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Henrotin, Yves ; Gharbi, Myriam ; Mazzucchelli, Gabriel ; Dubuc, Jean-Emile ; De Pauw, Edwin ; Deberg, Michelle

ABSTRACT

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Fibulin 3 Peptides Fib3-1 and Fib3-2 Are Potential Biomarkers of Osteoarthritis

Yves Henrotin,1 Myriam Gharbi,1 Gabriel Mazzucchelli,1 Jean-Emile Dubuc,2 Edwin De Pauw,1 and Michelle Deberg 1

Objective. This study was undertaken to identify new biomarkers of osteoarthritis (OA) by proteomics analysis and to develop specific immunoassays to detect and quantify them.

Methods. Proteomics analysis was performed in urine samples from 10 women (mean ± SD age 76.0 ± 5.0 years) undergoing knee replacement surgery due to severe OA and 5 healthy women (mean ± SD age 25.6 ± 2.6 years). Protein content was analyzed by 2-dimensional differential gel electrophoresis. Protein spots that exhibited an OA:control abundance ratio of ≥1.5 were identified by mass spectrometry. Specific enzyme-linked immunosorbent assays were developed and validated in serum obtained from 236 healthy subjects ages 20–64 years and from 76 patients with severe radiologic knee OA (mean ± SD age 68.8 ± 11.9 years). Immunohistochemical analysis was performed on articular cartilage from tibial plateaus.

Results. Thirteen proteins within spots that were significantly modified between groups were identified. Two peptides of fibulin 3, named Fib3-1 and Fib3-2, were of particular interest. Two antisera directed against these peptides were used to develop immunoassays. Compared with age-matched healthy subjects, median levels of serum Fib3-1 and Fib3-2 were elevated in OA patients (54.6 pM versus 85.1 pM [P < 0.0001] and 144.4 pM versus 191.4 pM [P < 0.0001], respectively). Using area under the receiver operating characteristic curve analysis, we demonstrated that Fib3-1 and Fib3-2 levels discriminate between OA and normal populations. Immunostaining revealed the presence of Fib3-1 and Fib3-2 in chondrocytes and in the extracellular matrix of the superficial layer of the fibrillated cartilage.

Conclusion. Our findings indicate that Fib3-1 and Fib3-2 are potential biochemical markers for the diagnosis of OA.

Osteoarthritis (OA) is a progressive disorder characterized by synovial inflammation, bone remodeling, and degradation of the extracellular matrix of articular cartilage (1,2). Currently, the diagnosis of OA is based on clinical and radiographic changes which occur late in disease progression. This method does not allow early detection of structural damage, and it is cumbersome to use in daily practice. Some of the available imaging methods lack reproducibility and sensitivity, and thus long-term followup of a large population is required to demonstrate the efficacy of a drug. This constitutes an impediment to new drug development. Therefore, there is an acute need for reliable biochemical markers that can facilitate the diagnosis of OA and inform prognosis, monitoring, and therapeutic strategies for the disease.

Biochemical markers of bone, synovium, or cartilage turnover have been proposed as tools for the diagnosis and prognosis of OA and for the monitoring of OA treatment (3–5). The following 5 serum and urinary biomarkers are receiving increasing attention: serum hyaluronic acid (HA), serum cartilage oligomeric matrix protein (COMP), urinary C-telopeptide of type II collagen...
lagen, serum collagenase-mediated type II collagen neoepitope, and serum and urinary Coll2-1 and its nitrated form (6). Higher baseline levels of serum COMP and serum HA were associated with incident knee OA, osteophytes, and joint space narrowing (7). However, at that time, there were no reliable biomarkers for early diagnosis, for the identification of different OA phenotypes, or for use as surrogate clinical end points (8). Further, evidence is emerging to support the idea that biomarkers perform better when used in combination. Therefore, the discovery of novel OA biomarkers is essential for drug discovery and development, for early diagnosis, and to facilitate individualized treatment to improve clinical outcomes.

Proteomic technology platforms have given researchers the ability to rapidly identify novel protein biomarkers in various biologic fluids, tissue types, and cell extracts. The first step consists of the identification of protein biomarkers using proteomic approaches. The second step involves the selection of a subset of biomarker candidates based on several criteria, such as an association with disease biology reported in the literature. The third step requires the production of antibodies and the validation of specific immunoassays for the quantification of biomarkers in biologic fluids. The last step of biomarker discovery involves the development of preliminary assays that confirm the bioanalytical measurements from the first step, as well as prequalifying the biomarker(s) in preclinical models or clinical trials, to initiate future marker qualification and development. In this study, we followed this strategy to identify new biomarkers of OA and to develop immunoassays for their quantification.

**MATERIALS AND METHODS**

**Proteomics analysis.** Preparation of urine samples. Urine samples were obtained from 10 women (with a mean ± SD age of 76.0 ± 5.0 years) undergoing knee replacement surgery due to severe OA (OA group) and from 5 healthy women (with a mean ± SD age of 25.6 ± 2.6 years) with no history of or current joint disease or trauma (control group). All OA patients met the American College of Rheumatology (ACR) criteria for OA (9) and had a radiologic score of 3 or 4 on the Kellgren/Lawrence scale (10). All samples in each group were pooled to avoid biologic variation. Urine samples were concentrated 100 times by ultracentrifugation on an Amicon Ultra-15 (Millipore) and depleted in albumin using a Montage Albumin Deplete kit (Millipore). Proteins were purified by precipitation using a PlusOne 2D Clean-up kit (GE Healthcare).

Labeling of proteins. The purified proteins were labeled on lysine residues with Cy3 or Cy5 CyDye DIGE Fluors (GE Healthcare). Three gels were made with labeling inversion to avoid technical variation. On the first and second gels, proteins from the pooled control sample were labeled with Cy3, whereas proteins from the OA sample were labeled with Cy5. On the third gel, Cy3 and Cy5 labeling were reversed. An internal standard comprising equal amounts of control and OA samples was labeled with Cy2 and loaded onto each gel. A total of 9 spot maps were obtained.

**Two-dimensional electrophoresis.** Protein samples (12.5 μg each) were pooled in isoelectric focusing (IEF) buffer (5 M urea, 2% CHAPS, 0.5% immobilized pH gradient buffer, 1% dithiothreitol [DTT], and a trace of bromphenol blue), which was loaded onto an Immobiline DryStrip (pH 3–10 nonlinear, 24 cm; GE Healthcare). IEF was carried out at up to 70,000 volts/hour overnight using a Protean IEF cell (Bio-Rad). The gels were equilibrated in reduction buffer (375 mM Tris HCl [pH 8.8], 6 M urea, 20% glycerol, 2% sodium dodecyl sulfate [SDS], and 130 mM DTT) for 12 minutes, followed by alkyla-
tion (375 mM Tris HCl [pH 8.8], 6 M urea, 20% glycerol, 2% SDS, and 135 mM iodoacetamide) for 12 minutes. The second dimension was run overnight at 20°C in an Ettan DALT system (GE Healthcare) at 1.5W per gel. Gels were scanned using a Typhoon 9400 scanner at the wavelengths corresponding to each CyDye. Image analysis was performed on DeCyder software (GE Healthcare), which was used for the simultaneous comparison of abundance changes across sample groups. Differentially expressed spots (P < 0.05 by Student’s t-test) with a Cy3:Cy5 intensity ratio of ≥1.5 were picked out of the gel in order to allow protein identification. Only spots of protein that were present on all spot maps were considered for differential analysis.

Protein identification. Protein spots were cut out of the polyacrylamide gel and washed twice for 5 minutes with an ammonium hydrogen carbonate (50 mM)–acetonitrile mixture (1:1). Gel pieces were incubated in DTT (10 mM) and NH₄HCO₃ (50 mM) for 40 minutes in a 56°C water bath. Proteins in the gel spots were alkylated for 1 hour in the dark with iodoacetamide (55 mM) in NH₄HCO₃ (50 mM). The gel spots were then washed twice as described above, dehydrated with acetonitrile, and dried for 15 minutes at room temperature. The gel spots were then rehydrated for 10 minutes on ice with trypsin in NH₄HCO₃ (25 mM) and incubated overnight at 37°C. Tryptic digestion was stopped in trifluoroacetic acid (1%)–acetonitrile (5%) solution. The gel pieces were then sonicated twice for 1 minute in order to release protein fragments.

The identity of the proteins was determined by tandem mass spectrometry (MS/MS) sequencing using an Ultimate 3000 Nano-LC system (Dionex) and analyzed online using an Esquire HCT Ultra electrospray ion-trap mass spectrometer (Bruker Daltonics). The peptide content extracted from each spot was trapped on a C18 precolumn (300 μm inner diameter × 5 mm) (Acclaim PepMap; Dionex) and desalted for 5 minutes at 30 μl/minute with 97.9% water, 2% acetonitrile, and 0.1% formic acid. Peptides were separated on a C18 analytic column (75 μm × 150 mm) (Acclaim; Dionex) with a solvent gradient of 30 minutes at a flow rate of 0.3 μl/minute (where solution A consisted of 0.1% formic acid and solution B consisted of 80% acetonitrile plus 0.1% formic acid, and B was increased from 7% at 0 minutes to 35% at 30 minutes). The mass range of 200–1,500 mass/charge was scanned in the MS to select the 3 most intense peptides (bearing +2 and +3 charge states) for sequence analysis.
or other inflammatory disease. At the time of donation, the subjects were not taking any medication known to modify arthritic disease or influence joint metabolism. They answered a short questionnaire regarding joint symptoms, and individuals with joint symptoms were not included in the study. The group included in the study consisted of 93 women and 143 men, ages 20–64 years. The women had a mean ± SD age of 41.5 ± 11.8 years, and the men had a mean ± SD age of 45.3 ± 11.3 years. Blood samples clotted during 1 hour at room temperature. Serum was separated from cells by centrifugation at 2,000 revolutions per minute for 10 minutes at 4°C. Serum samples were immediately stored at −80°C until analyzed.

Serum samples were obtained from 76 patients (ages 39–89 years) who were diagnosed as having severe knee OA according to the ACR clinical and radiologic criteria. This group was composed of 52 women with a mean ± SD age of 69.5 ± 11.7 years and 24 men with a mean ± SD age of 66.0 ± 12.3 years. All subjects were Caucasian. Their mean ± SD body mass index was 28.4 ± 5.2 kg/m². Of these OA patients, 21 had unilateral symptomatic and radiologic knee OA, and 55 had bilateral OA. All subjects underwent placement of a prosthesis. At the time of the study, none was taking any medication known to modify arthritic disease or influence joint metabolism. Medications taken included mainly treatments for cardiovascular disease, antidepressants, and hormonal supplements. Serum samples were collected at the time of surgery and stored at −80°C until analyzed. Written informed consent was obtained from all study subjects, and the study was approved by the ethics committee of the Catholic University of Leuven (Leuven, Belgium) (B4032007964).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5 for Windows. The nonparametric
Mann-Whitney U test was used to compare OA patients and healthy subjects of the same age range. To determine the influence of sex and aging on Fib3-1 and Fib3-2 concentrations, we performed a regression analysis. To determine which subclasses of healthy subjects grouped by age were different from the others, we used one-way analysis of variance. P values less than 0.05 (2-tailed) were considered significant. Sensitivity and specificity for the diagnosis of OA were analyzed using area under the curve (AUC) of the receiver operating characteristic (ROC) curve analysis with leave-one-out validation.

RESULTS

Results of proteomics analysis. The protein distributions in the urine samples from control subjects and those from OA patients were compared. On 3 gels, a mean ± SD of 570 ± 48 spots were detected, and 333 of these spots were common to all spot maps. Of these 333 spots, 40 had a ratio of change in abundance between the OA and control groups of ≥1.5. Of these 40 spots, protein identification by MS revealed a significant difference in abundance between groups (P < 0.005) in 18 of the patients (Figure 1 and Table 1). More specifically, MS analysis allowed the identification of 2 tryptic fragments containing specific sequences of fibulin 3. These 2 sequences, 331–345TCQDINECETTNCR and 377–387CVCPVSNAMCR, were named Fib3-1 and Fib3-2, respectively. Results of MS/MS fragmentation of these peptides are available on the Arthritis & Rheumatism web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

Validation of immunoassays for Fib3-1 and Fib3-2. Antiserum specificity. Two antisera, AS88 and AS94, with high specificity for Fib3-1 and Fib3-2, respectively, were selected. Antiserum did not recognize complete fibulin 3 or BSA and did not cross-react between Fib3-1 and Fib3-2 (Figure 2).

Enzyme-linked immunosorbent assay (ELISA) for Fib3-1. The lower detection limit of the assay, defined as the concentration corresponding to 3 SD above the mean of ≥10 determinations of the 0 calibrator, was 5.2 pM. The working range of this assay was established by calculating the coefficients of variation of each calibrator in 10 independent calibration curves. The coefficients of variation were <10% between 12.5 pM and 400 pM. The within-run (intraassay) variation, assessed by measuring 3 sera ≥10 times in the same run, and the between-run (interassay) variation, assessed on 8 plates, were <11%. Linearity was shown by diluting samples serially and comparing the observed values with the expected values. A typical recovery rate of 85.8–104.0% was noted in a range of dilution between 2 and 8 fold.

Spiking recovery, determined by the addition of known quantities of Fib3-1 peptide (from 25 pM to 250 pM) to the sample, ranged from 92.3% to 103.2%. Serum samples were twice-diluted in standard buffer.

ELISA for Fib3-2. The lower detection limit of the assay was 8 pM, and the working range was 12.5–400 pM. The coefficients of variation of intraassays and interassays were <11%. The dilution curves were parallel to the standard curve with a typical recovery rate of 80.4–105.1% in a range of dilution between 2 and 8 fold. Spiking recovery was between 101.3% and 112.4%. Serum samples were twice-diluted in standard buffer.

Fib3-1 and Fib3-2 levels in the healthy study population. In 11 of 236 serum samples (4.6%), Fib3-1 was under the limit of quantification of the immunoassay; therefore, the value of the lower limit of quantification was used for these samples (in nonparametric statistical analysis). The median concentration of Fib3-1 was 58.5 pM (interquartile range [IQR] 46.2–73.9 pM). Linear regression showed a decrease in Fib3-1 concentration with age in women (r = 0.72). Median Fib3-1 levels were higher in premenopausal than in postmenopausal women (70.6 pM versus 57.1 pM; P = 0.017). No variation in Fib3-1 levels with age was found in men.

Neither age nor sex modified the serum levels of the Fib3-2 fragment. The median concentration of Fib3-2 in serum samples from healthy subjects (n = 236) was 140.8 pM (IQR 126.5–156.3 pM). Comparisons
between premenopausal and postmenopausal women did not reveal any difference in Fib3-2 concentration.

**Fib3-1 and Fib3-2 levels in the OA study population.** Compared with 140 age-matched healthy subjects, OA patients had higher levels of Fib3-1 and Fib3-2 ($P < 0.0001$). The median Fib3-1 concentration was 54.6 pM in healthy subjects and 85.1 pM in OA patients, and the median Fib3-2 concentration was 144.4 pM in healthy subjects and 191.4 pM in OA patients (Figure 3). No correlation was found between the concentrations of Fib3-1 and Fib3-2 in either the healthy or OA study populations.

### Table 1. List of identified proteins and their ratio of abundance in OA patients to abundance in controls*

<table>
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<tr>
<th>Protein</th>
<th>Swiss-Prot accession no.</th>
<th>No. of peptides matched</th>
<th>Sequence coverage, %</th>
<th>Mowse score</th>
<th>Abundance ratio (OA:controls)</th>
<th>$P^†$</th>
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<tr>
<td>$\beta$-actin</td>
<td>P60709</td>
<td>3</td>
<td>6</td>
<td>111</td>
<td>4.1</td>
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<td>$\alpha_1$-microglobulin/bikunin precursor</td>
<td>P02760</td>
<td>3</td>
<td>5</td>
<td>107</td>
<td>2.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fibulin 3</td>
<td>Q12805</td>
<td>2</td>
<td>5</td>
<td>97</td>
<td>2.2</td>
<td>&lt;0.001</td>
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<tr>
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<td>Q9BRQ8</td>
<td>2</td>
<td>3</td>
<td>59</td>
<td></td>
<td></td>
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<tr>
<td>Zinc-$\alpha_2$-glycoprotein precursor</td>
<td>P25311</td>
<td>3</td>
<td>8</td>
<td>46</td>
<td>2.0</td>
<td>&lt;0.001</td>
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<tr>
<td>Fibulin 3</td>
<td>Q12805</td>
<td>1</td>
<td>2</td>
<td>45</td>
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<tr>
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<td>P25311</td>
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<td>21</td>
<td>85</td>
<td>2.0</td>
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<tr>
<td>Polymeric Ig receptor</td>
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<td>9</td>
<td>141</td>
<td>1.8</td>
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<td>gp36b</td>
<td>Q12907</td>
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<td>10</td>
<td>85</td>
<td>1.5</td>
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<td>73</td>
<td>-1.7</td>
<td>0.007</td>
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<tr>
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<td>4</td>
<td>6</td>
<td>120</td>
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<td>8</td>
<td>151</td>
<td>-2.3</td>
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<td>Serpin B3</td>
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<td>94</td>
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<td>6</td>
<td>77</td>
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<td>&lt;0.001</td>
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</table>

* Proteins listed more than once were identified in more than 1 spot (13 different proteins were identified in 18 spots). Proteins in the same spot have the same abundance ratio and statistical value. Sequence coverage refers to the percentage of identified sequences out of the total protein sequence. The Mowse score is $-10 \log (P)$, where $P$ is the probability that the observed match is a random event. Protein scores are derived from ion scores as a nonprobabilistic basis for ranking protein hits. OA = osteoarthritis.

† By Student's $t$-test.

Sensitivity and specificity of Fib3-1 and Fib3-2 for the diagnosis of OA. Using area under the ROC curve analysis, the specificity and sensitivity of the tests were determined (Figure 4). The AUC for Fib3-1 was 0.75 (95% confidence interval [95% CI] 0.67–0.82) ($P < 0.0001$), and the AUC for Fib3-2 was 0.83 (95% CI 0.77–0.90) ($P < 0.0001$). The optimal cutoff was determined as the value corresponding to the greatest sum of sensitivity and specificity of the test for discriminating OA patients from healthy subjects. At a cutoff point of 71.15 pM, the specificity was 77.1%, and the sensitivity...
was 68.4% for the Fib3-1 assay. At a cutoff point of 164.0 pM, the specificity was 85.7%, and the sensitivity was 74.6% for the Fib3-2 assay.

Results of immunohistochemical analysis. Localization of Fib3-1 (Figures 5A, C, E, and G) and Fib3-2 (Figures 5B, D, F, and H) was performed using antisera AS88 and AS94, respectively. In normal cartilage (Figures 5A and B), antibodies labeled a thin layer of flattened chondrocytes at the cartilage surface. A weak staining of the superficial zone of the extracellular matrix was also observed. A similar pattern of staining was observed with both antisera. However, staining of the superficial layer was more intense with AS88 antisemur. In OA cartilage with fibrillations, strong labeling was observed in the extracellular matrix and in cell clusters (Figures 5C and D). The inner zone of cartilage did not appear to be labeled with antisera. A clearly distinct limit appeared between the trabecular bone zone, which was stained, and the overlying cartilage (Figures 5E and F). A strong inhibition of staining was observed when antiserum preincubated with standard peptide was used for the labeling (Figures 5G and H).

DISCUSSION

Differential proteomics analysis has been used to compare the composition of joint tissue, serum, and synovial fluid in OA and normal subjects (for review, see ref. 12). In this study, we compared the proteomic profiles of urine samples from healthy young subjects with those from patients with OA. Particularly in biologic fluids like serum, highly abundant proteins, such as albumin or immunoglobulins, are present in quantities that can be measured in milligrams per milliliter and constitute >95% of the total proteins. Proteins of interest as potential biomarkers are usually present in serum at levels that are measured in nanograms per milliliter or picograms per milliliter, making them difficult to detect among abundant proteins. The urinary proteome has received increasing attention in the proteomics field for its simplicity compared to serum, as well as its potential in biomarker discovery. In urine, MS-based technology has been used in targeted approaches in order to characterize particular peptides (13). To the best of our knowledge, this is the first study to analyze urine samples using a differential proteomics strategy to identify new biomarkers of OA.

MS analysis identified 13 proteins whose levels were decreased or increased by ≥1.5 fold in OA patients compared to healthy controls. Some of them had previously been identified and found to be elevated in the synovial fluid of OA or RA patients compared to controls. These included mainly kininogen, zinc-α2-glycoprotein, and α1-microglobulin (14–16). Interestingly, 2 spots that were mostly increased in the proteome of OA patients contained specific sequences of fibulin 3, which was the only extracellular matrix protein found to be significantly modified in the urinary proteome of OA patients.

Fibulin 3 is a member of a family of extracellular matrix proteins characterized by tandem arrays of epidermal growth factor–like domains and a C-terminal fibulin-type module. It is encoded by the EFEMP1 gene, which encodes a 493-amino acid protein with a molecular mass of 55 kd. In adults, fibulin 3 is widely distributed in various tissue types. It is also present in blood vessels of different sizes, and is capable of inhibiting vessel development and angiogenesis both in vitro and in
vivo (17). During development, fibulin 3 is expressed in the mesenchyme, giving rise to cartilage and bone, and plays a role in organizing the development of the skeletal system (18).

Moreover, fibulin 3 is intimately associated with tissue inhibitor of metalloproteinases 3 (TIMP-3), an inhibitor of metalloproteinases involved in the pathogenesis of OA. The interaction between fibulin 3 and TIMP-3 was previously investigated in macular degenerative diseases, and studies have shown that the proteins colocalize in vivo in human placenta sections and form a complex in situ (19–21). Recently, investigators showed that overexpression of fibulin 3 in the clonal murine cell line ATDC5 negatively regulates chondrocyte differentiation (22). The overexpression of fibulin 3 suppressed chondrocyte differentiation by inhibition of cartilage nodule formation, proteoglycan production, and matrix gene expression, and selectively maintained the expression of SOX9 but suppressed the expression of SOX5 and SOX6. However, the role of fibulin 3 in the homeostasis of mature cartilage and in the pathophysiology of OA remains unexplored.

Fibulin 3 levels have been found to be elevated in OA cartilage (23,24). In the present study, we demonstrated that the levels of 2 fibulin 3 fragments, called Fib3-1 and Fib3-2, were increased in urine and serum samples from OA patients compared to healthy controls. Based on these findings, we have developed 2 immunoassays for measuring Fib3-1 and Fib3-2 specifically in serum. These 2 immunoassays have good analytic performance with respect to precision, recovery, linearity, and specificity. Antisera were specific for Fib3-1 or Fib3-2 amino acid sequences and did not cross-react with complete fibulin 3. This clearly indicates that our immunoassay measures fibulin 3 fragments and not native fibulin 3.

In women, a decrease in Fib3-1 concentration was observed with age in the healthy study population. This decrease with aging in women could be explained by the diminution of estrogen levels that occurs with menopause, since it is known that fibulin 3 expression is up-regulated by estrogens (25). Despite the decrease in Fib3-1 concentration with aging, we observed an increase in this concentration in the OA subjects compared to age-matched or younger healthy subjects. This indicates that fibulin 3 turnover is regulated differently in aging than in OA. In contrast, Fib3-2 concentrations remained constant from age 20 years to age 64 years in both healthy men and healthy women. This suggests that the increase in Fib3-2 seen in OA patients is related to the disease and is not a consequence of aging. Fib3-1 and Fib3-2 serum levels were not affected by the extent of the disease. Indeed, no difference was found between patients with unilateral and those with bilateral knee OA. Finally, Fib3-1 and Fib3-2 serum levels were not correlated.

Area under the ROC curve analysis is commonly used to evaluate the ability of a test to discriminate between patients and healthy subjects and is recommended by the National Institutes of Health–sponsored Biomarkers Working Group to classify a biomarker as a diagnostic biomarker (26–28). The accuracy of a diagnostic test is acceptable if the AUC value is >0.75. The area under the ROC curve for serum levels of Fib3-1 and Fib3-2 were 0.751 and 0.838, respectively. A test with an AUC value of 0.80–0.90, as in the case for Fib3-2 serum level, is considered to be good at separating subjects with a disease from those who do not have the disease. The potential of Fib3-2 serum level as a diagnostic biomarker needs to be confirmed in larger cohorts of patients.

Immunostaining of cartilage revealed that Fib3-1 and Fib3-2 were located in the extracellular matrix and cell clusters of the fibrillar zone but were absent in the surrounding unfibrillated zones. This observation indicates that fibulin 3 fragments measured in serum are generated, at least in part, in degraded cartilage. However, the question of whether the increase in fibulin 3 fragments in the serum and urine of OA patients is significant remains. Indeed, fibulin 3 is not cartilage specific, and it is found in other tissue types, including bone. However, our immunohistochemical analysis showed that the concentrations of Fib3-1 and Fib3-2 were not increased in OA subchondral bone, suggesting that fibulin 3 degradation in OA is a process that occurs mainly in cartilage. Further studies are needed to better understand the role of fibulin 3 in the structure and metabolism of cartilage.

The main limitation of this study is the lack of information about the size and the form of the circulating fibulin 3 fragments. Further, we lack information about the role played by fibulin 3 in OA, making the data interpretation speculative. One other limitation of this study is that it was performed using samples from patients with severe, end-stage OA, who are not representative of the general OA population. Further studies investigating the relationship between OA severity and the level of fibulin 3 fragments are required to qualify these biomarkers as burden-of-disease biomarkers.

In conclusion, we have designed 2 immunoassays to quantify specific peptides of fibulin 3 fragments in serum. The concentrations of these 2 specific fragments...
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were not correlated and probably reflected different biologic processes. The levels of one of the peptides, Fib3-1, were associated with aging and hormonal status, while the levels of the other, Fib3-2, were not modified by sex, age, or menopause. Despite these differences in course between the 2 fragments, both were increased in the serum of OA patients and are potentially good biomarkers of OA.

AUTHOR 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. Dr. Henrotin had full access to all of the data and the accuracy of the data analysis.

Study conception and design. Henrotin, Gharbi, De Pauw, Deberg.

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Analysis and interpretation of data. Henrotin, Gharbi, Mazzucchelli, Deberg.

ADDITIONAL DISCLOSURES
Authors Gharbi and Deberg are employees of Artialis SA.

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