Increased serum levels of microfibrillar-associated protein 4 (MFAP4) are not associated with clinical synovitis in rheumatoid arthritis but may reflect underlying cardiovascular comorbidity

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Abstract

Objective

To study circulating MFAP4 in rheumatoid arthritis (RA) and its associations with clinical phenotype.

Methods

Early RA (ERA): 47 patients with newly diagnosed, treatment naïve RA were included. Serum MFAP4, clinical and laboratory disease variables were recorded serially during 12 months of intensive synovitis suppressive treatment. Long-standing RA (LRA): 317 patients participated, all receiving DMARD treatment. Disease activity, autoantibody status, extra-articular manifestations and cardiovascular morbidity were recorded. Paired serum and synovial fluid samples were obtained from 13 untreated ERA patients. Healthy blood donors served as reference points. MFAP4 was quantified by AlphaLISA immunoassay. Univariate, multivariate and mixed effects regression models were applied in the statistical analysis.

Results

ERA: MFAP4 increased from baseline and was significantly elevated at the 12-month follow-up, 17.8 U/l [12.6;24.1] vs. healthy controls, 12.7 U/l [9.5;15.6], p<0.001. MFAP4 did not correlate with joint counts or C-reactive protein. LRA: MFAP4 was increased, 25.9 U/l [20.4;33.7] vs. healthy controls, 17.6 U/l [13.7;21.2], p<0.0001, but did not correlate with disease activity measures or presence of extra-articular manifestations. Notably, MFAP4 correlated inversely with smoking (p<0.0001) and presence of antibodies against cyclic citrullinated peptides (anti-CCP), p=0.005. There was a positive association with systolic blood pressure, p=0.001 and co-occurrence of three cardiovascular events and/or risk factors, p<0.0001. The serum:synovial fluid MFAP4 ratio was 2:1.

Conclusion

MFAP4 increases from diagnostic baseline despite intensive treatment but does not associate with synovitis at early or late stages of RA. Correlation patterns indicate that increased MFAP4 may reflect enhanced RA-related vascular remodelling.

Key words

microfibrillar-associated protein 4, rheumatoid arthritis, disease activity, co-morbidity, extra-articular manifestations

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Introduction

Joint deformities due to fibrous synovial hypertrophy and osteochondral destruction are cardinal characteristics of chronic persistent rheumatoid joint inflammation (1, 2). Extra-articular manifestations develop in around 40% of newly diagnosed RA patients during follow-up for 12 years (3). While the aetiology is unknown there is strong evidence that genetic, epigenetic and environmental effectors are implicated in the pathogenesis (4-6). Besides smoking, prediagnostic or early subclinical lung inflammation and periodontal tissue inflammation are being considered as disease triggers (1). Within recent years, cohort and population studies have shown that RA is associated with increased cardiovascular morbidity and mortality of complex origin, including traditional cardiovascular disease (CVD) risk factors, genetic determinants and systemic inflammation (7-9). The expanding insight into the pathogenesis of RA and the availability of narrowly directed biologic disease modifiers have generated a need for biomarkers reflecting the current state of important joint and extra-articular disease pathways.

Extracellular matrix (ECM) proteins of joints and adjacent structures are targets as well as adaptive defense mediators in inflammatory rheumatic diseases. A growing body of evidence from experimental and clinical studies suggests that the altered remodelling of joint tissues in arthritis can be assessed by measuring soluble molecular by-products released during cartilage, bone and soft connective tissue turnover (10, 11). Recently, microfibrillar-associated protein 4 (MFAP4) has attracted attention as a candidate fibrosis marker (12-15). MFAP4 is considered to be a human homologue of the extracellular 36 kDa microfibril-associated glycoprotein 36, which was first isolated from porcine aorta (16). The N-terminus contains an Arg-Gly-Asp (RGD) sequence, which serves as ligand motif for cellular integrin receptors (16, 17). MFAP4 forms oligomers of 3–6 dimers and has been suggested to be a Ca^{2+}-dependent adhesive protein, which associates with elastin, fibrillin and collagen in the ECM (18). MFAP4 is particularly prevalent in elastin rich tissues (19, 20). MFAP4 is implicated in elastic fibre assembly (18, 21, 22) and smooth muscle cell driven tissue hyperplasia in *e.g.* airways and arteries (17, 23). MFAP4 in serum correlates positively with early liver fibrosis grade as well as established cirrhosis (12) and cardiovascular death (24). Taken together, these findings support that MFAP4 is involved in tissue remodelling responses to various kinds of injury. We hypothesised that MFAP4 in serum or synovial fluid may reflect rheumatoid synovitis activity or RA-related extra-articular pathologies involving elastin rich connective tissues. We opted to study MFAP4 in newly diagnosed and long-standing RA and possible associations with joint inflammation and systemic comorbidity, cardiovascular disease in particular.

Materials and methods

Written informed consent was obtained from all study participants. The trials were carried out in accordance with the Declaration of Helsinki and were-approved by the Regional Scientific Ethical Committees (S-20090051, 19980024 and NCT00209859).

Study populations

• *Early RA (ERA)*

Forty-seven patients aged 18–71 years with newly diagnosed RA according to the 1987 American College of Rheumatology criteria (25) were included during April 1998 through September 1999. All had persistent soft tissue swelling in at least one joint for a minimum of 6 weeks but less than 12 months. Exclusion criteria were a previous history of arthritis, treatment with disease-modifying anti-rheumatic drugs (DMARDs), past or present malignancy, kidney or liver disease, oral or intraarticular glucocorticoid therapy within 4 weeks before inclusion and pregnancy or lactation. Oral methotrexate (MTX) 7.5 mg weekly was initiated at inclusion. During 48 weeks of clinical follow-up and blood sampling at 6-week intervals, MTX dosage was increased to maximum 20 mg weekly aiming at maximal synovitis suppression. Patients with high disease activity who were unable

to await the onset of MTX action were offered prednisolone 7.5 mg orally per day. Joint aspirations were performed as needed, followed by intraarticular glucocorticoid injection. Mild analgesics were allowed on demand.

• *Long-standing RA (LRA)*

From May 2009 through April 2010, 317 patients with long-standing RA were recruited at random among 741 attending the Rheumatology Clinics at Odense University Hospital (n=147), Vejle Hospital (n=159) and King Christian Xth Hospital for Rheumatic Diseases in Graasten (n=11). All participants fulfilled the 1987 American College of Rheumatology classification criteria for RA (25) and were 19–84 years of age, disease duration ≥5 years. Two were on glucocorticoid steroid (GCS) monotherapy, 25 on GCS plus a synthetic DMARD (sDMARD) and 3 on a biologic DMARD (bDMARD). S- or bDMARD monotherapy was administered to 168 and 18 patients respectively. Seventy-four were treated with an sDMARD plus a bDMARD, 18 were on triple therapy with an sDMARD plus a bDMARD and GCS. The s- plus bDMARD combination was unknown in 1 patient. Eight patients were DMARD drug free upon inclusion. Exclusion criteria were pregnancy or lactation, current infection, chronic kidney or liver disease, past or present malignancy with the exception of non-melanoma skin cancer, severe asthma, chronic obstructive pulmonary disease or interstitial lung disease (ILD) requiring treatment.

• *Paired synovial fluid and blood samples*

Pretreatment serum and synovial fluid samples were collected simultaneously from 13 patients with early RA who underwent knee synovial fluid needle aspiration followed by glucocorticoid injection.

Control subjects

Two control groups matched with the ERA and LRA cohorts according to time of blood sampling were included to avoid potential effects of different storage decay on MFAP4. Serum from eighty-six healthy female and male blood donors aged 23–64 years were obtained from the blood bank at Odense University Hospital in 1999 and served as reference for the ERA study population. One hundred and twenty, self-reportedly healthy male and female blood donors aged 20–70 years were recruited in 2006 and served as controls for the LRA cohort. They were evenly distributed according to decades and gender and without current medication besides over the counter drugs.

Table I. Baseline demographic, clinical and laboratory characteristics of patients with early (ERA) and longstanding RA (LRA).

Characteristic	ERA (n=47)	LRA (n=317)	*p*-value LRA *vs*. ERA
Sex (M/F)	14/31	92/225	0.77
Age (years)	55 [45;62]	61 [52;69]	**<0.01**
Anti-CCP (pos/neg)	27/9	213/104	0.34
Smokers / non-smokers	21/24	95/221	**<0.01**
C-reactive protein (mg/l)	18 [10;46]	5 [2;9]	**<0.01**
Swollen Joint Count (28/56)	9 [4;12]	0 [0;1]	**<0.01**
Tender Joint Count (28/56)	6 [2;11]	2 [0;5]	**<0.01**
Visual Analogue Scale - Doctor (0-100 mm)	36 [22;54]	NA	NA
Visual Analogue Scale - Pain (0-100 mm)	49 [25;72]	NA	NA
Visual Analogue Scale - Global (0-100 mm)	53 [27;74]	30 [13;54]	**<0.01**
DAS28-CRP	NA	2.8 [2.2;3.8]	NA
HAQ score (0-3)	0.5 [0.3;1.0]	0.6 [0.1;1.1]	0.74

Results are presented as medians [inter-quartile ranges].
DAS28: Disease Activity Score 28; HAQ: Health Assessment Questionnaire; NA: not assessed.

Outcome measures

• *ERA*

The following disease activity and disability variables were recorded upon inclusion and at each visit for a 12-month follow-up (Table I): Health Assessment Questionnaire (HAQ), Visual Analogue Scales (VAS, 0–100 mm) – pain, physician and global patient assessment, a 56-joint count (tender and swollen joints), antibodies against cyclic citrullinated peptides (anti-CCP) and C-reactive protein (CRP).

• *LRA*

At inclusion, demographic, clinical and laboratory details were obtained (Table I). Disease activity and disability measures included Disease Activity Score in 28 joints (DAS28-CRP), a 28-joint count, serum CRP, HAQ score, Visual Analogue Scales (VAS, 0–100 mm) – global patient assessment, anti-CCP and extra-articular RA manifestations including rheumatoid nodules, interstitial lung disease, vasculitis, episcleritis/scleritis, neuropathy and Sjogren's syndrome. Details regarding cardiovascular co-morbidity were extracted from relevant databases in the National Patient Registry and from hospital databases. Diagnosis codes were selected according to the International Classification of Diseases version 8 (ICD8) from 1980 to 1993 and ICD10 from 1994 to 2010. Diagnosis or data, which were recorded included heart disease, peripheral vascular disease, stroke, use of blood pressure medication or statins, systolic blood pressure (BP), diastolic BP, levels of blood glucose, total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides.

Laboratory analyses

• *Blood biochemistry*

Serum from patients was obtained from non-fasting blood samples. Prior to centrifugation at 2000 g for 15 minutes at 4°C, the blood samples were allowed to clot at room temperature. Following centrifugation, the serum was stored at -80°C until analysis.

Serum MFAP4 was measured using an AlphaLISA immunoassay (Perkin Elmer, USA) as described previously (20). The inter-assay coefficient of variation (cv) was 6.6% and the intra-assay cv was 3.2%. The reference interval for serum MFAP4 in the ERA control population was 6.7-23.5 U/l (2.5 and 97.5 percentiles) with a 95% confidence in-

terval of 6.1;7.5 U/l and 21.2;24.5 U/l for lower and upper limit, respectively. The reference interval for the LRA control population was 8.6-32.5 U/l (95% CI 7.8;9.6 and 26.8;33.7 U/l).

Anti-CCP antibodies and serum CRP (mg/l) were measured according to standard laboratory measures applied in the participating departments.

• *Synovial fluid*

Synovial fluid was stored at -80°C. To reduce viscosity, hyaluronidase treatment (bovine testicular hyaluronidase, Sigma H3884) was performed. Briefly, upon centrifugation for 30 minutes at 400 g, the supernatants were incubated for 4 hours at 37°C with 2 µl hyaluronidase (1 mg/ml in 10 mM TRIS, 10 mM sodium acetate, pH 6.0), to 200 µl synovial fluid or serum and subsequently centrifuged for 10 minutes at 15.000 g. The supernatants were analysed for MFAP4 by AlphaLISA immunoassay.

Statistics

The Mann-Whitney test was applied for comparison between groups. Descriptive statistics concerning demographics and disease variables are presented as medians and inter-quartile ranges in square brackets. Variables were first studied in univariate regression and subsequently in multivariate analyses with adjustment for age, sex and smoking status. In the ERA subset a mixed effects regression model with adjustment for age, gender, smoking and anti-CCP status was applied for analysis of the MFAP4 pattern during follow-up. For normalisation of data we used logarithmic transformation of MFAP4. Potential outliers were identified by calculating z-scores. Five MFAP4 outliers were found in the LRA study population and one in the ERA subset. Outliers, except for those identified during model check of the regression analysis, were included in subsequent analyses. p-values ≤0.05 were considered statistically significant. The statistical analysis was performed using STATA v. 13.

Results

Baseline demographic, clinical and laboratory characteristics in the ERA and LRA subsets are summarised in Table I. The early and long-standing rheumatoid arthritis subsets were comparable with regard to gender distribution, HAQ score and anti-CCP status. The LRA individuals were older, while the proportion of smokers was higher in the ERA subset (p<0.01). Patients with ERA had higher disease activity as reflected by higher numbers of tender and swollen joint counts, VAS global patient assessment score and CRP (p<0.01). Occurrence of extra-articular manifestations and cardiovascular events and/or risk factors is presented in Table II. A total of 86 LRA patients (27%) had clinically detectable extra-articular manifestations. Among these, 76 (24%) had one, while 9 (3%) had two and a single patient had 3 extra-articular manifestations. One hundred and thirty-eight (44%) of the LRA patients presented with one or more cardiovascular events and/or risk factors; 74 (23%) had one, 38 (12%) had two, 18 (6%) had three, 6 (2%) had four and 2 (1%) had five cardiovascular events and/or risk factors.

MFAP4 in ERA

MFAP4 was slightly higher in ERA patients than in healthy controls at baseline, although not reaching statistical significance, 14.0 U/l - IQR [11.4;18.1] *vs*. 12.7 U/l [9.5;15.6], p=0.09. During 12 months of synovitis suppressive treatment, MFAP4 exhibited a temporal increase (Fig. 1) as assessed in a mixed effects regression model where a positive correlation between MFAP4 and time was observed (p<0.001).

By the end of the follow-up period, MFAP4 was significantly higher compared to the control population, 17.8 U/l [12.6;24.1] *vs*. 12.7 U/l [9.5;15.6], p<0.001. Age, gender, smoking and anti-CCP status did not emerge as significant covariates in the ERA study population. MFAP4 did not correlate with CRP, swollen or tender joint counts.

MFAP4 in LRA

MFAP4 was increased in LRA patients, 25.9 U/l [20.4;33.7] compared to healthy control subjects, 17.6 U/l [13.7;21.2], p<0.0001). MFAP4 was significantly higher in anti-CCP negative patients than in anti-CCP positive, 28.8 U/l [23.0;36.1] *vs*. 24.5 U/l - [19.3;32.1], p=0.003.

Table II. Occurrence of extraarticular manifestations and cardiovascular conditions in patients with longstanding RA (LRA).

	Total (%) (n=317)
Extraarticular manifestations	
Rheumatoid nodules	69 (22%)
Episcleritis/scleritis	9 (3%)
Vasculitis	7 (2%)
Sjögren's syndrome	7 (2%)
Interstitial lung disease	5 (2%)
Neuropathy	4 (1%)
Cardiovascular events and risk factors	
Use of blood pressure medication	112 (35%)
Use of statins	49 (15%)
Heart disease	49 (15%)
Stroke	17 (5%)
Peripheral vascular disease	11 (3%)
Systolic blood pressure ≥140 mmHg	113 (36%)
Diastolic blood pressure ≥80 mmHg	176 (56%)
Blood glucose*	4 (1%)
Total blood cholesterol*	199 (63%)
Blood HDL	26 (8%)
Blood LDL*	164 (52%)
Blood triglycerides*	60 (19%)

*Above local reference ranges.
HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

Non-smokers had higher levels of serum MFAP4 compared to smokers, 27.6 U/l [22.3;35.9] *vs*. 22.3 U/l [17.1;29.2], p<0.0001. However, MFAP4 was significantly increased as compared with healthy controls in RA smokers as well as non-smokers, p<0.0001 (Fig. 2). MFAP4 was higher in patients receiving MTX, 26.3 U/L [20.8;35.2] *vs*. those receiving other kinds of synthetic DMARDs, 23.4 U/L [19.2;28.5], p=0.01, but MFAP4 in both subsets was significantly higher than in healthy controls, p<0.0001. MFAP4 did not associate with MTX dose. MFAP4 was significantly elevated in patients with systolic blood pressure above 140 mm Hg compared to lower levels, 28.8 U/l [23.0;36.3] *vs*. 24.9. U/l [19.4;31.7], p=0.001.

With adjustment for age and gender, MFAP4 associated negatively with smoking (p<0.0001) and anti-CCP positivity (p=0.005). Univariate regression analyses did not show significant correlations between MFAP4 and CRP, swollen or tender joint counts, VAS global patient assessment score, HAQ

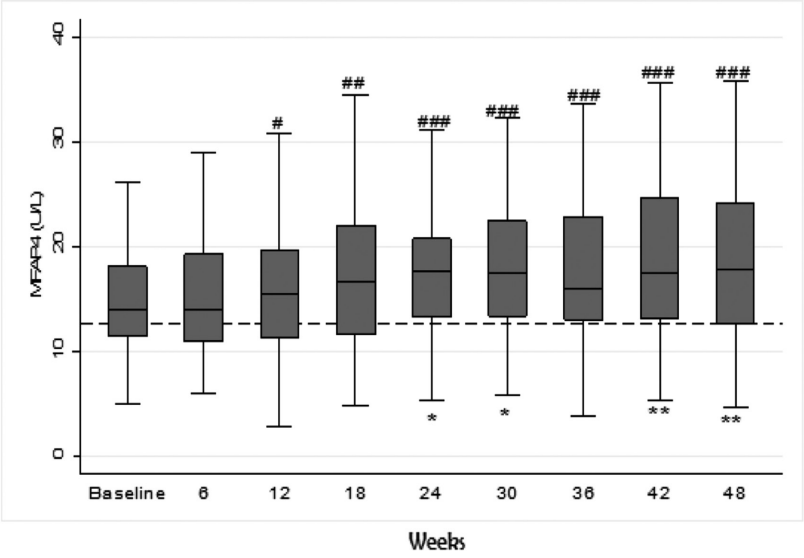

Fig. 1. MFAP4 levels (median – interquartile ranges) during 48 weeks follow-up in the early RA (ERA) study population. Dotted line equals the median MFAP4 in healthy subjects. Outliers are excluded. Using Mann-Whitney test MFAP4 levels in RA patients were compared with baseline: *p<0.05 and **p<0.01 and with controls: $^{\#}p$=0.01, $^{\#\#}p$=0.001 and $^{\#\#\#}p$<0.0001.

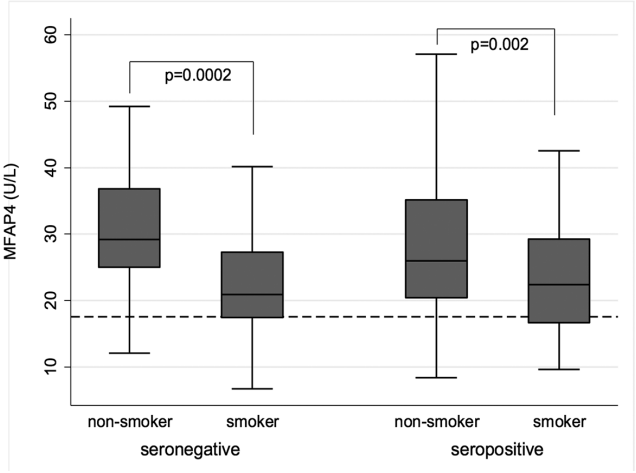

Fig. 2. MFAP4 levels (median – interquartile ranges) according to anti-CCP and smoking status in the long-standing RA study population (LRA). Dotted line equals the median MFAP4 in healthy subjects. Outliers are not depicted.

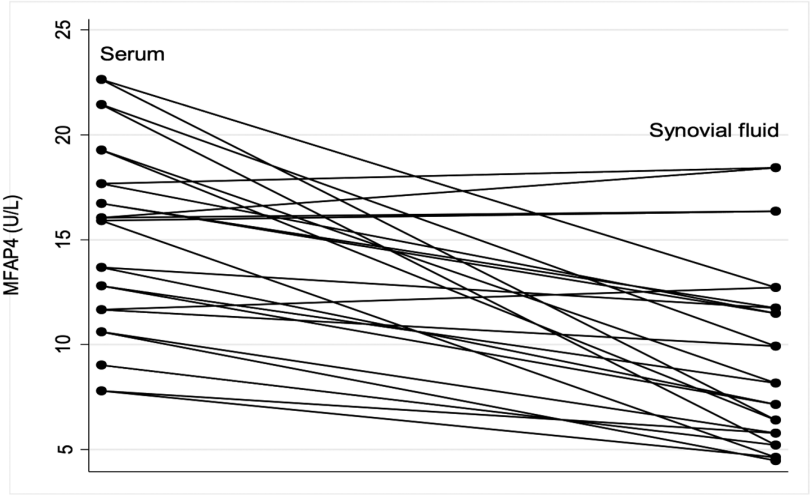

Fig. 3. Pretreatment MFAP4 levels in paired serum and synovial fluid samples from 13 patients with early RA. The serum concentration was twice that recorded in synovial fluid (p=0.007).

score, DAS28-CRP or extra-articular manifestations. Following stratification according to anti-CCP status, no significant correlations were found between MFAP4 and the above mentioned measures or with extra-articular manifestations. Conversely, MFAP4 correlated positively with use of statins (p=0.02), stroke (p=0.08), systolic blood pressure (p<0.001) and levels of HDL cholesterol (p<0.01). When applying multivariate analysis with adjustment for age, gender and smoking, only systolic blood pressure emerged as significant, p=0.001. MFAP4 did not correlate with single or total number of extra-articular manifestations. Similarly, MFAP4 did not correlate with the total number of cardiovascular events and/or risk factors. However, a significant positive correlation was found between MFAP4 and co-occurrence of three cardiovascular events and/or risk factors, p<0.0001. In the long-term storage population (controls for ERA), MFAP4 was 12.7 U/l [9.5;15.6] *vs*. controls for LRA, 17.6 U/l [13.7;21.2], p<0.0001.

MFAP4 in paired serum and synovial fluid samples

Serum MFAP4 was significantly higher than in synovial fluid with a ratio of approximately 2:1; 8.2 U/l [5.8;11.8] *vs*. 16.0 U/l [11.7;17.7], p=0.007 (Fig 3).

Discussion

In this first and exploratory study on circulating MFAP4 in RA we report that the serum concentration is increased at early and late stages of the disease with an incremental pattern during the first year post diagnosis despite DMARD treatment aiming at synovitis suppression. MFAP4 did not correlate with clinical and laboratory disease activity measures or presence of extra-articular manifestations and the synovial fluid level was only half of that in serum. MFAP4 associated positively with systolic blood pressure and co-occurrence of 3 cardiovascular events and/or risk factors, while correlating negatively with anti-CCP and smoking.

Although serum MFAP4 was increased above the level of controls in both ERA and LRA, the absence of associations with core disease variables and the low

synovial fluid concentration do not favor a role for MFAP4 as a sero-marker of clinically detectable RA synovitis. This finding may reflect that MFAP4 is not overexpressed in or released from the synovial membrane in adequate amount to influence the serum level or that the molecule is degraded along with other matrix constituents within the inflammatory infiltrate before reaching the blood stream.

MFAP4 correlated negatively with smoking and anti-CCP status, which are well-documented risk factors in RA (26, 27). The relationship between serum MFAP4 and smoking in RA patients accords with a recent report by Sækmose *et al*. that MFAP4 is decreased in healthy smokers (28). The inverse association between MFAP4 and smoking in patients with LRA may reflect that the structural integrity of elastic tissue is compromised by smoking leading to MFAP4 depletion (29, 30). This is supported by a recent report by Mölleken *et al*. that MFAP4 is not elevated in murine or human lung fibrosis (14). Since severe interstitial lung disease was an exclusion criterion in the present cohorts it is reasonable to assume that the increased MFAP4 levels recorded in both RA cohorts are not due to confounding fibrotic lung disease.

Cardiovascular co-morbidity is a leading cause of premature death in rheumatoid arthritis (31). While MFAP4 did not associate with single cardiovascular events and/or risk factors or extra-articular manifestations in RA, serum MFAP4 was significantly elevated in patients with systolic blood pressure exceeding 140 mm Hg as compared with lower levels and correlated positively with co-occurrence of any three cardiovascular events and/or risk factors. Available evidence indicates that traditional risk factors as well as systemic inflammation and anti-CCP positivity predispose to RA related cardiovascular disease (9). Vascular smooth muscle cells are a major source of arterial MFAP4 (20, 23). Although the MFAP4 response to systolic blood pressure in otherwise healthy subjects has not been studied, the higher MFAP4 level in hypertensive LRA compared with normotensives may reflect an increased arterial wall vulnerability to pulsatile pressure leading to vascular smooth muscle cell activation and increased MFAP4 expression as has been reported for collagen in hypertensive patients (32, 33). Anti-CCP antibody positivity is by most authors considered to be a risk factor for cardiovascular morbidity in RA (26), *e.g*. by causing endothelial dysfunction independent of other CVD risk factors (34). Thus, the lower MFAP4 in anti-CCP positive versus seronegative LRA patients and the negative correlation between MFAP4 and anti-CCP positivity may reflect that anti-CCP antibodies suppress MFAP4 production as has been reported for cartilage type II collagen regeneration in RA (35) or that these pro-inflammatory autoantibodies enhance vessel wall elastolysis and collateral MFAP4 depletion.

Some critical issues need to be considered. The two RA cohorts were recruited with 10-year intervals implying a risk of systematic error due to different epitope decay. In order to address this concern, we used separate control groups that were matched with the ERA and LRA cohorts according to time of blood sampling. MFAP4 was higher in RA patients currently receiving MTX versus treatment with other DMARDs. However, MFAP4 was increased above control levels in both treatment subsets and there was no correlation between MFAP4 and MTX dose. Although clearly relevant, we did not consider including parallel measurement of markers of endothelial dysfunction or vessel wall remodelling in this first study on MFAP4 in RA. Strengths of the study include its combined prospective and cross-sectional design comprising well characterised RA patients at early and late stages. Moreover, MFAP4 has not previously been studied in RA, and the size of the study populations enabled us to do stratifications according to core disease variables.

To conclude, this study does not support a role for MFAP4 as a useful sero-marker for clinical synovitis in early or late stage RA. The incremental serum levels from diagnostic baseline independent of disease activity or treatment and the correlation pattern with CVD risk factors may suggest that elevated MFAP4 in RA patients reflects enhanced myomedial remodelling in elastic artery walls. Further studies are needed to elucidate the potential of MFAP4 as a risk indicator for cardiovascular morbidity in RA.

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