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Fib3-3 as a Biomarker for Osteoarthritis in a Rat Model with Metabolic Dysregulation

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Abstract

Objective. Fibulin-3 is a glycoprotein highly expressed in osteoarthritic cartilage and inhibits angiogenesis and chondrocyte differentiation. Recent studies have indicated that fibulin-3 has potential value as a biomarker in osteoarthritis. The aim of the present study is to examine the role of 3 fibulin-3 peptides (Fib3-1, Fib3-2, and Fib3-3) and a type II collagen degradation product in a rat osteoarthritis model with systemic metabolic alterations combined with local cartilage damage. *Design*. Forty, 12-week-old male, Wistar rats were randomly divided over 2 groups: a standard or a high-fat diet inducing metabolic dysregulation. After 12 weeks, articular cartilage damage was induced on the femoral condyles (groove model), in 1 knee joint in 14 rats of each diet group. At endpoint, blood was collected and serum was isolated. Enzyme-linked immunosorbent assay on all selected fibulin-3 fragments was performed from serum samples in addition to immunohistochemical analysis for Fib3-3. *Results*. Serum concentrations of Fib3-3 were increased by 29.9%, when cartilage damage was induced in addition to a high-fat diet. Fib3-3 was also associated with an increased histological total joint degeneration ($r = 0.435$) and cartilage degeneration ($r = 0.435$). Immunostainings demonstrated increased Fib3-3 in the superficial cartilage of animals with high-fat diet and/or cartilage damage. *Conclusions*. In the rat groove model combined with high-fat diet–induced metabolic dysregulation an increased Fib3-3 concentration was observed systemically, which is associated with local joint degeneration. This suggests that systemic Fib3-3 concentrations can indicate the status of joint degeneration and function as a biomarker in osteoarthritis.

Keywords

osteoarthritis; biomarker, articular cartilage, animal model

Introduction

Osteoarthritis (OA) is the most common joint disorder in the middle aged and older population and is characterized by progressive cartilage degeneration.[1,2] By the time the disease is diagnosed with radiographs, joint tissue degeneration is already well established and in most cases considered irreversible.[3] Currently, there are no disease-modifying OA drugs (DMOADs) available for the treatment of OA.[4,5] Therefore, there is a need for biomarkers that can act as an early warning of the initiation of matrix breakdown and provide earlier diagnosis on (progression of) OA.[6-8] A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention and could thereby inform on the prognosis, monitoring, and therapeutic strategies.[9] Fibulin-3 fragments are previously proposed to be a potential prognostic and diagnostic biomarker for OA.[10,11] Fibulin-3 is a member of a family of extracellular matrix proteins, and the fibulin-3 epitopes (Fib3-1, Fib3-2, and Fib3-3) contain a specific sequence of fibulin-3.[3,10] Fibulin-3 is widely distributed in various connective tissues, including blood vessels, bone, ligaments, and cartilage[3] and is important in skeletal development, which inhibits angiogenesis and chondrocyte differentiation.[12,13] Moreover, the overexpression of fibulin-3 suppresses chondrocyte differentiation by inhibition of cartilage nodes formation, proteoglycan production, and matrix gene expression.[14] Fibulin-3 also stimulates certain tissue inhibitor of metalloproteinases (TIMP-1 and TIMP-3), and inhibits the matrix metalloproteinases (MMP2,

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MMP-3, and MMP-9) all involved in the remodeling and pathogenesis of OA.[13]

Type II collagen is the major structural protein in cartilage and collagen degradation is an important feature of cartilage breakdown during OA.[15] Therefore a degradation product of type II collagen can be used as a specific OA biomarker that can assess both disease progression and activity.[16] One of these collagen products is Coll2-1NO_2, the nitrated form of collagen-derived fragments reflecting the oxidative-related cartilage extracellular matrix degradation and is increased in the process of OA.[17] As nitration requires oxidative stress, the concentration of Coll2-1NO_2 may indicate the extent of oxidative stress in articular cartilage and the level of inflammation in the synovium.[18] Elevated levels of Coll2-1NO_2 have been observed in chronic inflammatory conditions, including established OA, but the effect of this marker in preclinical and preradiographic phase of OA is still unknown.[19]

The aim of the present study was to determine the concentrations of 3 fibulin-3 circulating epitopes and Coll2-1NO_2 as a biomarker in a preclinical rat model with a metabolic phenotype induced by a high-fat (HF) diet in addition to local cartilage damage, resulting in an increased inflammatory state and mild OA. This is the first time Fib3-3 was studied in a preclinical model of OA. Furthermore, we investigated if tissue specific changes were associated with the histological changes in this model.

Methods

Animal Model

Forty Wistar rats (12 weeks old, male), were randomly divided over 2 groups: 20 rats were fed a high-fat diet (D12492i, Research Diets, Inc., New Brunswick, NJ, USA) while the other animals received a standard diet (801730, SDS, Essex, UK). After 12 weeks, cartilage damage was induced on both femoral condyles in 1 knee joint according to the rat groove model[20] in 14 rats of each diet group. Remaining animals served as a control group for each diet. A more detailed description of the performed study is given by de Visser *et al*.[21] The study was approved by the Utrecht University Medical Ethical Committee for animal studies (DEC 2013.III.12.086) and ARRIVE guidelines were fully complied with.

Serum Samples to Identify Fibulin Fragments

At endpoint, 24 weeks after the start of the study and 12 weeks postsurgery, blood was sampled from the lateral tail vein in all rats. One hour after collection, the whole blood was separated by centrifugation for a period of 15 minutes at 3000 RCF and serum was collected. All samples were stored in 50-µL aliquots at −80°C on analysis. All serum samples were quantified in duplicate by specific competitive enzyme-linked immunosorbent assays (ELISAs) (Artialis SA, Liege, Belgium). In total, 33 samples were included in the analysis, 7 samples were missing because collected blood volumes were too small. The immunoassays for measuring Fib3-1, Fib3-2, Fib3-3, and Coll2-1NO_2 have been developed using polyclonal antibodies (AS88, AS94, poly33-11, and D37, respectively). All fibulin-3 epitopes did not recognize complete fibulin-3 and did not cross-react between each other.[11] For Fib3-1, Fib3-2, and Coll2-1NO_2, the coating and the competition were made using a bovine serum albumin (BSA)–coupled Fib3-1, Fib3-2, or Coll2-1NO_2 peptide. For Fib3-3, a streptavidin coating was performed and the uncoupled Fib3-3 peptide was used for the competition. Serum samples were diluted 8 times in phosphate buffered saline BSA 0.2% Tween-20 0.1% buffer for Fib3-1 Fib3-2, and Coll2-1NO_2 and 4 times in NaCl 0.9% BSA 7% for Fib3-3. For each fragment, the association with metabolic parameters (total body weight, glucose, total cholesterol, insulin, and homeostatic model assessment–insulin resistance [HOMA-IR]) together with the histological joint degeneration scores[22] were assessed.

Histopathological and Immunohistochemical Examination of the Knee Joint

Joint degeneration was assessed as previously described using the OARSI (Osteoarthritis. Research Society International) histopathology score for rats according the guidelines.[22] Coronal plane sections of paraffin-embedded sections of 5 µm thickness were stained with hematoxylin and eosin to visualize characteristics of inflammation and inflammatory cells and safranin-O to envision cartilage damage and the amount and distribution of the glycosaminoglycans. The parameters of the histologic scoring include cartilage matrix loss width, cartilage degeneration, cartilage degeneration width, osteophytes, synovial membrane inflammation, calcified cartilage, and subchondral bone damage.

Immunohistochemistry for Fib3-3 was performed to visualize cells that express Fib3-3 in the articular cartilage. Antigen retrieval was performed by proteinase-free chondroitinase ABC (0.4 units/L; Sigma) in 0.1 M Tris HCl, 60 mM sodium acetate, pH 8.0, for 30 minutes at 37°C. All sections were blocked for nonspecific binding with 10% normal goat serum (Jackson ImmunoResearch) and 1% BSA diluted in 50 mM Tris, 138 mM NaCl (Tris buffered saline [TBS]), pH 7.6, following antigen retrieval. Next, sections were incubated overnight with primary antibody poly33-11 (1:1000; Artialis SA), diluted in blocking buffer containing 0.1% of Tween-20, at 4°C. Subsequently, the antibody was visualized with horseradish peroxidase conjugated to goat anti-rabbit immunoglobulins (DAKO) 30 minutes at room temperature following a 5-minute conversion of diaminobenzidine (DAKO). Sections were counterstained with Ehrlich's hematoxylin and isotype control staining was carried out.

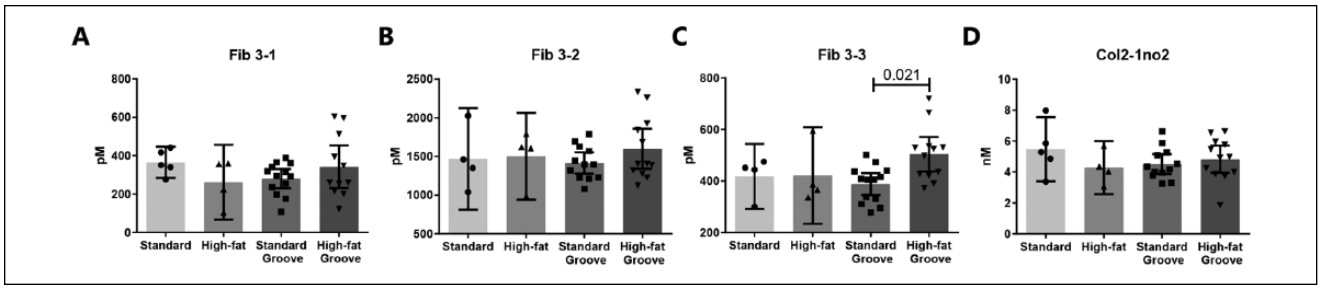

Figure 1. Serum levels of selected biomarkers. Mean serum concentrations at endpoint are presented for Fib3-1 (**A**), Fib3-2 (**B**), Fib3-3 (**C**), and Coll2-1NO$_2$ (**D**) with 95% confidence interval of the mean. Statistical differences were tested by a 1-way analysis of variance with Bonferroni correction.

The articular cartilage of the tibia compartment directly opposite of the induced grooves on the femoral condyles was specifically focused on, as this region was not directly damaged by groove surgery. The total number of positive chondrocytes were counted and expressed as a percentage of the total number of chondrocytes present in the articular cartilage.

Statistical Analysis

The serum concentrations for the fibulin-3 epitopes and Coll2-1NO$_2$ are presented as mean with 95% confidence interval of the mean. Differences between the different study groups were performed by a 1-way analysis of variance with Bonferroni correction. To identify the correlation between the individual serum marker and the histological outcome parameters a Pearson correlation was performed. For immunohistochemistry, data are presented as mean percentage of positive cells with 95% confidence interval of the mean. Differences in activity between study groups were performed by a 1-way analysis of variance with Bonferroni correction (SPSS statistics 21, IBM Corp. Armonk, NY, USA). For all tests, P values <0.05 were considered statistically significant.

Results

Serum Concentrations in the Rat Groove Model of OA

Serum samples collected at endpoint were tested for three different fibulin-3 epitopes (Fib3-1, Fib3-2, and Fib3-3) and Coll2-1NO$_2$. In rats with HF diet–induced metabolic dysregulation without groove surgery, no differences were observed compared with the animals on a standard diet for all selected biomarkers (**Fig. 1**). When cartilage damage was induced in HF diet rats, only a statistical significant increase of Fib3-3 was observed compared with the rats with groove surgery on a standard diet (**Fig. 1**; $P = 0.021$).

Correlation of Biomarkers With Histological Joint Degeneration

Looking at the correlation of serum concentration from the four selected biomarkers with the determined metabolic parameters (body weight, glucose, insulin, HOMA-IR, and cholesterol), only serum values of Fib3-3 showed a borderline significant positive correlation with the total body weight ($r = 0.316$; $P = 0.073$) and an unexpected negative correlation with total cholesterol ($r = -0.503$; $P = 0.003$) at endpoint.

Next, when looking at the correlation of serum values with the histological joint degeneration, again no associations were found for Fib3-1, Fib3-2, and Coll2-1NO$_2$. Interestingly, only for Fib3-3 there was a statistical significant positive correlation with the total joint degeneration as determined by the OARSI score using this model (**Fig. 2A**). Expectedly, this observed correlation was specifically cartilage driven as there was also a positive statistical significant correlation with the histological cartilage degeneration score (**Fig. 2B**). On the other hand, no statistical correlations were observed with the synovial membrane inflammation ($P = 0.279$) or osteophyte formation ($P = 0.297$).

Expression of Fib3-3 in Articular Cartilage of the Tibia

To confirm the local presence of Fib3-3 in the articular cartilage as indicated by the enhanced systemically serum concentrations, immunohistochemical staining for Fib3-3 was performed on paraffin embedded slides of the tibia. Expression of Fib3-3 was present in the superficial zone of the articular cartilage of the tibia opposite of the induced grooves on the femoral condyles in all rats. Besides, there were some positive cells observed in the middle zone, but not in the deep zone, of the articular cartilage in all study groups. The HF diet group showed more positive stained cells compared to the standard diet–fed rats, although not statistically different (**Fig. 3A-C**). Groove surgery in combination to a HF diet resulted in an increased expression

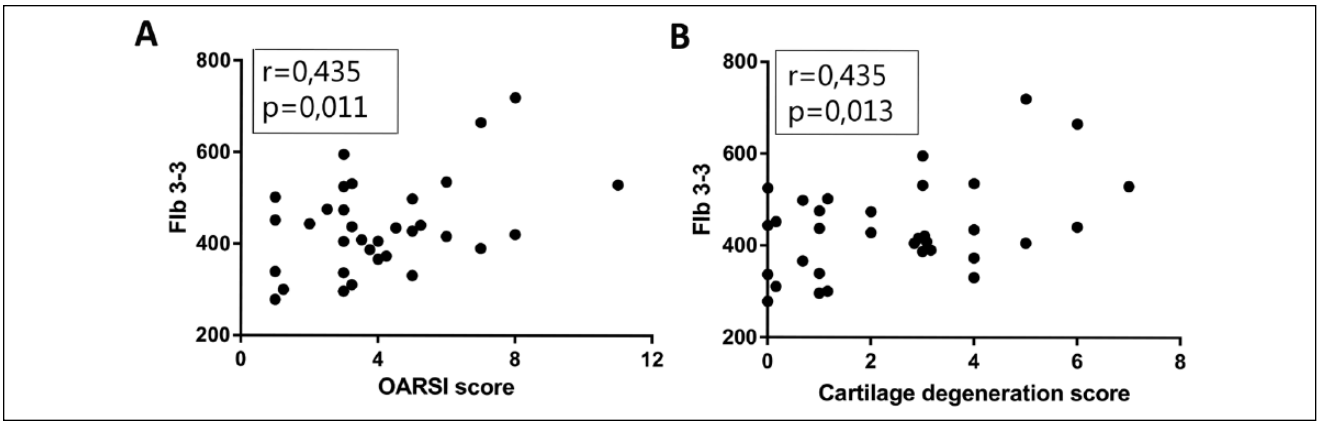

Figure 2. The association between the serum Fib3-3 concentration and histological total joint degeneration, as assessed by the rat OARSI (Osteoarthritis. Research Society International) score (**A**), and the cartilage degeneration score, a subscore of the rat OARSI score (**B**) is presented. Associations were determined by the Pearson correlation.

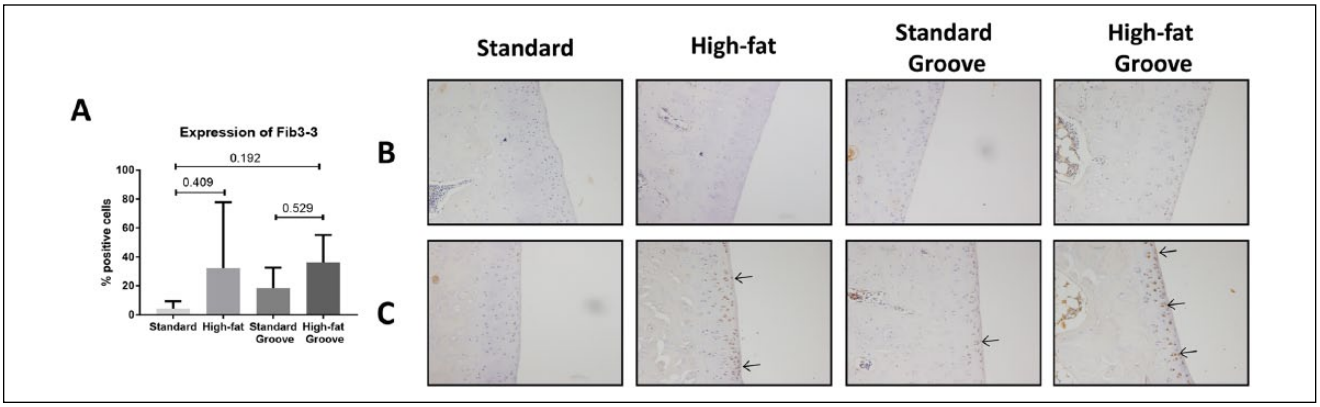

Figure 3. Expression of Fib3-3 on the articular cartilage of the tibia opposite of the surgically placed grooves as determined by immunohistochemistry on paraffin embedded knee joints (**A**). Data are presented as a percentage of positive cells within the articular cartilage of the tibia compartment with 95% confidence interval of the mean. Statistical differences were tested by a 1-way analysis of variance with Bonferroni correction. Representative images of Fib3-3 expression are presented in the articular cartilage of the tibia compartment. The least expression (**B**) as well as the highest expression (**C**) for each study group is presented.

compared with the nonsurgical and surgical rats on a standard diet (**Fig. 3A-C**).

Discussion

The current study shows increased Fib3-3 serum concentrations that was positively correlated with the histological joint degeneration in the rat groove model of OA combined with metabolic dysregulation. This increase in Fib3-3 was not only observed systemically in serum samples, but also locally with increased expression in the chondrocytes mostly in the superficial zone of the articular cartilage. Other epitopes of fibulin 3, Fib3-1, and Fib3-2, as well as the nitrated form of a collagen-derived fragment, the Coll2-1NO_2 marker, were not sensitive to either joint degeneration or metabolic dysregulation.

Fibulin-3 is an extracellular glycoprotein broadly expressed throughout the body and important in skeletal development.[13] Expression is increased in young-adults, while aging results in decreased levels of fibulin-3.[23,24] A knockout model of *Efemp1* (the encoded gene of fibulin-3) in mice causes early onset aging by a disruption of elastic fibers in connective tissues.[25] This could be indicative for the role of fibulin-3 in repair or remodeling processes.

In articular cartilage, fibulin-3 has a similar distribution as lubricin, but does not have the same function, and is involved in cartilage and joint homeostasis.[23,25] Whereas, in the osteoarthritic situation, in both humans and mice, increased levels of fibulin-3 were previously observed.[10,11,23] In a study with middle-aged overweight and obese women, representing the metabolic phenotype of OA, a positive association for fibulin-3 concentrations with the incidence

of clinical knee OA was reported.[11] Limitation of studies solely using serum samples is the lack of tissue specific changes that occur in the process of OA. Another limitation of studying OA in humans is to identify OA patients in its early phase, as diagnosis is only possible when structural changes are already present. Therefore end-stage human OA patients are often compared with healthy individuals.

The use of animal models provides the opportunity to collect and use specific joint tissues, especially in earlier stages of the disease.[26] On the other hand, findings from OA animal models are only translatable to the human situation to a certain extent.[27]

This is the first time the role of Fib3-3 was studied in a preclinical model of OA. The selected animal model, combining the surgical placement of grooves on the femoral condyles with a HF diet, represents the human situation of overweight and obese individuals at risk to develop OA.[21] It was demonstrated that this model resulted in, inflammatory driven, mild joint degeneration as seen in early OA without destabilizing the joint. Although the observed joint degeneration 12 weeks postsurgery is still mild, a distinct increase is observed for Fib3-3 in serum samples. This increase in systemic serum concentration of Fib3-3 is most likely a reflection of the local Fib3-3 expression in the articular cartilage. With specific activity in the superficial zone of the articular cartilage that is triggered by the HF diet–induced metabolic dysregulation, as indicated by immunohistochemistry and the correlation with histological assessed joint degeneration.

The fibulin-3 expression in the articular cartilage was previously reported in the superficial zone in both healthy and aging human cartilage together with OA cartilage in mice.[23] In human end-stage OA, fibulin-3 expression was increased in all zones of the articular cartilage. In our rat model, the observed Fib3-3 expression was restricted not only to the superficial zone, as seen in mice, but was also expressed in the middle zone of the articular cartilage, especially when increased joint degeneration was seen. Although differences were observed in histological joint degeneration when local cartilage damage was induced in addition to a HF diet, no difference in expression of Fib3-3 was observed by immunohistochemistry compared with nonsurgical HF diet controls. This observation could be explained by the fact that a HF diet resulted in increased chondrocyte proliferation and clustering in the articular cartilage, a specific histological observation that is not reflected in the OARSI score of histological joint degeneration.[22] Also, Fib3-3 was observed not only in the articular cartilage but also in the subchondral bone of the tibia compartment (data not shown). This expression was specifically seen in the HF diet rats and could possibly be a marker of subchondral bone remodeling in osteoarthritic joints.

Conflicting results are presented for Coll2-1NO_2, the nitrated form of Coll2-1, in human OA studies. In end-stage human OA increased serum levels of Coll2-1NO_2 are observed whereas in a population of middle-aged overweight and obese women at risk for knee OA, decreased urinary levels are presented.[17,28] It was suggested that Coll2-1NO_2 is directly related to the level of inflammation in the synovium.[17] In the current model, no differences in serum concentrations or correlations with histological joint degeneration were detected for Coll2-1NO_2 in the given time frame. On the other hand, the model only shows minimal joint degeneration as seen in early OA, with limited joint inflammation, and is as such apparently not severe enough to detect specific systemic changes for this marker.

In summary, we showed that Fib3-3, an epitope of fibulin-3, could be detected in the circulation of rats, and increased levels of Fib3-3 were associated with the increased local joint degeneration and cartilage degeneration in knee joints with induced local cartilage damage in combination with HF diet feeding. This increased concentration of Fib3-3 in serum samples is not solely a reflection of the induced systemic metabolic changes by a HF diet, supported by the local expression of Fib3-3 in the chondrocytes of the articular cartilage in the animals on a HF diet and/or induced cartilage damage. This makes Fib3-3 a potential biomarker for OA, that could detect early degenerative changes in joints that are at risk to develop OA.

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Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Y. E. Henrotin is the founder of Artialis SA, a spin-off of the University of Liège.

Ethical Approval

The study was approved by the Utrecht University Medical Ethical Committee for Animal Studies (DEC 2013.III.12.086) and ARRIVE guidelines were fully complied with.

Animal Welfare

The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation.

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