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Title: Histologic and transcriptional evidence of subclinical synovial inflammation in rheumatoid arthritis patients in clinical remission.

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ABSTRACT:

Introduction: Patients with rheumatoid arthritis (RA) in clinical remission may have subclinical synovial inflammation. Here, we sought to determine the proportion of patients in remission or low disease activity at the time of arthroplasty that have histologic or transcriptional evidence of synovitis, as well as clinical features that distinguish patients with subclinical synovitis.

Methods: We compared disease activity scores with 28 joint counts (DAS28) to synovial histology features in 135 patients with RA undergoing arthroplasty. We also compared DAS28 to RNA-Seq data on a subset of 35 patients.

Results: 14% of patients met DAS28 criteria for clinical remission (DAS28<2.6) and another 15% met criteria for low disease activity (DAS28<3.2). Histologic analysis of synovium revealed synovitis in 27% and 31% of samples from patients in remission and low disease activity respectively. Patients with low disease activity (DAS28<3.2) and synovitis also exhibited increased CRP and anti-citrullinated peptide antibody (CCP) levels compared to those without synovitis. 183 genes were differentially expressed in synovium of patients with subclinical synovitis compared to those with low inflammatory synovium. 86% of these genes were also differentially expressed in synovium of patients who were clinically active (DAS28≥3.2).

Conclusion: 31% of patients with low clinical disease activity exhibit histologic evidence of subclinical synovitis, which was associated with increased CRP and CCP levels. Synovial gene expression signatures of clinical synovitis are present in patients with subclinical synovitis.

INTRODUCTION:

An improved understanding of remission in long-standing rheumatoid arthritis (RA) is important to identify patients that may safely discontinue treatment. A primary concern with discontinuing medication is that disease activity may return and treatment response may be difficult to recapture. Several groups have studied discontinuation of biologics in long-standing RA and found that approximately 50% of patients flare within one year[1-4]. Thus, the American College of Rheumatology (ACR) treatment guideline recommends lifelong treatment, even in patients in clinical remission[5].

As measured by ultrasound imaging, 43% of patients in remission have evidence of increased power Doppler signal[6]. The clinical relevance of subclinical synovitis is underscored by the fact that patients with subclinical synovitis on imaging are at risk for flare[7] and can continue to accrue radiographic damage[8]. These radiographic studies[6, 9, 10] suggest that clinical remission is distinct from immunologic remission; however, the histological and transcriptional features of ongoing synovitis during clinical remission are unknown.

To gain insights into the cellular and transcriptional mechanisms underlying RA, we recently performed histologic and RNA sequencing (RNA-Seq) analysis of RA synovium[11]. Clustering of synovial gene expression data identified three synovial subtypes of RA, including low-inflammatory, high-inflammatory, and mixed subsets[11]. Though these synovial subsets were significantly associated with autoantibodies such as CCP, RF and systemic markers of inflammation such as CRP and ESR, they were not associated with clinical features such as swollen and tender joint counts or pain scores.

Arthroplasty represents a unique opportunity to examine the synovium of RA patients with longstanding disease with various levels of disease activity. Here, we sought to determine (i) the proportion of patients in DAS28 remission at the time of arthroplasty that exhibit synovial inflammation, (ii) the clinical features that might distinguish patients with subclinical synovial inflammation from those without, and (iii) the transcriptional features of subclinical synovitis.

METHODS:

Study Setting

This was approved by the Hospital for Special Surgery and Rockefeller University ethical review boards (#2014-233 and DOR0822). All included patients signed informed consent. Patients over the age of 18 undergoing total hip arthroplasty (THA) or total knee arthroplasty (TKA) as previously described[12], who met classification criteria for RA using the ACR/EULAR 2010 or 1987 criteria, and had both preoperative DAS28-ESR and complete histology scores available were included in this analysis.

Clinical Data

Pre-operative data collected included age, sex, comorbidities, duration of disease, and medications. Disease activity was measured using DAS28-ESR and scores were classified as high= DAS28 ≥5.1, moderate= DAS28≥3.2- <5.1, low= DAS28 ≥2.6-<3.2, remission= DAS28<2.6 [13]. Rheumatoid factor (RF) and anti-CCP antibodies, joint counts (excluding the surgical joint), and inflammatory markers were measured pre-operatively. Since clinical CCP assays are reported as values up to 250, CCP was also measured by our group, using a research-grade Bio-Plex CCP assay[14] (Bio-Rad, Hercules, CA, USA) that utilizes multiple citrullinated peptides conjugated to an individual Bio-Plex bead and is read as mean fluorescent intensity (MFI). Medication use, such as methotrexate, disease modifying anti-rheumatic drugs (DMARDs), and biologics, was recorded.

Histology

Synovium obtained at arthroplasty was examined by gross inspection and areas that appeared inflamed, that is, opaque and dull, were selected for histology. If no area appeared inflamed by gross inspection, the femoral aspects of the medial and lateral gutters, and the central supratrochlear region in the suprapatellar pouch were selected. Hematoxylin and eosin stains were performed on synovial tissue collected at the time of arthroplasty and synovium was assessed for 10 features as described [11], including lymphocytes, plasma cells, lining hyperplasia, binucleate plasma cells, Russell bodies, fibrin, neutrophils, synovial multinucleated giant cells, detritus, and mucin. Binucleate plasma cells, Russell bodies, fibrin and neutrophils were all scored as either absent or present. Mucin was scored as none, slight, moderate or marked. Lymphocytes were scored as either none, mild, moderate or marked. Synovial lining hyperplasia was categorized as normal, mildly increased, or markedly increased (>4 cells thick). Plasma cells were classified as <10%, 10-50% or > 50% of infiltrating lymphocytes. Additionally, the 10 histology features were summarized into a single score of high, mixed, or low inflammatory synovium using our machine learning derived algorithm as described [11]. A full description of the histology scoring system, with example images can be found at https://www.hss.edu/pathology-synovitis.asp. Similarly, the algorithm to summarize the 10 histology features into a single summary score can be found at https://predictrasubtypes.shinyapps.io/shiny/.

Data Analysis

Pre-operative patient characteristics are summarized as percentages for categorical variables. The distribution of continuous features was assessed for normality using the Shapiro-Wilk test. Continuous characteristics are summarized by mean ± standard deviation or median [interquartile range], as appropriate. Pearson's chi-squared test was used to compare the frequency of the individual histology feature scores and summary histology score according to DAS28 category. For samples with sparse cell counts, we collapsed disease activity categories to DAS28< or ≥3.2 and used the Fisher's exact test. Mann-Whitney tests were used to compare clinical features of patients, and their disease activity scores, with or without subclinical synovitis and other histology features.

RNA-Sequencing

RNA-Seq datasets were generated on bulk RNA isolated from arthroplasty synovial samples as previously described[11]. The data set can be accessed at ImmPort (accession no. SDY1299). Thirty-five of the samples in this dataset had available DAS28 ESR and were included in this analysis. We used DESeq2 to normalize the data and genes with p value less than 0.01 using Benjamini-Hochberg to adjust for multiple comparisons were considered differentially expressed. We evaluated Gene ontology term enrichment of significantly differentially expressed genes using GoMiner [15] (Database Build 2011-11, Application Build 469).

RESULTS:

Clinical remission is uncommon in RA patients undergoing arthroplasty

Clinical characteristics of 135 patients, according to disease activity score, are presented in Supplemental Table 1. Despite relatively long disease duration and aggressive therapy with DMARDs including biologics, at the time of arthroplasty remission was uncommon, with 14% of patients (n=19) meeting DAS28 criteria for remission. Drug free remission was exceedingly rare and was seen in only 2 patients (1.5%). Fifty-three percent had moderate disease activity and 18% had high disease activity, and therefore 71% of all patients met ACR-EULAR criteria for medication dose escalation. There was no significant difference in age or disease duration between the disease activity categories, and there was no correlation of disease duration with disease activity (Spearman's p= -0.07).

Histologic evidence of subclinical synovitis

While most patients in DAS28 remission had low inflammatory synovium, some were markedly inflamed. A sample image of highly inflamed synovium from a patient in DAS28 remission is presented in Figure 1A. We quantitated the frequency of ten individual histologic features of synovitis according to DAS28 score (Figure 1B). While there was a trend of increasing binucleate plasma cells, plasma cells, and Russell bodies with increasing disease activity, none of the individual histology features were significantly more common in any disease activity group. When we applied our histology scoring algorithm to summarize the 10 histology features into a single score, we again note a non-significant trend of increasing frequency of high inflammatory synovitis with increasing DAS28 score. Of note, more than half of all patients with moderate and high disease activity had low inflammatory synovium.

This likely represents expected sampling error since RA does not affect all joints equally at a given time (i.e. it is possible to have active RA related inflammation in some joints while having low inflammation in the arthroplasty joint). On the other hand, 16% of synovial samples from patients in DAS28 remission had the mixed synovial inflammatory subtype while 11% had the high inflammatory subtype (Figure 1C). We next grouped patients with mixed and high inflammatory synovium, and labelled these subclinical synovitis according to histology score. Using this definition, 27% of patients in DAS28 remission had histologic evidence of subclinical synovitis. Similarly, 31% of those with DAS28 low disease activity had histologic evidence of subclinical synovitis (Figure 1D).

Clinical features of patients with and without histologic subclinical synovitis

We compared clinical features of patients DAS28<2.6 (remission, n=19) (Figure 2A) or DAS28<3.2 (low disease activity and remission, n=39) (Figure 2B) with and without histologic evidence of subclinical synovitis. Age, disease duration and ESR were not different in patients with and without synovitis. CRP was significantly increased in patients with synovitis in both remission and in those with DAS28<3.2. There was a trend of increased CCP level in those with histologic evidence of subclinical synovitis in DAS28 remission (Figure 2A), and this finding was statistically significant when patients with DAS28<3.2 were included (Figure 2B).

Transcriptional features of subclinical synovitis

In our prior work, we found that histologic features of synovial samples could be used to predict gene expression subtype [11]. Here we sought to identify gene expression features of subclinical synovitis in order to provide insight into the

immune processes that remain active in patients with low disease activity. Our previous analysis identified three robust synovial gene expression clusters, characterized by low, mixed and high inflammatory gene expression. Principal component analysis of the 500 genes with most variable expression across all samples is presented in Figure 3A. Principal component one and principal component two capture 41.2% and 9.8% of the variance, respectively. Classifying the data according to our previously defined synovial gene expression clusters demonstrates that principal component one is associated with increasing inflammation (Figure 3A, top panel). However, when the samples are classified according to DAS28 category, it becomes clear that while patients with high inflammatory gene expression are more likely to have DAS28≥3.2, patients with low inflammatory gene expression have striking variability in disease activity scores (Figure 3A, lower panel). This result is anticipated and reminiscent of our finding that there are substantial proportions of patients with low inflammatory histology scores at all levels of disease activity (Figure 1C), which we attribute to the heterogeneity of joint involvement in any given patient with RA. We therefore grouped all samples with low inflammatory gene expression irrespective of disease activity and categorized those as the reference low inflammatory group. We then compared samples with gene expression evidence of either mixed or high inflammatory synovitis from patients with DAS28<3.2 or DAS28≥3.2 to the reference low inflammatory group (Figure 3B). We identified 183 differentially expressed genes in the DAS28<3.2 group (Supplemental Table 2) and 3,194 differentially expressed genes in the DAS28≥3.2 group (Supplemental Table 3) with adjusted p values less than 0.01. The vast majority (86%) of the differentially expressed genes in the DAS28<3.2 group were also differentially expressed in the DAS28≥3.2 group. A

heat map of 100 genes that were most significantly differentially expressed in samples with DAS28<3.2 relative to low inflammatory synovium is presented in Figure 3C. Pathways significantly enriched in the DAS28<3.2 group relative to low inflammatory synovium included lymphocyte activation and C-C chemokine binding (Supplemental Table 4) and B cell genes: MS4A1 (CD20), MZB1 (marginal zone B cell 1), CD5, TNFRSF17 (BCMA, B cell maturation factor), CD27, CD79A, as well as immunoglobulin genes. They also included CD8 T cell signature genes such as CD8A, CD8B, GZMK (granzyme K), GZMM (granzyme M), as well as IL2RB, IL2RG, GATA3, the canonical Th2 transcription factor, and chemokine genes such as CXCL10, CXCL12, CXCR6, CCR2, CCR4, and CCR6. 706 pathways were significantly enriched in the DAS28≥3.2 group (Supplemental Table 5) and additionally included pathways such as myeloid leukocyte activation, natural killer cell mediated immunity and wound healing.

DISCUSSION:

Here we analyzed data from a cohort of 135 long-standing RA patients undergoing arthroplasty to investigate the cellular and transcriptional features of subclinical synovitis in RA. We did not find a significant difference in the frequency of 10 histologic features typical of inflamed synovium in subgroups based on their disease activity. While it is expected that patients with high DAS28 scores do not necessarily have highly inflamed synovium in all joints at any given time, the finding of synovitis in patients with low disease activity was not anticipated. We found that 27% and 31% of patients in remission and low disease activity, respectively, had inflamed synovium according to our histology scoring algorithm.

Given the interest in determining whether there are RA patients that can safely discontinue DMARD treatment, we examined whether there were any clinical features that distinguish patients in remission or low disease activity either with or without subclinical synovitis. Though there was no difference in disease duration or age, patients with subclinical synovitis possessed increased anti-CCP antibodies, suggesting that the level of these autoantibodies might be useful in predicting the likelihood of achieving both clinical and histologic remission.

Our analysis of RNA-Seq data was consistent with our analysis of histology scores in relation to DAS28, that is, patients with low inflammatory synovium had variable DAS28 scores, highlighting the fact that not all joints are uniformly affected by inflammation individual patients with RA. Using low inflammatory samples as a reference group, we identified 183 differentially expressed genes in patients with low disease activity (DAS<3.2). The vast majority of these genes were also differentially expressed in patients with DAS28≥3.2. Interestingly, we identified a robust signature of B cell activation in patients with gene expression evidence of either mixed or high inflammatory synovium and DAS28<3.2. This observation parallels our finding of increased anti-CCP antibody levels in patients with histologic evidence of synovitis and DAS28<3.2, raising the possibility that the synovium may be a niche for anti-CCP antibody producing cells in patients with subclinical synovitis. In particular, our gene expression analysis identified B cell signature genes such as TNFRSF17/BCMA and MS4A1 (CD20), which are targets of approved agents that limit B cell mediated inflammation (such as rituximab and belimumab). We hypothesize that there may be a role for these drugs, specifically to dispel persistent

inflammation in RA patients with subclinical synovitis, prior to treatment discontinuation.

Our study has several important limitations. First, is the small sample size of only 19 patients in clinical remission and 20 patients with low disease activity at the time of arthroplasty. This highlights the fact that remission is uncommon even in those with long-standing disease. Additionally, patients referred to a tertiary care hospital and requiring treatment with arthroplasty, represent a bias in the patient population studied. Since most patients do not receive their rheumatologic care at our institution and were referred for the purpose of surgery, we only had access to disease activity scores for the majority of patients at the presurgical screening visit and do not know the duration or stability of their disease activity. Patients with longer duration of remission may be less likely to have ongoing subclinical synovitis. Analysis of tissue from one joint in a polyarticular disease suffers from sampling error, since not all joints are affected equally at any given time, making it difficult to connect either histology or transcriptional data to composite disease activity scores that include joint counts. Further, even within individual joints, histologic studies have demonstrated heterogeneous involvement of the synovium, and thus sampling bias within the synovium of individual joints may have also confounded our results.

In summary, we observe a discordance between clinical and immunologic disease activity in a subset of long-standing RA patients in DAS28 clinical remission. Of patients in clinical remission, 27% continued to have histologic evidence of subclinical synovitis. Gene expression profiling of patients with subclinical synovitis demonstrate evidence of ongoing inflammation characterized by retention of CD8 T

cells and B cells as well as chemokine production. CCP and CRP levels may be useful for identifying RA patients more likely to have subclinical synovitis.

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FIGURE LEGENDS:

Figure 1: Histologic and transcriptional evidence of subclinical synovitis in RA patients in DAS28 remission at the time of arthroplasty

A. A sample image of H&E stained synovium from a RA patient in DAS28 remission at the time of arthroplasty reveals immune cell infiltrates and other histologic features of inflammation. Left panel: 5x magnification, Right panel: 20x magnification. B. Frequency of 10 synovial histologic features of 135 patients with various disease activity scores. C. Frequency of low, mixed or high inflammatory summary histology score of patients with various disease activity score levels. D. Frequency of synovitis (either mixed or high inflammatory) or no synovitis in patients with various disease activity scores. There was no difference in the frequency of any histology feature according to DAS28 category using Pearson's chi-squared or Fisher's exact test after adjusting for multiple comparisons.

Figure 2: Clinical features of RA patients with and without histologic features of subclinical synovitis

A. Clinical features of patients with remission (DAS28<2.6) (n=19) with or without histologic evidence of synovitis. B. Clinical features of patients with low disease activity (DAS28<3.2) (n=39) with or without histologic evidence of synovitis. Data presented are median and interquartile range. P values were calculated using Mann-Whitney test, NS=not significantly different.

Figure 3: Transcriptional features of subclinical synovitis

A. Principal component analysis of the top 500 most variably expressed genes detected by RNA-Seq from 35 RA patient synovial samples. Upper panel: samples

are classified according to gene expression cluster: low, mixed or high inflammatory. Lower panel: samples are classified according to DAS28 category. B. Principal component one versus DAS28. The line divides low inflammatory synovium (as defined by gene expression cluster) from the rest of the samples, which are classified as either DAS28<3.2 or DAS28≥3.2. C. Heat map of two-way hierarchical clustering of the top 100 most significantly differentially expressed genes of DAS28<3.2 relative to low inflammatory synovium of 35 RA patient synovial samples.