Diurnal rhythm of cytokines in rheumatoid arthritis may be reflected in *in vitro* cultures of collected mononuclear cells

Sirs,

Rheumatoid arthritis (RA) is characterized by diurnal rhythm; both in laboratory and clinical settings (1, 2). Elevated levels of cytokines, *e.g.*, IL-6, TNF-α and IL-10, are both found in serum and synovial fluid in patients with active RA. In patients with RA serum IL-6 levels are substantially elevated and in sequential measurements its concentration demonstrates a significant diurnal rhythm (1). In context with that RA-patients also report a diurnal rhythm of their clinical symptoms, *e.g.*, morning stiffness and increasing grip strength (1). Thus, when performing studies in RA on cytokines it is of crucial importance to plan collection of biosamples in respect to the described circadian rhythm, *i.e.*, the nadirs and zeniths for measured biomarkers. However, we were not able to find any information on whether the harvest time of blood lymphocytes from patients with RA would influence the capacity of lymphocytes in functional *in vitro* studies. Therefore, we collected serial blood samples (8:00 a.m. after overnight fasting, 12:00 and 16:00) from nine patients (three males; six females), with active RA to estimate whether diurnal variation is reflected in functional *in vitro* mononuclear cell experiments.

All participants were in need of disease modifying anti-rheumatic drugs (DMARD). However, they were not allowed to have been treated with glucocorticosteroids or any DMARDs prior to the study. A rheumatologist made clinical assessments and each patient evaluated their morning stiffness and joint pain on a 10-grade visual analogue scale. The study protocol was approved by the Hospital Ethics Committee.

The samples were processed by the same biologist and mononuclear cells were isolated by our laboratory standard methods. The cells were incubated at 37°C (5% CO_2) without stimulation for 24 hours for analysis of IL-6 and 48 hours for analysis of IL-10, by using Quantikine ELISA kit (R&D, Minneapolis, USA). Haemoglobin, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were also measured.

Results: The mean concentrations of IL-6 in cell supernatants decreased during the day, from 544±331 pg/ml to 428±295 pg/ml while the concentration of IL-10 increased from 13.4±4.4 pg/ml to 59.2±40.7 pg/ml (Fig. 1). However, neither reached statistical significance (Wilcoxon test for paired data and Friedman test serial data). No correlation was found between the levels of measured cytokines and a variety of clinical and laboratory parameters indicating the inflammatory activity (Spearman Rank correlation).

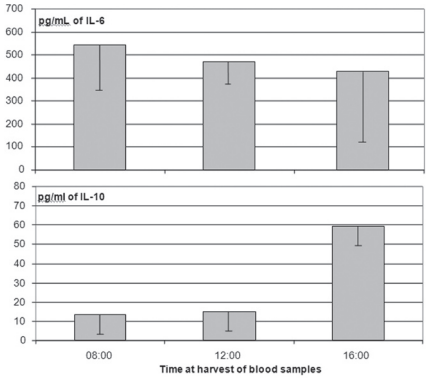

Fig. 1. Mean concentration of IL-6 and IL-10 in cell supernatants when lymphocytes (blood samples) were harvested at three different time points during the day (08:00, 12:00 and 16:00 hours) from 9 patients with active rheumatoid arthritis.

The circadian rhythm, which characterizes rheumatoid arthritis (RA), may eventually be reflected in functional lymphocyte *in vitro* studies. At least, the present study demonstrated a trend of diurnal rhythm in the functional activity of lymphocytes, depending on the time of harvest.

Several cytokines and hormones have a diurnal variation in healthy humans (1, 3, 4), *e.g.*, IL-6, IL-1, melatonin and cortisol (4). In contrast, the TNF-α and IL-10 dose not shows a diurnal rhythm in healthy individuals (3, 5). However, some studies indicate that IL-10 has a zenith between 20:00 and 2:00 a.m. and a nadir between 07:00 and 13:00h (6).

The level of serum IL-6 in RA has been shown to have significant diurnal variation (1) and correlation has been documented between IL-6 and various biomarkers (2, 7, 8), *e.g.*, ESR and CRP, as well as with clinical symptoms (7). While TNF-α do not seem to have diurnal variation in RA (1). On the other hand, TNF-α has been shown to correlate with parameters for inflammation (9), while IL-10 has not been related to disease activity of RA (10). We have not been able to find any information on the relationship of cytokine levels in cell supernatants and clinical symptoms or biomarkers in RA, nor did we find in the present study any correlation between IL-6 or IL-10 in cell supernatants with clinical symptoms or inflammatory markers.

In the present study, the concentration of IL-6 in the cell supernatants did show a weak diurnal variation, with decreased levels in the samples which were taken in the afternoon. Furthermore, the IL-10 levels were substantially raised in the samples that were harvested in the afternoon. As in the case of IL-6, the difference did not reach significant levels, probably due to the small study cohort.

The results from our study indicated that the harvest time of biosamples, in the case of blood lymphocytes, may have influenced the lymphocyte functional activity in *in vitro* studies. The mechanism behind this phenomenon is unknown, but it is of interest to conduct further analyses in an extended cohort of patients to elucidate further the circadian rhythm in RA. Meanwhile, this observation may call for standardizing the harvest time of biosamples, when lymphocytes from patients with active RA are to be used in functional *in vitro* studies.

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