Inflammation and vascularisation markers of arthroscopically-guided finger joint synovial biopsies reflect global disease activity in rheumatoid arthritis

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ABSTRACT

Objective. *To analyse whether synovial markers of the clinically dominant metacarpophalangeal (MCP) joint reflect global disease activity measures in rheumatoid arthritis (RA).*

Methods. *Arthroscopically-guided synovial biopsies from the dominant metacarpophalangeal (MCP) joint of 10 patients with RA (DAS28 >3.2) were stained for determination of the synovitis score, CD68, vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1α (HIF-1α). MRI and ultrasound were used to calculate the RAMRIS and US7 score respectively. Arthroscopy of the same joint was repeated in 6 patients after 6 months.*

Results. *The synovitis score significantly correlated to DAS28 (Spearman r=0.74), CRP (r=0.69), and US7 (r=0.66); sublining CD68 macrophages to CRP (r=0.6); HIF-1α to DAS28 (r=0.77), CRP (r=0.73); and VEGF to DAS28 (r=0.753) and RAMRIS (r=0.663). All patients showed a reduction of the DAS28 after 6 months (mean±SD: 5.2±1.5 vs. 2.75±1.1; p<0.05). There were three patients with a good EULAR response, and only these showed declining sublining CD68 macrophages in the control biopsy (χ^2 test: LR 8.3, p=0.05). Two of the remaining patients with increasing CD68 sublining macrophages showed a deterioration of the RAMRIS.*

Conclusion. *Some histological findings in arthroscopically-guided biopsies of the dominantly affected MCP joint reflect global disease activity measures and their changes in RA patients. Moreover, repeated MCP synovial biopsy may distinguish true responders from individuals with residual disease activity, who are not readily recognised by clinical means.*

Introduction

Synovial tissue analysis in RA has increased our understanding of disease mechanisms, and can be used in early phase trials to assess response to treatment (1, 2). Markers such as sublining CD68 macrophages have consistently been shown to be excellent disease activity parameters of rheumatoid arthritis (RA), potentially even superior to clinical composit measures such as the DAS28, which may be more liable to placebo effects (3-5). A study by Kraan *et al.* involving 9 patients suggested that comparable histological signs of inflammation in clinically inflamed joints are found irrespective of their localisation (6). This justified that synovial biopsies are usually collected from large joints such as the knee. However, the knee joint might not be affected in a considerable number of patients at the time of sampling. Thus, other large inflamed joints are recommended alternative choices (1). We noted that a number of RA patients refer to an MCP joint as their most severely affected joint in terms of pain and functional disability, especially in early disease, often in the absence of considerable clinical inflammation of larger joints. This is in accordance with systematic analyses of the topic (7). Uninflamed large joints may be biopsied, but display less severe inflammation (8), potentially not representing overall disease activity. In these cases, synovial sampling of the dominant MCP joint is an attractive option. However, to our knowledge, there are no systematic studies evaluating whether the changes observed relate to overall disease activity. Furthermore, there is theoretical concern that extensive sampling in such a small joint could preclude longitudinal analyses because of synovial scaring, or is not well tolerated by patients. Earlier studies have established the safety of singular MCP joint arthroscopy in RA (9, 10). Therefore, we assessed whether singular and repeated analysis of macroscopically inflamed synovial tissue, gathered from few targeted biopsies of MCP-joint synovial tissue, reflects measures of global disease activity such as the DAS28 or imaging procedures in RA patients.

Patients and methods

10 consecutive patients with RA based on 2010 ACR/EULAR criteria with (1) a DAS28 >3.2, who (2) required initiation of DMARD therapy (n=4, methotrexate) or a switch of medication (n=6 patients with methotrexate additionally received adalimumab), (3) indicated an MCP joint as their most severely af-

fected and painful joint, and (4) gave their full informed written consent into the study, were recruited. All patients received an ultrasound examination with determination of the US7 score (11) and a Gadolinium-enhanced MRI (either 3.0 Tesla MRI (n=6) or alternatively 0.2 Tesla MRI in case of claustrophobia (n=4)) of the hand with determination of the RAMRIS score (12). Within one week, arthroscopy was carried out as described previously with 1.9 diameter arthroscope and local anaesthesia (Karl Storz, Tuttlingen, Germany) (9, 10). Briefly, 2 laterodorsal portals were created after skin incision for introduction of the arthroscopic camera and the biopsy forceps, respectively. A total of 6 synovial biopsies were obtained under visual control from macroscopically inflamed areas and snap frozen in Tissue-Tek (Sakura Finetek Germany, Staufen, Germany). The study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the ethics committee of the Medical Faculty of Heinrich-Heine-University (study number 3390).

Synovial tissue

3-5 µm serial sections were prepared from snap-frozen synovial tissue, Haematoxylin Eosion (HE) stained (Merck, Darmstadt, Germany) and evaluated prior to immunohistochemical staining of parallel sections. In each patient, a representative biopsy with the best morphology including a lining layer was selected for further analysis. Immunohistochemistry was carried out with monoclonal mouse anti-CD68 antibody (Dako, Glostrup, Denmark); anti-VEGF (Millipore, Billerica, USA), and anti-HIF-1α (Abcam, Cambridge, UK) antibody representing vascularisation; or IgG1 isotype control and secondary antibody contained within Dako Real Detection system, essentially according to manufacturer's instructions (all Dako). Stained sections were coded and evaluated at random by an observer blinded to the diagnosis and clinical data on a microscope (Axioskop 2 plus, Carl Zeiss, Jena, Germany) with digital camera (Nikon DS Vi 1, Nikon, Düsseldorf, Germany) and image acquisition software (NIS-Elements F, Nikon). HE stained sections were used for determination of the synovitis score according to Krenn (13). This is a semiquantitative 4-point sum-scale which considers lining layer hypertrophy, inflammatory infiltrate, and density of resident cells. CD68 of the sublining layer, VEGF, and HIF-1α were assessed by digital image analysis, essentially as previously described (14). Briefly, images were photographed in 100 x magnification and stored in TIF-format (resolution of 1600 x 1200). ImageJ (http://rsbweb.nih.gov/ij/index.html) was used to select regions of interest, *i.e.* sublining layer or total biopsy and the image was thresholded to highlight the stained areas, but not the respective isotype controls. For all samples stained with the same antibody, the same settings were used. After conversion of the photograph to an 8 bit binary image, the stained area was calculated as a fraction of the selected region.

Table I. Correlation of MCP joint synovial analyses with disease activity measures.

	DAS28	CRP*	US7[¥]	RAMRIS[§]
Synovitis score[¶]	**0.74 (*p*=0.014)**	**0.691 (*p*=0.019)**	**0.661 (*p*=0.038)**	0.327 (NS)
lining layer	0.289 (NS)	0.410 (NS)	0.239 (NS)	-0,200 (NS)
inflammatory infiltrate	**0.789 (*p*=0.007)**	**0.707 (*p*=0.015)**	0.494 (NS.)	0.539 (NS)
resident cells	0.412 (NS)	0.357 (NS)	0.601 (NS)	0.194 (NS)
Sublining CD68	0.553 (NS)	**0.599 (*p*=0.001)**	0.455 (NS)	-0,164 (NS)
VEGF	**0.753 (*p*=0.012)**	0.565 (NS)	0.620 (NS)	**0.663 (*p*=0.037)**
HIF-1α	**0.766 (*p*=0.01)**	**0.73 (*p*=0.01)**	0.564 (NS)	0.345 (NS)

*C-reactive protein, [¥]7 joint ultrasound score (11), [§]rheumatoid arthritis magnetic resonance imaging score (12), [¶]according to (13); NS: not significant.

Correlations of RA disease activity measures and synovial analyses of 10 MCP joint arthroscopies with the synovitis score and digital image analysis calculating the fraction of stained are of the total biopsy for vascular endothelial growth factor (VEGF) and hypoxia inducible factor 1α (HIF-1α), or the sublining region for CD68. Correlations were calculated according to Spearman, significant results are printed in bold.

Table II. Individual DAS28 responses of patients with follow-up synovial biopsies after 6 months (T_0, T_1) and corresponding sublining CD68 staining.

Patient number	DAS28		EULAR response	sublining CD68		RAMRIS*	
	T_0	T_1		T_0	T_1	T_0	T_1
1	5.6	1.2	good	47	12	34	22
2	3.2	1.7	good	13	1	26	26
4	5.1	3.2	good	9	1	13	7
6	5.7	4.4	moderate	24	27	37	40
7	6.5	3.3	moderate	7	8	71	82
10	3.5	2.6	moderate	3	9	34	22

*rheumatoid arthritis magnetic resonance imaging score (12).

Disease Activity Score of 28 joints (DAS28), EULAR response based on the DAS28, digital image analysis of CD68 staining, and MRI RAMRIS score of the hand in initial (T_0) and control biopsies after 6 months (T_1). A decline in sublining CD68 was only noted in those patients with a good EULAR response (χ^2 test: LR 8.3, *p*: 0.05).

Statistical analysis

Correlations were calculated according to Spearman. χ^2 test was used for analysis of a cross table including patients with a good EULAR response and declining CD68 staining. $p<0.05$ was considered significant. SPSS 21 was used for analyses.

Results

Correlations of synovial markers to disease activity

We were interested to determine whether markers of synovial inflammation relate to established measures of systemic disease activity (DAS28), and global inflammation (CRP). Table

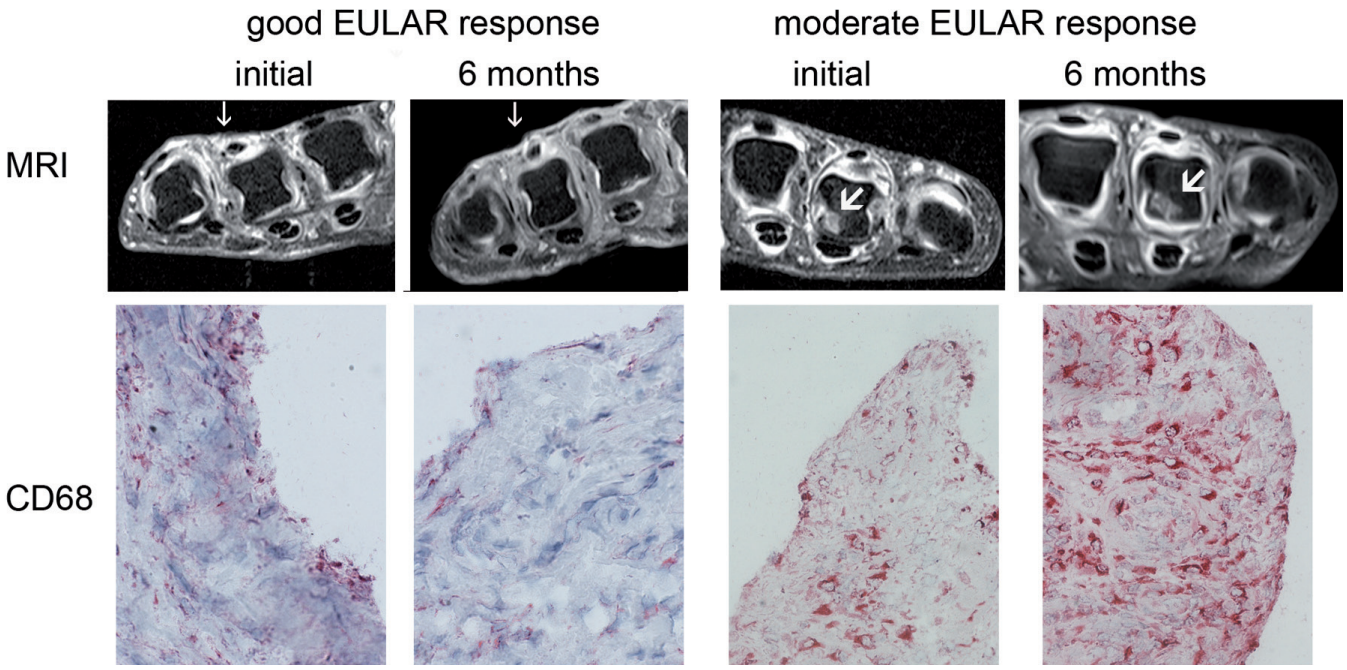

Fig. 1. MRI, synovial CD68 staining and EULAR response in individual patients.
Illustration of axial T1 MRI images of the hand and corresponding synovial membrane CD68 staining of one patient with a good EULAR response (P2, left panel: reduction in synovitis (arrow)) and a patient with only moderate response (P6, right panel: progressive erosions (arrow)) illustrating a progressive erosion in MRI and increasing sublining CD68 staining despite a moderate EULAR response.

I shows that the synovitis score correlated to the DAS28 and CRP. This was also true for the inflammatory infiltrate, but not for the other subscores. Similarly, CD68 staining correlated to CRP. Because vascularisation is a crucial event in RA pathophysiology and readily assessed by imaging procedures, we were interested to analyse how markers of vascularisation relate to global disease activity. As can be seen in Table I, HIF-1α and VEGF correlated to the DAS28, while only HIF-1α also related to CRP. Next, we assessed how the synovial markers related to global measures of modern imaging procedures. Correlations of the synovitis score with the US7 ultrasound score and of VEGF with the RAMIRS MRI score were observed (Table I). When considering the subscores of the respective imaging methods, VEGF staining correlated to bone marrow oedema (0.676, p=0.032) and erosions scores (0.695, p=0,026) of the RAMRIS, and synovitis (0.854, p=0.002) and power Doppler scores (0.676, p=0.032) of the US7. Correlations of the histological synovitis score to the US7 were largely due to correlations with the synovitis subscore of the US7 (0.694, p=0.026).

Individual changes in synovial tissue analysis

Furthermore, we were interested to explore the possibility that histological changes in the joint after 6 months relate to treatment response. In our cohort, 3 out of 6 patients fulfilled the criteria for a good EULAR response (P1, P2, and P4; Table II). Only these patients had declining sublining CD68 macrophages in the control biopsy, however statistical significance was not reached (χ^2 test: LR 8.3, p=0.05). Moreover, two of the remaining patients (P6, P7) had an increase in sublining CD68 macrophages and deteriorated in the RAMRIS score (Table II, Fig. 1) despite a moderate EULAR response with a reduction of the DAS28.

Safety of MCP biopsy

Patients were followed-up for 14.4±5.6 months. Adverse events consisted in light to moderate pain during the arthroscopy in 2 cases, temporary swelling of the hand and impeded motion of the MCP joint for up to one week in 15 cases. There were no serious adverse events (any permanent damage, damage to nerves or vessels, infections, thrombosis, embolism). Of the 10 patients initially enrolled, 6 consented into a second arthroscopy, one patient had to be excluded due to a heart attack 5 months after the initial arthroscopy, one patient was lost to follow-up, and 2 declined a second arthroscopy, because they felt to be in remission. Scaring after the first arthroscopy was minimal and no problems were encountered introducing the arthroscope a second time into the joint.

Conclusion

In the current study, we demonstrate that established histological assessments of the synovial membrane, e.g. sublining CD68 macrophages (3-5) and the synovitis score (13), as well as markers for vascularisation out of a single dominant MCP joint consistently relate to the DAS28, a widely used score of RA disease activity, or the serum CRP, a marker for systemic inflammation. Importantly, this was true in spite of our approach to limit the number of synovial biopsies in order to preserve enough synovial membrane for a second analysis under therapy. Arthroscopical guidance of biopsies permitted sampling of macroscopically inflamed or non-scared areas, limiting

sampling errors. This was especially important for the second biopsy, where discontinuous scaring of the synovium was observed in all cases, possibly due to the first biopsies, or effective treatment. Thus, the singular or repeated collection of few targeted MCP-joint biopsies with initial quality control of HE stained sections, may represent an alternative to more extensive sampling with pooling of biopsies from larger joints (1, 14), and still permit an adequate appraisal of overall RA disease activity. We are careful not to overinterpret these findings, because the patient number was limited and histological synovial markers were rather arbitrarily chosen and necessarily incomplete, given the small sample size out of MCP joints. Depending on the underlying question, the detection of additional markers such as CD4 and CD20 may be useful. Furthermore, high field MRI was not possible in all patients due to claustrophobia. Despite the recent demonstration of excellent sensitivity of low field MRI (15), different field strengths and the use of contrast agents have to be considered when interpreting this data. However, there are a few interesting implications. First, MCP biopsies may be used to further validate MRI or ultrasound technology, especially the concept of "silent progression", wherein patients with clinical improvement (*e.g.* DAS28) show a lack of improvement or deterioration of imaging findings. Interestingly, the global sumscores used for the assessment of ultrasound and MRI in the present study related to distinct histological markers. This reinforces the hypothesis that imaging of joint inflammation acquired by different techniques highlights distinct cellular or mechanistic aspects (16). More studies comparing histological data to imaging procedures are warranted to improve our interpretation of these images.

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