CONCISE REPORT

Real-time quantitative PCR to detect changes in synovial gene expression in rheumatoid arthritis after corticosteroid treatment

Danielle M Gerlag, David L Boyle, Sanna Rosengren, Tony Nash, Paul P Tak, Gary S Firestein

Ann Rheum Dis 2007;**66**:545–547. doi: 10.1136/ard.2006.059790

Synovial biomarkers are increasingly important in the development of novel therapeutic agents for the treatment of rheumatoid arthritis (RA). To identify biomarkers correlating with changes in clinical disease activity, real-time quantitative PCR (Q-PCR) was used to evaluate changes in synovial gene expression after treatment with corticosteroids. Patients with active RA received either oral prednisolone (n = 10, 60 mg daily for the first week and 40 mg daily for the second week) or placebo (n = 11) for 14 days. Real-time Q-PCR was used to quantify gene expression of tumour necrosis factor (TNF)α, IL1β, IL8 and matrix metalloproteinase (MMP) 1 in synovial tissue samples obtained through an arthroscopic procedure before and after treatment. mRNA levels were reported as relative expression units compared with a cell-based standard. Statistical analysis was performed using an analysis of covariance model. Prednisolone markedly decreased IL8 and MMP1 expression compared with placebo, and the CIs excluded the likelihood of no effect. A trend towards reduction was seen in IL1β and TNFα mRNA expression in the prednisolone group, although CIs included the value for no effect. These data suggest that Q-PCR can be used to measure synovial mRNA expression of mediators implicated in the pathogenesis of RA in small proof-of-concept trials.

After preclinical evaluation of potential new drugs for rheumatoid arthritis (RA), clinical trials in humans are required to assess efficacy and safety. Improved therapy, ethical aspects and the growing number of novel therapeutic agents have increased the need for trials generating a higher density of data in smaller groups of patients. Limited numbers of patients and a shorter study period could be used in early development to assess the effect of a therapeutic intervention on biomarkers that are potentially predictive of clinical efficacy.[1 2] The aim of this study was to examine the utility of real-time quantitative PCR (Q-PCR) as a method to detect changes in gene expression in a small proof-of-concept clinical trial on small synovial tissue samples. Patients with RA were treated with a well-known effective treatment, and real-time Q-PCR technique was used to quantify gene expression in serial synovial biopsy specimens obtained after active treatment with prednisolone compared with placebo.

PATIENTS, MATERIALS AND METHODS

Patients

Twenty-one patients, aged 18–85 years, with RA according to the 1987 criteria of the American College of Rheumatology were included in the study.[3] All had active disease at enrolment, defined by the presence of ⩾6 tender and ⩾6 swollen joints out of the 28 joints assessed. In addition, patients had one of the following: erythrocyte sedimentation rate ⩾28 mm/h, serum levels of C reactive protein ⩾1.5 mg/dl or morning stiffness ⩾45 min. They had to be stable for at least 28 days on disease modifying antirheumatic drugs.

Study design

Following informed consent, eligible patients were randomised to receive either oral prednisolone 60 mg daily for the first week, followed by 40 mg daily during the second week (comparable to the dosages used in the COBRA study,[4] n = 10), or matching placebo for 2 weeks (n = 11) in a 1:1 ratio.[5] Clinical assessment included Disease Activity Score 28 (DAS28). An independent assessor performed the clinical evaluation.

Arthroscopy

All patients underwent synovial tissue sampling at the Academical Medical Center, University of Amsterdam, Amsterdam, The Netherlands, by needle arthroscopy of an actively inflamed joint (knee, ankle or wrist), performed under local anaesthesia before and after 14 days of treatment, as described previously in detail.[6 7] During each procedure, biopsy specimens were taken from six or more sites of the joint to minimise sampling error.[8 9] The biopsy specimens were snap frozen in liquid nitrogen and stored until RNA isolation.

RNA isolation and complementary DNA synthesis

RNA from synovium and peripheral blood monocytes (PBMCs) was isolated using RNAStat-60 (TelTest, Friendswood, Texas, USA). PBMC were stimulated in vitro with Concanavalin A (Sigma Chemical Co, St Louis, Missori, USA) for 24 h to induce mRNA transcription. The RNA content was determined with RiboGreen (Molecular Probes, Eugene, Oregon, USA), and up to 500 ng per reaction was reverse-transcribed in a final volume of 50 µl. The resulting synovial complementary DNA (cDNA) was stored at −80°C. PBMC-cDNA was diluted in fourfold steps to yield a set of standards representing mRNA acquired from between 24 and 100 000 cells.

Real-time Q-PCR

Q-PCR was performed as described previously in detail.[8] mRNA levels were quantified using the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, California, USA). Predeveloped sequence detection reagents specific for human tumour necrosis factor (TNF) α, IL1β and IL8, including forward and reverse primers as well as a fluorogenic TaqMan FAM/TAMRA-labelled hybridisation probe, were used. Matrix metalloproteinase (MMP) 1 was amplified with forward

Abbreviations: c-DNA, complementary DNA; CE, cell equivalent; C(t), threshold cycle number; DAS, disease activity score; MMP, matrix metalloproteinase; PBMC, peripheral blood monocyte; Q-PCR, quantitative PCR; RA, rheumatoid arthritis; REU, relative expression units; TNF, tumour necrosis factor

Table 1 Geometric means of synovial mRNA levels before and after treatment with prednisolone or placebo

	Prednisolone (before treatment)	Prednisolone (after treatment)	Placebo (before treatment)	Placebo (after treatment)
IL1β				
Geometric mean	0.001829	0.000993	0.001412	0.001479
CI low	0.000663	0.000485	0.000621	0.000668
CI high	0.005047	0.002034	0.003212	0.003276
TNF				
Geometric mean	0.349658	0.197523	0.277621	0.366998
CI low	0.126729	0.096473	0.122033	0.165688
CI high	0.964744	0.404419	0.631577	0.812898
IL8				
Geometric mean	0.009089	0.004387	0.008108	0.012567
CI low	0.003294	0.002142	0.003564	0.005673
CI high	0.025077	0.008981	0.018446	0.027835
MMP1				
Geometric mean	40.26167	3.861284	12.28034	33.79457
CI low	14.59228	1.88599	5.398034	15.25717
CI high	111.0863	7.905792	27.93737	74.85481

CI low, confidence interval lower limit; CI high, confidence interval upper limit.
All values are expressed in relative expression units, normalised to glyceraldehyde-3-phosphate dehydrogenase (GADPH) message.

primer: TTT CAT TTC TGT TTT CTG GCC A; reverse primer: CAT CTC TGT CGG CAA ATT CGT and detected byprobe: 6FAM-AAC TGC CAA ATC GGC TTG AAG CTG CT-TAMRA. The fluorescent signal was plotted versus cycle number, and the threshold cycle (C(t), the cycle number at which an increase above background fluorescence could be reliably detected) was determined for each sample.

In order to relate message levels for the cytokines, MMP1 and glyceraldehyde-3-phosphate dehydrogenase to known standard, fourfold dilutions of PBMC-cDNA were included. Standard curves were generated by linear regression using log(C(t)) versus log(cell number). The PBMC equivalent (cell equivalent (CE)) number for synovial samples was calculated from C(t) values using the PBMC standard curve. Data are expressed as the ratio between inflammatory mediator-CE and glyceraldehyde-3-phosphate dehydrogenase (GADPH) CE, yielding the relative expression unit. Each PCR run also included non-template controls containing all reagents except cDNA. These controls generated a C(t) >40 (ie, message below detection level) in all experiments.

Statistical analysis

An analysis of covariance was used to control for initial differences in the values of the baseline samples in this small group of samples. The model fitted included terms for treatment as a fixed effect and the baseline measurement as a covariate, with the aim to estimate a treatment difference. The geometric mean was taken instead of the mean or median, because the data were skewed to the right. A normal distribution was reached after transforming the data to a logarithmic scale. The geometric mean was obtained by taking the exponent of the mean of the transformed data.

RESULTS

Demographic features

Eight male and 13 female patients were included into the trial. Of these, 10 received prednisolone and 11 received placebo treatment. Demographic features were comparable in both groups. The mean age was 49.4 (range 32–63) years for patients in the prednisolone group and 55.7 (range 37–69) years in the placebo group. The median disease duration was 17 (range 4–107) and 22 (range 5–92) months for the prednisolone and placebo group, respectively. Use of disease-modifying antirheumatic drugs was the same in both groups. All patients had active disease with comparable disease activity scores DAS28 in both groups: 6.27 (range 4.59–7.98) for the prednisolone group and 5.98 (range 4.56–7.59) for the placebo group.

Clinical results

A strong positive effect of oral prednisolone treatment on the DAS28 (SD) was found, with a decrease of 2 units (from 6.27 (0.95) to 4.11 (1.43)). The mean DAS28 of the placebo group did not change after treatment (5.98 (0.99) before vs 5.68 (1.31) after treatment).

Cytokine mRNA expression

The mRNA yield of the samples was 5.1 (1.13) μg. Table 1 shows the mean (SD) relative expression units before and after treatment.

The effect of prednisolone treatment on the cytokine mRNA expression of IL1β and TNFα in the synovial tissue showed a trend towards reduction after treatment, although confidence intervals included the value for no effect (fig1 A,B). Prednisolone markedly decreased the expression levels of IL8 and MMP1 mRNA compared with placebo (fig 1C,D).

DISCUSSION

Biomarker-based clinical trials are becoming increasingly important as a means to evaluate the efficacy of novel therapeutic agents for RA. While peripheral blood is readily available for analysis, the target organ, (the synovium), likely provides the most accurate assessment of disease activity. As a result, several investigators have focused on the analysis of serial synovial tissue samples.[10–15] Among the techniques that have been developed, immunohistochemistry to evaluate cell populations or protein expression in the inflamed synovium correlates well with subsequent changes in clinical disease activity.[5 16]

Whereas protein is most relevant to function, analysis of RNA transcript levels may provide additional evidence of drug efficacy or information on the mechanism of action in proof-of-concept clinical trials. This study was designed to determine the utility of a Q-PCR technique, developed specifically for the analysis of small synovial biopsy samples,[9] to measure mRNA expression of genes implicated in the pathogenesis of RA after oral prednisolone or placebo treatment. A marked clinical effect was seen in patients treated with prednisolone, but not in the placebo group.

Expression of two biomarkers (IL8- and MMP1-mRNA) significantly decreased in the corticosteroid-treated patients compared with those treated with placebo. A trend was also observed in IL1β and TNFα gene expression in the prednisolone group. This difference did not reach statistical significance, possibly because of a relatively small sample size or relatively low transcript abundance. The more prominent effect observed in MMP1 is especially interesting, because its expression represents the integration of multiple proinflammatory cytokines, including IL1 and TNFα, which regulate this family of proteases.

This is the first study demonstrating that changes in expression of genes determined by Q-PCR in serial synovial biopsies can differentiate between effective and ineffective treatment. Synovial mRNA levels of IL8 and MMP1 in particular could be useful as biomarkers in clinical proof-of-concept studies, especially in combination with immunohistochemistry to quantify the synovial cell infiltrate. More recent developments in synovial biopsy techniques that obtain larger specimens might also improve the power of cytokine

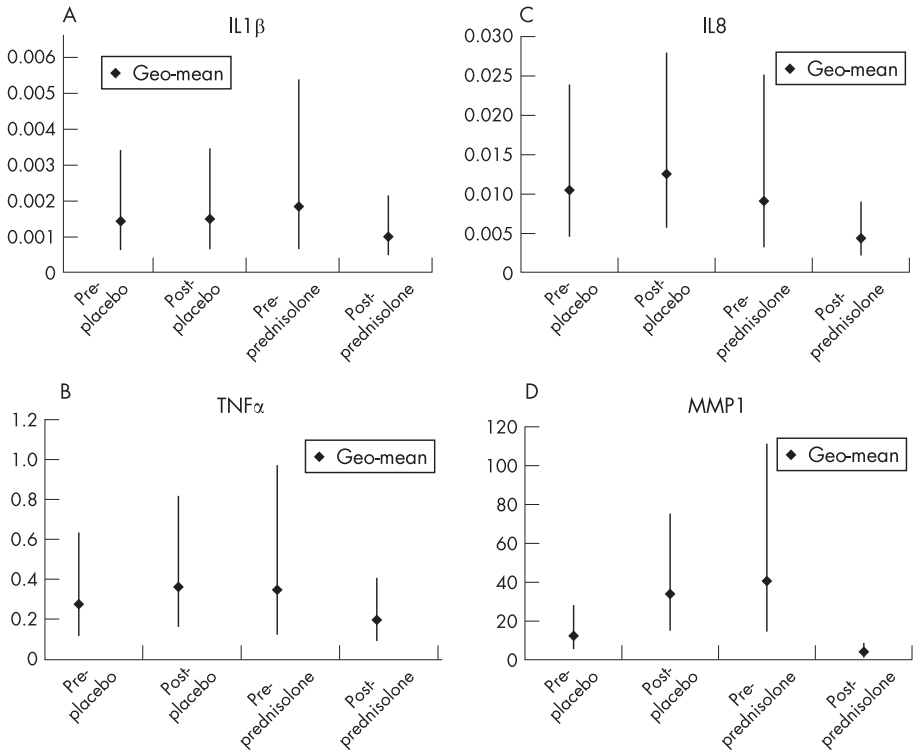

Figure 1 Geometric mean (♦) of (A) ILIβ, (B) tumour necrosis factor α (TNFα), (C) IL8 and (D) matrix metalloproteinase (MMP) 1 mRNA expression of genes, with their confidence interval (CI) before (pre) and after (past) treatment with prednisolone or placebo. Numbers are expressed as relative expression units (REUs).

mRNA expression for relatively low abundance transcript species.

ACKNOWLEDGEMENT

We thank Dr CA Wijbrandts for her statistical support.

Authors' affiliations

Danielle M Gerlag, Paul P Tak, Division of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
David L Boyle, Sanna Rosengren, Gary S Firestein, Division of Rheumatology, Allergy and Immunology, UCSD School of Medicine, La Jolla, California, USA
Tony Nash, Astra Zeneca, Macclesfield, UK

Competing interests: TN worked for Astra Zeneca when the study was performed, and owns Astra Zeneca stock.

The clinical trial was sponsored by Astra Zeneca. DMG was sponsored by the Dutch Arthritis Association.

Correspondence to: Professor P P Tak, Division of Clinical Immunology and Rheumatology, F4-218, Academic Medical Center, University of Amsterdam, PO Box 22700, 1100 DE, Amsterdam, The Netherlands; p.p.tak@amc.uva.nl

Accepted 1 September 2006
Published Online First 19 September 2006

REFERENCES

1 **Ilyin SE**, Belkowski SM, Plata-Salaman CR. Biomarker discovery and validation: technologies and integrative approaches. *Trends Biotechnol* 2004;**22**:411–16.
2 **Biomarkers definitions working group**. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther* 2001;**69**:89–95.
3 **Arnett FC**, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;**31**:315–24.
4 **Boers M**, Verhoeven AC, Markusse HM, van de Laar MA, Westhovens R, van Denderen JC, *et al*. Randomised comparison of combined step-down prednisolone, methotrexate and sulphasalazine with sulphasalazine alone in early rheumatoid arthritis. *Lancet* 1997;**350**:309–18.
5 **Gerlag DM**, Haringman JJ, Smeets TJ, Zwinderman AH, Kraan MC, Laud PJ, *et al*. Effects of oral prednisolone on biomarkers in synovial tissue and clinical improvement in rheumatoid arthritis. *Arthritis Rheum* 2004;**50**:3783–91.
6 **Kraan MC**, Reece RJ, Barg EC, Smeets TJ, Farnell J, Rosenburg R, *et al*. Modulation of inflammation and metalloproteinase expression in synovial tissue by leflunomide and methotrexate in patients with active rheumatoid arthritis. Findings in a prospective, randomized, double-blind, parallel-design clinical trial in thirty-nine patients at two centers. *Arthritis Rheum* 2000;**43**:1820–30.
7 **Kraan MC**, Reece RJ, Smeets TJ, Veale DJ, Emery P, Tak PP. Comparison of synovial tissues from the knee joints and the small joints of rheumatoid arthritis patients: implications for pathogenesis and evaluation of treatment. *Arthritis Rheum* 2002;**46**:2034–8.
8 **Dolhain RJ**, Ter Haar NT, De Kuiper R, Nieuwenhuis IG, Zwinderman AH, Breedveld FC, *et al*. Distribution of T cells and signs of T-cell activation in the rheumatoid joint: implications for semiquantitative comparative histology. *Br J Rheumatol* 1998;**37**:324–30.
9 **Boyle DL**, Rosengren S, Bugbee W, Kavanaugh A, Firestein GS. Quantitative biomarker analysis of synovial gene expression by real-time PCR. *Arthritis Res Ther* 2003;**5**:R352–60.
10 **Tak PP**. Lessons learnt from the synovial tissue response to anti-rheumatic treatment. *Rheumatology (Oxford)* 2000;**39**:817–20.
11 **Bresnihan B**, Tak PP. Synovial tissue analysis in rheumatoid arthritis. *Baillieres Best Pract Res Clin Rheumatol* 1999;**13**:645–59.
12 **Ruderman EM**, Weinblatt ME, Thurmond LM, Pinkus GS, Gravallese EM. Synovial tissue response to treatment with Campath-1H. *Arthritis Rheum* 1995;**38**:254–8.
13 **Cunnane G**, Madigan A, Murphy E, Fitzgerald O, Bresnihan B. The effects of treatment with interleukin-1 receptor antagonist on the inflamed synovial membrane in rheumatoid arthritis. *Rheumatology (Oxford)* 2001;**40**:62–9.
14 **Youssef PP**, Haynes DR, Triantafillou S, Parker A, Gamble JR, Roberts-Thomson PJ, *et al*. Effects of pulse methylprednisolone on inflammatory mediators in peripheral blood, synovial fluid, and synovial membrane in rheumatoid arthritis. *Arthritis Rheum* 1997;**40**:1400–8.
15 **Ulfgren AK**, Andersson U, Engstrom M, Klareskog L, Maini RN, Taylor PC. Systemic anti-tumor necrosis factor alpha therapy in rheumatoid arthritis down-regulates synovial tumor necrosis factor alpha synthesis. *Arthritis Rheum* 2004;**43**:2391–6.
16 **Haringman JJ**, Gerlag DM, Zwinderman AH, Smeets TJ, Kraan MC, Baeten D, *et al*. Synovial tissue macrophages: a sensitive biomarker for response to treatment in patients with rheumatoid arthritis. *Ann Rheum Dis* 2005;**64**:834–8.