Biochemical changes of the pericellular matrix and spatial chondrocyte organization—Two highly interconnected hallmarks of osteoarthritis

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Abstract
During osteoarthritis, chondrocytes change their spatial arrangement from single to double strings, then to small and big clusters. This change in pattern has recently been established as an image-based biomarker for osteoarthritis. The pericellular matrix (PCM) appears to degrade together alongside cellular reorganization. The aim of this study was to characterize this PCM-degradation based on different cellular patterns. We additionally wanted to identify the earliest time point of PCM-breakdown in this physiopathological model. To this end, cartilage samples were selected according to their predominant cellular pattern. Qualitative analysis of PCM degradation was performed immunohistochemically by analysing five main PCM components: collagen type VI, perlecan, collagen type III, biglycan, and fibrillin-1 (n = 6 patients). Their protein content was quantified by enzyme-linked immunosorbent assay (127 patients). Accompanying spatial cellular rearrangement, the PCM is progressively destroyed, with a pericellular signal loss in fluorescence microscopy for collagen type VI, perlecan, and biglycan. This loss in protein signal is accompanied by a reduction in total protein content from single strings to big clusters (P < .001 for collagen type VI, P = .003 for perlecan, and P < .001 for biglycan). As a result of an increase in the number of cells from single strings to big clusters, the amount of protein available per cell also decreases for collagen type III and fibrillin-1, where total protein levels remain constant. Biochemical changes of the PCM and cellular rearrangement are thus highly interconnected hallmarks of osteoarthritis. Interestingly, the earliest point in time for a relevant PCM impairment appears to be at the transition to small clusters.

KEYWORDS
biglycan, cartilage, osteoarthritis, pericellular matrix, perlecan, spatial chondrocyte organization

Abbreviations: ANOVA, analysis of variance; BC, big clusters; DS, double strings; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; MMP, matrix metalloproteinase; OA, osteoarthritis; PBS, phosphate-buffered saline; PCM, pericellular matrix; SC, small clusters; SS, single strings.
Osteoarthritis (OA) is a common degenerative joint disease characterized by the irreversible destruction of the articular cartilage that affects a big proportion of the elderly population. Since there is no defined degenerative cut-off threshold to formulate the diagnosis of OA, the condition is difficult to quantify. It has recently been shown that the chondrocytes within the articular cartilage are arranged in distinct spatial patterns along the arciform collagen fiber network, following a vertical orientation in the ascending/descending part of the collagen arcades, and a horizontal orientation in the apex of the arcs in the superficial zone. The predominant physiological cellular pattern found in the human femoral condyle is that of single strings. With the onset of OA, the cellular spatial organization changes by rearranging first from single to double strings, followed by small and finally by big clusters. Interestingly, these spatial changes are not only a hallmark of OA, but they also seem to depend on, and to be highly specific to localized tissue degeneration. Big clusters, in particular, are, for example, often localized near cartilage fissures in the upper layer. Spatial cellular organization has therefore been suggested for use as an image-based biomarker for local tissue degeneration at the cellular level.

In the immediate surrounding of the chondrocytes, a unique and highly specialized area is present, termed the pericellular matrix (PCM). It has been suggested that this area acts as a mechanosensitive cell–matrix interface, protecting the chondrocytes from apoptosis and modulating the cellular biosynthetic response to outside stimuli. Interestingly, the PCM is not particular and exclusive to articular cartilage, but it seems to be a general feature of connective tissue: the presence of a PCM has also been described in the intervertebral disc, temporomandibular cartilage and disc, meniscus, tendons, and cricoarytenoid cartilage. Perlecan (also known as heparan sulfate proteoglycan 2), collagen VI, biglycan, fibrillin-1, and collagen type IX, and collagen type III are key components of the PCM. With OA onset and progression, a cascade of inflammatory and catabolic processes is triggered, which leads to an upregulation as well as elevated activity of various matrix metalloproteinases (MMPs). This ultimately triggers collagen fibril denaturation and loss of proteoglycans. Of note, even though an upregulation in the collagen expression is present in OA-affected cartilage, an overall decrease of collagen type II of the extracellular matrix is observed, particularly in the upper fibrillated area of the advanced osteoarthritic samples. Similar observations have been made for PCM-collagen type VI. It can thus be assumed that the PCM is destroyed during the course of OA, which has also been suggested for the PCM in the intervertebral disk and meniscus. Collagen type VI and perlecan are thus well established and investigated PCM components in both healthy and OA affected tissue; several studies have emphasized their involvement and connection to the biomechanical properties of the PCM. Little information is currently available with respect to other minor compositional PCM constituents, such as collagen type III, biglycan, and fibrillin-1.

Collagen type III has been shown to be present in a diffuse pattern in the pericellular region, in the close vicinity of the chondrocytes within the articular cartilage, as well as in the intervertebral disc. Even though collagen type III is a feature of tissue repair in damaged tissue, low amounts of collagen type III are detected in both normal adult cartilage and OA-affected cartilage. Similar observations have been made for biglycan, which has been described to have a pericellular localization where it is directly attached to the collagen fibrils. This may indicate its possible involvement in modulating morphogenesis and differentiation. Also, pericellularly localized, fibrillin-1 regulates the viscoelastic properties of the tissue, as it is involved in the cell-matrix mechanotransduction and the cell-matrix adhesion. Fibrillin-1 has been shown to interact with perlecan in weight-bearing tissues by regulating the bioavailability of transforming growth factor-β1 and playing a role in bone morphogenetic protein-signalling.

In order to paint the big picture with respect to PCM degradation and to better understand the underlying mechanisms, these minor and often overlooked PCM components and the synergistic role they play in the degradative process of the pericellular region need to be elucidated. In a previous study, we had already shown that the PCM progressively loses its characteristic biomechanic properties alongside cellular rearrangement. We had further demonstrated that, accompanying these pattern changes, the two main PCM components—collagen type VI and perlecan—undergo progressive destruction. In the present study, the current knowledge of PCM degradation on the basis of cellular spatial organization is further expanded upon by additionally investigating its components collagen type III, biglycan, and fibrillin-1. We also wanted to identify the point in time in the physiopathological model of spatial cellular rearrangement when the PCM structure breaks down. The hypothesis was that the PCM integrity is progressively disrupted during the course of cellular rearrangement, and that total protein content decreases with increasingly pathological spatial cellular arrangement. We also analyzed whether or not the observed breakdown of the PCM is a generalized process occurring within the entire tissue or only within isolated superficial areas of the cartilage.

2 MATERIALS AND METHODS

2.1 Cartilage harvest

For this experimental and fundamental research study (evidence level IIb), tissue was obtained from patients undergoing total knee arthroplasty in the Department of Orthopaedic Surgery of the University Hospital of Tuebingen, Germany, and in the Winghofer clinic, Rottenburg a.N., Germany, for end-stage OA of the knee. Ethical committee approval was obtained before commencement of the study (project number 674/2016BO2). Articular cartilage samples for patients with degenerative and posttraumatic OA were used and samples from patients with any other
inflammatory pathology (eg, rheumatoid arthritis) were completely excluded. Samples were collected from a total of 139 patients, with 78 women (aged 67 (35-88) years) and 61 men (68 (49-89) years) (for sex—age distribution and frequency, see Figure S1). Articular cartilage coming exclusively from femoral condyles was used for the immunohistological and biochemical analyses in the present study (Figure S2A).

2.2 | Cartilage preparation and sectioning for immunolabelling

Following intraoperative resection, the tissue was transported and stored in serum-free Dulbecco’s Modified Eagle’s Medium (Gibco, Life Technologies, Darmstadt, Germany) with 2% (v/v) penicillin-streptomycin and 1.2% (v/v) amphotericin B. Two types of tissue sections were used: perpendicular sections (side view) and tangential sections (top-down view). For the side-view analysis, cartilage-bone cuboid samples (~1 cm in width, 3-4 cm in length) were cut from the resected condyle along the kinematic uniaxial movement direction of the knee. These cuboids were fixed in 4% (w/v) paraformaldehyde/phosphate-buffered saline (PBS) (pH 7.0) for 24 hours at 4°C, followed by decalcification in 20% (w/v) ethylenediaminetetraacetic acid in PBS for several days at 37°C, with the solution being changed every other day. Following decalcification, samples were cut at a thickness of 35 µm with a Leica cryotome type CM3050S (Leica Biosystems, Wetzlar, Germany). For the side-view analysis, cutting was performed perpendicularly to the articular surface to obtain a full-thickness view of all cartilage layers, including the subchondral bone in a single lateral view (Figure S2B). Sections for the top-down views were performed directly on the resected tissue from the femoral condyle at 35-µm thickness in parallel to the articular surface (Figure S2C).

2.3 | Cartilage immunostaining

Cartilage sections for both side views and top-down views were processed and labeled for collagen type VI, perlecgn, collagen type III, biglycan (n = 6 patients with paraformaldehyde-fixed cartilage), and fibrillin-1 (n = 6 patients unfixed cartilage). As fibrillin-1 is a calcium-binding protein, the ethylenediaminetetraacetic acid decalcification procedure used for the side-view analysis led to an overall loss of immunoreactivity. No decalcification process was therefore used for fibrillin-1 staining; rather, the cartilage was harvested from the bone directly. Histological sections were pretreated with 0.2% (w/v) collagenase type XI (Sigma-Aldrich, Taufkirchen, Germany) for collagen type VI and fibrillin-1 staining and with 0.1% (w/v) hyaluronidase (Sigma-Aldrich) for perlecgn, collagen type III, and biglycan staining in PBS for 1 hour at 37°C, followed by three washing steps with PBS. To reduce unspecific antibody binding, sections were blocked for 30 minutes in 5% (w/v) bovine serum albumin and 0.3% (v/v) Triton X-100 in PBS. This was followed by incubation with the primary monoclonal antibodies at a dilution of 1:100 in 2.5% (w/v) bovine serum albumin-PBS at 4°C overnight: collagen type VI (rabbit anti-collagen VI, ab-182744; Abcam, Cambridge, UK), perlecgn (mouse anti-perlecgn, sc-377219; Santa Cruz Biotechnology Inc., Dallas, TX), collagen type III (rabbit anti-collagen type III, ab-7778; Abcam), biglycan (rabbit anti-biglycan, HPA003147; Sigma-Aldrich), and fibrillin-1 (mouse anti-fibrillin-1, MAS-12770; Thermo Fisher Scientific, Waltham, MA). All antibodies were used as specified by the manufacturers and no separate validation of their immunohistochemical binding properties and specificity was performed. Sections were rinsed with PBS and incubated with secondary antibodies at a dilution of 1:100 (Alexa Fluor 555 goat anti-rabbit immunoglobulin G [IgG], a-21429; Thermo Fisher Scientific, or Alexa Fluor 594 goat anti-mouse IgG, ab-150116; Abcam) for 2 hours in complete darkness. Nuclei of the cells were stained with 4’,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Darmstadt, Germany) at a dilution of 1:1000 in PBS for 5 minutes. Fluorescent staining was visualized with a Carl Zeiss Observer Z1 fluorescence microscope (Carl Zeiss Microscopy, Jena, Germany) equipped with MosaiX image acquisition software (Carl Zeiss Microscopy).

2.4 | Optical assessment of PCM integrity

The analysis for structural integrity of the PCM was performed using a blinded optical approach to the histological side views, as previously described by Hofmann et al.25 Due to the arciform collagen fiber and cell orientation, these sections allow for better visualization of the developing patterns, especially in deeper cartilage layers where the orientation of the cells is perpendicular to the articular surface rather than parallel to it as in the superficial zone.

Two rectangular regions of interest of approximately 500 × 500 µm were selected at random from each mosaic image per cellular pattern. PCM integrity assessment was performed as previously described.25 The percentage of the circumference of the cell that was surrounded by the staining signal for the PCM was thereby determined for the immunolabelled PCM components collagen type VI, perlecgn, collagen type III, biglycan, and fibrillin-1. The percentages of the PCMs that were encountered were ordinally classified into one of the following three groups: less than 25%, 25% to 75%, and more than 75%. A weighted arithmetic mean was then calculated for each staining and section for further analysis.

2.5 | Biochemical quantification of the PCM: enzyme-linked immunosorbent assay (ELISA)

Discs of articular cartilage from 127 patients (approximately 30 discs per target analysis) were cut, sorted, and assigned to their predominant cellular pattern organization as described previously.39 Using a custom-fabricated device, cartilage was cut in the horizontal
plane (ie, parallel to the superficial zone) to obtain discs of 300 µm in thickness and 4 mm in diameter. These discs were then stained with 4 µM calcein-AM fluorescent dye (Cayman Chemical, Ann Arbor, MI) in Dulbecco's modified Eagle's medium for 30 minutes at 37°C. Stained explants were then visually classified and grouped according to their predominant cellular spatial patterns as visualized under a fluorescence microscope (Leica DM IMRE, Wetzlar, Germany). Pooled discs were snap-frozen with liquid nitrogen and stored at −70°C until further use. These frozen and grouped discs were then subjected to protein extraction through crushing with a pestle and mortar under liquid nitrogen and placement on ice for 15 minutes in a homogenization buffer (25 mM Tris-HCl pH 7.5, 100 mM NaCl, 1% (v/v) IGEPAL CA-630). Extracted proteins were subjected to centrifugation at 15 000g for 15 minutes at 4°C, and collection of the supernatant containing the protein phase then followed. The total protein concentration across samples was normalized by using the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA).

To quantify protein content of the specific PCM components, sandwich ELISAs were performed for each distinct cellular pattern. For collagen type III, a total of 10 µg of protein was used. For biglycan (BIOZOL Diagnostica Vertrieb GmbH, Eching, Germany) and fibrillin-1 (LifeSpan Biosciences Inc., Seattle), 30 µg was used following the manufacturer’s protocol. For collagen type VI and perlecan, the data from a previous publication were used. While collagen type VI, perlecan, collagen type III and biglycan absorbance was measured with an EL 800 reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany), a chemiluminescent plate reader (Infinite 200 PRO; Tecan Group Ltd., Männedorf, Switzerland) was used for fibrillin-1 ELISA. Three independent measurements of the ELISAs were performed for each spatial pattern.

### 2.6 Statistical analysis

The values obtained from the two regions of interest per pattern and side-view histological section were averaged for further analysis.
Distributions of variables were assessed by histograms. The weighted arithmetic mean of the averaged cellular PCM integrity in the histological analyses was calculated as previously described. Data are graphically presented as boxplots and bar diagrams. Differences between the independent variables were calculated by way of Kruskal-Wallis test or analysis of variance (ANOVA) as appropriate, with post hoc testing by Mann-Whitney U test or t test for independent samples. Alpha adjustment on the basis of a significance level of 0.05 was performed for each calculation using the Benjamini-Hochberg procedure. Statistical analyses were performed using SPSS statistical software 22 (IBM Corp., Armonk, NY).

3 | RESULTS

In our top-down histological qualitative analysis, in healthy tissue areas ascribed to single strings, the PCM signal was intact and well-defined for collagen type VI, perlecan, collagen type III, biglycan, and fibrillin-1 staining (Figure 1B1-F1). With the cellular patterns shifting along the physiopathological chain from double strings to small clusters and eventually to big clusters, clear changes in staining intensity and localization could be detected for collagen type VI, perlecan, and biglycan (Figure 1B2-C2,E2): the PCM contour around the chondrocytes softened, and the PCM staining of the three components was no longer representative of the pericellular region, rather it was scattered randomly between the cells. Interestingly, signal intensity remained constant for collagen type III and fibrillin-1, although the structural integrity of the PCM also appeared to be compromised (Figure 1D1-D4,F1-F4).

To further elaborate these findings, the PCM signal was analyzed in side views of the cartilage (Figures 2 and 3). While no major changes could be observed from single to double strings, the number of cells per tissue area increased slightly from double strings to small clusters, and increased greatly from small to big clusters (Figure 3). This increase in the number of cells was accompanied by a reduction in the number of cells that were encompassed by an intact PCM. At the same time, the number of cells with a scattered PCM increased. Moreover, when analyzing the averaged PCM integrity per cell based on their cellular organization, a significant decrease of collagen type VI, biglycan and fibrillin-1 (Figures 4A and 4D,E, Table 1) was observed. Perlecan and collagen type III signaling around the cells also decreased, without, however, reaching statistical significance (Figure 4B,C).

To measure the total protein content in the tissue, ELISAs were performed. Complementary to the results from the immunohistochemical analysis, the quantitative ELISAs showed that the more pathological the cellular pattern, the more the protein content in collagen type VI, perlecan, and biglycan decreased (Figures 5A, 5B, and 5D). The earliest point in the sequence of events at which a significant reduction could be observed was at the transition from single strings to small clusters for collagen type VI (P = .016) and perlecan (P = .008), and at the transition from single strings to double strings for perlecan (P = .008).

FIGURE 2 Evaluation of signal presence in the pericellular matrix (PCM) and optical readout in immunohistochemical sections. Side view of the cartilage with its underlying subchondral bone after perpendicular sectioning and immunolabelling. Representative areas were selected for optical readout of PCM integrity after PCM staining (in this case, collagen type III antibody) (white) and nuclear staining with 4',6-diamidino-2-phenylindole (DAPI) (blue). A, Aerial view of the entire tissue section. Scale bar = 2000 µm. (B-E) Enlarged selected tissue areas showing small clusters (B), single strings (C), big clusters (D), and double strings (E). Scale bar 50 µm [Color figure can be viewed at wileyonlinelibrary.com]
from double strings to big clusters in the case of biglycan \( (P < .001) \) (Table 2). While the lowest amounts of protein were measured in big clusters for all of the aforementioned PCM components, collagen type III and fibrillin-1 displayed a constant total protein content throughout all subsequent cellular rearrangements (Figures 4C and 4E).

4 | DISCUSSION

The aim of the present study was to investigate the degeneration of the PCM on the basis of local cellular spatial organization by analysing five main components of the pericellular region: collagen type VI, perlecan, collagen type III, biglycan, and fibrillin-1. We hypothesized that the PCM becomes progressively structurally impaired and that the protein content of these components is concomitantly reduced. The progressive impairment of collagen type VI and perlecan with increasingly pathological spatial organization had been described previously,\(^2\_4,3\_9\) and thus served as a reference experiment. It was shown previously that collagen type VI, which is initially pericellularly well defined in strings and in double strings, shows a much more diffuse signal in big clusters, where its intracellular signal is lost. Perlecan in strings has a signal both extracellularly and intracellularly. Both signals are mostly lost in degenerative cartilage. The staining signals of the antibodies for collagen type III and fibrillin-1, however, are only slightly impaired in their structural integrity, and they remain at a relevant signal intensity. Biglycan is initially present in the PCM and has a slight intracellular signal. Later, its fluorescence signal is no longer present immediately surrounding the cells, and its total protein content is reduced. The signal now encircles the lacuna in big clusters at the interface to the interterritorial matrix. Moreover, the biglycan signal directly adjacent to the nucleus greatly increases. Since the PCM is evidently disrupted in the course of OA, the question arises: why are its collagen type VI and perlecan components so clearly being reduced, while collagen...
Type III and fibrillin-1 remain present at a similar level to their initial conditions, and biglycan mostly disappears from the PCM, is shifted to the boundary of the lacunae, and its levels increase near the nuclei?

Since proteolytic enzymes are elevated in osteoarthritic cartilage, a loss of structural proteins such as collagen type VI and perlecan only appears natural and can be considered to be the synergetic result of the various catabolic MMPs. The loss of perlecan

![Figure 4](image)

**Figure 4** Changes in average pericellular matrix (PCM) integrity are associated with cellular spatial rearrangement. Boxplots showing the calculated weighted arithmetic means for the PCM for collagen type VI (A), perlecan (B), collagen type III (C), biglycan (D), and fibrillin-1 (E) for each of the cellular organizational patterns. A significant decrease for collagen type VI, biglycan, and fibrillin-1 was observed with cellular reorganization. For perlecan and collagen type III, a decreased tendency was observed but without reaching statistical significance. *P < .05.

**Table 1** Semiquantitative differences of the PCM components in side-view immunohistochemical analyses

| Comparisons | Collagen VI | Perlecan | Collagen III | Biglycan | Fibrillin-1 |
|-------------|-------------|----------|--------------|----------|------------|
| All groups  | 0.045<sup>a</sup> | 0.592<sup>a</sup> | 0.523<sup>a</sup> | 0.019<sup>a</sup> | 0.014<sup>a</sup> |
| SS-DS       | 0.631<sup>b</sup> | 0.609<sup>b</sup> | 0.873<sup>b</sup> | 0.522<sup>b</sup> | 0.100<sup>b</sup> |
| SS-SC       | 0.046<sup>b</sup> | 0.873<sup>b</sup> | 0.523<sup>b</sup> | 0.051<sup>b</sup> | 0.064<sup>b</sup> |
| SS-BC       | 0.045<sup>b</sup> | 0.133<sup>b</sup> | 0.523<sup>b</sup> | 0.019<sup>b</sup> | 0.014<sup>b</sup> |
| DS-SC       | 0.233<sup>b</sup> | 0.592<sup>b</sup> | 0.523<sup>b</sup> | 0.439<sup>b</sup> | 0.522<sup>b</sup> |
| DS-BC       | 0.046<sup>b</sup> | 0.592<sup>b</sup> | 0.523<sup>b</sup> | 0.019<sup>b</sup> | 0.035<sup>b</sup> |
| SC-BC       | 0.046<sup>b</sup> | 0.592<sup>b</sup> | 0.523<sup>b</sup> | 0.019<sup>b</sup> | 0.100<sup>b</sup> |

Note: Statistically significant P-values are denoted in bold.
Abbreviations: BC, big clusters; DS, double strings; SC, small clusters; SS, single strings.

<sup>a</sup>Kruskal-Wallis test.
<sup>b</sup>Mann-Whitney U test.
**FIGURE 5** Quantification (ELISA) of the five major pericellular matrix components (collagen type VI, perlecan, collagen type III, biglycan, and fibrillin-1) analyzed according to the locally dominant cellular spatial organization. Bar diagrams displaying protein content as measured by ELISAs with homogenized cartilage for collagen type VI (A), perlecan (B), collagen type III (C), biglycan (D), and fibrillin-1 (E). Prior to analysis, cartilage samples had been grouped according to the locally predominant spatial cellular pattern. From single strings to big clusters, a significant decrease in collagen type VI, perlecan, and biglycan can be observed. No changes with spatial cellular rearrangement were observed in the content of collagen type III and fibrillin-1. Data displayed as mean ± standard deviation. Images (A) and (B) taken from.³⁹

* P < .05; ** P < .01; *** P < .001.

ELISA, enzyme-linked immunosorbent assay; BC, big clusters; DS, double strings; ELISA, enzyme-linked immunosorbent assay; SC, small clusters; SS, single strings

**TABLE 2** Protein content differences of the PCM components as a function of cellular spatial organisation measured by ELISAs

| Comparisons  | Collagen VI  | Perlecan  | Collagen III | Biglycan  | Fibrillin-1 |
|--------------|--------------|-----------|--------------|-----------|-------------|
| All groups   | 0.017⁵       | 0.004⁵    | 0.160⁵       | <0.001⁵   | 0.269⁵      |
| SS-DS        | 0.739⁶       | 0.458⁥    | 0.891⁶       | 0.565⁵    | 0.266⁵      |
| SS-SC        | 0.016⁶       | 0.003⁥    | 0.219⁥       | 0.079⁵    | 0.905⁵      |
| SS-BC        | <0.001⁶      | 0.003⁥    | 0.586⁥       | <0.001⁵   | 0.905⁵      |
| DS-SC        | 0.311⁶       | 0.008⁥    | 0.160⁥       | 0.077⁵    | 0.266⁵      |
| DS-BC        | 0.058⁥       | 0.005⁥    | 0.353⁥       | <0.001⁵   | 0.269⁵      |
| SC-BC        | 0.014⁥       | 0.790⁥    | 0.133⁥       | 0.079⁵    | 0.905⁵      |

Note: Statistically significant P-values are denoted in bold.
Abbreviations: ANOVA, analysis of variance; BC, big clusters; DS, double strings; SC, small clusters; SS, single strings.

⁵Data taken from Danalache et al.⁢³⁹
⁶ANOVA.
⁶⁺Test for independent samples.
from the pericellular region, in particular, might additionally be attributed to an inflammatory change in b1 integrin, as it has been suggested to mediate interactions between the cell surface and pericellcan.\textsuperscript{41}

In accordance with the presented data, biglycan has been previously described to have a mainly pericellular localization in healthy cartilage.\textsuperscript{32} Recently, Phillips et al\textsuperscript{42} showed that intra-articular injected biomimetic proteoglycans are capable of diffusing into OA affected cartilage and being deposited into the pericellular region of the chondrocytes. Complexes of biglycan with other small proteoglycans such as matrilin-1 and decorin have been indicated to act as a link between collagen type VI microfibrils and the extracellular components aggrecan and collagen type II.\textsuperscript{53} In late stages of OA, it has been proposed that biglycan is involved in the regulation of collagen type I organization.\textsuperscript{24} Once the collagen type VI triple helix is irreversibly degraded, as observed in advanced OA stages, the possibility to create a close link between PCM and ECM is lost. It is possible that biglycan presence, therefore, shifts to the boundary between big clusters and the surrounding ECM, where collagen type I is localized, as also observed during immunostaining. The observed presence of intracellular staining might indicate a compensatory effort of the chondrocytes to restore the overall proteoglycan content occurring as a result of the ongoing tissue damage.\textsuperscript{44} Moreover, Barreto et al\textsuperscript{45} showed that biglycan effectively induces the upregulation of proteinase in early OA stage (stage I), but that this effect is lower in advanced OA stages (stage VI), a condition in which most of the PCM components have already suffered from degradation. As in our ELISA, total biglycan protein content has been shown in radioimmunoassays to be reduced in osteoarthritic cartilage.\textsuperscript{25}

One of the most interesting observations of the present study is that collagen type III and fibrillin-1, in both the qualitative histological and the quantitative protein analyses, show constant expression, and thus appear to be unaltered. As collagen type III is known to be present at healing and repair sites of various tissues, it is conceivable that its constant levels and presence might represent the response to the ongoing tissue and matrix damage occurring throughout the course of the disease. In conjunction with our presented data, even though collagen type III is a feature of tissue repair, low levels of collagen type III have been previously detected in normal adult cartilage, with levels increasing in osteoarthritic cartilage.\textsuperscript{30,31} Hosseinia et al\textsuperscript{31} actually hypothesized that collagen type III might act as a glue in the final attempt to retain and stabilize the vast collagen matrix from further damage, in a similar fashion to wound healing processes occurring in collagen type I based tissues such as tendons.\textsuperscript{46} With respect to fibrillin-1, this protein has also been described to be pericellularly located in articular cartilage\textsuperscript{34} as well as in tendons,\textsuperscript{47} regulating transforming growth factor-β bioavailability,\textsuperscript{37} which plays a critical role in joint homeostasis as well as OA development. Fibrillin-1 is a calcium-binding protein, and calcium ions actually protect the full-length fibrillin-1 from proteolysis by catabolic enzymes,\textsuperscript{48} possibly explaining the constant and unaltered presence of fibrillin-1 observed throughout our study. Interestingly, in the absence of calcium, the microfibrils change their native organization, becoming loose and diffuse (own data not shown). When interpreting the constant levels of collagen type III and fibrillin-1, it has to be borne in mind that the more pathological the spatial pattern, the higher the number of cells in the tissue.\textsuperscript{25} This increase in cells can be highly attributed to cells with an impaired PCM, as shown by the optical readout for the categories 25% to 75% or less than 25% PCM signal circumference. Even though the total protein content remains similar for collagen type III and fibrillin-1, it must be assumed that the overall protein amount available per PCM decreases.

With respect to the point in time of PCM breakdown, no relevant changes in protein content and distribution could be observed between strings and double strings. Clear differences were visible between small and big clusters, as well as at the transition from double strings to small clusters, which makes this the earliest point in time where relevant OA-triggered changes take place. Notably, functional impairment with respect to the elasticity modulus of the PCM can be detected at the transition from strings to double strings.\textsuperscript{37} This is still a stage where the cartilage appears to be macroscopically intact.

If it is assumed that an intact functional unit is the prerequisite for intact cartilage metabolism and resilience, and that in bigger clusters the architecture of a proper PCM is not permitted, then regenerative therapies would have to aim at maintaining and/or restoring spatial organization and these intact units. Three interesting questions thereby arise (a) Are single strings necessary for intact cartilage metabolism and function, or are double strings also a spatial pattern that allows similar or possibly even better cartilage performance? (b) Can this process of spatial cellular rearrangement be reversed? (c) Until what point in time would this be possible? Further research will elucidate these questions. For now, it can be derived from the present data that when evaluating the therapeutic success of regenerative therapies, a strong focus should be put on maintaining and/or restoring the PCM.

5 STUDY LIMITATIONS

The results of the mathematical analysis of the PCM derived from the histological sections presented in this study are based on a highly subjective procedure. We, therefore, limited the variable to an ordinal scale of only three categories. All counts were performed by the same observer and in a blinded fashion, and inaccuracies should have thereby been averaged out due to the sample size. Moreover, no entirely healthy cartilage was assessed, rather only the cartilage from patients who had undergone total knee arthroplasty for OA. The “healthy” reference tissue was also derived from osteoarthritic cartilage. OA is, however, a condition that affects the entire joint. Nonetheless, it is noteworthy that in many cases the results are clear and highly significant. Analysing entirely healthy cartilage as a baseline might have therefore yielded even stronger differences than those described in the present study. It also needs to be made clear that conclusions drawn from the immunohistochemical analyses
were based on the signal intensity under the microscope and from the ELISA on the band strength. Both techniques depend on specific antibody binding to epitopes present in the tissue. Indeed, while in many cases a difference in signal or band intensity also signifies a change in protein content via a change in available epitopes for the antibody, it is also conceivable that epitopes get selectively destroyed while the core protein itself is still left in place. It is similarly possible that the proteins get fragmented and lose their function, yet the epitopes remain in the tissue thus allowing antibody binding to still take place.

6 | CONCLUSION

During OA, with spatial cellular rearrangement the PCM is progressively destroyed. The collagen type VI, perlecan, and biglycan components are especially affected, while signal intensity in immunohistochemical analyses and protein content analysis for collagen type III and fibrillin remain unaltered. First clear changes seem to take place at the transition from double strings to small clusters, where the morphological features of the functional units start to look impaired, and protein content decreases. In big clusters, intact functional units can no longer be detected.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

M.D. designed the study, performed the experiments, did the statistical analyses, and wrote the manuscript. A.L.E. performed the experiments, helped with the statistical analyses, and co-wrote the manuscript. J.M.W. helped with the experiments and co-wrote the manuscript. M.S. provided the cartilage samples, helped interpret the data, and critically revised the manuscript. Finally, U.K.H. designed and supervised the study, helped with the statistical analyses, and wrote the manuscript. All authors read and approved the final manuscript.

ETHICS STATEMENT

Project number of the ethics committee of the University of Tuebingen: 674/2016BO2.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.