Association between adiponectin and cartilage degradation in human osteoarthritis


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Objective: Conflicting findings raise questions about the role of adiponectin in osteoarthritis (OA). The current study aimed to investigate in OA patients the association between the production of adiponectin and the grade of cartilage destruction, and to provide functional evidence for a potential role of adiponectin in OA.

Design: The expression of adiponectin was examined by immunohistochemistry in cartilage obtained from healthy individuals (n = 2; ages 56 and 41 years; 1 male and 1 female) and OA patients (n = 11; ages 64–79 years; 2 male and 9 female). The association between its production in chondrocytes and the grade of cartilage destruction was established on full-depth cartilage biopsies. The functional activity of adiponectin in OA cartilage was determined from the relation between the expression of adiponectin, its receptor, cartilage-specific components and factors involved in matrix degradation, and from the chondrocyte response to the full-length or the globular form of adiponectin.

Results: Adiponectin was not detected in healthy cartilage. Conversely, the adipokine was up-regulated in damaged tissue, but no strong association with the grade of cartilage destruction was found. We showed a positive correlation between adiponectin and mPGES or MMP-13 while AdipoR1 was related to the expression of type 2 collagen, aggrecan and Sox9. The full-length form of adiponectin but not the globular isoform, stimulated the production of PGE2 and MMP-13 activity in cultured human chondrocytes.

Conclusions: The elevated level of adiponectin found in chondrocytes from OA patients might contribute to matrix remodelling during OA, the full-length isoform being the single active form.

Introduction

Although extensive studies aimed to identify the mechanisms underlying articular cartilage changes associated with osteoarthritis (OA), the pathogenesis of this degenerative joint disease remains unclear. It is well recognized that OA develops in the highly metabolic and inflammatory environment of adiposity. In the last decade, a new hypothesis based on the relation between obesity and OA has emerged. The sole role of biomechanical loading cannot explain the increased risk for OA in non-weight-bearing joints among overweight persons, and recent studies indicate that adiposity rather than simply excess in body mass is detrimental to the joint. More especially, some adipokines are thought to be implicated in the development and the progression of OA. In fact, it is now well recognized that adipose tissue plays a critical role as an active endocrine organ through the release of various adipokines in an integrated network that maintains interactions between fat and other organs. These adipose-derived proteins are required for normal physiological homeostasis, but impaired production may be involved in obesity-related disorders.

Adiponectin mediates its biological effects through two receptors, namely AdipoR1 and AdipoR2. This adipokine has a modular structure comprising of an N-terminal collagenous domain with multiple collagen triple helix repeats, followed by a C-
terminal C1q-like globular domain which has similar folding to
polypoly with Tumor Necrosis Factor-α (TNFα)8,9. A truncated form of
adiponectin restricted to the globular domain can be generated by
leukocyte elastase secreted from activated monocytes and/or
neutrophils10. The adipokine exhibits insulin-sensitizing proper-
ties11 and reduced serum levels are found in obesity12,13. Adiponectin
is considered as an anti-inflammatory mediator especially with
regard to atherosclerosis13, but in some chronic inflammatory/
autoimmune diseases it may have pro-inflammatory effects and its
production correlates with inflammatory markers and disease ac-
tivity. Thus, serum adiponectin levels have been shown to be
strongly associated with radiographic changes in patients with
rheumatoid arthritis (RA)14,15 and to be predictive of radiographic
progression in early RA15. The adipokine was reported to stimulate
the production of IL-616, IL-817 and prostaglandin E2 (PGE2)18 by
human synovial fibroblasts, suggesting its potential contribution to
the pathogenesis of synovitis in RA. Adiponectin may also be
considered as a marker of joint degradation or local inflammatory
processes. Indeed, increased serum levels of adiponectin were
detected in female patients with erosive compared with non-
erosive OA of the hands19. The adipokine is found in the synovial
fluid of human OA-affected joints and its articular levels are posi-
tively correlated with degenerative fragments of aggrecan20,21. Whether
adiponectin plays a pro- or anti-inflammatory role in OA
is still the subject of debate and its effects on cartilage are far to be
fully elucidated. For Chen et al., adiponectin may have a protective
role on cartilage by up-regulating the Tissue Inhibitor of
Metalloproteinase-2 (TIMP-2) and down-regulating IL-1β-induced
MMPs and pro-inflammatory mediators22. The recent data
reporting the stimulating effect of adiponectin on the production of
chemokines, cytokines and matrix-degrading enzymes both in syn-
ovial fibroblasts and in chondrocytes further support the inflam-
matory and the destructive activity of adiponectin in the joint13–25.

In order to further understand the contribution of adiponectin
to cartilage changes associated with human OA, we investigated
the expression of adiponectin in cartilage obtained from OA pa-
tients in relation to the grade of cartilage destruction. We further
sought to identify the functional significance of adiponectin
expression in chondrocytes. As AdipoR1 functions as a high-
affinity receptor for globular and full-length adiponectin26 and is
strongly expressed in chondrocytes27,28, we determined whether
both forms of adiponectin are able to modulate inflammation
and cartilage destruction in OA. After washing tibial plateaus in sterile phosphate buffered saline
(PBS), full-depth standardized cartilage biopsies (between 4 and 9
biopsies for each patient) were collected using a biopsy punch
(5 mm diameter). The samples were then cultured for 2 days at
37°C in a humidified atmosphere of 5% CO2 in Dulbecco’s Modified
Eagle Medium/Nut Mix F12 (DMEM/Ham’s F12 medium) supple-
menced with l-glutamine (2 mM), penicillin (0.1 U/ml), strepto-
mycin (100 ng/ml) and amphotericin B (250 ng/ml). The tissue
specimens were thereafter fixed for 24 h in 4% paraformaldehyde
and conditioned media were stored at −80°C prior to analysis.

Adiponectin treatment of human chondrocytes

Cartilage samples collected from six OA patients were washed in
sterile phosphate buffer saline (PBS) and then cut into small pieces.
Chondrocytes were isolated after a sequential digestion of the
extracellular matrix with pronase (0.15%, w/v) for 2 h and colla-
genase (0.2%, w/v) overnight at 37°C. After centrifugation, cells
were suspended in Dulbecco’s Modified Eagles Medium/Ham’s F-12
(DMEM/Ham’s 12) supplemented with 10% (v/v) foetal calf serum
(FCS), 2 mM l-glutamine, penicillin (0.1 U/ml), streptomycin
(100 ng/ml) and 250 ng/ml amphotericin B (InVitrogen, Cergy-
Pontoise, France), then seeded as primary chondrocytes culture in
75 cm2 culture flasks at high density (2 × 104 cells/cm2). They were
expanded for 10–12 days in monolayer in a humidified atmosphere
containing 5% CO2, and the culture medium was changed every 3–
4 days.

Confluent primary chondrocytes were then incubated with human recombinant full-length or globular adiponectin (R&D
Systems) at 0.2, 1 and 5 μg/ml for 24 h in 1% FCS containing
medium. The culture supernatants were then kept at −80°C until
analysed.

Histological assessment

The full-length cartilage biopsies fixed in paraformaldehyde
were decalcified in rapid bone decalifier (RDO, Eurobio, Les Ulys,
France) for 1 h, and further fixed in 4% paraformaldehyde. Cartilage
specimens were then dehydrated in a graded series of alcohol and
embedded in paraffin.

For all cartilage samples, haematoxylin–eosin–safran (HES),
and safranin-O-fast-green stainings were performed on serial sec-
tions (5 μm) to determine histological grading. The severity of OA
cartilage lesions was evaluated for each biopsy by two independent
observers, and was graded using the Mankin score29.

Immunohistochemical analysis

Paraffin sections (5 μm) from cartilage specimens were depar-
affinized in Tissue Clear (Bayer Diagnostic, Puteaux, France) and
rehydrated in a graded series of ethanol. Antigen retrieval was
performed by heating the sections in a citrate buffer (10 mM,
ph = 6) up to 60°C for 30 min. The endogenous peroxidase activity
was blocked by incubating the sections in H2O2 (0.3%). After
neutralization of non-specific sites with bovine serum albumin
(BSA, 4% (w/v)) for at least 1 h, sections were incubated overnight
at 4°C with a goat polyclonal antibody of the human adiponectin
(R&D Systems). After washing twice in PBS, the corresponding
biotinylated rabbit anti-goat IgG (Dako) was applied for 30 min at
room temperature. The signal was amplified with preformed
avidin-biotinylated horseradish peroxidase complexes for 45 min
at room temperature (EnVision kit, Dako), and staining was
developed with 3,3′-diaminobenzidine (0.05% in hydrogen
peroxide). Counterstaining of nuclei was performed with haema-
toxylin, and sections were dehydrated in graded ethanol, cleared in

Materials and methods

Patients and samples

Specimens of human OA articular cartilage were obtained from
tibial plateaus of patients undergoing total knee replacement sur-
gery (n = 11; 2 male and 9 female; ages 64–79 years, mean
70.9 ± 5.3 years; BMI 22–54 kg/m2, mean 33.0 ± 8.8 kg/m2). All
patients were evaluated by an orthopaedic surgeon and diagnosed
for knee OA according to the criteria of the American College of
Rheumatology30. For comparison with diseased tissues, normal
knee joint cartilage was obtained from transplant donors (n = 2;
ages 56 and 41 years; 1 male and 1 female) through agreement with
the Agence de la Biomedecine. The human study described here
was conducted in conformity with the declaration of Helsinki
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Gene expression analysis

Total ribonucleic acid (RNA) was extracted from freshly isolated chondrocytes using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. The integrity of the isolated RNA was assessed by ethidium bromide staining on agarose gel and concentration was determined by measurements of the absorbance at 260 nm on a NanoDrop ND-1000 Spectrophotometer (Labtech, Palaiseau, France). RNA samples were then reverse transcribed for 90 min at 37°C using oligo-dT primers (100 pmol) and M-MLV reverse transcriptase (200 U) (Invitrogen, Cergy-Pontoise, France). Gene expression was analysed by quantitative real-time polymerase chain reaction (PCR) (Lightcycler Roche, Mannheim, Germany) using the SYBRgreen master mix system (Qiagen, Courtaboeuf, France) according to the manufacturer’s protocol. The gene-specific primer pairs optimized for this method are described in Table 1. As a control of the amplification specificity, melting curve analysis was performed for each PCR experiment. Amplified products were also visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide. Quantification was achieved using a specific calibration curve obtained from serial dilutions of a PCR positive standard with a known amount of corresponding purified PCR products. For standardization of gene expression levels, messenger RNA (mRNA) ratios relative to RP29 chosen as housekeeping gene were calculated.

Release of adiponectin and PGE2

The concentrations of adiponectin in conditioned media of cultured cartilage specimens were determined in duplicate by a sandwich enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (R&D Systems, Lille, France). The concentrations of PGE2 released by untreated and adiponectin-stimulated chondrocytes were also measured in duplicate by a competitive enzyme immunoassay using commercially available kit (R&D Systems, Lille, France). According to the manufacturer, the detection limit was 250 pg/ml and 41.4 pg/ml for adiponectin and PGE2 respectively. The interassay coefficient of variation was 7.8% and 10.6% for adiponectin and PGE2 respectively, and the intraassay coefficients of variation were less than 5%.

Determination of MMP-13 activity

MMP-13 activity was quantified in culture supernatants using a fluorogenic substrate. Briefly, latent MMPs were activated by adding 50 μl of culture supernatant to 50 μl of 1.5 mM 4-amino-phenylmercuric acetate (APMA (Sigma)) diluted in a Tris-HCl (50 mM, pH 7.5) reaction buffer containing 15 mM NaCl and 5 mM CaCl2. The fluorogenic MMP-13 substrate MCA-Pro-Cho-Gly-Nva-His-Ala-Dpa-NH2 (Calbiochem, Nottingham, UK) was then added to a final concentration of 10 μM. After 4 h of incubation in darkness at 37°C, MMP-13-induced cleavage of the substrate was measured with a Fluostar Optima™ spectrofluorimeter (BMG Labtechnologies, Champigny-sur-Marne, France) at excitation and emission wavelengths of 325 nm and 393 nm, respectively. Data were expressed in arbitrary units of fluorescence.

Statistical analysis

Statistical analysis was conducted with SPSS software (SPSS Inc., Chicago, IL, USA). In order to study the release of adiponectin from OA cartilage in relation to the grade of cartilage destruction, 84 biopsies were collected from 11 OA patients. The samples were split up into two subgroups according to the BMI of the patients (n = 37 biopsies for BMI < 30 kg/m² and n = 47 biopsies for BMI ≥ 30 kg/m²). The statistical analysis was performed by comparing the production of adiponectin for each histological score (mild, moderate and severe). As the data did not fulfill the assumption of a normal distribution implicit in the analysis of variance, Friedman repeated measures analysis of variance on ranks was used to compare median-values of the three histological groups of cartilage destruction. When this global-test was statistically significant (P < 0.05), a pair-wise post-hoc analysis was performed. For these tests, the power analysis included a post-hoc pair-wise correction (0.0167) as a control for alpha inflation.

The analysis of statistical correlation between the expression of adiponectin or its receptor AdipoR1 (independent variables) and that of genes encoding cartilage matrix components or inflammatory factors (dependent variables) were performed with chondrocytes freshly isolated from the 11 OA patients by the Spearman test of rank correlation.

The effect of adiponectin on cultured chondrocytes was examined with cells isolated from six OA patients and experiments were performed in triplicate for each patient. Statistical differences between adiponectin-treated and untreated chondrocytes were determined with mean-values obtained from the triplicate experiments (n = 6) using one-way analysis of variance (ANOVA) followed by a Student’s t test. P value less than 0.05 was considered significant for differences and correlations.

Results

Relationship between adiponectin expression and the grade of cartilage destruction

Before examining the grade-dependent expression of adiponectin in OA cartilage, we compared healthy human articular cartilage with articular cartilage obtained from OA patients. No adiponectin immunostaining was detected in normal cartilage whereas many adiponectin positive chondrocytes were found in OA cartilage [Fig. 1(A)]. Adiponectin expression was most prominent in superficial and middle zones of OA cartilage, but rarely found in the deep layer.
This OA-specific expression pattern of adiponectin in cartilage prompted us to determine whether the adipokine level was associated with the grade of cartilage destruction. Five to nine full-depth standardized cartilage biopsies were sampled in tibial plateaus collected from 11 OA patients (n = 84). The production of adiponectin was determined by ELISA in conditioned media from cultured tissue samples to improve the quantitative analysis of adiponectin expression compared to immunohistochemical score. At the end of the culture period, each specimen was stained with safranin-O-fast-green to evaluate the severity of OA cartilage lesion. Cartilage samples exhibited variability in their histological appearance ranging from surface irregularities to marked middle cracks. Proteoglycan loss was evident histologically in moderately damaged OA samples, but the overall thickness of the cartilage was preserved. By contrast, specimens with advanced cartilage destruction, exhibited a severe proteoglycans loss and cartilage erosion. Overall, 84 biopsies were divided into three groups according to the histological severity grade: Mankin scores between 1–4, 5–9 and 10–14 were considered as mild (n = 18), moderate (n = 44) and severe OA (n = 22), respectively. In addition, two groups of patients were considered according to the BMI values (obese vs non-obese patients). Our data indicated that a grade-dependent increase in the production of adiponectin was found for non-obese patients (BMI < 30 kg/m², n = 5), but the difference did not reach statistical significance [Fig. 1(B)]. For obese patients (BMI ≥ 30 kg/m², n = 6), specimens with mild OA displayed reduced levels of adiponectin when compared to samples with moderate (P = 0.0075) or severe (P = 0.0103) OA histological score. However, the synthesis of adiponectin did not increase anymore for the most severe OA grade.

**Associations between adiponectin, its receptors and extracellular matrix, degradative enzymes and inflammatory mediators**

The potential pathophysiological role of adiponectin in OA cartilage was evaluated through the relationships between the mRNA levels of the genes encoding adiponectin or its receptors (AdipoR1 and AdipoR2) on one hand, and aggrecan, type 2 collagen, Sox9, MMP-13, mPGES and iNOS on the other hand. The correlation analysis revealed a significant positive association between the mRNA levels of adiponectin and those of mPGES or MMP-13 [Fig. 2]. By contrast, no relationship was found between adiponectin and the cartilage-specific genes. As AdipoR2 was barely detected in chondrocytes from OA patients, the associations have been examined with AdipoR1. Unlike the associations found with adiponectin, we failed to demonstrate any correlation between the mRNA levels of AdipoR1 and mPGES or MMP-13 whereas aggrecan, Sox9 and type 2 collagen were strongly related to the gene expression of the receptor [Fig. 3].

**Effects of adiponectin on chondrocytes**

To determine whether the correlations previously found in freshly isolated chondrocytes obtained from OA patients are causal...
relationships, we next investigated whether the adipokine as its full-length or globular form was able to modulate inflammation or catabolic activity in chondrocyte. For more physiological relevance, the recombinant protein was used at concentrations found in the synovial fluid from OA patients (0.2, 1 and 5 μg/ml). Immunoassay analysis of culture supernatants indicated that stimulation of primary human chondrocytes with the highest dose of the full-length adiponectin increased the MMP-13 activity \( (P < 0.001) \) and the PGE2 synthesis \( (P = 0.0012) \) [Fig. 4]. By contrast, neither the lower concentrations nor the globular form of adiponectin did not induce any significant changes in MMP-13 activity and PGE2 production. In all experimental conditions, no detectable amount of NO was found in the culture supernatant of adiponectin-stimulated chondrocytes. The lack of any response in chondrocytes treated with heat-inactivated adiponectin indicated that endotoxins did not account for the stimulatory effect of adiponectin.

Fig. 2. Correlations between the mRNA expression of adiponectin and that of the microsomal prostaglandin E synthase (mPGES), the matrix metalloprotease-13 (MMP-13), the aggrecan, Sox9 and collagen type 2 in cartilage from OA patients \( (n = 11) \).

Fig. 3. Correlations between the mRNA expression of adiponectin receptor 1 (AdipoR1) and that of the microsomal prostaglandin E synthase (mPGES), the matrix metalloprotease-13 (MMP-13), the aggrecan, Sox9 and collagen type 2 in cartilage from OA patients \( (n = 11) \).
Adiponectin is up-regulated in OA chondrocytes, but without strong association with the grade of cartilage destruction

We report here the first demonstration of the expression of adiponectin at sites of cartilage destruction. Indeed, the adipokine is not detected in healthy cartilage, but is up-regulated in damaged tissue. However, ELISA measurements which provide quantitatively accurate information on adiponectin levels in cultured OA cartilage supernatants indicate that the increase in adiponectin production depends on the BMI of the patients and is not strongly related to the grade of cartilage destruction. A statistically significant increase is found only in specimens obtained from patients with BMI > 30 kg/m². Cartilage samples with moderate lesions and collected from obese OA patients release high amounts of adiponectin, but the production does not increase anymore when damage is getting worse. As was previously shown for RA, these findings suggest that adiponectin may play different physiopathological roles depending on the stages of OA. The adipokine may be more associated with cartilage destruction in patients with established disease compared with those with early OA, obesity being an aggravating factor. The current data provide somewhat conflicting information over previous data published by Koskinen et al. They showed a positive association between radiological OA severity and the amount of adiponectin released by OA cartilage samples in culture media. Variations in the study design, and more especially in the index severity used to investigate the link with adiponectin production, might explain this discrepancy. Indeed, the current study looked for cartilage damage through histological grading by the Mankin score while Koskinen et al. referred to a radiological score of the whole joint to evaluate OA. In addition, these authors studied OA patients undergoing knee replacement surgery indicating that the range of OA severity includes only severe OA. We assumed actually that our experimental approach based on measurements performed with an individual sample is more suitable to establish the relation between adiponectin production and cartilage changes. Our data reveal actually that various cartilage specimens obtained from a single joint produce different amounts of adiponectin, suggesting that adiponectin levels in cultured cartilage supernatant cannot be related to an overall radiological score.

The full-length form of adiponectin exhibits inflammatory and destructive activities for cartilage

Because of the up-regulation of adiponectin in OA cartilage compared to healthy tissue, we next sought to elucidate its potential contribution in OA by examining the effect of both adiponectin isoforms on chondrocyte function. Studying the globular form is essential as especially this isoform has a high-binding affinity for AdipoR1 which is strongly expressed in human OA cartilage.

In accordance with previous studies, we showed that adiponectin stimulates MMP-13 activity. Our data indicated also that the adipokine increases the production of PGE₂ in chondrocytes, as was previously demonstrated upon exposure of synovial fibroblasts to adiponectin. Interestingly, this stimulatory effect of adiponectin on MMP-13 activity and PGE₂ release matches well with the positive association found in the current study between the mRNA expression in freshly isolated OA chondrocytes of adiponectin and that of MMP-13 and mPGE₂. The pro-inflammatory effect of adiponectin has been previously observed in human synovial fibroblasts. Thus, the adipokine stimulates the production of chemokines and cytokines, and human synovial fibroblasts increase the protein secretion of proMMP-1, MMP-3, MMP-10 and MMP-12 in response to adiponectin. Beside, the adipokine up-regulates numerous MMPs in human chondrocytes including MMP-1, -3, -13, and -12 in response to adiponectin. All these findings support the role of adiponectin in inflammation through the activation and recruitment of inflammatory cells into the joint, and in cartilage destruction. Beside, we showed for the first time that the globular form is unable to induce any response in human chondrocyte, suggesting that the potential pro-inflammatory effect of adiponectin is due to the full-length form only.

The expression of AdipoR1 in OA cartilage is strongly associated with that of matrix components

Most studies assign to adiponectin destructive effects, but very few examined its potential contribution to the synthesis of
extracellular matrix. Delessa Challa et al. demonstrated that adiponectin up-regulates the expression of type 2 collagen, aggrecan and Sox9 in cultured chondrocytes. Surprisingly, our data indicate that the expression of these genes in OA cartilage is not associated with adiponectin but rather with the mRNA levels of its receptor AdipoR1. The elevated levels of AdipoR1 found in damaged cartilage area compared to non-lesional area suggest that adiponectin may be involved in the repair attempts induced by cartilage destruction during OA. The adipokine might also contribute to the chondrocyte hypertrophy-like changes observed during OA. A phenotypic shift in the mature chondrocyte to a cell type that displays many characteristics of hypertrophic cells is a typical feature of OA and is associated with the progressive cartilage breakdown. The adiponectin-mediated increase in the mRNA expression of type 2 collagen, aggrecan and Sox9, as well as its stimulatory effect on Runx2 and type 10 collagen found by Challa et al. suggest that adiponectin may drive chondrocyte changes towards the hypertrophic stage.

As discussed above, the strength of the current study is the use of individual cartilage specimens to establish, on the same sample, the link between the production of adiponectin and the grade of cartilage degradation. This experimental approach has however some limitations especially when considering the OA disease. First, the different grades of cartilage destruction cannot be extrapolated to the different stages of the disease. Adiponectin might be better related to OA severity if both the synovial fluid level and an overall OA score are determined. Second, a cartilage sample with severe damage may locally influence the release of adiponectin from a neighbouring specimen with a lower Mankin score.

Conclusions

In summary, the current study provides further insights on the production of adiponectin in human OA cartilage. The adipokine is not detected in healthy tissue but is up-regulated in cartilage from OA patients. The close correlation found in OA cartilage between the expression of the gene encoding adiponectin and that of the genes encoding mPGES and MMP-13, as well as the stimulatory effect of physiological concentrations of the full-length adiponectin, but not the globular form, on the production of PGE2 and MMP-13 activity, further support the role of this adipokine in cartilage destruction. However, our data demonstrated also that the mRNA level of AdipoR1 is strongly associated with the mRNA expression of cartilage-specific components, suggesting that adiponectin is rather involved in matrix remodelling. The lack of any strong association between the production of adiponectin and the grade of cartilage destruction may explain the discrepancies observed in the various studies dealing with the relation between the adiponectin levels and the severity of the disease. Further investigations on the expression of adiponectin and its receptors during the course of experimental models of OA would be helpful to determine whether the adipokine is protective in mild or moderate OA and actively participate in the process of cartilage degradation in severe OA, depending partly on the expression of AdipoR1.

Authors’ contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. NP had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. P-JF, AA and CG contributed to the acquisition and analysis of the data. DaM, AB, PP, JYJ and DM participated in the design of the study and were involved in the interpretation of the data. DM recruited also the patients and provided the cartilage specimens. NP participated in the coordination of the study and in the interpretation of the data, and drafted the manuscript. DaM, AB, PP, JYJ and DM revised the manuscript critically for important intellectual content and all authors gave their final approval of the version to be submitted.

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Competing interest

The authors have no competing financial interests or other conflicts of interest to declare.

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