Clinical and molecular classification of very early arthritis patients
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LOCAL SYNOVIAL ENGAGEMENT OF ANGIOGENIC TIE2 DRIVES THE DEVELOPMENT OF PERSISTENT AND EROSIVE RHEUMATOID ARTHRITIS IN EARLY ARTHRITIS PATIENTS

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ABSTRACT

Disease outcome in patients with rheumatoid arthritis (RA) and other forms of inflammatory arthritis is markedly improved by early and aggressive treatment. However, little is known about local early changes in synovial cellular and molecular processes which predict disease diagnosis and outcome in RA. Serum levels of angiogenic factors are associated with inflammation and the development of erosive disease in patients with RA, and synovial markers of angiogenesis are differentially expressed in early RA and spondyloarthritis (SpA) patients. Here, we examined the role of synovial vascular endothelial growth factor (VEGF) and angiopoietin (Ang) signalling in disease diagnosis and outcome in a prospective study of 50 disease-modifying antirheumatic drug (DMARD) naïve early arthritis patients. Quantitative analysis of VEGF, VEGF receptor (VEGFR), Ang-1, Ang-2, Tie2 and activated phospho (p)-Tie2 was examined by immunohistochemical analysis combined with computer-assisted digital imaging. Expression of Ang-1, Tie2 and p-Tie2 was comparable between the patients with RA at baseline and undifferentiated arthritis (UA) patients who fulfilled ACR criteria over time, but was significantly higher in these patients than SpA patients or patients who remained UA. In patients with UA at baseline, expression and engagement of Tie2 by Ang-1 was significantly related to development of persistent disease, while in RA, the degree of Tie2 activation robustly predicted the development of erosive disease. Our study provides the first evidence of involvement of a specific biochemical signaling pathway, local engagement of synovial Tie2 by Ang-1, in the earliest phases of disease in RA which contributes to both disease development and progression. Early therapeutic targeting of Tie2 signaling may be useful in improving outcome in arthritis.
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology characterized by synovial inflammation in multiple joints with hyperplasia of the synovial intimal lining layer, influx of inflammatory cells and neovascularisation. In a large proportion of patients chronic inflammation results in cartilage and bone destruction, disability, and co-morbidity.[1,2] In RA, early and aggressive treatment regimens have shown to prevent or decrease joint destruction, improve functional outcome, and decrease mortality risks.[3–7] Despite the importance of making an early diagnosis and estimation of prognosis in the individual patient in determining the choice of treatment, both in RA and other inflammatory joint diseases, in about one third of the patients in the rheumatology outpatient clinic, no diagnosis can be made at presentation with currently practiced clinical and serological evaluations, indicating a need for new diagnostic and prognostic markers.[8–10]

Analysis of synovial tissue, the primary site of inflammation in RA, might identify specific markers for RA which have diagnostic and prognostic value, as well as provide insight into cellular and molecular processes that contribute to pathogenesis. In established RA, the number of macrophages infiltrating the synovial sublining layer is tightly associated with disease activity, and changes in their number are predictive of effective clinical responses to therapy.[11,12] In healthy individuals with circulating rheumatoid factor (RF) and/or anti-citrullinated protein/peptide antibodies (ACPA), the cellularity and cellular composition of synovial tissue is identical to autoantibody-negative healthy individuals.[13] Also, the cellular composition of patients with UA who do not develop RA is remarkably similar to those who develop RA, as well as to patients with established RA, and no synovial biomarker has been identified which is predictive of eventual fulfillment of classification criteria of RA or related to development of persistent or destructive arthritis (van de Sande et al, chapter 5 this thesis). However, a trend towards a difference in vascularisation between patients with UA at baseline who developed RA after follow up and those who remain UA was observed, consistent with previous findings suggesting that angiogenesis might be differentially regulated in different early arthritis patients groups.[14–17]

Angiogenic processes are thought to play a key role in the initiation and perpetuation of synovial inflammation in RA, as well as in joint erosion. Neovascularisation allows for the influx of inflammatory cells in synovial tissue, and provides nutrients and oxygen to the hyperplastic synovium.[18] In particular, vascular endothelial growth factor (VEGF) and the angiopoietins (Ang) -1 and -2, signaling via their tyrosine kinase receptors VEGFR and Tie2, respectively, play an important role in angiogenesis and are expressed in the synovium of inflammatory arthritis patients.[17,19–21] VEGF produced by synovial stromal fibroblast-like synoviocytes (FLS) and perivascular cells promotes endothelial cell proliferation, and in combination with Ang-1, stabilizes new blood vessels.[22] Ang-1 can also promote FLS proliferation and matrix metalloproteinase (MMP) secretion.[23] Ang-2 can antagonize Ang-1 activation of Tie2, destabilizing the vasculature, but also has unique pro-inflammatory capacities, such as cooperating with TNF to induce endothelial cell adhesion protein expression.[24,25]

In RA, serum levels of VEGF, Ang-1 and Ang-2 are related to clinical inflammatory parameters and blood flow in inflamed joints as measured by ultrasound.[26] Less clear is the relationship between angiogenic pathways and disease progression. In one study, no relationship was observed between VEGF and joint destruction was shown in early RA.[27] However, other studies of early arthritis patients have indicated that serum levels of Ang-1 and VEGF are related
to inflammation and joint destruction at baseline and after 1 year of follow up.[28,29] Also, Ang-2 correlates with disease activity and cardiovascular disease in recent-onset RA.[30] While it is unclear from these studies how VEGFR and Tie2 signaling contributes to disease onset and progression in RA, genetic or pharmacological inhibition of neoangiogenesis and signaling via either of these receptors diminishes pathology in animal models of RA.[31–36] Here we investigated the expression and activation of angiogenic pathways in the synovial tissue of a prospective cohort of disease-modifying antirheumatic drug (DMARD) naïve early arthritis patients.

MATERIAL AND METHODS

Study Patients
Fifty patients consecutively included in our prospective early arthritis cohort between August 2002 and July 2006 with synovial tissue biopsy samples available for analysis were enrolled in this study. We selected patients based on diagnosis after 2 years of follow-up according to classifying criteria.[49,50] Patients were classified as having UA if no classifying diagnosis for RA, SpA or other forms of arthritis could be made. All patients with RA, SpA and undifferentiated arthritis UA were included. All patients had arthritis of at least one knee, ankle or wrist joint with arthritis duration of less than 1 year. All patients were DMARD-naïve, and none were taking corticosteroids or received intra-articular steroid injections. This study was approved by the institutional review board at the Academic Medical Center, University of Amsterdam, and performed according to the declaration of Helsinki. All study patients provided written informed consent.

Study Design
At inclusion we collected demographics, disease duration, and disease activity parameters. X-rays of hands and feet were made. Paired serum and arthroscopic synovial tissue biopsy samples were collected. Diagnosis was made after 2 years of follow up according to established classifying criteria for RA [49] and SpA ESSG criteria.[50] Patients were classified as UA if no classifying diagnosis could be made. In addition, patients were classified as having self limiting, persistent disease or having persistent erosive disease.[47] Erosions score was based on presence or absence of erosions on X-rays of hands and feet at 2 year follow up defined by a Sharp van de Heijde erosion score of >0.[51]

Disease Activity Parameters
At inclusion we assessed disease activity by 68 tender and 66 swollen joint score, patients visual analog scale (VAS) of global disease activity (scale 0-100mm), VAS of pain (scale 0-100mm), erythrocyte sedimentation rate (ESR), and serum levels of C-reactive protein (CRP). Local disease activity of the knee joint was assessed by a patients’ VAS of disease activity and pain (scale 0-100 mm). Knee joint pain and swelling was scored by an assessor on a scale 0-3.

Measurement of Autoantibodies and Cytokines in Patient Serum
The presence of IgM-RF and ACPA in patient serum, collected at inclusion, was measured using IgM-RF (Sanquin, Amsterdam, The Netherlands) and anti-CCP2 (Eurodiagnostica, Arnhem,
The Netherlands) ELISA kits, respectively, and VEGF, Ang-1, and Ang-2 were measured using standard quantitative sandwich ELISA (RnD Systems).

**Arthroscopic Synovial Tissue Biopsy Analysis**

All patients underwent arthroscopic synovial tissue biopsy sampling of a knee, wrist or ankle joint.[52] Six synovial tissue biopsies were collected for immunohistochemistry as described earlier [53] to correct for sampling error. The synovial biopsy samples were snap-frozen en bloc in TissueTek OCT (Miles, Elkhart, IN) immediately after collection. Cryostat sections (5 μm) were cut and mounted on Star Frost adhesive glass slides (Knittelgläser, Braunschweig, Germany). Sealed slides were stored at -80°C until use for immunohistochemistry. The sections were fixed with acetone, and endogenous peroxidase activity blocked by immersion in 0.3% hydrogen peroxide and 0.1% sodium azide in phosphate–buffered saline (PBS). Slides were incubated overnight at 4°C with primary antibody diluted in 1% bovine serum albumin/PBS. Primary antibodies used in this study were polyclonal rabbit antibodies specific for Tie2, Ang-1, Ang-2, VEGF, VEGFR (all from Santa Cruz Biotechnology) and murine monoclonal antibodies recognizing phosphorylated (p)-Tie2 (Cell Signaling, Beverly, MA). As a negative control, irrelevant/isotype matched immunoglobulins were applied to the sections instead of the primary antibody, or the primary antibody was omitted. Sections were washed with PBS and incubated with goat anti-mouse or swine-anti-rabbit -horseradish peroxidase (HRP)-conjugated antibodies (Dako, Glostrup, Denmark), followed by incubation with biotinylated tyramide and streptavidin-HRP, and development with aminoethylcarbazole (Sigma, St. Louis, MO).[54] Slides were counterstained with Mayer’s hematoxylin and mounted in Kaiser’s glycerol gelatin (Merck, Darmstadt, Germany).

After staining of the slides the sections were analyzed by digital image analysis. All sections were analyzed in random order by trained readers (DD, GS, MS) who were blinded with regard to the patient’s clinical characteristics. The analysis was performed using a computer-assisted image analysis algorithm, as previously described in detail.[11] Images were acquired and analyzed using a Syndia algorithm on a Qwin-based analysis system (Leica, Cambridge, UK). Expression and/or phosphorylation of proteins was calculated as the number of positive cells/mm² or the median integrated optical density (IOD) per mm² tissue corrected for cellularity. Relative phosphorylation values were obtained by dividing p-Tie2 IOD by total Tie2 IOD.

**Statistics**

Statistical analysis was performed using SPSS V.16.0 (Chicago, IL) software. For comparison of differences in expression or phosphorylation of markers values between the different diagnostic groups Kruskall Wallis test was used. The Mann-Whitney U test was used to compare differences in expression or phosphorylation of markers between outcome groups and 2 diagnostic groups. Correlations were examined by Spearman’s rank correlation coefficient. Univariable logistic regression was performed to evaluate the predictive value of the separate markers. In addition, backward multivariable logistic regression analysis was performed. Explained variance according to Nagelkerke is reported. Results were considered significant if P < 0.05.
RESULTS

Composition of patient cohort

Of the 50 included patients, 19 patients were diagnosed with RA at baseline and after 2 years of follow up (RA-RA), 8 patients had UA at baseline but fulfilled 1987 ACR criteria of RA at 2 year follow up (UA-RA), 16 patients with UA at baseline remained UA at 2 years follow up (UA-UA), and 7 patients had SpA at 2 year follow up (SpA). In addition, of the 24 patients who had UA at baseline, 12 patients had self-limiting disease after 2 years of follow up, while 12 had persistent non-erosive disease or persistent erosive disease. Of the 27 patients who fulfilled 1987 ACR criteria of RA at 2 years follow up, 10 patients developed erosive disease, and 2 patients were lost to follow up and were excluded form the analysis. Baseline patient characteristics in the different subgroups are shown in Table 1 (diagnostic subgroups), Table 2 (all UA with self-limiting or persistent disease) and Table 3 (all RA with or without erosive disease).

Enhanced synovial Ang-1 expression precedes the fulfillment of RA classification criteria

For comparison of our patient cohort at baseline with previously published studies, where serum concentrations of VEGF and Ang-1 failed to predict fulfillment of RA classification criteria in patients with UA, but were elevated in patients with progressive disease, we first assessed patient serum concentrations of VEGF, Ang-1, and Ang-2 (Figure 1A). We detected serum concentrations of VEGF and Ang-1 that were similar to previous reports.[17,28] However, we observed no differences in concentrations of VEGF or Ang-1 between any of the different diagnostic groups (UA-UA, RA-RA, UA-RA, and SpA) (Figure 1A). Ang-2 levels, however, were slightly and significantly lower in patients diagnosed with SpA at baseline, compared to those diagnosed with RA ($P=0.05$) (Figure 1A).

Table 1. Baseline patient characteristics.

<table>
<thead>
<tr>
<th></th>
<th>UA-UA (n=16)</th>
<th>RA-RA (n=19)</th>
<th>UA-RA (n=8)</th>
<th>SpA (n=7)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>39 (20-67)</td>
<td>52.5 (22-82)</td>
<td>54 (43-66)</td>
<td>44 (20-56)</td>
<td>0.19</td>
</tr>
<tr>
<td>Female (n)</td>
<td>12</td>
<td>10</td>
<td>7</td>
<td>0</td>
<td>0.002</td>
</tr>
<tr>
<td>Dis. duration</td>
<td>3.75 (1-10)</td>
<td>5 (1-12)</td>
<td>3.5 (1-11)</td>
<td>2 (1-12)</td>
<td>0.38</td>
</tr>
<tr>
<td>VAS glob (0-100)</td>
<td>63 (9-93)</td>
<td>37 (11-99)</td>
<td>46 (32-98)</td>
<td>35 (17-91)</td>
<td>0.82</td>
</tr>
<tr>
<td>TJC 68 (n)</td>
<td>3 (0-37)</td>
<td>16 (5-36)</td>
<td>13 (3-25)</td>
<td>4 (0-11)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SJC 66 (n)</td>
<td>1 (1-19)</td>
<td>8 (4-41)</td>
<td>6 (1-20)</td>
<td>2 (1-5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>34 (4-85)</td>
<td>32 (3-91)</td>
<td>30 (14-77)</td>
<td>11 (3-47)</td>
<td>0.23</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>11 (2-58)</td>
<td>12 (3-133)</td>
<td>17 (3-42)</td>
<td>7 (1-46)</td>
<td>0.73</td>
</tr>
<tr>
<td>ACPA pos (n)</td>
<td>2</td>
<td>10</td>
<td>3</td>
<td>0</td>
<td>0.01</td>
</tr>
<tr>
<td>IgM RF pos (n)</td>
<td>2</td>
<td>13</td>
<td>1</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

UA= undifferentiated arthritis, RA= rheumatoid arthritis, SpA= spondylarthritis, VAS = visual analog scale of global disease activity; TJC 68 = 68 tender joint count; SJC 66 = 66 swollen joint count; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; IgM-RF positive = immunoglobulin M rheumatoid factor positive; ACPA positive = anti-citrullinated protein antibody positive. Median and range are shown. Groups were compared by Kruskal-Wallis test. A $P$-value of $>0.05$ was considered significant.
Next we performed immunohistochemical analyses to detect synovial expression of VEGF, Ang-1, and Ang-2. While no specific staining was observed with irrelevant control antibodies (data not shown), staining was observed in synovial intimal lining and sublining layers with antibodies recognizing VEGF, Ang-1 and Ang-2 (Figure 1B). VEGF expression was observed predominantly in the synovial sublining of patients in all diagnostic groups. Ang-1 expression was observed in the intimal lining layer, the synovial sublining, and in sublining vasculature. Staining in the same synovial regions was observed for Ang-2 in all diagnostic groups, but Ang-2 expression was more readily evident in patients with SpA. Use of digital imaging analysis to quantify expression revealed that there was no significant difference in VEGF expression
Table 2. Baseline patient characteristics persistent versus self-limiting disease.

<table>
<thead>
<tr>
<th></th>
<th>UA self-limiting (n=12)</th>
<th>UA persistent (n=12)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>43 (20-67)</td>
<td>48 (28-66)</td>
<td>0.76</td>
</tr>
<tr>
<td>Dis. Duration (months)</td>
<td>4 (1-10)</td>
<td>5 (1-11)</td>
<td>0.66</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>26 (4-68)</td>
<td>36 (14-85)</td>
<td>0.27</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>11 (2-58)</td>
<td>22 (3-42)</td>
<td>0.21</td>
</tr>
<tr>
<td>TJC 68 (n)</td>
<td>3 (0-19)</td>
<td>8 (2-37)</td>
<td>0.05</td>
</tr>
<tr>
<td>SJC 66 (n)</td>
<td>1 (1-18)</td>
<td>5 (1-20)</td>
<td>0.03</td>
</tr>
<tr>
<td>VAS global (0-100)</td>
<td>56 (9-86)</td>
<td>51 (32-98)</td>
<td>0.38</td>
</tr>
<tr>
<td>IgM RF positive (%)</td>
<td>8</td>
<td>16</td>
<td>0.49</td>
</tr>
<tr>
<td>ACPA positive (%)</td>
<td>8</td>
<td>33</td>
<td>0.10</td>
</tr>
</tbody>
</table>

UA = undifferentiated arthritis, VAS = visual analog scale of global disease activity; TJC68 = 68 tender joint count; SJC66 = 66 swollen joint count; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; IgM-RF positive = immunoglobulin M rheumatoid factor positive; ACPA positive = anti-citrullinated protein antibody positive. Median and range are shown. Groups were compared by Kruskal- Wallis test. A P-value of > 0.05 was considered significant.

Table 3. Baseline patient characteristics erosive versus non-erosive RA.

<table>
<thead>
<tr>
<th></th>
<th>RA non-erosive (n=15)</th>
<th>RA erosive (n=10)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>54 (22-58)</td>
<td>53 (24-82)</td>
<td>0.88</td>
</tr>
<tr>
<td>Dis. Duration (months)</td>
<td>4 (1-12)</td>
<td>5 (1-10)</td>
<td>0.86</td>
</tr>
<tr>
<td>DAS28</td>
<td>5.9 (3.3-6.7)</td>
<td>5.0 (2.9-6.6)</td>
<td>0.13</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>27 (7-76)</td>
<td>37 (3-91)</td>
<td>0.67</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>16 (3-133)</td>
<td>9 (3-114)</td>
<td>0.62</td>
</tr>
<tr>
<td>TJC 68 (n)</td>
<td>18 (4-38)</td>
<td>8 (2-23)</td>
<td>0.08</td>
</tr>
<tr>
<td>SJC 66 (n)</td>
<td>8 (1-41)</td>
<td>7 (3-16)</td>
<td>0.39</td>
</tr>
<tr>
<td>VAS global (0-100)</td>
<td>50 (25-98)</td>
<td>27 (11-76)</td>
<td>0.12</td>
</tr>
<tr>
<td>IgM RF positive %</td>
<td>43</td>
<td>66</td>
<td>0.27</td>
</tr>
<tr>
<td>ACPA positive %</td>
<td>50</td>
<td>44</td>
<td>0.80</td>
</tr>
</tbody>
</table>

RA = rheumatoid arthritis; VAS = visual analog scale of global disease activity; TJC68 = 68 tender joint count; SJC66 = 66 swollen joint count; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; IgM-RF positive = immunoglobulin M rheumatoid factor positive; ACPA positive = anti-citrullinated protein antibody positive. Median and range are shown.

between the diagnostic groups (Figure 1C). Expression of Ang-1 was comparable between the patients with RA at baseline (RA-RA) and the patients who fulfilled ACR criteria over time (UA-RA). In contrast, expression of Ang-1 was significantly higher in RA-RA (P=0.03) and UA-RA (P=0.02) patients compared to SpA patients. Ang-1 expression in UA-RA patients was also elevated compared to patients that remained UA (UA-UA) after 2 years of follow up (P=0.01). For Ang-2, expression was elevated in SpA patients compared to those with RA (P=0.003).
The Ang-1/Tie2 axis is specifically activated in synovial tissue of RA patients before diagnostic criteria are met

We next examined synovial expression of VEGFR and Tie2. VEGFR expression was observed in the sublining vessels of patients in all diagnostic groups (Figure 2A). Tie-2 expression was seen in the intimal lining layer, the synovial sublining and in blood vessels. Quantitative analysis revealed that there were no significant differences in VEGFR expression between different diagnostic groups (Figure 2B). Expression of Tie2 was comparable between the patients with RA at baseline and UA-RA patients who fulfilled ACR criteria over time. However, Tie2 expression in both of these patient groups was elevated compared to patients with SpA ($P=0.001$). UA-RA patients also demonstrated increased Tie2 expression compared to UA-UA patients ($P=0.001$). As Ang-1 expression was elevated in these same RA-RA and UA-RA diagnostic groups, we examined the activation status of Tie2 using phospho (p)-specific antibodies. Activated Tie2 was observed primarily in the intimal lining layer and in vessels, but could also be observed in synovial sublining tissue (Figure 2A). Activated Tie2 was significantly elevated in RA-RA ($P=0.001$) and UA-UA patients ($P=0.003$) compared to SpA patients (Figure 2B). Importantly, p-Tie2 levels were also increased in UA-RA patients compared to UA-UA patients ($P=0.006$), although the relative activation of Tie2 (p-Tie2/Tie2) did not differ between diagnostic groups. These data suggest that Ang-1/Tie2 signaling is specifically activated in RA, even before patients fulfill ACR criteria of RA, while Ang-2 is specifically up-regulated in SpA. In confirmation of this, logistical regression analysis demonstrated that synovial Ang-1 and p-Tie2 expression was significantly related to the development of RA, with explained variances ($R^2$) of 46% ($P=0.03$) and 57% ($P=0.03$), respectively.

Expression of Ang-1 and Tie2 is related to the development of persistent disease in UA patients

We next investigated the serum and synovial levels of the same angiogenic markers in relationship to disease persistence in all patients diagnosed with UA at baseline (UA-RA, n=8; UA-UA, n=16). Twelve of these patients had self-limiting disease, and 12 developed persistent disease. Serum levels of VEGF, Ang-1 and Ang-2 did not differ between the two outcome groups (Figure 3A). Also, no differences were observed in synovial tissue, as detected by digital imaging analyses (Figure 3B). Synovial VEGFR expression was also similar between outcome groups, but patients who developed persistent disease had significantly elevated synovial expression of Tie2 ($P<0.05$) and active p-Tie2 ($P<0.01$) (Figure 4). To better understand the relationship between each of the angiogenic markers and the persistence of disease, we performed linear regression analysis. Serum levels of VEGF, Ang-1 and Ang-2 had no predictive value for the development of persistent disease (data not shown). However, synovial expression of Ang-1 ($R^2=34\%, P=0.04$) Tie2 ($R^2=40\%, P=0.04$) and p-Tie2 ($R^2=48\%, P=0.04$), were significantly related to development of persistent arthritis. Backward logistic regression analysis demonstrated that in a combined model incorporating Ang-1 and p-Tie2, the explained variance increased to 68% ($P=0.048$).

Relative engagement of synovial Tie2 is related to the development of erosive disease in RA patients

To evaluate the relationship between angiogenic markers and development of joint destruction over time, we grouped all patients with RA after 2 years follow up (RA-RA, n=19; UA-RA, n=8)
Figure 2. Vascular endothelial growth factor receptor (VEGFR), Tie2 and phosphorylated (p) Tie2 in the different outcome groups. Representative immunohistochemical staining (A), and quantitative analysis of expression in synovial tissue (B). UA-UA = undifferentiated arthritis at baseline and after follow up, RA-RA=rheumatoid arthritis at baseline and after follow up, UA-RA= undifferentiated arthritis at baseline who develop rheumatoid arthritis after follow up, SpA=spondyloarthritis. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the box mark the median value, and lines outside the boxes denote the 10th and 90th percentiles. Lines connecting data sets indicate statistically significant differences between groups. ** P < 0.005
Figure 3. Serum (A) and synovial tissue (B) expression of vascular endothelial growth factor (VEGF), angiopoietin 1 (Ang-1), and angiopoietin 2 (Ang-2) in undifferentiated arthritis patients with self-limiting or persistent arthritis after 2 years of follow up. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the box mark the median value, and lines outside the boxes denote the 10th and 90th percentiles. Lines connecting data sets indicate statistically significant differences between groups. * $P < 0.05$.

Figure 4. Synovial tissue expression of vascular endothelial growth factor receptor (VEGFR), Tie2 and phosphorylated (p) Tie2 in undifferentiated arthritis patients with self-limiting and persistent disease after follow up. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the box mark the median value, and lines outside the boxes denote the 10th and 90th percentiles. Lines connecting data sets indicate statistically significant differences between groups. * $P < 0.05$ ** $P < 0.01$. 
based on the absence or presence of erosive disease. Two patients were lost to follow up, 10 patients developed erosive disease, and 15 patients had persistent non-erosive disease. Serum levels of VEGF, Ang-1, and Ang-2 did not differ between patient outcome groups (Figure 5A), nor did synovial expression of the angiogenic factors (Figure 5B). Additionally, VEGFR, Tie2, and p-Tie2 levels were similar between outcome groups. However, the relative activation of Tie2 was significantly elevated in patients who developed erosive disease ($P < 0.01$) (Figure 6). Linear regression analysis revealed that the relative activation of Tie2, but not other markers, was significantly related to the development of erosive disease in RA ($R^2=49\%, P=0.03$).

FIGURE 5. Serum and synovial tissue expression of vascular endothelial growth factor (VEGF), angiopoietin 1 (Ang-1) en angiopoietin 2 (Ang-2) in rheumatoid arthritis (RA) patients with non-erosive or erosive RA after follow up. Erosive disease was defined by a Sharp-van der Heijde erosion score ≥ 1 at two year follow up. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the box mark the median value, and lines outside the boxes denote the 10th and 90th percentiles.

DISCUSSION

One of the challenges in current patient care in early arthritis clinics is starting patient tailored treatment as soon as possible. Timely patient tailored treatment is aimed at remission hereby preventing joint destruction and optimizing functional outcome with a minimum of (potential) harmful side-effects. Treatment decisions are guided by the clinical diagnosis, the biochemical or molecular characteristics of the specific disease and the estimated risk of development of joint destruction. Recently developed models have used clinical parameters to aid in this process.[8,10] Lately, increasing effort is put into the identification of biochemical markers with potential diagnostic or prognostic value, which might even serve as therapeutic targets that can guide personalized treatment decisions.[37]

ACPA and rheumatoid factor are serological parameters that are quite specific for the diagnosis of RA. In many RA patients, the onset of clinical symptoms is preceded by up to
several years by the appearance of circulating autoimmune IgM-RF and ACPA, indicating a weakening of peripheral tolerance in the adaptive immune system. Recently, ACPA has been incorporated in the novel 2010 ACR/EULAR RA criteria.[38,39] Also, these autoantibodies have been associated with a higher risk of development of destructive disease.[40] Synovial fluid concentrations of cytokines derived from, or indicative of, activated T lymphocytes, including IFNγ, IL-2, IL-4, and IL-17 were shown to be elevated in patients with UA prior to their development of RA.[41] A similar profile of T cell cytokines also predominate the serum of individuals with no complaints of synovitis but who later develop RA.[42] Previously, other serological markers such as VEGF,[28] IL-32,[43] MMPs,[44] and soluble granzyme B [45] have also been associated with destructive disease in RA. Examining synovial tissue in early arthritis patients, we previously observed a significant difference in synovial tissue expression of macrophages and plasma cells at the group level between early RA and non-RA patients.[46] However, in an independent study on consecutive patients with early arthritis, we were unable to identify these same differences between RA and UA patients (van de Sande, chapter 5 this

Figure 6. Synovial tissue expression of vascular endothelial growth factor receptor (VEGFR), Tie2, and phosphorylated (p) Tie2 in rheumatoid arthritis (RA) patients with non-erosive RA or erosive RA. Erosive disease was defined by a Sharp-van der Heijde erosion score ≥ 1 at two year follow up. Relative Tie2 phosphorylation was calculated as the ratio of IOD phosphorylated protein to IOD of total protein (arbitrary units). Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the box mark the median value, and lines outside the boxes denote the 10th and 90th percentiles. Lines connecting data sets indicate statistically significant differences between groups. ** P < 0.01
thesis). Together, besides the validated prediction models based on clinical parameters,[10,47] no novel serological or synovial immunohistochemical biomarker has been strong enough to guide initial treatment decisions in the individual patient.

Here, we provide the first biochemical evidence that changes occur in synovial tissue prior to our ability to determine a disease diagnosis. Our results demonstrate that expression of Ang-1, and associated Tie2 activation, is increased in RA compared to SpA, while Ang-2 expression is more prominent in SpA. In previous studies of this patient cohort, we did not identify significant differences in expression of cellular and vascular markers between RA patients with self-limiting, persistent or persistent-erosive disease (van de Sande, chapter 5 this thesis), although vWF expression was significantly different between UA-RA patients and UA-UA patients. In this current study, we did not observe a difference in cellular infiltrate between the different diagnostic groups at baseline. Additionally, although vWF expression, representative of synovial tissue vascularity, was significantly increased in SpA compared to RA, we observed no significant correlation between expression of angiogenic markers and vWF (data not shown), indicating that difference in the expression and engagement of Ang-1 and Tie2 is not secondary to differences in the level of synovial inflammation and vascularization. Together, this suggests that Ang-1/Tie2 signaling is specifically involved in pathogenic processes active in RA, from the earliest phases on, even before ACR criteria of RA are met, and the resultant activation of Tie2 promotes the development of persistent and erosive disease.

Differences in microscopic and macroscopic synovial tissue vascularisation between RA and SpA patients have been a consistent finding in previous studies.[14–17] Not only does the number of vessels vary between these patients groups, but differences in vessel morphology are also observed. Ang-2, which is known to be involved in vessel destabilization and the sprouting of new vessels, is highly expressed in psoriatic arthritis compared to RA, and increased expression of Ang-2 is associated with the appearance of tortuous vessels observed in SpA synovial tissue, compared to the more straightened vascularization observed in RA.[16,17] The exact mechanisms by which local Ang-1 stimulation of Tie2 contributes to the development of persistent erosive RA remain to be elucidated, but may reflect processes independent of direct effects on endothelial cells. For example, in vitro stimulation of RA FLS with Ang-1 induces activation of MAP kinase, PI3-K and NFκB signaling pathways, protecting FLS against apoptosis, promoting FLS migration, and stimulating production of pro-MMPs and degradation of cartilage matrix.[23] In murine models of RA, blockade of Tie2 not only decreases synovial angiogenesis, but decreases arthritis development and severity, as well as joint destruction. Here, decreased joint destruction was associated with decreased synovial RANKL expression, independently of synovial inflammation status.[33]

Our study demonstrates that an increased relative engagement of Tie2 is prospectively related to the development of joint destruction suggesting that this pathway plays an important role in joint destruction in early arthritis patients. Increases in the expression of levels of Ang-1, Tie2 and pTie2 in patients with persistent arthritis compared to patients with self-limiting arthritis, and increased pTie2/Tie2 ratio in RA patients who develop erosions over time, may be useful predictive indicators of disease outcome. These markers could explain up to 68% of the variance in outcome, which is quite extensive when comparing to previous studies applying immunohistochemical synovial tissue analysis as a predictive biomarker for response to treatment.[48] However, our findings indicate that other variables are involved as well, and that
combinations of different clinical or biological parameters will improve the predictive value of synovial Tie2 engagement, making it possible to guide treatment decisions in individual patients. As important, our study suggests that targeting Ang-1 and Tie2 therapeutically at the earliest stages of disease may be useful in improving outcome in arthritis.

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