

RESEARCH ARTICLE

Synovitis biomarkers: *ex vivo* characterization of three biomarkers for identification of inflammatory osteoarthritis

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Abstract

Objective: Characterize biomarkers measuring extracellular matrix turnover of inflamed osteoarthritis synovium.

Methods: Human primary fibroblast-like synoviocytes and synovial membrane explants (SMEs) treated with various cytokines and growth factors were assessed by C1M, C3M, and acMMP3 in the conditioned medium.

Results: TNF α significantly increased C1M up to seven-fold ($p=0.0002$), C3M up to 24-fold ($p=0.0011$), and acMMP3 up to 14-fold ($p<0.0001$) in SMEs. IL-1 β also significantly increased C1M up to five-fold ($p=0.00094$), C3M four-fold ($p=0.007$), and acMMP3 18-fold ($p<0.0001$) in SMEs.

Conclusion: The biomarkers C1M, C3M, and acMMP-3 were synovitis biomarkers *ex vivo* and provide a translational tool together with the SME model.

Keywords

Neo-epitope biomarkers, personalized medicine, synovial membrane

History

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Introduction

Osteoarthritis (OA) is a painful degenerative joint disease, which can be divided into different phenotypes dependent on different drivers of disease. One phenotype of OA involves synovial inflammation, which results in an altered turnover of the extracellular matrix (ECM) of the joint and with a possible faster progression of OA (Ayril et al., 2005; de Lange-Brokaar et al., 2012). As patients with different OA phenotypes may respond differently to treatment, development of biomarkers for use in personalized medicine is needed (Karsdal et al., 2014). One method to assess and profile an inflammatory OA phenotype could be to apply serological biomarkers (Siebuhr et al., 2014). Consequently, there is a need for well-characterized biomarkers, as well as translational *in vitro* or *ex vivo* systems that can apply such biomarker tools from *in vitro* to man. As synovial inflammation (synovitis) is a common denominator of inflammatory

arthritis, biomarkers assessing synovitis are potential biomarkers of inflammatory OA. However, currently there is a lack of synovitis biomarkers and translational models for the investigation of direct tissue turnover and tissue-related effects of for example novel anti-inflammatory treatments.

The synovial membrane is normally a thin membrane consisting of a lining and a sublining layer of cells. The lining consists of 1–3 layers of fibroblast-like synoviocytes (FLS) and macrophage-like synoviocytes (MLS) that overlay the sublining (Smith, 2011). In the healthy synovium, the FLS express Cadherin-11 (Cad-11) as the only cell type, whereas MLS express CD14 and CD68 (Chang et al., 2011; Daghestani et al., 2015; Pessler et al., 2008). The lining layer is composed mainly of type III collagen, while the sublining consists mainly of type I collagen (Fox & Warnock, 2011; Smith, 2011). In synovitis, the FLS in the lining layer becomes hyperplastic and the sublining is infiltrated with immune cells (Beffa et al., 2013; Noss & Brenner, 2008). The immune cells activate the MLS, which among others produce tumor necrosis factor α (TNF α) and interleukin (IL)-1 β , which are potent activators of FLS (Bondeson et al., 2006; Scanzello & Goldring, 2012). Both FLS and MLS have been shown to play key roles in driving the synovial inflammation, both able to stimulate inflammation and affect the ECM turnover, through the secretion of cytokines, chemokines, and matrix metalloproteinases (MMPs) (Blom et al., 2007; Bondeson et al., 2006; Juarez et al., 2012; Kiener et al., 2010; Tian et al., 2013). However, whether FLS or MLS alone is the main driver of synovitis or they together drive the inflammation is unknown.

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Connective tissue turnover can be quantified by the neo-epitope biomarkers C1M and C3M, which are MMP-derived degradation fragments of collagen types I and III, respectively (Barascuk et al., 2010; Leeming et al., 2011). C1M and C3M have previously been demonstrated to be elevated in serum from rheumatoid arthritis (RA) patients and reduced in serum of RA patients receiving tocilizumab in combination with methotrexate (Bay-Jensen et al., 2014; Siebuhr et al., 2013). Furthermore, both C1M and C3M were found elevated in serum of patients with inflammatory OA (Siebuhr et al., 2014). These results indicate that circulating C1M and C3M can be used as biomarkers for synovitis. However, whether C1M or C3M can be directly released from the synovium remains to be investigated. C1M and C3M are generated mainly by MMP-2 and -9 (Barascuk et al., 2010; Leeming et al., 2011). MMP-3 has previously been associated with activated FLS in traditional tissue culture settings and is together with MMP-2 the main activator of MMP-9 (Bottini & Firestein, 2012; Pasternak & Aspenberg, 2009). Despite data from traditional cultures, there are limited data on the active form of MMP-3 in *ex vivo* cultures. FLS are traditionally cultured either on plastic or on a simple two-dimensional ECM coating. This two-dimensional matrix is very different from the complex natural ECM environment, whereas *ex vivo* cultures in three dimensions reflect the *in vivo* state more accurately (Hansen et al., 2015). A biomarker of the active form of MMP-3 (acMMP3) has recently been developed and is thought to be a biomarker of synovitis. acMMP3 is generated when the pro-peptide of MMP-3 is cleaved off, and has been shown to be released *ex vivo* from human OA synovial membrane explant (SME) cultures (Sun et al., 2014), indicating this biomarker as a biomarker of synovitis.

In this study, we investigated if the three biomarkers C1M, C3M, and acMMP3 were produced and released from activated FLS *in vitro* and *ex vivo*, which would further strengthen their role as biomarkers of synovitis. We tested this by adding cytokines and growth factors, which have been shown to be present in the inflamed joint, to two model systems: (1) an *in vitro* model with primary human FLS cultured on collagen and (2) an *ex vivo* model with human OA SMEs.

Methods

Human tissue

Synovial membrane biopsies used for the isolation of FLS and synovial tissue culture were collected from OA patients undergoing total knee replacement at Gentofte Hospital, Denmark. This was approved by the Danish scientific ethical commission (H-D-2007-0084) and all patients gave written informed consent prior to surgery.

Fibroblast-like synoviocytes isolation

FLS were isolated from synovial membrane biopsies. Initially, the visible fat tissue was removed manually using a scalpel, the synovial tissue was then washed in phosphate-buffered saline (PBS) and cut into small pieces. The minced synovial tissue was then incubated for 2 h in Roswell Park Memorial Institute (RPMI)-1640, 1% penicillin and streptomycin (P/S), 10% fetal calf serum (FCS), and 1 mg/mL collagenase type 2

(Worthington, Irving, TC, LS004176) (20 mL/g synovial tissue) at 37 °C, 5% CO₂. Afterwards, the digested tissue was filtered through a 70-µm cell strainer and centrifuged for 5 min at 220g. The cell pellet was re-suspended in RPMI-1640, 1% P/S, 10% FCS. Cells that had adhered to the flask over night were cultured for three passages before use. The morphology of the FLS was assessed in light microscopy during isolation and culture with no changes observed. Additionally, FLS was stained for Cad-11 using immunofluorescence after 14 d of culture with >95% positive stain indicating the cultured cells were homogenous. Cells at passage three to six was used for experiments.

Cell and tissue culture

The isolated FLS were plated 20 000 cells/well on human fibroblastic collagen type I and III (Sigma Aldrich, St. Louis, MO, C2249) coated 96-well plates 1 d prior to experiment and cultured for 14 d in Dulbecco's Modified Eagle Medium (DMEM)-GlutaMAX™, 1% P/S and 2% FCS. The FLS were untreated (w/o) or treated with 10 ng/mL TNFα, IL-1β, Oncostatin M (OSM), insulin growth factor-1 (IGF-1), transforming growth factor β-2 (TGFβ-2), or 1 ng/mL IL-6.

Synovial membranes for tissue culture were cleaned for visible fat tissue as described above and cut into SMEs of 30 ± 2 mg. Synovial membranes from a total of seven patients were used with 1–4 technical replicates per patient depending on the size of the biopsy. For each experiment, a total of six replicates per treatment were included. The SMEs were either used for experiment directly or pre-incubated for 2 d before the addition of treatment. They were cultured for 21 d in DMEM-GlutaMAX™, 1% P/S and treated with 10 ng/mL TNFα, IL-1β, TGFβ-2 or left untreated (w/o). As negative control, metabolically inactivated (MI) SMEs were included. The SMEs were metabolically inactivated through three repeated freeze and thaw cycles in liquid nitrogen and 37 °C water bath.

Conditioned media were collected and new medium with treatments was added every 2nd and 3rd day for both isolated FLS and SMEs. The conditioned media were used for biomarker measurements, western blot, and gelatin zymography.

Viability

The Alamar blue assay (Life Technologies, Carlsbad, CA, DAL1100) was used to determine the FLS and SMEs metabolic activity. FLS's metabolic activity was measured on the last day of experiment, while SMEs' metabolic activity was measured once a week. FLS or SMEs were incubated with 10% Alamar blue in medium for 1.5 h (SME) or 2 h (FLS) at 37 °C, 5% CO₂. Hereafter the conditioned Alamar blue medium was transferred to a black 96-well plate and the fluorescence were read at 540 nm with 590 nm as a reference. Four wells without cells or explants were included every time as background; this background was subtracted from the remaining measurements.

Histology

SMEs were fixated in 4 % formaldehyde after 21 d of culture. The SMEs were then dehydrated using the Sakura Tissue Tek VIP E150 followed by paraffin embedding. The SMEs were

cut into 5 μm thick sections (Microm HN 355/360). The slides were stored at 4 °C until use. The sections were stained with hematoxylin and eosin.

Biomarker measurements

Three in-house neo-epitope biomarkers, C1M, C3M, and active MMP-3 (acMMP3), were measured in the conditioned media with competitive enzyme-linked immunosorbent assay (ELISA) as described previously (Leeming et al., 2011; Sun et al., 2014; Veidal et al., 2010). In short, each biomarker was measured on a 96-well streptavidin coated plate. The plate was coated with the appropriate biotinylated synthetic peptide for 30 min at 20 °C in an optimized assay buffer. A volume of 20 (C1M and C3M) or 30 μL (acMMP3) of conditioned media together with 100 μL of optimized assay buffer containing a monoclonal antibody specific for the antigen was incubated 1 h at 20 °C (C3M) or overnight at 4 °C (C1M and acMMP3). After each incubation step, the plate was washed five times in TBST (25.5 mM Tris-Base, 50 mM NaCl, 0.03% Bronidox L5 and 1 mM Tween 20). For visualization, 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) (C1M and C3M) or TMB sense (acMMP3) was added and incubated for 15 min. The reaction was stopped by adding 100 μL of 0.1 M H_2SO_4 to the plate. The absorbance was measured at 450 nm with 650 nm as a reference. The concentration was calculated from a standard curve plotted using a 4-parametric mathematical fit model. Values measured below the detection limit of the assay were assigned the lower limit of detection (LLOD).

Western blot

Secretion of MMP-3 (all forms of MMP-3) was detected in conditioned media with western blot. Equal volume of conditioned media was run on a 4–12% Bis-Tris gel. The separated proteins were transferred to a nitro cellulose membrane using the iBlot[®] Dry blotting system (Invitrogen, Waltham, MA, IB1001) according to the direction of the manufacturer. The membrane was blocked in 5% skimmed milk in TBST for 1 h at room temperature (RT). MMP-3 was detected with incubation with anti-MMP-3 (Abcam, Cambridge, UK, Ab38916) in 5% skimmed milk in TBST overnight at 4 °C. The membrane was washed three times in TBST and incubated with HRP-conjugated anti-rabbit (Jackson, West Grove, PA, 111-035-003) diluted in 5% skimmed milk for 1 h at RT, followed by another three washes in TBST. The final visualization was done in two different ways: (1) using light sensitive films and (2) using digital imaging. For both methods, GE Amersham[™] ECL western blotting system was used according to the direction of the manufacturer. For method (1), the membrane was exposed to Amersham hyperfilm[™] ECL for 1 min and developed on a film processor from 3 M Health Care. For method (2), the digital imager C-Digit from LI-COR was used to digital process the light emission.

Gelatin zymography

Detection of MMP-2 and MMP-9 was performed with gelatin zymography. An equal volume of conditioned media was run

on a SDS-PAGE with 4% stacking gel and 10% separating gel with 0.2 mg/mL gelatin. The gel was rinsed once in washing buffer (0.1 M Tris Base, 13.3 mM CaCl_2 , 0.35 μM ZnCl_2 , 6.2 mM sodium azide, pH 7.5) and incubated in incubation buffer (washing buffer with 1% Triton X-100) for 2 d at 37 °C. The gel was stained for 15 min with comassie blue, destained 1 h with fast destaining buffer (20% methanol, 16.6% ethanol, 7% acetic acid, and 0.6% diethyl ether). If destaining was not sufficient, the gel was destained further for up to 24 h with 7% acetic acid.

Statistics

Statistical differences between w/o and cytokine treatments were tested using one-way analysis of variance (ANOVA), with Dunnet's multiple test in GraphPad Prism Version 6.03 (GraphPad Software, Inc., San Diego, CA).

Results

Isolated FLS on collagen were not able to generate C1M

We tested whether FLS could generate C1M *in vitro* using isolated FLS from OA synovial membranes. The FLS were plated on type I and III collagen-coated plates to provide collagen substrate to generate C1M. The FLS were stimulated with six different cytokines and growth factors (TNF α , IL-1 β , OSM, IGF-1, TGF β -2, or IL-6) to investigate, which stimulus would induce the release of C1M. We found that the FLS were metabolically active after 14 d of culture when comparing the w/o group to the blank control (no cells) ($p < 0.0001$) (Figure 1). FLS stimulated with TNF α , IL-1 β , or TGF β -2 were significantly more metabolically active than w/o ($p < 0.0001$) (Figure 1). OSM, IGF-1, and IL-6 did not affect the metabolic activity of the FLS compared with w/o after 14 d of treatment (Figure 1). Immunofluorescence staining against Cad-11 showed that FLS treated with TNF α , IL-1 β , and TGF β -2 increased in proportion to the number of cells after 14 d in culture (data not shown).

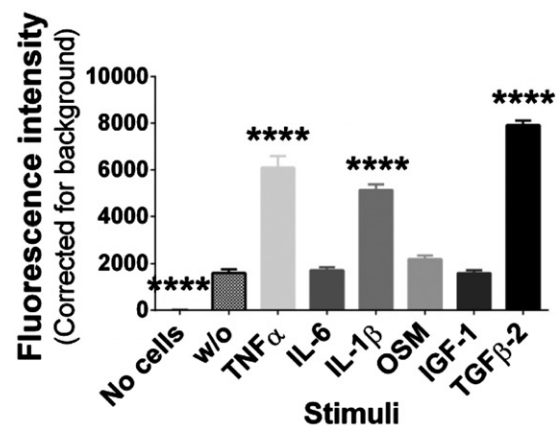


Figure 1. TNF α , IL-1 β , and TGF β -2 increased FLS metabolic activity *in vitro*. The FLS metabolic activity was determined with Alamar blue after 14 d of culture. The FLS were untreated (w/o) or treated with 10 ng/mL tumour necrosis factor α (TNF α), interleukin (IL)-1 β , oncostatin M (OSM), insulin growth factor-1 (IGF-1), transforming growth factor β -2 (TGF β -2), or 1 ng/mL IL-6. Wells with no cells were included as negative control. Data shown is pooled data from three independent experiments with FLS from three different patients. Data are presented mean \pm SEM. **** $p < 0.0001$.

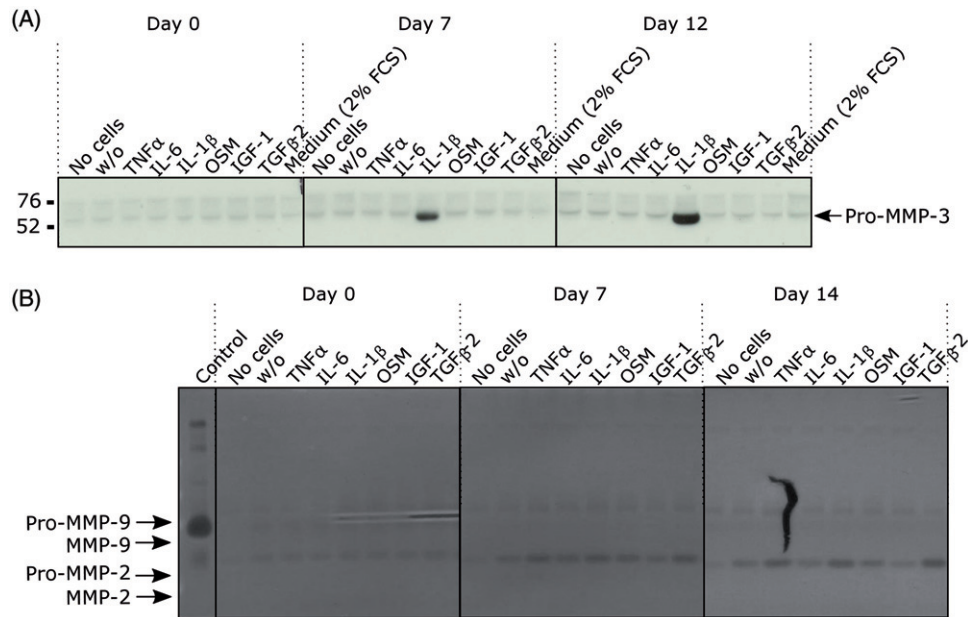


Figure 2. MMP-2, -3 or -9 were secreted by FLS, but not in the active form. Conditioned media from primary FLS untreated (w/o) or treated with 10 ng/mL TNF α , IL-1 β , OSM, IGF-1, TGF β -2, or 1 ng/mL IL-6 from days 0, 7, and 14 were used to detect the secretion of total MMP-3 with western blot (A) and total MMP-2 and -9 with gelatin zymography (B). (B) Control: bovine cartilage explants treated with 10 ng/ml oncostatin M and 20 ng/ml TNF α for 17 d used as ladder, as this is known to express both MMP-2 and MMP-9 (Sondergaard et al., 2006).

C1M was measured in the conditioned media from the primary FLS collected at day 0, 5, 9, and 14. No measurable levels of C1M were detected at any time points or with any of the treatments (data not shown).

Western blotting showed that IL-1 β increased the secretion of pro-MMP-3 after 7 d of treatment, but no band for the active MMP-3 was detected (Figure 2A). A band of \sim 76 kDa was detected in all conditions including the medium containing 2% FCS and the negative control (no cells), indicating unspecific binding to a protein in the FCS (Figure 2A). Additionally, no bands for active MMP-2 and -9 were detected in the gelatin zymography. However, TNF α , IL-1 β , and TGF β -2 tended to increase the secretion of pro-MMP-2 after 14 d of culture (Figure 2B). The amount of pro-MMP-9 detected was the same in all conditions including the negative control (no cells) indicating that secreted pro-MMP-9 could not be detected in FCS (Figure 2B). Because the activated FLS only secreted the pro-forms of MMP-2 and -3 and not the activated form, it was not possible to use the *in vitro* FLS model to characterize C1M.

In contrast synovial membrane explants secrete and activate MMPs

To investigate whether *ex vivo* conditions would generate active MMPs, we used SMEs from a total of seven patients. These SMEs were generated from synovial membrane biopsies from OA patients undergoing total knee replacement. Figure 3(A) illustrates the variation of the biopsies which can vary in size and amount of visible fat. To limit the amount of fat in the individual SMEs, excess visible fat was removed using a scalpel. An individual SME was then cut from the cleaned synovial biopsy and weight, before added into a 96-well plate for culturing (Figure 3B and C). In culture, we

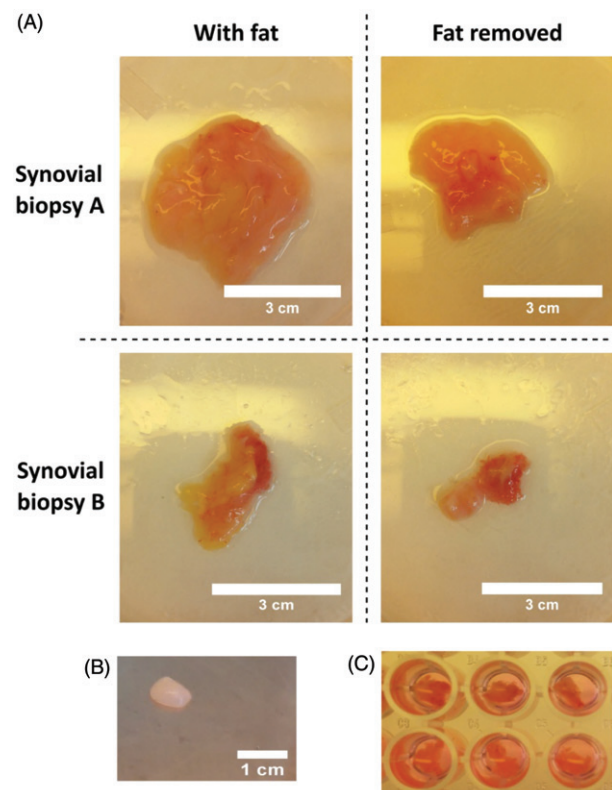


Figure 3. Preparation of synovial biopsies to synovial membrane explants. (A) When synovial biopsies are received from the hospital the fat is initially removed with a scalpel. Here are two biopsies from two different patients (A and B) showed before and after the fat was removed. Scale is 3 cm. (B) Small explants of 30 ± 2 mg are cut with the scalpel and the wet weight determined after the fat is removed. Scale is 1 cm. (C) Cut explants are transferred directly to a well containing culture media. Before start of the experiment the explants are washed 3 times in culture media.

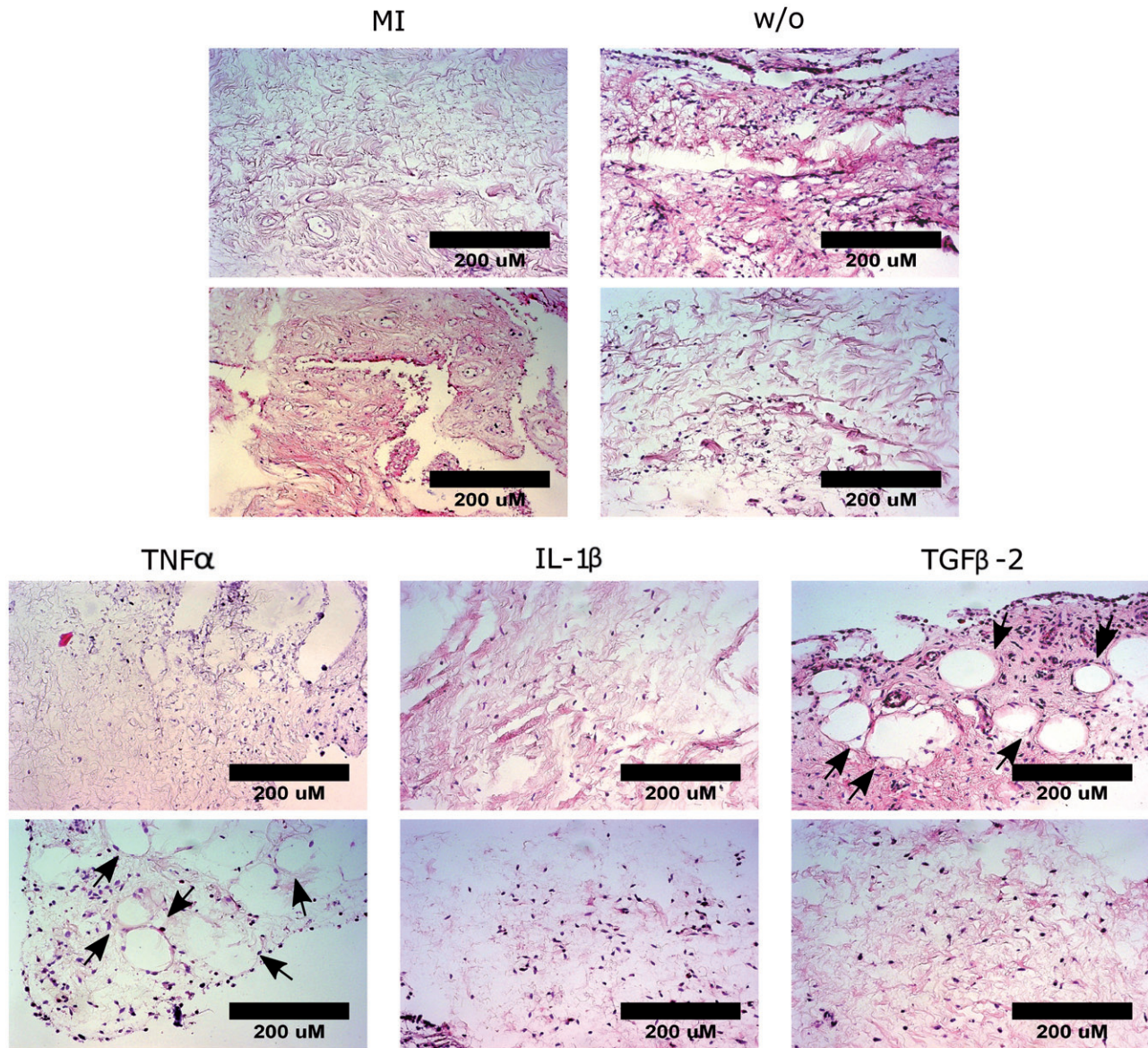


Figure 4. Hematoxylin and eosin staining of synovial membrane explants (SMEs). SMEs were fixated and embedded in paraffin, cut, and stained with hematoxylin and eosin after 14 d of culture. Here are two different explants from each treatment shown. The arrows indicate adipocytes found within the SMEs. The scale is 200 μ M.

tested the three stimuli found to stimulate proliferation and secretion of MMP-2 in the isolated FLS; $\text{TNF}\alpha$, $\text{IL-1}\beta$, and $\text{TGF}\beta$ -2. After 21 d of culture, the SMEs were fixated and embedded in paraffin for histology. Hematoxylin and eosin staining showed that despite most fat was removed prior to cutting the explants some fat remained within a few of the explants (arrows in Figure 4). Additionally, it was discovered that the amount of collagen within the explants after 14 d of culture varied within the treatment groups. However, MI, w/o, and $\text{TGF}\beta$ -2 tended to contain slightly more collagen compared with $\text{TNF}\alpha$ and $\text{IL-1}\beta$ -treated SMEs (Figure 4). The paler staining of the collagen could indicate that collagen had been degraded during the 21 d of culture. To assess if active MMPs were present, we used the conditioned media for gelatine zymography. We found that the conditioned media from the SME *ex vivo* tissue culture, in contrast to FLS, both secreted and activated MMP-2 and -9, and $\text{TNF}\alpha$ and $\text{IL-1}\beta$ increased both the activation and the secretion of MMP-9 in

two independent experiments (Figure 5B and D). This pattern was the same for secretion and activation of MMP-3. The background secretion level of pro-MMP-3 was low and no or little activation was detected for w/o. $\text{TNF}\alpha$ and $\text{IL-1}\beta$ increased the secretion and the activation of MMP-3 after 7 d compared with w/o (Figure 5A and C). $\text{TGF}\beta$ -2 did not increase the secretion or activation of MMP-9 or MMP-3 compared with w/o.

Synovial membrane explants release biomarkers: C1M, C3M, and acMMP3

We measured C1M, C3M, and acMMP3 in the conditioned media from SMEs at days 2, 7, 14, and 21 in the first experiment and at days 0, 5, 7, 10, 14, and 21 in the second experiment. The first experiment did not include a pre-incubation period of 1 d and does, therefore, not have a day 0 as the second experiment. The two experiments were

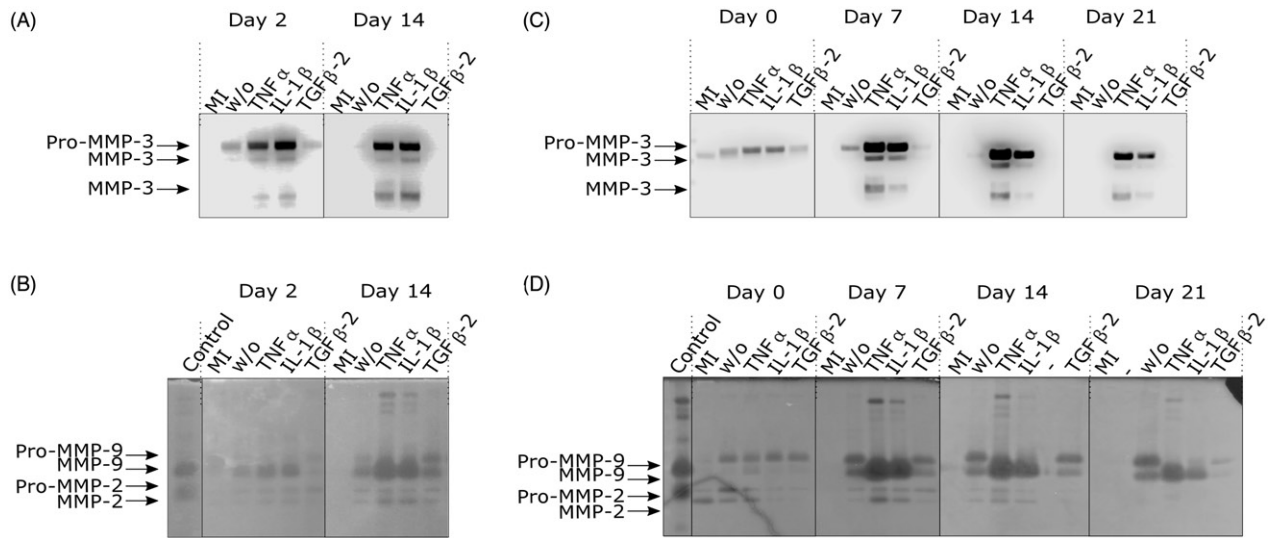


Figure 5. TNF α and IL-1 β induce activation of MMP-3 and -9 in SMEs. Pooled conditioned medium from synovial membrane explants (SMEs) either metabolically inactivated (MI), untreated (w/o), or treated with 10 ng/ml TNF α , IL-1 β , or TGF β -2 were used to detect total MMP-3 with western blot (B + D) and total MMP-2 and -9 with gelatin zymography (A + C). The results shown are from two independent experiments with synovial tissue from four (A and D) and three (B and D) OA patients, respectively. (A + C) Control: bovine cartilage explants treated with 10 ng/ml oncostatin M and 20 ng/ml TNF α for 17 d used as ladder, as this is known to express both MMP-2 and MMP-9 (Sondergaard et al., 2006).

otherwise identical. The SMEs were metabolically active throughout both experiments, but no difference was measured between treatments (data not shown).

After 7 d of TNF α treatment, the release of C1M was increased five-fold compared with w/o ($p = 0.027$) in the first experiment and six-fold compared with w/o in the second experiment ($p < 0.0001$). Additionally, TNF α increased the release of C1M four-fold compared with w/o at day 5 ($p = 0.0068$) and seven-fold compared with w/o at day 10 ($p = 0.0002$) in the second experiment (Figure 6A and D). Furthermore, IL-1 β increased C1M four-fold compared with w/o at day 7 in the first experiment, although not significantly (Figure 6A). In the second experiment, IL-1 β significantly increased the release of C1M five-fold compared with w/o at day 5 ($p = 0.0013$), four-fold compared with w/o at day 7 ($p = 0.0082$), and five-fold compared with w/o at day 10 ($p = 0.0094$) (Figure 6D).

The release of C3M into the conditioned media followed a similar pattern as C1M: TNF α induced a significant 24-fold compared with w/o increased release of C3M at day 7 in the first experiment ($p = 0.0011$) and 13-fold compared with w/o at day 10 ($p = 0.0002$), and 14-fold compared with w/o at day 14 ($p = 0.0001$) in the second experiment (Figure 6B and E). At day 7, IL-1 β increased the release of C3M 13-fold compared with w/o in the first experiment and seven-fold compared with w/o in the second experiment, although not significantly (Figure 6B and E). In the second experiment, the release of C3M was significantly increased four-fold compared with w/o at day 5 ($p = 0.007$) and eight-fold compared with w/o at day 10 ($p = 0.025$).

The level of C1M and C3M decreased again after 14 d of culture, but both TNF α and IL-1 β tended to postpone the release. This was, however, only significant for IL-1 β , which increased the release of C3M five-fold compared with w/o at day 14 ($p = 0.049$) in the first experiment (Figure 5A, B, D, and E). As expected, neither TGF β -2

nor MI increased the release of C1M and C3M (Figure 6A, B, D, and E).

The release of acMMP3 was significantly increased three-fold compared with w/o at day 2 by TNF α ($p = 0.017$) and four-fold compared with w/o by IL-1 β ($p = 0.0088$) in the first experiment. TNF α additionally increased the release of acMMP3 three-fold compared with w/o at day 7 ($p < 0.0001$), 14-fold compared with w/o at day 14 ($p = 0.0006$), and nine-fold compared with w/o at day 21 ($p < 0.0001$) (Figure 6C). IL-1 β increased acMMP3 in the conditioned media three-fold compared with w/o at day 7, 18-fold compared with w/o at day 14, and 14-fold compared with w/o at day 21 ($p < 0.0001$) (Figure 6C). In the second experiment, no difference was detected at day 0 (Figure 6F). At day 5, in the second experiment, acMMP3 was significantly increased three-fold compared with w/o by TNF α ($p = 0.022$) and four-fold compared with w/o by IL-1 β ($p = 0.0015$). At day 7, TNF α increased acMMP3 in the conditioned media eight-fold compared with w/o ($p < 0.0001$) and IL-1 β five-fold compared with w/o ($p = 0.017$). At days 10 and 14, TNF α increased the release of acMMP3 11-fold compared with w/o ($p < 0.0001$ and $p = 0.0024$, respectively) (Figure 6C and F).

Discussion

It is becoming evident that OA has different phenotypes and that an inflammatory phenotype is one of those. Consequently, there is a medical need for the identification of synovitis-derived biomarkers that can easily identify this inflamed subpopulation. Here we present an *ex vivo* characterization of three biomarkers of synovitis, which may be used in translational science by measurements in the *ex vivo* model of synovitis. We found:

- (1) FLS on collagen coating do not activate the MMPs they secrete.
- (2) The pro-inflammatory stimuli (IL-1 β and TNF α) lead to the activation of MMPs in the SME culture.

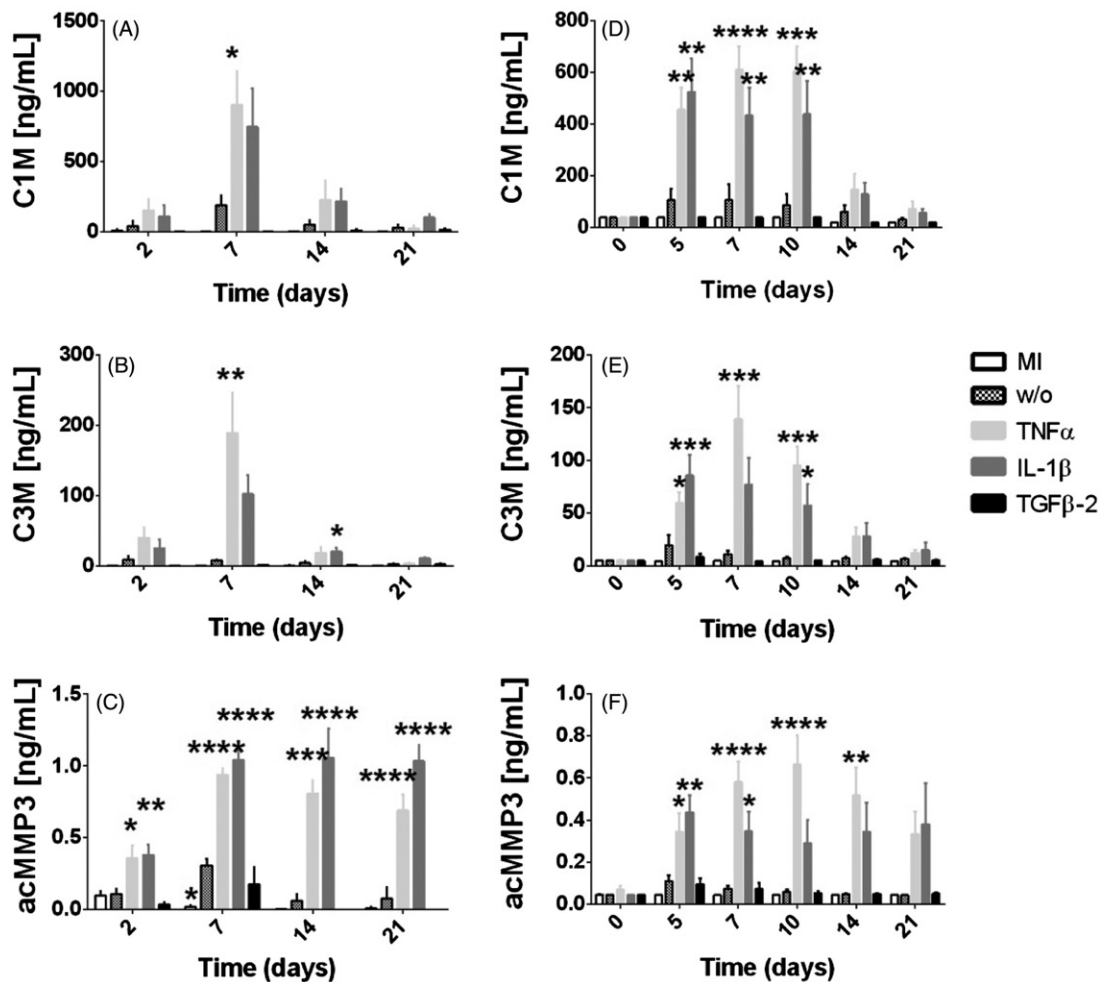


Figure 6. TNF α and IL-1 β stimulated release C1M, C3M, and acMMP3 from the SMEs. Concentrations of C1M (A + D), C3M (B + E), and acMMP3 (C + F) were measured with ELISA in the conditioned medium from SMEs either metabolically inactivated (MI), untreated (w/o), or treated with 10 ng/ml TNF α , IL-1 β , or TGF β -2. The biomarker measurements are shown from two independent experiments with synovial tissue from four (A–C) and three (D–F) OA patients, respectively. Data are presented mean \pm SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

- (3) Biomarkers of synovitis (C1M, C3M, and acMMP3) were increased with pro-inflammatory stimuli in the SME culture.

Isolated FLS in a two-dimensional cell culture have been used to study the FLS's involvement in OA and RA pathology for many years. This has provided valuable information to the field regarding FLS intracellular signalling, expression, and secretion of cytokines and MMPs, and interaction with other cell types in the joint (Alaaeddine et al., 1997; Hardy et al., 2013; Nair et al., 2012). We found that the isolated FLS were insufficient to study direct tissue degradation with neo-epitope degradation biomarkers as they did not activate the MMPs they produced in contrast to the SME cultures (Figure 7). In addition, the FLS cultures do not contain the ECM which is needed to produce biomarkers of tissue degradation. MLS have previously been shown to be responsible for FLS's cytokine and MMP production by using CD14+ depleted synovial cell cultures (Bondeson et al., 2006). This together with the data presented here could indicate that MLS are necessary co-conspirators for FLS to produce active MMPs and degradation of their surrounding ECM. However, this needs further investigation. Another possible explanation for the lack of active MMPs could be that

the isolated FLS undergo a phenotypic change during the isolation making the FLS unable to activate the MMPs. However, already back in 1993, Winchester et al. (1993) found that FLS from an inflammatory environment maintained their expression pattern through multiple passages. *Ex vivo* models provide an environment with higher *in vivo* likeness and enhance the ability to perform translational research compared with *in vitro* cultures (Hansen et al., 2015). The SME cultures provide ECM, FLS, and MLS. In particular, it has been demonstrated that the macrophages in general are involved in fibrosis and tissues turnover processes (Scanzello & Goldring, 2012; Wynn & Barron, 2010). Here the *ex vivo* SME model generated the neo-epitope biomarkers: C1M, C3M, and acMMP3 and their release was increased in response to TNF α and IL-1 β that simulated a pro-inflammatory environment of inflammatory OA in contrast to the isolated FLS (Figure 7). TNF α and IL-1 β are the main activators of the synovium and known to stimulate secretion of MMP-3 and MMP-9 (Lambert et al., 2014; Sun et al., 2014; Tolboom et al., 2002). In agreement, we found that increased active MMP-3 and -9 correlated with the collagen type I and III MMP-degradation. In contrast, TGF β -2 did not increase active MMP-3, -9, or the MMP-mediated

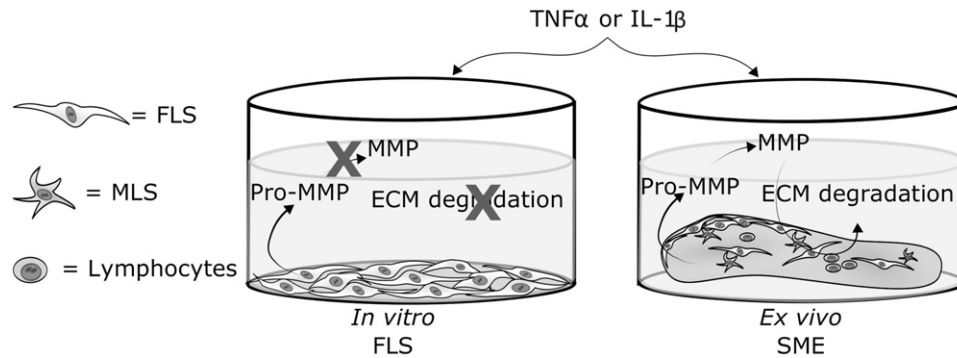


Figure 7. Synovial tissue generates active MMPs and degrades the ECM in response to $\text{TNF}\alpha$ or $\text{IL-1}\beta$ *ex vivo*. *In vitro* primary fibroblast-like synoviocytes (FLS) cultured on type I and III collagen coating do not generate active metalloproteinases (MMPs), and, therefore, cannot generate extracellular matrix (ECM) degradation biomarkers like C1M, C3M, and acMMP3. *Ex vivo* cultured synovial membrane explants (SMEs) contain multiple cell types in their natural three-dimensional ECM, and generate active MMPs and release degradation biomarkers: C1M and C3M and the biomarker of activated MMP3, acMMP3, in a pro-inflammatory environment. MLS = macrophage-like synoviocytes.

degradation of type I or III collagen. This was expected as $\text{TGF}\beta$ is an anabolic factor, able to stimulate expression of genes involved with collagen formation and tissue inhibitors of MMPs in isolated FLS (Remst et al., 2014). We have previously shown that acMMP3 is released endogenously from un-stimulated SMEs at levels of 0.1 ng/mL, which was similar to what we found here (Sun et al., 2014). However, the current data are the first to document that this level is increased in response to $\text{TNF}\alpha$ and $\text{IL-1}\beta$. Surprisingly, we found that $\text{TNF}\alpha$ and $\text{IL-1}\beta$ increased the release of acMMP3 and active MMP-2 and -9 throughout the culture period compared with w/o, while the $\text{TNF}\alpha$ and $\text{IL-1}\beta$ induced increase of C1M and C3M declined in the later time points. It is not known why this is, but a possible explanation could be the lack of accessible cleavage sites and that the ECM of the SMEs was destroyed in the later time points.

SME cultures have previously been used to investigate cytokine secretion or gene expression profile of the synovium either alone, in response to activated mesenchymal stem cells, or blockage of the Toll-like receptor 2 to elucidate the pathology of OA (Beekhuizen et al., 2011; Ultraigh et al., 2011; van Buul et al., 2012). Furthermore, an equine SME model was recently used to characterize the anti-inflammatory treatment effects of platelet-rich plasma on cytokine and growth factor secretion (Ríos et al., 2015). The present model has two advantages: (1) the human origin and (2) the use of translational biomarkers, making this model a translational model able to provide information of the synovial ECM turnover in the joint.

Limitations

This study did not include clinical measurements of serum, but it has previously been shown that C1M, C3M, and acMMP3 are elevated in serum from OA and RA patients (Bay-Jensen et al., 2014; Siebuhr et al., 2014; Sun et al., 2014). Furthermore, collagen types I and III are not exclusively found in the synovium and could potentially be generated in other connective tissues. However, the current study demonstrates that C1M and C3M can be released from the inflamed synovial tissue and consequently affect the serological pool of these biomarkers. It has not been investigated whether the levels of synovial inflammation

endogenously will affect the biomarker levels of the SMEs, but it may possible contribute to the variation. Information regarding the inflammatory status of the patient could be useful to investigate how the levels of biomarkers in explants reflected the different inflammation levels. The SMEs contains multiple cells type but the specific cell constituents were not determined in this study. It is a limitation to the model as different cell types could have different effects on the biomarker release. Future experiments could characterize the cell type composition of the SMEs. Another limitation of the study is that the expression and secretion profile of the isolated FLS have not been followed through the passages and compared with the SME and/or MLS to elucidate if there is a phenotypic change. However, the FLS used here were isolated using a widely used method and the differences between FLS and SME emphasize that it is important to consider what models to use when doing translational research.

Conclusions

In conclusion, these data show that the serological markers C1M, C3M, and acMMP3 are released from the synovial membrane under inflammatory conditions, and thus are biomarkers of synovitis. In addition, the *ex vivo* SME model may provide an important tool for translational science in rheumatology by enabling characterization of synovitis biomarkers *ex vivo* in response to different treatments. The biomarkers reflect the synovial tissue turnover and can be compared with serological measurements *in vivo*.

Declaration of interest

Anne-Christine Bay-Jensen and Morten Karsdal are full-time employees and shareholders in Nordic Bioscience. Anne Sofie Siebuhr is full-time employee of Nordic Bioscience and Christoph Ladel is full-time employee of Merck Serono, whereas Cecilie Kjelgaard-Petersen and Thorbjørn Christiansen report no declarations of interest. The authors would like to acknowledge The Danish Research Foundation for funding Nordic Bioscience and the D-Board consortium for supporting this work. The D-Board project has received funding from the European Union's Seventh Framework

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Author contributions

CFKP, ACBJ, CL, ASS and MK have planned and designed the experiment. CFKP conducted the experiment and measured the biomarkers. TC was responsible for providing the human synovial membranes. All authors were involved with data analysis, scientific discussion and interpretation of the data. Additionally, all authors were involved with drafting the manuscript, approving the manuscript prior to submission and have agreed to be accountable for all published data.

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