CD46 could be detected in only two of the five investigated donors. Both CD46 positive donors had a history of hip OA, but the third investigated OA donor and the FNF donors were CD46 negative. Since we found in a previous study that there was no CD35 expression in cartilage, it was not further addressed here.¹⁵ Like CD35, CD46 also inhibits the opsonins C3b and C4b, whereby CD46 promotes the proteolysis of these opsonins as a cofactor.³⁶ Possibly these opsonins play no major role in OA, but rather in rheumatoid cartilage disorders. In contrast, CD55 inhibits the upstream cleavage of C3 and C5 by antagonizing formation of the respective C3 and C5 convertases as prerequisites for ongoing complement cascade activation and terminal MAC formation. CD55 is also found in a soluble form,³⁶ possibly an explanation for the observed immunostaining of chondrocyte lacunae. CD59 interferes directly with the formation of the MAC complex.³⁶ Its deficiency has been shown to promote the OA phenotype in a mouse model.⁸ Investigation of a higher number of OA and non-OA samples of different grades would indicate whether downregulation of cytoprotective CD55 and CD59 might be directly associated with different OA stages (early or late) in human beings.

Limitations of this Study

This study is limited by the heterogenous donor collective and their high age, particularly in the FNF subgroup. So, age-related influences could not be excluded. For the determination of the cell parameters in culture, the OA group was rather small ($n = 4$). The classification “OA” and “FNF” was based on clinical diagnosis; therefore, some FNF samples might also have OA, which leads to the high macroscopical and histological scores observed in several FNF patients. There is also the limitation that the FNF samples, which represent the controls, are derived from hip fracture patients. These samples are not entirely healthy due to the fact that the most common reason for this event is osteoporosis.¹⁹

For histology, only one sample could be included from each donor, since the majority of cartilage was needed to be harvested for cell isolation. So, the best cartilage area of each femoral head was selected. This could be the reason why the Mankin scoring provided generally lower scoring numbers, suggesting higher cartilage quality, and hence, superior results than the macroscopical score. In this study, the macroscopical score is more predictive than the Mankin score, concerning cartilage quality. In this respect, sampling and time-consuming processing for histology could be omitted in future.

Conclusion

The self-designed scoring system is applicable for an easy grading of cartilage samples by inexperienced observers for experimental purposes. We are not expecting that our macroscopical scoring system will also find clinical application in arthroscopy of the hip joint. For our coworkers in the laboratory setting, who did not have XRs, etc., available, this score was accepted as helpful and practical. Experiments with human-derived chondrocytes are superior to using animal-derived samples due to interspecies differences.^{37,38} Therefore, the facts reported here for FNF- versus OA-derived chondrocytes, which are the main chondrocyte sources of human origin for research, help to assess their peculiarities and its potential influence on experiments. With regard to further characterize the extent of complement activation during OA progression, other complement factors such as C3, C4, and C5 and the split fragments such as C3a and C5a should also be included in future studies.

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Author Contribution

Conceived and designed the experiments: JB, OG, GS-T, CM, MJ. Analyzed the data: JB, BK, TS, GS-T. Wrote the first draft of the manuscript: OG, GS-T. Contributed to the writing of the manuscript: ZH, SA. Agree with manuscript results and



conclusions: JB, OG, BK, CM, MJ, ZH, SA, TS, GS-T. Jointly developed the structure and arguments for the paper: GS-T, BK, MJ. Made critical revisions and approved final version: JB, OG, MJ, ZH, SA, GS-T. All authors reviewed and approved the final manuscript.

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