The stromal component in rheumatoid arthritis: CD55 expression, cell death and beyond
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STROMAL CELL MARKERS ARE DIFFERENTIALLY EXPRESSED IN THE SYNOVIAL TISSUE OF PATIENTS WITH EARLY ARTHRITIS

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ABSTRACT

Objectives
Previous studies have shown increased expression of stromal markers in synovial tissue (ST) of patients with established rheumatoid arthritis (RA). Here, ST expression of stromal markers in early arthritis in relationship to diagnosis and outcome was studied.

Methods
ST from 67 patients included in two different early arthritis cohorts and 7 non-inflammatory controls was analysed using immunofluorescence to detect stromal markers CD55, CD248, fibroblast activation protein (FAP) and podoplanin. Diagnostic classification (gout, psoriatic arthritis, unclassified arthritis (UA), parvovirus associated arthritis, reactive arthritis and RA) and outcome (resolving/persistent) were determined at baseline and after follow-up, and related to the expression of stromal markers.

Results
We observed expression of all stromal markers in ST of early arthritis patients, independent of diagnosis or prognostic outcome. Expression of FAP and podoplanin was significantly higher in patients with early RA compared to non-inflammatory controls. Significantly greater expression of FAP was found in anti-citrullinated peptide antibody (ACPA)-negative RA patients and in patients with UA fulfilling classification criteria for RA after follow-up compared to patients with resolving disease and patients with persistent disease who did not fulfil classification criteria for RA after follow-up.

Conclusions
Stromal cell markers CD55, CD248, FAP and podoplanin, are expressed in ST in the earliest stage of arthritis. Expression of FAP is higher in early UA patients who fulfil classification criteria for RA over time and in ACPA-negative RA compared to resolving or non-RA arthritides. These results suggest that significant fibroblast activation occurs in RA in the early window of disease.
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting synovial tissue (ST) in multiple joints leading to joint destruction, deformity and disability.[1] Classifying patients in an early stage of the disease is important, as early appropriate treatment can reduce or even prevent joint destruction.[2] Unfortunately, at presentation not all patients with early symptoms of arthritis can be diagnosed and therefore remain unclassified,[3,4] resulting in a delay in optimal treatment, indicating a need for new diagnostic and prognostic markers.

Stromal cells play an important role in organising the structure of ST by producing extracellular matrix components, recruiting infiltrating immune cells and secreting inflammatory mediators. Furthermore, these cells may contribute to the persistence of inflammation and joint damage in RA.[5,6] Currently known stromal markers include CD55, CD248, fibroblast activation protein (FAP), podoplanin and VCAM-1.[7-10]

Previous work has suggested the presence of cytokines in the synovial compartment of some patients with short duration RA that is rich in stromal growth factors.[11,12] We hypothesised that subpopulations within the stromal compartment might become activated and expanded during the inflammatory processes occurring in early disease, defining a profile that may be specific for RA. We chose to examine an established intimal lining layer stromal marker (CD55)[9] and more recently discovered markers described both in RA and cancer, where the role of tumour-associated fibroblasts has become prominent. These include CD248,[14,15] a synovial sublining glycoprotein marker expressed in perivascular and cancer stromal cells and in the RA synovium; FAP,[7,16,17] a cell surface protein with exoenzyme activity and an important role in epithelial cancers that is highly expressed in established RA synovium; and podoplanin (gp38),[8,13] an intimal lining layer glycoprotein marker with roles in lymph node stromal networks and epithelial to mesenchymal transition. Following initial screening of tissue samples in one early arthritis cohort, we validated and expanded our observations in an independent cohort.

METHODS

Cohorts and synovial tissue

Synovial tissues of patients included in two different early arthritis cohorts in Amsterdam and Birmingham were used in this study. All patients were naïve to treatment with disease-modifying antirheumatic drugs (DMARDs) and corticosteroids at inclusion. In the Birmingham cohort (BEACON), patients who develop synovitis in at least one joint referred by general practitioners were seen within 2 weeks. Patients were recruited if symptom duration, defined as any symptom attributed by the assessing rheumatologist to inflammatory joint disease (pain, stiffness and/or swelling) was no greater than 3 months. Newly presenting patients fulfilling classification criteria for RA with symptom duration of >3 months were also recruited as a control population.[18,19] Consenting patients underwent ultrasound guided synovial biopsy of an inflamed joint at baseline as previously described.[20] Diagnostic and prognostic outcomes were assigned after 18-months of follow-up.
In the Amsterdam cohort (Synoviomics),[21] patients presenting with arthritis of at least one knee, ankle or wrist joint with duration of less than 1 year were included. [22] Of note, 93.8 % of the Synoviomics patients included in the present study had symptom duration of no greater than 3 months. Arthritis duration was defined as the time from the first clinical signs of arthritis, irrespective of which joint was initially affected, determined by an experienced rheumatologist; data on symptom duration were recorded, enabling the use of the AMC cohort for validation purposes. At inclusion, synovial tissue was collected during a mini-arthroscopy procedure as previously described.[23] Diagnostic and prognostic outcomes were assigned after 2 years of follow up.

In both cohorts, patients were classified as having RA according to the 2010 ACR/EULAR classification criteria for RA,[18,19] psoriatic arthritis (PsA) according to the CASPAR criteria for PsA,[24] parvovirus arthritis based on clinical diagnosis plus serological testing, and other diagnoses by characteristic clinical features, including the presence of a pre-existing infectious episode (reactive arthritis) and uric acid crystals in synovial fluid (gout). Patients were classified as having unclassified arthritis (UA) if they did not meet any classification criteria. Patients were classified as having resolving disease if they had no clinical evidence of synovial swelling and had not taken DMARDs or received glucocorticoid treatment in any form in the preceding 3 months.

We included ST of 7 individuals with mechanical joint symptoms undergoing exploratory arthroscopy during which no evidence of synovial pathology was found macroscopically or on subsequent histological analysis.

All studies were approved by local medical ethical committees and patients gave written, informed consent to participate.

**Immunofluorescence and confocal microscopy**

During establishment of ultrasound guided biopsy techniques in Birmingham in 2007, tissue processing protocols for histological analysis were harmonised with the AMC Synoviomics project, leading to a common protocol for tissue recovery, processing and storage. Synovial tissue samples were snap-frozen in Tissue-Tek OCT medium (Miles, Elkhart, IN) immediately after collection. In order to account for heterogeneity, six to eight biopsies from different areas of the joint were combined in one block of tissue. Cryostat sections (5mm) were cut, mounted on Star Frost adhesive glass slides (Knittelplaser, Baunschweig, Germany) and stored at -80°C.

All tissue staining, image acquisition and quantification took place in Birmingham and was blinded to clinical outcomes. Prior to use, slides were thawed at room temperature (RT) for 30 min. After fixation in acetone the sections were washed in PBS and blocked with 10% normal human serum for 30 min at RT. Incubation with primary antibodies was performed overnight at 4°C in blocking solution. As primary antibodies, anti-CD55 (mouse IgG2a, clone BU84; University of Birmingham, UK), anti-CD248 (mouse IgG1 supernatant, clone B1 35.1 [14]), anti-FAP (mouse IgG1, clone F11-24; eBioscience), anti-gp38 (mouse IgG1, clone D2-40; AbD Serotec, Kidlington, UK), antiCD31 (endothelial cell marker; mouse IgG2a, clone HEC7; Thermo Scientific, Loughborough, UK), anti-CD68 (macrophage marker; mouse IgG2b, clone Y1/82A; BD Pharmingen, Oxford, UK) were used. Tissue sections were incubated for 1 hour at RT and bound primary antibodies were detected with goat antibodies against mouse IgG1 conjugated with fluorescein (FITC), IgG2a conjugated with rhodamine (TRITC), and IgG2b conjugated with cyanine 5 (Cy™ 5) (all Southern
Biotech, Birmingham, AL). To increase signal from FITC-channel, slides were stained for 30 min at RT with goat anti-FITC Alexa-488 antibody (Invitrogen, Paisley, UK). All sections were co-stained with Hoechst solution (Sigma-Aldrich Company Ltd., Gillingham, UK) to visualize cell nuclei. As a negative control, a mixture of anti-IgG1, anti-IgG2a and anti-IgG2b secondary fluorochrome-conjugated antibodies followed by anti-FITC Alexa-488 were applied to the sections after omission of the primary antibodies. From different parts of the tissue sections, 1-8 images were acquired with confocal scanning microscope Zeiss LSM 510 and ZEN pro 2011 imaging software (Zeiss, Welwyn Garden City, UK). Settings within one staining experiment remained unchanged. For each image, the number of pixels with intensity from 30 to 255 of every fluorescent channel was quantified with ZEN pro 2011 and divided by a manually defined area (μm²) only including tissue zones containing cells. The average number of fluorescent pixels with intensity 30-255 per unit area (pixel/UA) from all photographs within one synovial tissue section was calculated. In addition, two researchers independently assessed the fluorescence level of every marker using a semiquantitative scoring system of grade 0-4 combining staining intensity and number of positive cells. Semiquantitative scores correlated well with unbiased pixel analysis scores (Spearman’s rho >0.7, p <0.001 for all markers, data not shown).

During the study, tissues were stained in total on three separate occasions, all performed in the Birmingham unit. These comprised [1] initial staining of Birmingham tissues, [2] staining of AMC and confirmatory restaining of Birmingham tissues and [3] staining of an extended panel of Birmingham and AMC tissues for FAP and podoplanin. In order to measure and account for any variation between staining of sections during different staining runs, we stained sections from the same 11 patients on each occasion for a range of four stromal (CD55, CD248, FAP and podoplanin), and two cellular (CD31, CD68) markers. To assess reliability of outcome variables between staining procedures, intraclass correlation coefficients were calculated (SPSS, IBM). Intraclass correlation coefficients were as follows reflecting good (ICC >0.7) internal consistency for all markers except CD248, which closely approached this level (ICC 0.69). CD55: ICC 0.77; FAP: ICC 0.74; podoplanin: ICC 0.74; CD31: ICC 0.89; CD68: ICC 0.96.

Statistical analysis
Using Prism (Graphpad, La Jolla, CA) software, differences between two groups were assessed using the Mann-Whitney U test; correlation of two outcome measurements was assessed with the Spearman rank-order correlation coefficient. A P-value <0.05 was considered statistically significant.

RESULTS
Patient characteristics
Baseline characteristics of the early arthritis patients from the two cohorts presenting with symptom duration of three months or less are shown in Table 1. Characteristics of two comparison groups including selected patients from Birmingham and Amsterdam cohorts are also shown: Comparison cohort 1 includes patients presenting with treatment naïve RA with symptom duration of greater than three months. Comparison cohort 2 includes patients presenting with symptom duration of three months or less who ultimately developed persistent disease with a diagnosis other than RA.
Chapter 6

Table 1. Baseline characteristics of early arthritis patients of the Birmingham and Amsterdam cohorts.

<table>
<thead>
<tr>
<th></th>
<th>Birmingham BEACON cohort</th>
<th>Amsterdam Synoviomics cohort</th>
<th>Comparison cohort 1</th>
<th>Comparison cohort 2</th>
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<tr>
<td></td>
<td>Resolver (n = 10) ≤3 months</td>
<td>RA (n = 11) ≤3 months</td>
<td>Resolver (n = 14) ≤3 months</td>
<td>RA (n = 13) ≤3 months</td>
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<td>Age (years)</td>
<td>36 (33-48)</td>
<td>51 (48-63)</td>
<td>53 (42.3-61)</td>
<td>51 (37-65)</td>
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<td>Female (%)</td>
<td>3 (30)</td>
<td>4 (36)</td>
<td>6 (43)</td>
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<td>ESR (mm/h)</td>
<td>8 (3.5-24.8)</td>
<td>58 (20-63)</td>
<td>35 (8-52)</td>
<td>34 (15-80.5)</td>
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<tr>
<td>CRP (mg/l)</td>
<td>7.5 (4.5-9.3)</td>
<td>26 (18-45)</td>
<td>11.5 (3.1-44)</td>
<td>5.3 (3.8-61.2)</td>
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<td>DAS28ESR</td>
<td>3.6 (2.6-4.2)</td>
<td>5.7 (4.4-6.7)</td>
<td>4.0 (2.5-4.6)</td>
<td>5.4 (4.2-5.8)</td>
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<td>Symptom duration (wks)</td>
<td>5.5 (4-7.5)</td>
<td>4 (3-6)</td>
<td>6.5 (4-10)</td>
<td>7 (4.5-9)</td>
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<td>RhF positive (%)</td>
<td>0 (0)</td>
<td>4 (36)</td>
<td>3 (21)</td>
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<td>ACPA positive (%)</td>
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<td>6 (55)</td>
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Values shown are median (interquartile range) or number (percentage). PsA = psoriatic arthritis, RA = rheumatoid arthritis, UA = unclassified arthritis, ESR = erythrocyte sedimentation rate, CRP = C-reactive protein, DAS28ESR = disease activity score in 28 joints calculated using the ESR, RhF: rheumatoid factor, ACPA: anti-citrullinated protein antibodies.

Initial screening of stromal markers in the Birmingham BEACON cohort

As an initial screen, we stained synovial tissues of 21 patients and seven controls for stromal markers. In this screening cohort, patients whose arthritis subsequently resolved included three patients with reactive arthritis, two with parvovirus arthritis, four with unclassified arthritis and one seronegative patient who fulfilled 2010 criteria for RA at baseline. 11 patients developed persistent RA; six of these were positive for ACPA; of the remaining five, two presented with unclassified arthritis but fulfilled classification criteria for RA after follow up (UA-RA).

Four stromal markers were examined: CD55, CD248, FAP and podoplanin (gp38). As shown in Figure 1, both FAP and podoplanin showed some evidence of differential expression between outcome groups: FAP normal vs persistent RA p=0.002; Podoplanin, normal vs resolving p=0.015; normal vs persistent RA p=0.002. No significant differences were seen between resolving and persistent diseases.

Examination of tissue cellular markers in a combined cohort
Initial screening results suggested that we should pursue staining of stromal markers in a larger cohort. We therefore exploited the common tissue collection and processing protocols in Birmingham and Amsterdam, and stained tissue from Amsterdam Synoviomics cohort patients with symptom durations in the same range as the BEACON cohort. Initial validation studies using Synoviomics samples alone suggested a similar pattern of staining was seen (data not shown). We therefore combined the data of the BEACON and Amsterdam Synoviomics cohorts. Also, to address the question of whether stromal markers are differently expressed in RA patients with symptom duration more or less than 3 months and whether stromal markers can differentiate persistent RA from persistent non-RA diseases, we undertook restaining of a larger combined panel. This panel included two control groups: baseline tissue from patients with longer duration, treatment naïve established RA and tissue from patients presenting in the first 3 months of symptoms who ultimately developed non-RA persistent disease (UA, PsA or gout, Table 1, comparison cohorts 1 and 2). Recognising that other cellular markers could provide...
important discriminating data and also an estimate of levels of tissue inflammation, we stained for CD31 (endothelial cells) and CD68 (macrophages).

Figure 2 shows these data in concise form, comparing groups of patients developing persistent RA or resolving disease presenting with less than 3 months symptom duration and normal controls, then comparing RA groups of less than and greater than 3 months symptom duration, using a comparison cohort of 11 patients presenting with treatment naïve RA (comparison cohort 1). No significant differences were seen between outcome groups for tissue

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**Figure 2. Expression of tissue markers in the combined Birmingham and Amsterdam cohorts.** (A-E) Expression of CD55, CD248, FAP, podoplanin, CD31 and CD68 (in pixels per square micrometer) in control patients (n = 7), baseline samples of early arthritis patients with resolving arthritis (n = 16-24) and persistent RA (n = 16-24) (left graph) and comparison of RA duration three months or less to untreated RA of greater than three months (n = 11) (right graph). Each dot represents a patient; median bars are shown. Significance of the comparisons was determined by the Mann-Whitney test. *p<0.05; **p<0.01.
Figure 3. Representative images of tissue staining. Multicolour confocal microscopy images are shown for tissue staining at baseline with FAP, CD55, podoplanin and CD68. (A) Patient presenting with unclassified arthritis (UA) who developed persistent RA. (B) Patient presenting with RA who developed persistent RA; (C) Patient presenting with resolving parvovirus arthritis. (D) Patient presenting with UA, who subsequently fulfilled RA criteria but whose arthritis then resolved.
expression of CD55, CD248, CD31 and CD68. Also, no significant differences were seen between the resolving group and persistent RA. However, both FAP and podoplanin staining levels were higher in both resolving and RA groups compared to normal. Figures 2C and 2D summarise all patient samples studied, including a final extended panel of short symptom duration disease undertaken to further explore FAP and podoplanin staining (FAP normal vs resolving p=0.036, normal vs persistent RA p=0.002; podoplanin, normal vs resolving p=0.041, normal vs persistent RA p=0.005). Representative images are shown in Figure 3. We therefore examined levels of FAP and podoplanin staining in more detail.

**FAP expression is significantly higher in an ACPA negative group of RA patients**

Figure 4 illustrates data for patients with symptom duration of three months or less, including a comparison group of patients who developed persistent disease but not RA (comparison cohort 2). Figures 4A and B show the effect of splitting the early RA group by ACPA status. FAP expression levels were significantly higher in patients with ACPA negative RA compared to patients whose arthritis resolved (p=0.03) or who developed non-RA persistent disease (p=0.02). Tissue from patients with ACPA positive disease demonstrated a broader range of FAP expression, showing no significant differences compared with resolving diseases or compared with non-RA persistent disease.

**Correlation of FAP expression with clinical variables in patients with early arthritis**

Because FAP expression levels appear to relate to clinical outcome, we tested whether FAP levels correlated with clinical variables including swollen and tender joint counts, DAS28ESR, ESR, CRP...
and symptom duration. In the entire cohort of patients with disease duration of three months or less (n=56), FAP expression levels demonstrated moderate positive correlation with ESR (r=0.30, p=0.027), CRP (r=0.33, p=0.013) and DAS28 (r=0.40, p=0.002). Correlation with the DAS28 should be regarded with some caution, as this disease activity index has not been widely validated outside its use in RA. Within the subgroup of patients who developed persistent RA, no correlations with clinical variables were seen, however the group size for these correlations are small.

**DISCUSSION**

The most prominent stromal cell of the ST is the fibroblast-like synoviocyte (FLS).[27,28] We previously showed that CD55 is a defining marker for FLS in the intimal lining layer where it can mediate contacts with CD97 on other immune cells and may be of primary importance in maintaining and amplifying synovial inflammation.[9,29,30] Other molecules markedly expressed by FLS in ST of patients with established RA are CD248 (also known as endosialin or TEM1) and podoplanin (gp38).[8,15] CD248 is expressed by FLS in synovium from patients with established PsA and RA, but is only weakly present in synovium from individuals with non-inflammatory knee problems.[15] It is believed to play a role in tissue remodelling by increasing proliferation and migration.[10] FAP, a cell surface-bound, type II transmembrane glycoprotein, belonging to the family of serine prolyl oligopeptidases, is expressed by activated fibroblasts associated with the granulation tissue of healing wounds and stroma of epithelial cancers. It has also been shown that FAP is strongly expressed in rheumatoid synovium.[7] Of these markers, podoplanin and FAP were found to be upregulated in early RA ST compared to tissue of healthy controls, but only FAP appeared to differ significantly from other forms of inflammatory joint disease or between patients who experienced a persistent versus a self-limiting course of disease.

RA FLS show a gene expression profile characteristic of myofibroblasts, and cells of the intimal lining layer in RA have been found to express α-smooth muscle actin (α-SMA) and type IV collagen.[31,32] It has therefore been suggested that RA FLS can undergo a process resembling epithelial-mesenchymal transition (EMT), whereby static epithelial cells lose cell-cell contacts, acquire mesenchymal features and manifest a migratory phenotype. This phenomenon is common to early developmental processes, tissue repair, fibrosis and carcinogenesis. Of interest, podoplanin and FAP are both hypothesized to be involved in EMT and are reported to be highly expressed in cells of the intimal lining layer in RA, with little expression in osteoarthritis synovium.[8,33] The role of podoplanin in inflamed synovial tissue is unclear, but it is possible that it could relate to interactions with infiltrating leukocyte sub-populations, as seen in podoplanin expressing fibroblast-like reticular cells and lymphatic endothelium of the lymph node.[13] Ultimately this could favour the formation of ectopic lymphoid structures.[34] Since we found upregulation of podoplanin in ST of patients with early RA, the involvement of this marker in an EMT-like differentiation of RA-FLS into myofibroblasts might be of more importance in the earlier stages of arthritis.

FAP is strongly expressed in ST of patients with destructive RA.[7] In our study, we showed a strong expression of FAP in ST of RA patients earlier in their disease course. High expression of this marker in the ST indicates the involvement of dysregulated extracellular matrix remodelling in the early stage of arthritis that could represent a potential therapeutic target in early disease.
One of the challenges in the treatment of early arthritis patients is starting patient tailored treatment as soon as possible.\cite{35-37} Personalized medicine in this patient group is aimed at remission, thereby preventing joint destruction and optimizing functional outcome with a minimum of potential harmful side-effects. Despite the importance of making an early diagnosis, in around one-third of rheumatology patients no diagnosis can be made at presentation, indicating a need for new diagnostic and prognostic markers. Our findings suggest that synovial stromal marker analysis could play a role in the guidance of treatment decisions in early arthritis patients where outcome is not possible to predict using existing clinical variables. Given these preliminary results, combination with other variables would be necessary in order to provide clear guidance for clinicians. Our findings and those of others mandate more extensive studies of candidate tissue markers alone and in combination for the prediction of diagnosis and prognosis in patients with early disease.\cite{38} In individuals at risk for developing RA, it would also be interesting to examine stromal markers in the synovium to test their ability to predict the development of RA, in combination with other clinical predictors.\cite{39}

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