Effects of adalimumab treatment on endothelial cell activation markers in the skeletal muscle of patients with rheumatoid arthritis

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Abstract
Objective
Patients with rheumatoid arthritis (RA), particularly those with severe disease, have increased risk of cardiovascular disease (CVD). Previous studies suggest that endothelial cell activation may contribute to this co-morbidity, and that treatment with tumour necrosis factor (TNF) inhibitors could reduce the risk of CVD in these patients. The aim of this study was to investigate endothelial cell activation markers in muscle tissue of patients after adalimumab treatment.

Methods
Patients with active RA who started treatment with adalimumab 40 mg every two weeks were included. Muscle biopsies taken before and 3 months after start of treatment were available from 11 patients (9 females, mean age 54.2 years, median disease duration 6.5 years, 91% anti-CCP positive, 7 on methotrexate [median dose 20 mg/week]). None of the patients had clinical signs of myopathy. IL-1α and HLA-DQ were investigated by immunohistochemistry. Quantification was performed by computer assisted image analysis.

Results
Disease activity, measured by DAS28 decreased (mean 5.5 vs. 4.1; p=0.018). A good or moderate EULAR response was seen in 6/11 patients. HLA-DQ was mainly expressed in endothelial cells in capillaries, whereas IL-1α was mainly seen in larger vessels. HLA-DQ expression decreased significantly after treatment (p=0.041). There was a similar trend for IL-1α, in particular in EULAR good/moderate responders.

Conclusion
Adalimumab treatment was associated with decreased expression of endothelial markers previously associated with severe systemic inflammation in RA. Our findings indicate a reduced endothelial activation in patients treated with anti-TNF drugs, which might contribute to a lower risk of cardiovascular co-morbidity.

Key words
rheumatoid arthritis, endothelial activation, interleukin-1α, HLA-DQ, CD31, cardiovascular disease, adalimumab
Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease, which affects 0.5–1% of the population. Without successful treatment, it leads to joint damage, impaired activity of daily living and increased mortality, especially from cardiovascular disease (CVD). It is well established that there is an association between RA and cardiovascular events (1), mainly due to an increased risk of myocardial infarction (2). The mechanisms behind the increased risk for CVD in patients with RA are not fully understood. However, patients with a more severe form of RA, those with extra-articular manifestations, seem to have a higher risk to develop CVD than others suggesting that the load of inflammation is a risk factor (3, 4). Notably, in a previous study, patients with extra-articular RA had an increased expression of Interleukin-1α (IL-1α) and Human Leukocyte Antigen DQ (HLA-DQ) in the endothelium of small vessels in skeletal muscle compared to RA patients without extra-articular manifestations, matched for age, sex and disease duration (5). Such endothelial activation has been suggested to contribute to systemic inflammation and vascular disease (6), which might confer a risk to develop premature atherosclerosis. Several vascular abnormalities have been reported to be more common among patients with RA compared to healthy controls, such as increased thickness of the intima and media of the carotid artery (7) increased vascular stiffness (8) and endothelial cell dysfunction (9). These findings fit well with the concept of an inflammatory process that leads to activation of vascular endothelium and damage to the vessel wall, which may lead to cardiovascular events (10). The expression of IL-1α and HLA molecules in endothelial cells of skeletal muscle may indicate a systemic inflammatory reaction and thus could decrease after effective anti-inflammatory treatment.

Treatment with tumour necrosis factor (TNF) inhibitors including adalimumab has led to major clinical improvement in many cases of severe RA (11-14). In addition to reduced joint inflammation and protection from radiographic damage, TNF inhibitors may also have an effect on RA associated vascular co-morbidity. In a population-based cohort from the same catchment area as the present study, patients with RA treated with biologics who had no previous history of cardiovascular disease had a reduced risk by an estimated 50% of developing cardiovascular disease compared to RA patients treated with non-biologic disease-modifying anti-rheumatic drugs (DMARDs) when adjusted for disease severity (15). A recent metaanalysis of published cohort studies showed a decreased risk of myocardial infarction and cardiovascular events in patients with RA treated with anti-TNF drugs (16), although there are some differences in study design and also heterogeneity in the results of published studies (17). To this date, there have been no controlled trials evaluating the effect of TNF inhibitors on the risk of CVD in patients with RA. In previous studies of endothelial dysfunction, treatment with TNF-inhibitors led to decrease of classic biomarkers of endothelial cell activation in RA (18). Reduction of levels of some other biomarkers of endothelial cell activation following anti-TNF therapy has also been described in patients with chronic inflammatory rheumatic disease different from RA (19). More importantly, improvement of endothelial function was observed following the initiation of adalimumab in RA patients that had been refractory to another anti-TNF antagonist (infliximab) (20). Persistence of improvement of endothelial function after 1 year of adalimumab therapy without increase of morphological atherosclerotic damage measured by carotid ultrasound was also observed in 34 patients with RA (21).

Taken together, observations from earlier reports are compatible with a generalised blood vessel and endothelial cell involvement in at least subgroups of RA patients. Microvessel involvement with endothelial cell activation could play a role in the pathogenesis of cardiovascular disease and potentially be reversible by aggressive anti-inflammatory treatment. The aim of this study was to investigate markers of endothelial cell activation in muscle biopsies...
from patients with RA before and after 3 months of treatment with adalimumab and to correlate these data to clinical outcome variables.

Patients and methods

Consecutive patients, seen in a single centre, who fulfilled the 1987 American College of Rheumatology (ACR) classification criteria for RA (22), and for whom treatment with adalimumab (Humira®) was indicated according to their rheumatologist, were enrolled. They had to have been non-responders to at least one DMARD. Additional inclusion criteria were: at least six swollen joints in 28-joint index, and a CRP >8 mg/L within the last three months. Patients were excluded if they had been treated with anti-TNF drugs in the last three months prior to inclusion, received intravenous corticosteroids within fourteen days before inclusion, and if they had ongoing treatment with oral high-dose corticosteroids (equivalent to ≥20 mg of prednisolone daily) or had completed such treatment less than fifteen days before inclusion. Patients with contraindications to muscle biopsy, such as severe bleeding disorder, extensive or refractory leg ulcers or severe peripheral vascular disease were also excluded.

Tissue samples, clinical evaluation and laboratory parameters

Muscle biopsies were performed before and after three months of treatment with adalimumab. Biopsies were taken from the tibialis anterior muscle and were obtained under local anaesthesia using a semi-open technique (23). At least three biopsy samples were taken from each patient, snap frozen in isopentane chilled with liquid nitrogen, and stored at -70°C. The second biopsy was taken in the contralateral leg.

All muscle samples were assessed without knowledge of the patient history for histopathological changes by an experienced neuropathologist, i.e. Dr I Nennesmo at the Division of Pathology, Karolinska University Hospital, Huddinge, Sweden. Muscle biopsies were evaluated using conventional histopathology and immunohistochemistry on serial sections to identify pathological changes. The first and last section of each series of consecutive sections was stained with Mayer’s haematoxylin and eosin (24), to confirm that the histopathology of the biopsies remained unchanged in the consecutive series of sections. These sections were also used for evaluation of the presence of degeneration, regeneration, atrophy, central nuclei, and mononuclear cell infiltrates. Patients were evaluated at baseline and after 3 months of treatment with adalimumab for RA disease activity, using standard measures (number of swollen joints, number of tender joints, rheumatoid factor [RF], C-reactive protein [CRP], health assessment questionnaire [HAQ] disability index, patient’s assessment of pain, patient’s global assessment of disease activity and physician’s assessment of disease activity). In addition, a standard physical examination was performed, and data on medications and cardiovascular risk factors such as smoking, current hypertension and history of cardiovascular events were recorded using a structured clinical interview.

Immunohistochemistry studies

The skeletal muscle biopsy specimens were frozen in pre-cooled isopentane, embedded in OCT compound (Tissue-Tek, Sakura Finetek BV, Zoeterwoude, The Netherlands) and stored at -70°C until sectioning was performed. Cryostat sections from the biopsies (6-8 μm) were placed on chrome gelatin-coated slides (Novakemi AB, Enskede, Sweden) and air dried for 30 minutes. The sections were initially fixed for 20 minutes with freshly prepared 2% formaldehyde (Sigma Chemicals, St Louis, MO, USA) at +4°C, washed twice in phosphate buffered saline (PBS) and then left to air-dry before storage at -70°C. Immunohistochemical staining was performed using standard avidin-biotin-peroxidase complex technique, as previously described (25) (for details on primary antibodies, see Table I). As secondary antibody, a biotinylated horse anti-mouse IgG1 antibody (Vector Laboratories, Burlingame, CA, dilution 1/320) was used.

Evaluation of muscle biopsy stainings

The sections were coded, and the investigator was blinded to the clinical information for each section. Whole tissue sections were assessed using computer assisted image analysis. The method has been described in detail previously

Table I. Antibodies used for immunohistochemical stainings*.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Dilution or concentration used for tissue staining</th>
<th>Isotype</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DQ</td>
<td>SK10</td>
<td>1/80</td>
<td>Mouse IgG</td>
<td>Becton-Dickinson, San Jose, CA</td>
</tr>
<tr>
<td>IL-1α</td>
<td>1277-89-7</td>
<td>1 mg/ml</td>
<td>Mouse IgG</td>
<td>Immunokontakt, Bioggo, Switzerland</td>
</tr>
<tr>
<td>IL-1β</td>
<td>2D8 combined with 1437-96-5</td>
<td>1 mg/ml</td>
<td>Mouse IgG</td>
<td>Immunokontakt, Bioggo, Switzerland</td>
</tr>
<tr>
<td>TNF*</td>
<td>2C8</td>
<td>5 mg/ml</td>
<td>Mouse IgG</td>
<td>Biodesign, Saco, ME</td>
</tr>
<tr>
<td>TNF*</td>
<td>Mab1 combined with Mab11</td>
<td>500 μg/ml</td>
<td>Mouse IgG</td>
<td>BD, PharMingen, San Diego, CA</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>84H10</td>
<td>1 mg/ml</td>
<td>Mouse IgG</td>
<td>AbD Serotec, UK</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>51-01C9</td>
<td>0.5 mg/ml</td>
<td>Mouse IgG</td>
<td>BD, PharMingen, San Diego, CA</td>
</tr>
<tr>
<td>CD31</td>
<td>6002-1</td>
<td>1/400</td>
<td>Mouse IgG</td>
<td>Monosan, Uden, The Netherlands</td>
</tr>
<tr>
<td>Negative control</td>
<td>X 0931</td>
<td>100 μg/ml</td>
<td>Mouse irrelevant IgG</td>
<td>Dakocytomation A/S, Glostrup, Denmark</td>
</tr>
</tbody>
</table>

HLA: human leukocyte antigen; IL-1α: interleukin-1α; IL-1β: interleukin-1β; *TNF: tumour necrosis factor (clone 2C8 is a non-neutralising antibody and Mab1 combined with Mab11 is neutralising antibodies); ICAM-1: intracellular adhesion molecule; VCAM-1: vascular cell adhesion molecule-1; CD31: endothelial cell marker.
Results

Clinical baseline characteristics
Fourteen patients with active RA were started on treatment with adalimumab 40 mg subcutaneously every two weeks. One patient was not re-biopsied at the follow-up visit due to the patient’s preference. From the remaining 13 cases, muscle biopsies of adequate histopathological quality taken before and after three months of treatment with adalimumab were available for evaluation from 11 patients, who were enrolled in this study (Table II). Seven of these patients were on methotrexate (MTX); median dose 20 mg/week, range 10–25 mg/week. The other four patients had previously been treated with MTX. Prednisolone was currently used by eight patients; median dose 5 mg/week, range 5–10 mg/day. Two of the patients had been treated with anti-TNF drugs before. One had stopped her previous anti-TNF treatment just over three months before the start of the study. The other had received two previous anti-TNF treatments, where the last treatment was stopped more than 18 months before inclusion. Both had discontinued anti-TNF treatment due to adverse events. Six patients were current smokers, two were former smokers and three had never smoked. None of the patients reported a history of cardiovascular events, but two had previously been diagnosed with hypertension. Six of the patients had a blood pressure over 140/80 mm Hg (mean 135/78; standard deviation [SD] for systolic pressure=15; SD for diastolic pressure=13) when examined at inclusion. Three patients used anti-hypertensive drugs. Diabetes or hyperlipidemia had not been diagnosed in any patient, but two patients had HbA1c just above the normal range, and three had moderate hypercholesterolemia, at inclusion. None were treated with statins. None of the patients had clinical signs of myositis or myopathy at baseline or after three months. Three of the patients had extra-articular involvement in the form of rheumatoid nodules at inclusion, but no current or previous history of vasculitis or other severe manifestations was recorded.

Muscle histopathological assessment
In the haematoxylin-eosin stained sections two samples showed centrally located nuclei (one at baseline and one other patient after three months of treatment). Minor inflammatory cell infiltrates surrounding or invading non-necrotic muscle fibers were seen in two patients at baseline, but in none of the patients after three months of treatment. No signs of fiber degeneration, regeneration or atrophy were seen.

HLA-DQ, IL-1α, and CD31 expression in endothelial cells of muscle tissue
HLA-DQ was mainly expressed in endothelial cells in capillaries, whereas IL-1α was mainly expressed in larger vessels. The area with positive staining for HLA-DQ was significantly reduced, compared to before treatment (median [M] 0.073%, IQR 0.027–0.121

RA clinical and laboratory outcomes
A good or moderate EULAR (26) response was seen in 6 out of the 11 patients. A clinically significant decrease in disease activity, measured by DAS28, was seen after the three-month period (mean change 1.40; p=0.018). Disability measured by HAQ (mean change 0.27; p=0.11) and CRP (median change 11 mg/L; p=0.29) were also reduced after 3 months, but the differences did not reach statistical significance.

Statistical analysis
In the comparison of baseline findings and after three months treatment, the paired t-test was used for parameters with a normal distribution (i.e. IL-1α and CD31 expression by computer assisted image analysis), the Wilcoxon sign rank test was used, and the results were presented as mean pairwise differences with 95% confidence intervals. For parameters without a normal distribution (i.e. HLA-DQ expression by computer assisted image analysis), the Wilcoxon sign rank test was used, and the results were presented as median pairwise differences and p-values between group differences. These comparisons were stratified by EULAR good/moderate responder status at 3 months after start of adalimumab. In an additional post-hoc exploratory analysis, the analyses were stratified by current smoking status at baseline.

We analysed correlations between changes (from inclusion to after three months of treatment) in clinical parameters (i.e. Disease Activity Score 28 [DAS28], CRP and ESR) and endothelial markers (i.e. IL-1α and HLA-DQ expression by computer assisted image analysis). For parameters with a normal distribution, Pearson’s correlation test was used, and for parameters without a normal distribution we used Spearman’s correlation test.

The study was approved by the regional research ethics committee in Lund, Sweden, and also approved as a phase IV clinical trial by the Swedish Medical Products Agency. The study was monitored according to a standard protocol by an independent agent. All participating patients gave their written informed consent to participate. This study is registered with ClinicalTrials.gov, number NCT01270087.

Table II. Baseline characteristics.

<table>
<thead>
<tr>
<th>n</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>9 female / 2 male</td>
</tr>
<tr>
<td>Age at inclusion (mean years; SD)</td>
<td>54.2 (10.9)</td>
</tr>
<tr>
<td>Disease duration (median years; IQR)</td>
<td>6.5 (3.5 to 15)</td>
</tr>
<tr>
<td>Rheumatoid factor seropositive</td>
<td>9/11 (82 %)</td>
</tr>
<tr>
<td>Anti-CCP positive</td>
<td>10/11 (91 %)</td>
</tr>
<tr>
<td>Prednisolone treated at inclusion</td>
<td>8/11 (73 %)</td>
</tr>
<tr>
<td>Methotrexate treated at inclusion</td>
<td>7/11 (64 %)</td>
</tr>
<tr>
<td>DAS28 (mean; SD)</td>
<td>5.3 (1.4)</td>
</tr>
<tr>
<td>CRP (mg/L) (mean; IQR)</td>
<td>1.44 (0.76)</td>
</tr>
<tr>
<td>CRP (mg/L) (median; IQR)</td>
<td>20 (13 to 43)</td>
</tr>
</tbody>
</table>

IQR: interquartile range; SD: standard deviation; DAS: disease activity score; HAQ: health assessment questionnaire; CRP: C-reactive protein.
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Fig. 1. Scatter diagram for endothelial HLA-DQ expression from computer assisted image analysis, baseline data and after three months of treatment for 11 patients.

Fig. 2a. Immunohistochemical staining of a skeletal muscle with capillaries positive for HLA-DQ in a patient at baseline. Magnification x 20.

Fig. 2b. Skeletal muscle with capillaries with positive HLA-DQ-staining after three months of adalimumab treatment in the same patient as Fig. 2a. Magnification x 20.

When patients were divided according to clinical response, a lower expression of IL-1α was seen in EULAR good/moderate responders after treatment compared to before treatment, but not in non-responders (mean difference -0.114% vs. 0.028%; p=0.041) (Table III). HLA-DQ expression decreased in both responders and non-responders (median difference -0.046% vs. -0.036%). There were no significant correlations between changes in DAS28 and changes in HLA-DQ or IL1-α (data not shown), but we found a strong correlation between decrease in HLA-DQ and decrease in CRP (Spearman’s r=0.74; p=0.01) and a similar trend was observed for IL1-α and CRP (Spearman’s r=0.60; p=0.05).

There was no major difference in baseline HLA-DQ expression between current smokers and non-smokers (M 0.084% vs. 0.073%; p=0.043), but not in current smokers (median difference 0.019%, p=0.345).

Other molecules in muscle tissue
Intracellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1), IL-1-β and TNF expression in endothelium and infiltrating cells were extremely limited, and detected in only occasional patients. There was no difference between baseline and follow-up samples (data not shown).

Discussion
Treatment with adalimumab was associated with decreased expression in muscle tissue of endothelial markers previously associated with extra-articular RA. The decreased HLA-DQ expression, which correlated with decreased CRP levels, might indicate reduced systemic endothelial activation in patients treated with adalimumab. This may in a longer perspective be of importance for development of CVD. Atherosclerosis is the main cause of CVD, and inflammation is now generally accepted as a major component in all stages of atherogenesis, from en-

vs. M vs. 0.023%, IQR 0.009–0.040; p=0.041) (Fig. 1, illustrated by representative examples in Figures 2A and 2B). There was a similar trend for IL-1α expression measured from baseline to a three-month follow-up (mean difference 0.049%; 95% confidence interval 0.069–0.168). Capillary density, measured as the percentage of CD31 positive area (Fig. 3), also tended to be lower after adalimumab treatment (Table III).
endothelial stress via vascular damage to plaque destabilisation and plaque rupture subsequently leading to atherothrombosis (27). Furthermore, a low degree of inflammation as measured by high sensitivity CRP is a risk factor for CVD (28). This is compatible with an independent effect of IL-6 on endothelial activation (29). Based on this, it would be expected that chronic systemic inflammation, as seen in many RA patients, could significantly aggravate atherogenesis. Indeed, striking similarities in the cellular and cytokine profiles of rheumatoid synovial lesions and atherosclerotic plaques have prompted speculations that shared inflammatory pathways in various rheumatic diseases may initiate and/or accelerate plaque formation (30).

In accordance with this, the increased risk of CVD events in patients with RA compared to the general population seems not to be completely explained by the traditional CVD risk factors (31). In this regard, a genetic component including HLA-DRB1* specific susceptibility has been associated with the presence of endothelial dysfunction in patients with RA (32).

Immunological pathways associated with chronic inflammation are important in autoimmune disease. The major histocompatibility complex (MHC) class II subtypes HLA-DR, -DQ and -DP are regulators of T-cell dependent immune responses, and abberant expression of these tissue antigens in the endothelium has been demonstrated in autoimmune disease such as RA and systemic lupus erythematosus (SLE) (6). Endothelial activation has been defined as “a quantitative change in the level of expression of specific gene products (i.e. proteins), which in turn endow endothelial cells with new capabilities that cumulatively allow endothelial cells to perform new functions” (33). Studies of patients with dilitated cardiomyopathy suggest an association between endothelial expression of MHC class II and diffuse endothelial dysfunction (342). In addition, expression of MHC class II and other vascular endothelial markers in cardiac microvessel in patients with CVD may be greater among those with inflammatory rheumatic diseases compared to those without (35). Endothelial up-regulation of MHC class II molecules in systemic autoimmune diseases may be a local phenomenon in affected organs, due to cytokine productions in inflammatory lesions, or a generalised vascular phenomenon, due to circulating cytokines. In patients with RA, increased expression of HLA-DQ in synovial microvessels in joints with active arthritis has been reported (36). By contrast, synovial HLA-DQ-expression in endothelial cells was not found in a group of patients with reactive arthritis (36), suggesting that this finding may be specific for microvessels of RA-patients. In a study of rheumatoid vasculitis, increased expression of HLA-DR, and of the adhesion molecules ICAM-1 and VCAM-1, was observed in muscle biopsy specimens from vasculitis patients with and without perivascular infiltrates, compared to controls with non-vasculitic RA and osteoarthritis (37). Furthermore, our previous study of muscle biopsies from patients with severe extra-articular RA showed increased endothelial expression of HLA-DQ in the absence of local inflammation, compared to RA controls without extra-articular disease (5). Although the patients in the present study did not have severe extra-articular RA, they had active severe joint disease, which could also be associated with systemic inflammation and vascular endothelial activation. Our data suggest that treatment with adalimumab may decrease such activation. Although the clinical response was variable in this limited sample, the observed effect on endothelial cell activation correlated well with reduction of CRP – an objective marker of systemic inflammation.

We also found a trend towards a decrease in expression of IL-1α, in particular among clinical responders. This is in line with previous studies that demonstrated that anti-TNF treatment may reduce IL-1 expression (38). IL-1α expression has been reported in arteriosclerotic lesions (39), specifically in endothelial cells of microves-

**Table III.** Endothelial markers; % of total tissue area.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Change from baseline to 3 months follow-up</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>Total n=11</td>
<td>0.122 (0.161) -0.049 (0.176)</td>
<td>0.38</td>
</tr>
<tr>
<td>(mean; SD)</td>
<td>Responders n=6</td>
<td>0.184 (0.200) -0.114 (0.206)</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>Non-responders n=5</td>
<td>0.047 (0.042) +0.028 (0.105)</td>
<td>0.58</td>
</tr>
<tr>
<td>HLA-DQ</td>
<td>Total n=11</td>
<td>0.073 (0.044–0.130) -0.036 (-0.061 to -0.001)</td>
<td>0.04</td>
</tr>
<tr>
<td>(median; IQR)</td>
<td>Responders n=6</td>
<td>0.073 (0.047–0.152) -0.046 (-0.097 to 0.018)</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>Non-responders n=5</td>
<td>0.073 (0.026–0.357) -0.036 (-0.208 to -0.014)</td>
<td>0.04</td>
</tr>
<tr>
<td>CD31</td>
<td>Total n=11</td>
<td>1.16 (1.36) -0.51 (1.41)</td>
<td>0.26</td>
</tr>
<tr>
<td>(mean; SD)</td>
<td>Responders n=6</td>
<td>1.09 (0.91) -0.44 (1.36)</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>Non-responders n=5</td>
<td>1.25 (1.89) +0.58 (1.35)</td>
<td>0.47</td>
</tr>
</tbody>
</table>
sels in atherosclerotic plaques (40), but not in normal blood vessels (39). IL-1α induces tissue factor like procoagulant activity and plasminogen activation inhibitor synthesis in human endothelial cell in vitro (41), indicating that increased IL-1α production may have a procoagulant effect on vascular endothelium which could promote CVD. Reduction of IL-1α may thus also be important in the prevention of atherosclerotic vascular disease.

A growing body of evidence supports the concept that treatment with TNF inhibitors has beneficial effects on the vascular system. Anti-TNF blockade has been associated with a reduction of different vascular/inflammatory markers, such as serum concentrations of vascular endothelial growth factor (VEGF), which is elevated in patients with RA and correlates with disease activity (42), as well as reduced angiogenesis in the synovium (42). Improved endothelium dependent vasodilation has also been described in patients treated with TNF-inhibitors (43), as well as reduction of intima-media thickness (44) and improved vascular elasticity expressed as significantly less arterial stiffness (45). It is tempting to speculate that diminished endothelial activation may be a short term effect of anti-TNF treatment. This may in a longer perspective have a beneficial effect on CVD events in anti-TNF treated patients. If this is a specific effect of TNF blockers or a consequence of decreased disease activity in general and if similar findings could be seen with other potent anti-rheumatic therapy is not known and could not be addressed by our study design. However, in a recent report, upregulation of pro-inflammatory pathways in endothelial cells was resistant to corticosteroids (46), suggesting that different modes of immunosuppression may have distinct effects on endothelial activation.

Previous studies have suggested that smokers with RA may be less likely to respond to anti-TNF treatment (47, 48). In the present study, we found a significant decrease in HLA-DQ expression in non-smokers, but not in current smokers, although baseline levels of expression were at least as high in smokers. This may indicate that some inflammatory pathways in smokers with RA are resistant to anti-TNF treatment. However, these results should be interpreted with caution due to the limited sample and the exploratory nature of this subanalysis.

Major strengths of this study are the repeated muscle biopsies and the well-established set of methods of immune histochemistry used, and also the careful characterisation of endothelial marker expression with computer assisted image analysis.

The main limitations of the present study are due to the small sample size, in particular in analyses stratified by clinical response.

**Conclusion**

In conclusion, treatment with adalimumab was associated with decreased expression of endothelial cell activation markers previously associated with severe systemic inflammation in RA. Our findings could indicate reduced systemic endothelial activation in patients treated with anti-TNF drugs, which might contribute to a reduced risk of cardiovascular co-morbidity.

**Acknowledgements**

We thank Eva Lindroos for excellent work with the sectioning and staining procedures and Associate Professor Inger Nennesmo for analysing slides for the presence of degeneration, regeneration, atrophy, centrally located nuclei, and inflammatory infiltrates. We also thank Jan-Åke Nilsson for his important comments on the statistical analysis, Dr Lida Marsal for her valuable aid in the patient recruitment and Ingrid Mattsson-GBorek for her excellent help with the illustrations.

**Key messages:**

- Adalimumab treatment was associated with decreased expression of endothelial markers associated with inflammation in RA.
- We found reduced endothelial activation in RA patients treated with anti-TNF drugs.
- Reduced endothelial activation might contribute to a lower risk of cardiovascular co-morbidity.

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