

Fibulin-3 fragments are prognostic biomarkers of osteoarthritis incidence in overweight and obese women



J. Runhaar † ‡^a, C. Sanchez ‡ §^a, S. Taralla ||, Y. Henrotin ‡ §^{*}, S.M.A. Bierma-Zeinstra † ‡ ¶

† Erasmus MC University Medical Center Rotterdam, Department of General Practice, The Netherlands

‡ D-BOARD Consortium, an European Committee FP7 Project, UK

§ Bone and Cartilage Research Unit, Arthropôle Liège, University of Liège, CHU Sart-Tilman, Belgium

|| Artialis SA, Liège, Belgium

¶ Erasmus MC University Medical Center Rotterdam, Department of Orthopedics, The Netherlands

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SUMMARY

Objective: To determine the association between three fibulin-3 peptides and the incidence of radiographic and clinical knee osteoarthritis (OA).

Design: Women between 50 and 60 years, with a BMI ≥ 27 kg/m², free of knee OA, were recruited. Using binary logistic regression, the association between baseline concentration of serum fibulin (Fib)3-1, Fib3-2 and Fib3-3 and incidence of clinical and radiographic knee OA after 30 months of follow-up was evaluated.

Results: Baseline and follow-up measurements were available for 241 women with a mean age of 55.9 ± 3.2 years and mean BMI of 31.7 ± 3.6 kg/m². None of the concentrations of the three Fib3 epitopes were associated with the incidence of medial or lateral joint space narrowing (JSN) ≥ 1.0 mm or the incidence of Kellgren & Lawrence (K&L) grade ≥ 2 after 30 months. All three Fib3 epitopes were associated with the incidence of the clinical and radiographic ACR-criteria and Fib3-1 and Fib3-3 also with chronic pain at follow-up. When adjusted for the other Fib3 peptide concentrations, only Fib3-1 was significantly associated to the incidence of the American College of Rheumatology (ACR)-criteria (OR 3.2 [1.2–8.7]) and chronic pain at follow-up (OR 3.0 [1.2–7.7]).

Conclusions: Baseline fibulin-3 concentrations are associated with the incidence of clinical knee OA among middle-aged overweight and obese women. Therewith, they meet the criteria of a prognostic biomarker according to the BIPED biomarker classification for OA. Further validation of the fibulin-3 epitopes seems warranted in order to better distinguish subgroups of individuals at increased risk for knee OA development.

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Introduction

Osteoarthritis (OA) is a progressive disorder characterized by synovial inflammation, bone remodeling, and degradation of the extracellular matrix of articular cartilage¹. 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.

* Address correspondence and reprint requests to: Y. Henrotin, Bone and Cartilage Research Unit, Arthropôle Liège, University of Liège, CHU Sart-Tilman, 4000 Liège, Belgium. Tel: 32-4-2427727.

E-mail address: yhenrotin@ulg.ac.be (Y. Henrotin).

^a Joint first authorship, based on equal contributions.

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^{2–4}. 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 points⁵. 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.

Originally discovered by proteomic to be increase in OA urine, the fibulin-3 epitopes (Fib3-1, Fib3-2 and Fib3-3) contain a specific sequence of fibulin-3, an extracellular glycoprotein widely

distributed in various connective tissues including blood vessels, bone, ligament and cartilage⁶. Fibulin-3 inhibits angiogenesis and chondrocyte differentiation⁷. More precisely, the overexpression of fibulin-3 suppresses chondrocyte differentiation by inhibition of cartilage nodes formation, proteoglycan production and aggrecan gene expression. Further, the overexpression of fibulin-3 selectively maintains the expression of Sox-9 but suppresses the expression of Sox-5 and Sox-6. Fibulin-3 also interacts with the tissue inhibitor of metalloproteinases (TIMP)-3, which is a matrix bound inhibitor of matrix metalloproteinases (MMPs)⁸. Fibulin-3 is known to be cleaved by MMP-1, -2, -3, -7, -9 and -12⁹.

Using immunoassays for Fib3-1 and Fib3-2, two specific amino acid sequences of fibulin-3, we demonstrated that their levels were increased in the serum of patients with severe knee OA compare to age-matched healthy subjects. Using the area under the receiver operating characteristic curve (ROC) analysis, Fib3-1 and Fib3-2 levels discriminated well normal and OA populations¹⁰.

The aim of this study was to determine the association between three fibulin-3 circulating epitopes and incidence of radiographic and clinical knee OA in middle-aged overweight and obese women, free of radiologic and symptomatic knee OA at baseline, using the PROOF cohort.

Methods

For this study, data of the PROOF study were used. This randomized controlled trial investigated the effects of a diet and exercise program and of oral glucosamine sulphate (double-blind and placebo-controlled) on incidence of knee OA, in a 2×2 factorial design^{11,12}. The PROOF study included middle-aged women (50–60 years) with a BMI ≥ 27.0 kg/m², but free of clinical and radiographic knee OA at baseline. Additional inclusion criteria were: mastering Dutch language, free of major-comorbidities and inflammatory rheumatic diseases, not under treatment for knee complaints, not using walking aids or glucosamine sulphate, and free of contraindications for MRI¹². In short, at baseline semi-flexed posterior-anterior knee radiographs were taken according to the metatarsophalangeal (MTP) protocol¹³ and scored using Kellgren & Lawrence (K&L) criteria¹⁴. Minimal joint space width was measured by visual reading for each tibiofemoral compartment¹⁵ and medial knee alignment angle was assessed for all knees¹⁶. All subjects underwent a physical examination, including determination of body weight and body height to calculate and stratify BMI (≥ 27 but < 30 kg/m² as overweight and ≥ 30 kg/m² as obesity), presence of Heberden's nodes, triceps skin fold (for calculation of fat percentage¹⁷), and waist circumference. Non-fasted serum was collected using a venepuncture and stored at -80°C . A questionnaire was filled in, containing questions on the number of days with knee pain, physical activity level (SQUASH¹⁸), knee complaints, the Knee Injury and Osteoarthritis Outcome Score (KOOS) score¹⁹, menopausal status, ethnicity, and co-morbidities. Using these data, mild symptoms were defined as present when a confirming answer was given to the question 'did you experience knee pain in the last 12 months?', chronic knee pain for an additional confirming answer to 'was the knee pain present on the most days of the last month?', and WOMAC pain, function and stiffness were calculated on a 0–100 scale using the KOOS scores. After 30 months, radiography measurements and the questionnaire were repeated.

Using the serum samples, total cholesterol, glycated hemoglobin (HbA1c) and three fibulin-3 peptides were determined. The sequence of Fib3-1, Fib3-2 and Fib3-3 were ³³¹TCQDINE-CETTNECR³⁴⁵, ³⁷⁷CVCPVSNAMCR³⁸⁷ and ⁴³⁰SGNENGEFYLR⁴⁴⁰, respectively. They were quantified in triplicate by specific competitive Enzyme-Linked Immunosorbent Assays (ELISAs) (Artialis SA, Liège, Belgium). The immunoassays for measuring

Fib3-1, Fib3-2 or Fib3-3 have been developed using polyclonal antibodies (AS88, AS94 and poly33-11, respectively) which did not recognize complete fibulin-3 and did not cross-react between each other. For Fib3-1 and Fib3-2, the coating and the competition were made using a bovine serum albumin (BSA)-coupled Fib3-1 or Fib3-2 peptide. For Fib3-3, a streptavidin followed by the biotinylated-peptide coating was performed and the uncoupled Fib3-3 peptide was used for the competition. The lower detection limit was 232 pM, 482 pM and 500 pM for Fib3-1, Fib3-2 and Fib3-3 assay, respectively. The within-run (intra-assay) precisions and the between-run (inter-assay) precision were below 11%. Linearity was shown by diluting samples serially and comparing the observed values with those expected¹⁰. Serum samples were twice-diluted in a PBS BSA 0.2% tween-20 0.1% buffer, pH 7.2 for Fib3-1 and Fib3-2, and six-diluted in NaCl 0.9% BSA 7% for Fib3-3.

The present study selected all subjects with baseline serum and complete follow-up data available. Baseline concentrations of all fibulin-3 fragments were checked for normal-distribution. For each fragment, the univariate association with age, BMI, menopausal status, years since menopause, fat percentage, waist circumference, total cholesterol, glycated hemoglobin (HbA1c), physical activity level, ethnicity, presence of Heberden's nodes, K&L grade 1 vs 0, varus alignment, history of knee injury, presence of mild symptoms, and the presence of OA in other joints was assessed. Factors with a *P*-value < 0.2 for their association with the fibulin-3 fragment were selected for a multivariable analysis, wherein those with a *P*-value < 0.05 were selected. Subsequently, the associations of the each of the three baseline fibulin-3 concentrations separately and all three together with the incidence of knee OA was determined using logistic regression. Stepwise adjustment was applied: no adjustments in model 1; adjustment for baseline age and BMI in model 2 additional adjustment for significant factors from the multivariable analysis and the randomization groups of the original trial (since data originate from a clinical trial, but were used as cohort data) in model 3. Several outcome measures were used for the incidence of knee OA at follow-up: incidence of medial joint space narrowing (JSN) ≥ 1.0 mm, lateral JSN ≥ 1.0 mm and of K&L ≥ 2 for incident radiographic knee OA and incidence of the radiographic + clinical ACR-criteria²⁰ and of chronic pain as incident clinical knee OA. To enable interpretation of the regression associations, Fibulin-3 peptides concentrations were standardized into z-scores. A z-score indicates the number of standard deviations (SDs) that the original value deviates from the population mean. The serum Fib3-1, Fib3-2 and Fib3-3 levels were logarithmically transformed before standardized into z-scores. Results for the regression analyses were also presented as odds ratios per SD increase in log Fib-3 and their corresponding 95% confidence intervals. Statistical analyses were performed with SPSS 20.0 (Chicago, IL). A *P*-value < 0.05 was defined as statistically significant in all logistic regression analyses.

Results

Immunoassays validation

As previously published, the antisera AS88 and AS94 have a high affinity for Fib3-1 and Fib3-2. Antisera did not recognize complete fibulin-3 or BSA and did not cross-react between Fib3-1 and Fib3-2¹⁰. Poly33-11 is specific for the Fib3-3 epitope without binding entire fibulin-3, BSA or Fib3-2 (Fig. 1). Linearity was shown by diluting serum samples serially and comparing the observed values with the expected values. For Fib3-3 assay, a recovery rate of 89.6% at 1:4, 102.9% at 1:8, 105.1% at 1:12 and 102.4% at 1:16 was observed ($n = 8$ performed in triplicate) when sera were diluted in NaCl 0.9% BSA 7%.

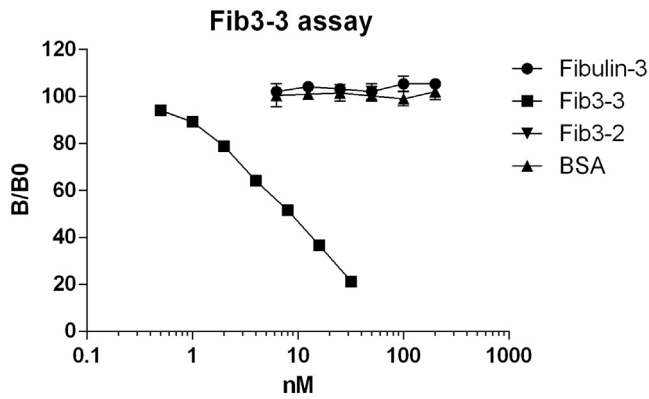


Fig. 1. Competitive inhibition of Poly-33-11 with the fibulin 3 peptides Fib3-3 and Fib3-2, complete fibulin-3, and BSA. B/B0 is the ratio of coated antigen bound in the presence of free antigen to coated antigen bound in the absence of free antigen.

Fibulin-3 epitopes in the PROOF study

Out of all women included in the PROOF study with bilateral K&L <2, 241 had complete follow-up data and a serum sample available and were selected for the present study. Baseline characteristics are given in Table I. Of all baseline factors presented in Table I, only physical activity level was significantly higher in those selected for the present study compared to those excluded (7097 ± 3798 vs 6246 ± 3335 , $P = 0.04$). Incidence of medial JSN ≥ 1.0 mm after 30 months was seen in 26 out of 241 women (11%), while lateral JSN was seen in 21 women (9%). Incidence of K&L ≥ 2 , the ACR-criteria and chronic pain after 30 months occurred in 22 (9%), 20 (8%) and 27 women (11%), respectively.

All three fibulin-3 concentrations showed a non-normal distribution at baseline. Therefore, log-transformed and subsequently Z-

Table I
Baseline characteristics of included subjects ($N = 241$)

	Mean \pm SD or percentage
Age (yr)	55.9 \pm 3.2
BMI (kg/m ²)	31.7 \pm 3.6
WOMAC pain (0–100)	6.1 \pm 10.2
WOMAC function (0–100)	5.9 \pm 9.7
WOMAC stiffness (0–100)	11.6 \pm 17.4
Joint space width	
Medial (mm)	4.87 \pm 0.67
Lateral (mm)	6.19 \pm 0.95
Postmenopausal	70%
Western ethnicity	96%
Heberden's nodes	28%
Unilateral and bilateral K&L 1	15% and 13%
Unilateral and bilateral Varus alignment	24% and 33%
Unilateral and bilateral History of knee injury	19% and 30%
Unilateral and bilateral Mild symptoms	29%
Unilateral and bilateral Total cholesterol (mmol/L)	15% and 14%
HbA1c (mmol/mol)	41%
Fat percentage (%)	24% and 17%
Waist circumference (cm)	6.1 \pm 1.1
Physical activity (SQUASH score)	39.1 \pm 7.1
Western ethnicity	42.9 \pm 4.8
OA in other joints	105 \pm 9
Serum Fib3-1 (nM)	7097 \pm 3798
Serum Fib3-2 (nM)	96%
Serum Fib3-3 (nM)	23%
	13.6 \pm 3.7
	19.7 \pm 8.9
	9.5 \pm 4.5

transformed concentration levels were used throughout the analyses.

Baseline Fib3-1 and Fib3-2 concentrations had only a borderline significant associations with Heberden's nodes ($P = 0.09$) and total cholesterol ($P = 0.06$) respectively in the multivariable analysis. Fib3-3 was significantly associated to HbA1c ($P = 0.02$) and borderline significant to ethnicity ($P = 0.05$) in the multivariable analysis. Hence, model 3 of the Fib3-3 analysis was additionally adjusted for baseline HbA1c level. Log-transformed baseline fibulin-3 concentrations did not differ between overweight and obese individuals (Fib3-1: 2.49 ± 0.01 vs 2.46 ± 0.01 [$P = 0.23$], Fib3-2: 2.49 ± 0.02 vs 2.48 ± 0.01 [$P = 0.62$], Fib3-3: 0.97 ± 0.02 vs 0.92 ± 0.02 [$P = 0.06$] for overweight and obese individuals, respectively).

The associations between the baseline concentrations of the three fibulin-3 epitopes separately and the different incident knee OA measures are presented in Table II and Fig. 2. None of the fibulin-3 epitopes were significantly associated to the incidence of any of the radiographic knee OA measures. Contrary, apart from Fib3-2 and incident chronic pain, all fibulin-3 epitopes were significantly associated to both clinical knee OA measures in the totally adjusted models. Model 3 showed no significant effects for adjustments for randomized intervention groups of the original trial (data not shown). When the analyses were adjusted for each of the baseline fibulin-3 concentrations, only Fib3-1 was significantly associated to incidence of the ACR-criteria (OR 3.21 95% CI 1.19–8.70 in model 3) and chronic pain (OR 3.05 95% CI 1.20–7.71 in model 3). Baseline concentrations of all three epitopes for subjects with (I) and without incidence (NI) ACR-criteria and for chronic pain at follow-up are presented in Fig. 3. The subjects with ACR-criteria incidence had a significant higher Fib3-1 (NI 13.5 ± 3.7 nM vs I 16.1 ± 3.7 nM; $P = 0.007$), Fib3-2 (NI 18.9 ± 6.8 nM vs I 24.1 ± 15.6 nM; $P = 0.0235$) and Fib3-3 (9.4 ± 4.5 nM vs 11.5 ± 4.4 nM; $P = 0.0290$) epitope concentrations at baseline than subjects with non-incidence. Those with a chronic pain incidence had significant higher Fib3-1 (NI 13.5 ± 3.5 nM vs I 16.2 ± 4.8 nM; $P = 0.008$), but not Fib3-2 or Fib3-3, serum levels than those with no chronic pain incidence.

Next, Fig. 4 presents the course of the epitope's concentrations over the 30 months follow-up period for subjects with and without incidence ACR-criteria and chronic pain at follow-up. No significant change of Fibulin-3 epitopes over 30 months was observed; Fib3-1: -0.90 ± 4.25 nM vs -2.51 ± 3.61 nM ($P = 0.19$), Fib3-2: -0.42 ± 6.45 nM vs -1.87 ± 7.754 nM ($P = 0.35$), Fib3-3: -1.4 ± 3.8 nM vs -2.7 ± 3.8 nM ($P = 0.19$) for NI and I of ACR-criteria, respectively. For non-incidence and incidence of chronic pain, epitope concentrations were: Fib3-1: 0.91 ± 4.37 nM vs -2.17 ± 3.33 nM ($P = 0.27$), Fib3-2: -0.42 ± 6.55 nM vs -1.92 ± 5.05 nM ($P = 0.20$), Fib3-3: -1.5 ± 3.8 nM vs -1.9 ± 3.9 nM ($P = 0.62$), respectively.

Discussion

We have designed three immunoassays to quantify specific epitopes of fibulin-3 in serum. As our antibodies fail to recognize the native fibulin-3, we anticipate that these epitopes are located in circulating fragments. Previously, we showed that Fib3-1 and Fib3-2 were not correlated and probably reflected different biologic processes. Fib3-1 was associated with aging and hormonal status, while Fib3-2 was not modified by sex, age, or menopause¹⁰. Immunostaining, revealed the presence of Fib3-1 and Fib3-2 in chondrocytes and in the extracellular matrix of the superficial layer of fibrillated cartilage, but were absent in the surrounding unfibrillated zone. Finally, Fib3-1 and Fib3-2 discriminated well

Table II
Associations (odd ratios with 95% CI) of three fibulin-3 fragments and incident knee OA measures at 30 months

	Incidence	Model 1*	Model 2†	Model 3‡
Medial JSN	11%			
Fib3-1		1.06 (0.61–1.84)	1.08 (0.61–1.91)	1.06 (0.59–1.91)
Fib3-2		0.66 (0.38–1.12)	0.65 (0.37–1.12)	0.65 (0.38–1.12)
Fib3-3		0.89 (0.57–1.37)	0.88 (0.56–1.36)	0.83 (0.53–1.32)
Lateral JSN	9%			
Fib3-1		0.76 (0.41–1.43)	0.71 (0.38–1.35)	0.65 (0.32–1.31)
Fib3-2		0.88 (0.50–1.53)	0.87 (0.49–1.52)	0.89 (0.50–1.58)
Fib3-3		0.98 (0.61–1.57)	0.98 (0.61–1.58)	0.90 (0.54–1.50)
K&L ≥2	9%			
Fib3-1		1.17 (0.68–2.04)	1.27 (0.70–2.30)	1.35 (0.73–2.51)
Fib3-2		1.17 (0.73–1.87)	1.16 (0.74–1.84)	1.20 (0.75–1.92)
Fib3-3		1.12 (0.70–1.78)	1.09 (0.68–1.75)	1.21 (0.73–2.00)
ACR-criteria	8%			
Fib3-1		2.33 (1.26–4.34)	2.29 (1.21–4.35)	2.70 (1.26–5.75)
Fib3-2		1.67 (1.07–2.62)	1.68 (1.07–2.65)	1.72 (1.07–2.78)
Fib3-3		1.69 (1.01–2.82)	1.70 (1.01–2.84)	2.00 (1.10–3.65)
Chronic pain	11%			
Fib3-1		2.26 (1.28–3.99)	2.41 (1.31–4.42)	2.62 (1.31–5.23)
Fib3-2		1.39 (0.92–2.09)	1.39 (0.92–2.08)	1.45 (0.95–2.22)
Fib3-3		1.43 (0.91–2.23)	1.44 (0.92–2.26)	1.72 (1.01–2.91)

Bold figures represent statistically significant odd ratio's.

* Unadjusted.

† Adjusted for age and BMI.

‡ Additionally adjusted for significant factors of multivariable analyses and randomized groups of the original study.

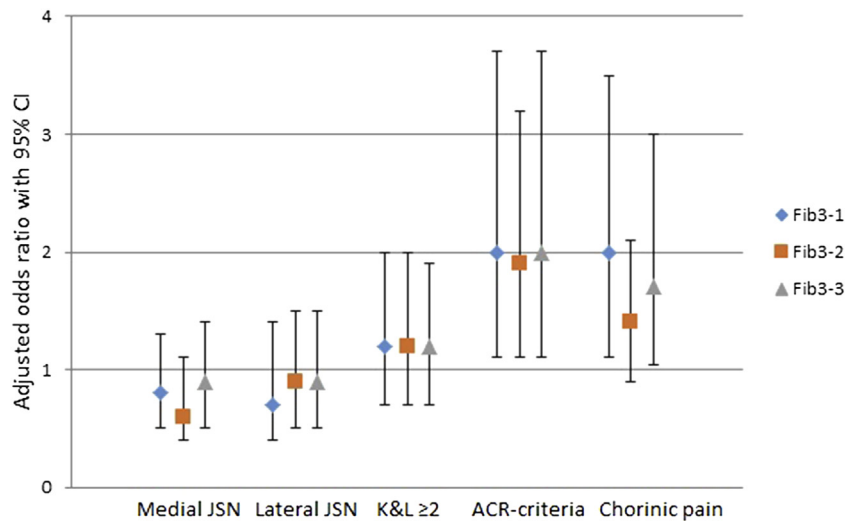


Fig. 2. Odds ratio's and 95% confidence intervals for all three fibulin-3 epitopes and the selected outcome measures.

subjects with knee OA from those who do not have the disease¹⁰. Until now, Fib3-3 was not investigated in animal model or human cohort.

In this study, baseline fibulin-3 epitope concentrations are associated to the incidence of knee OA defined by the radiological and clinical ACR-criteria among middle-aged overweight and obese women. In contrast, fibulin-3 epitopes are not associated with the knee JSN or the K&L score individually. Interestingly, Fib3-1 epitope was also related to chronic pain suggesting a relationship between fibulin-3 epitopes and pain in OA patient. This means that fibulin-3 epitopes predict more clinical than radiologic features in this population. We have then tried to find in the literature a rationale for this association, but fibulin-3 remains unexplored in OA. Therefore, our interpretation is speculative and based on observation in other disease conditions. Fibulin-3 is considered as a key elastogenic protein which control the growth of elastic fibers in ligament. In particular, with other fibulins,

fibulin-3 binds the monomeric form of elastin (tropoelastin) *in vitro* and is shown to be involved in various aspects of elastic fiber development *in vivo*²¹. In OA joint, ligament laxity is commonly observed, in early-stage OA, leading to joint instability and then increased strain on joint tissue²². It's a major risk factor in the development of OA²³. Joint instability is a determinant of pain in OA^{24,25}. Therefore, we can emphasize that a degradation of fibulin-3 is associated with ligament hyperlaxity, joint instability, and subsequently with pain.

Therewith, fibulin-3 epitopes meet the criteria of a prognostic biomarker according to the BIPEd biomarker classification for OA²⁶. Further validation of the fibulin-3 fragments seems warranted in order to better distinguish subgroups of individuals at increased risk for knee OA development. Of course, a better characterization of the circulating fragments are necessary to better understand fibulin-3 metabolism in healthy and OA conditions, but also to better interpret the data provided by the immunoassays.

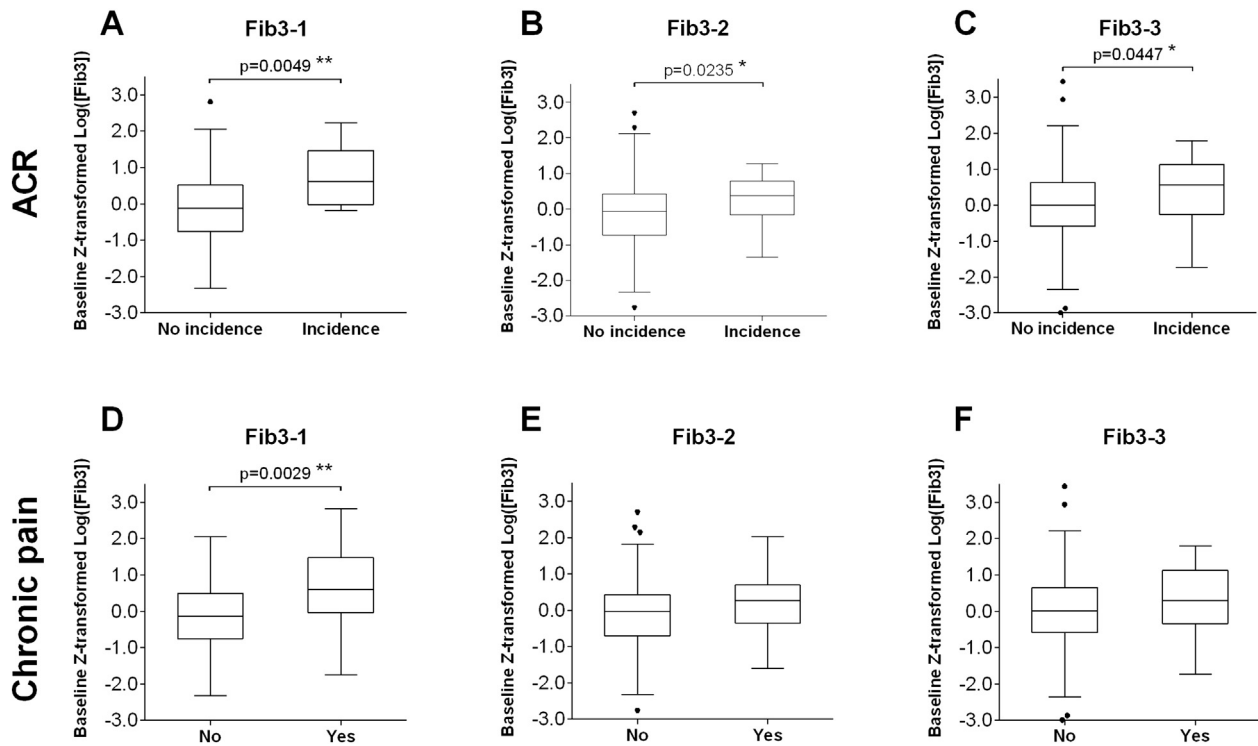


Fig. 3. Mean of log- and Z-transformed baseline concentrations for subjects with and without incidence of ACR-criteria (upper row) and chronic pain at follow-up (lower row) for fib3-1 (panels A and D), fib3-2 (panels B and E) and fib3-3 (panels C and F). Statistical significance between incidence or no incidence with Mann–Whitney test.

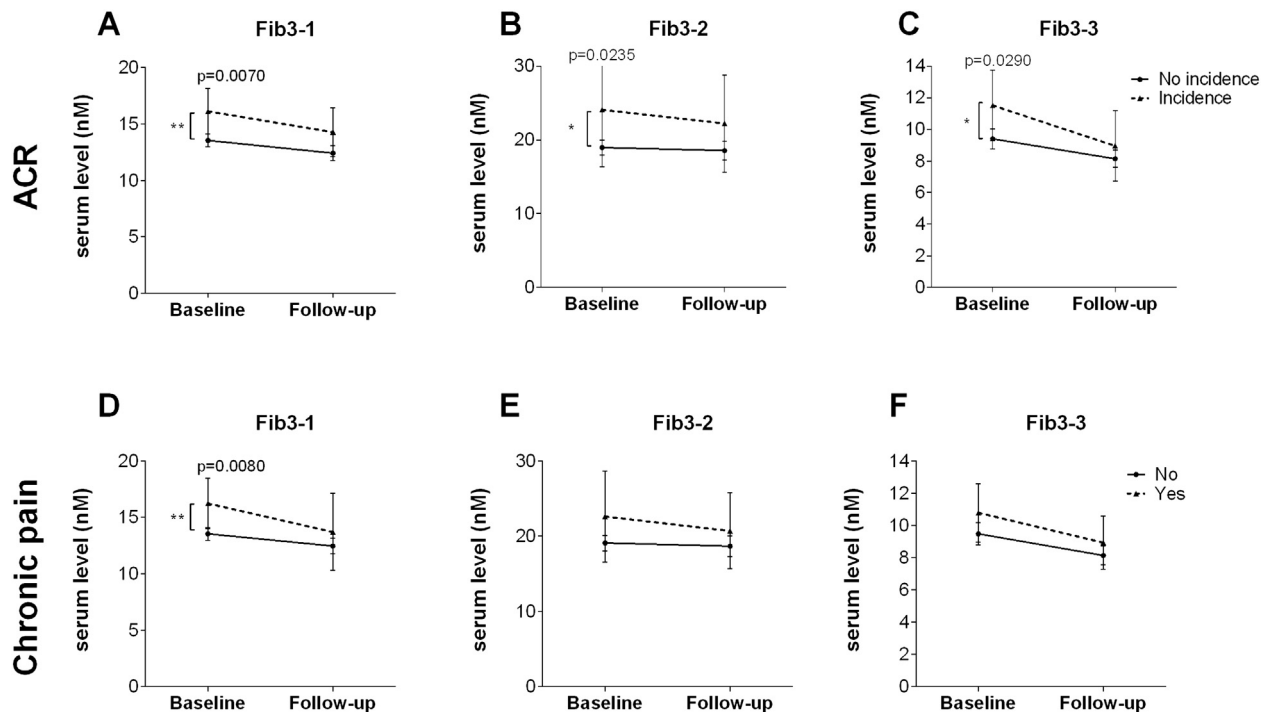


Fig. 4. Mean baseline and 30 months follow-up concentrations with 95% confidence intervals of fib3-1 (in nM, panels A and D), fib3-2 (in nM, panels B and E) and fib3-3 (in nM, panels C and F) for subjects with and without incidence of ACR-criteria (upper row) and chronic pain at follow-up (lower row). Asterisks mark statistical significance for untransformed baseline concentrations between incidence or no incidence with Mann–Whitney test ($P < 0.05$).

Therefore, the main limitation of this study is the lack of information about the size and the form of the circulating fibulin-3 epitopes. Further, we lack information about the role played by fibulin-3 in OA, making the data interpretation speculative. Fibulin-

3 is a ubiquitous protein suggesting that peptides released in the blood stream probably come from different tissues. The tissue specificity of an epitope is not required by OARSI to classify a biomarker in the prognostic biomarkers category. Therefore,

despite this lack of specificity, Fib-3 fragment can be considered as potential prognostic biomarkers. However, we recognize that this lack of tissue specificity could be a major concern to predict disease incidence or progression in a general population developing concomitant diseases. In this study, patients have been selected on the basis of strict criteria, excluding patients with severe vascular diseases and major co-morbidities. Further, Fib-3 peptides did not differ between overweight and obese individuals and are not correlated with the BMI, suggesting that they are not linked to comorbidity like an increased adiposity in these subjects. Therefore, we can consider that Fib fragments changes reflect OA disease condition. One other limitation of this study is that it was performed using samples from overweight and obese women, who are not representative of the general OA population. It is difficult to extrapolate the predictive value of fibulin-3 epitopes to other OA subphenotypes. Further studies investigating the relationship between OA incidence and progression and the level of fibulin-3 epitopes are required to qualify these biomarkers as prognosis biomarkers in general population. Finally, the incidence of OA is low, but common, in this cohort. This means that the number of subjects who developed OA represented a small sample, limiting the power of the statistical analysis. The follow-up of this cohort will allow to increase OA population and perhaps, to confirm these findings.

In this study of overweight and obese middle-aged women at risk for developing knee OA, high serum Fib3-1, Fib3-2 and Fib3-3 levels were significantly associated with increased risk of incidence of symptomatic knee OA 2.5 years later. It is in the early pre-clinical phase that distinguishing subjects who are at risk to develop symptomatic knee OA from those who are not, has the highest priority.

Author contribution

JR contributed to the conception and design of the study including collection and assembly of data, analysis and interpretation of data and critical revision of the article for important intellectual content.

CS contributed to the laboratory work, to the interpretation of data and to the critical revision of the article for important intellectual content.

ST contributed to the laboratory work.

SBZ contributed to conception and design of the study including obtaining of funding, analysis and interpretation of data and critical revision of the article for important intellectual content.

YH contributed to the interpretation of data and to the critical revision of the article for important intellectual content.

All authors approved the final version of the manuscript.

Competing interest statement

Y Henrotin is the founder and president of the university spin-off Artialis sa.

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