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Pre-analytical and biological variability in circulating interleukin 6 in healthy subjects and patients with rheumatoid arthritis

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Abstract

Interleukin (IL)-6, a key player in the inflammatory response, may be a useful biomarker in rheumatoid arthritis (RA). The aim was to determine analytical variability, a reference interval in healthy subjects, and long- and short-term variation in serum and plasma IL-6 in healthy subjects and RA patients. An enzyme-linked immunosorbent assay from R&D was used for determination of serum and plasma IL-6. The IL-6 concentration did not depend on the type of anticoagulant used or the 3-h time delay between sampling and processing or repeated freeze–thaw cycles. The median plasma and serum IL-6 in 318 healthy subjects were 1.3 pg ml^{-1} (range 0.33–26) and 1.4 pg ml^{-1} (range 0.25–23), respectively. The median coefficient of variation in plasma IL-6 in 27 healthy subjects during 1 month, and repeated after 6 and 12 months were 27%, 31% and 26%, respectively. No significant long-term changes were observed in serum IL-6 over a 3-year period (14%, $p = 0.33$). Exercise (cycling) increased serum IL-6 in healthy subjects but not in RA patients. In conclusion, circulating IL-6 is stable regarding sample handling and shows little variation over time. Changes in IL-6 concentrations $>60\%$ (2 times the biological variation) are likely to reflect changes in disease activity and not only pre-analytical or normal biological variability.

Keywords: *Biomarker, biological variation, IL-6, rheumatoid arthritis*

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Introduction

Interleukin (IL)-6 plays a major role in the pathogenesis of several chronic inflammatory diseases such as rheumatoid arthritis (RA) (Choy & Panayi 2001, Ishihara & Hirano 2002), type 2 diabetes (Spranger et al. 2003), inflammatory bowel

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disease (Atreya & Neurath 2005) and cancer (Caruso et al. 2004). IL-6 is produced by a variety of cell types such as T and B cells, macrophages, megakaryocytes, neutrophils, osteoblasts, chondrocytes, synoviocytes, fibroblasts, keratinocytes, adipocytes, and skeletal, smooth muscle, mast, endothelial, glial and cancer cells (Akira et al. 1993, Mohamed-Ali et al. 1997, Fried et al. 1998, Nagaraju et al. 1998, Starkie et al. 2001). IL-6 regulates the immune response (induces T-cell growth and cytotoxic T-cell differentiation), inflammation (Kamimura et al. 2003, Smolen et al. 2005), the acute-phase response (synthesis of C-reactive protein (CRP), fibrinogen, serum amyloid A and haptoglobin) and haematopoiesis (acts synergistically with IL-3 to support the formation of multilineage blast cell colonies in haematopoiesis and induces macrophages and megakaryocyte differentiation).

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease characterized by synovitis and progressive joint destruction. The pathogenesis is not fully understood, but the cytokines tumour necrosis factor (TNF)-α, IL-1β and IL-6 are involved in both inflammation and the increased bone resorption in RA patients (Udagawa et al. 1995, Papanicolaou et al. 1998, Wong et al. 2003, Franchimont et al. 2005, Smolen et al. 2005). IL-6-deficient mice ($IL\text{-}6^{-/-}$) are resistant to antigen- (Ohshima et al. 1998, Boe et al. 1999) and collagen-induced arthritis (Sasai et al. 1999). Elevated concentrations of IL-6 are found in serum or plasma and synovial fluid of patients with RA (Houssiau et al. 1988, Swaak et al. 1988, Guerne et al. 1989, Dasgupta et al. 1992, Manicourt et al. 1993, Wendling et al. 1993, Georganas et al. 2000). Serum and plasma IL-6 levels in RA patients are related to clinical measurement of disease activity (Madhok et al. 1993, Sack et al. 1993) and radiographic progression of joint destruction (Kotake et al. 1996). Several studies have shown that IL-6 inhibition is a therapeutic target in RA (McInnes & Liew 2005). A humanized anti-IL-6Rα monoclonal IgG_1 antibody (tocilizumab) has shown promising efficacy by inhibiting disease activity and joint destruction in phase I and II clinical trials of RA patients (Nishimoto et al. 2000, 2003, 2004, Choy et al. 2002, Maini et al. 2006, Straub et al. 2006).

A 'biomarker' (biological marker) can be defined as 'A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention' (Atkinson 2001). The use of serum or plasma concentrations of IL-6 has not yet received Food and Drug Administration approval for use as a biomarker in patients with RA or any other disease. It is unknown if knowledge of the serum or plasma IL-6 level in an individual patient is so reliable that it can be used to enable clinical decisions that will improve outcome of the patient. IL-6 is not disease specific, since its production, for example, by inflammatory cells is elevated in several diseases characterized by inflammation (Scheller J et al. 2006). Several retrospective clinical studies of RA patients indicate that serum or plasma concentrations of IL-6 may be useful as a 'prognosticator' and may have a role in monitoring of RA patients during treatment with disease modifying antirheumatic drugs (DMARDs) (Crilly et al. 1995) and targeted treatment with either IL-6 inhibitors or TNF-α inhibitors (Barrera et al. 2001, Drynda et al. 2002, Popa et al. 2005a,b).

Circulating biomarkers are subject to variability arising from sampling procedures and biological variation, and this variability must be determined and adjusted for in the interpretation of the concentrations of circulating IL-6. The aims of this study were to determine: (1) the analytical variability in IL-6; (2) differences in serum and

plasma IL-6; (3) a reference interval in healthy subjects; (4) the diurnal, day-to-day, week-to-week, year-to-year variations in healthy subjects; (5) the diurnal variations in RA patients; and (6) the effect of light exercise.

Materials and methods

IL-6 ELISA

Serum and plasma concentrations of IL-6 were determined by a commercially available human IL-6 high-sensitive enzyme-linked immunosorbent assay (ELISA) (Quantikine HS, high sensitivity, R&D Systems, Abingdon, Oxon, UK) in accordance with the manufacturer's instructions. A monoclonal mouse antibody against human IL-6 has been pre-coated onto a microplate. Standards and serum or plasma samples were pipetted into the wells, incubated for 2 h and any IL-6 present was bound by the immobilized antibody. After washing away any unbound substances, an alkaline phosphatase-labelled polyclonal antibody specific for IL-6 was added to the wells and incubated for 2 h. Following a wash to remove any unbound antibody–enzyme reagent, a substrate solution (nicotinamide adenine dinucleotide phosphate) was added to the wells. After an incubation period of 1 h, an amplifier solution (alcohol dehydrogenase and diaphorase) was added to the wells and colour develops in proportion to the amount of IL-6 bound in the initial step. The colour development was stopped after 30 min and the optical densities of the colour were measured using a microplate reader (Spectra II, Wallac, Austria or a EL_X 808, Bio-Tek Instruments Inc., Vermont, USA) at 490 nm. All steps were performed at room temperature (RT). Serum or plasma (100 μl) was used for each sample and standards. All samples and standards were analyzed in duplicate, and the mean value was used for statistical calculations. The coefficient of variation (CV) of the duplicates was low, in most cases between 0 and 5%. Re-analysis was done if the CV of the mean value was $>10\%$. All samples from each healthy subject and each RA patient were analyzed on one ELISA plate. Samples with values higher than the highest standard (10 pg ml^{-1}) were diluted. Three internal control samples were analyzed on each plate to confirm assay precision. A standard curve was created with linear function and reliable numerical values were between 0.156 pg ml^{-1} and 10 pg ml^{-1}.

Methodological variations

Preanalytical handling. Serum and EDTA plasma samples were obtained at RT and centrifuged within 30 min at 3000*g* for 10 min at 4°C and stored at −80°C until analysis, unless otherwise stated below.

Detection limit. The detection limit was assessed by calculating the limit of the blank (a signal 3 standard deviation (SD) above the mean for a calibrator that is free of analyte) ($n=33$).

Intra- and interassay coefficient of variation. Three controls with low, medium and high IL-6 concentration and three different plasma pools from three patients with RA were analyzed 37 times on the same ELISA plate, each to assess intra-assay precision. The interassay CV was calculated for the low (0.55 pg ml^{-1}), medium (3.13 pg ml^{-1}) and high (5.82 pg ml^{-1}) controls measured on 120–125 different ELISA plates over a

period of 22 months. Five different batches of the IL-6 ELISA were used in this period, and the controls from R&D were provided separately.

Recovery. Plasma in EDTA from six different RA patients (concentration 0.78, 1.9, 2.2, 3.1, 4.0 and 4.1 pg ml^{-1}) were diluted 1:2, 1:4 and 1:8 in the highest standard (10 pg ml^{-1}). Undiluted and diluted samples were measured in triplicates.

Different types of blood samples (anticoagulation). Corresponding samples of serum, heparin plasma, citrate plasma, and EDTA treated plasma (all stored at RT, the latter also stored on ice for 30 min) were collected from ten healthy and ten RA patients. After centrifugation the serum and plasma were aliquoted (except the 5 mm stratum above the buffy coat). IL-6 concentrations in citrate plasma were corrected by a factor 1.11 for buffer volume.

Delay and temperature before centrifugation. Corresponding serum and plasma samples were collected from ten healthy subjects and five RA patients. From each individual, eight serum and eight EDTA plasma tubes were kept at RT, and a similar number was kept at 4°C. After 30, 45, 60 min, 2, 3, 4, 6 and 24 h, one serum and one EDTA tube were centrifuged and the serum and plasma were stored at −80°C until analysis.

Repeated freeze–thaw cycles. EDTA plasma samples were collected from six healthy subjects and six RA patients. Each sample was separated and aliquoted into ten tubes and stored at −80°C. Sample 1 was not thawed until assay. Samples 2–10 were thawed/refrozen 2–10 times prior to assaying.

Subjects. All the healthy subjects were recruited among hospital staff and all RA patients used in these studies fulfilled the ACR revised criteria (Arnett et al. 1987).

Biological variations

Preanalytical handling. Serum and EDTA plasma samples were obtained at RT centrifuged within 2 h at 2500*g* for 10 min at 4°C and stored at −80°C until analysis, unless otherwise stated below.

Reference interval (normal range). IL-6 was determined in serum and plasma samples from 318 healthy, volunteer blood donors (median age 47 years, range 18–64, 122 women and 196 men), who were well characterized. A health questionnaire was filled out and approved before every blood donation, and donation was not allowed during disease periods.

Short-term (diurnal) variation. The study included three groups: (1) healthy subjects ($n=16$); (2) patients with early untreated RA (disease duration <6 months, $n=10$); and (3) patients with long-term erosive RA (disease duration 7–10 years, $n=11$). During the daytime the subjects were allowed to do normal activities (e.g. dressing, washing, walking) but they did not participate in heavy physical activities such as exercise or running. Serum was collected at seven time points during a 24-h period (day 1: 10 AM, 1 PM, 4 PM, 7 PM, 10 PM, and day 2: 7 AM and 10 AM), allowed to

clot and centrifuged at 4000*g* for 10 min at RT. On day 2, the patients were not allowed out of bed or any meal before blood sample collection at 7 AM.

Subjects. The healthy subjects were recruited among hospital staff. All RA patients fulfilled the ACR revised criteria (Arnett et al. 1987).

Variation during 3 weeks. IL-6 was determined in plasma samples collected at 8 AM on day 1, 2, 8, 15, and 22 in 38 subjects recruited among hospital staff. In addition, IL-6 was determined in plasma collected at 2 PM on day 8.

Variation during 12 months. The 3-week variation study was repeated after 6 months (2nd round) and 12 months (3rd round), using the same patients ($n=27$) throughout this timeframe.

Variation during 3 years. Serum IL-6 was determined in samples collected between 8 AM and 10 AM at five time points during a 4-week period (day 1, 8, 15, 22 and 29) in 30 healthy women (premenopausal ($n=11$), perimenopausal ($n=8$) and postmenopausal women ($n=11$)) and again in 21 of the women after 3 years at five time points during a 4-week period (day 1, 8, 15, 22 and 29). They had no medical history of endocrinological, gastrointestinal, hepatic or renal diseases and were not taking any medicine. They underwent a physical examination and a general biochemical health screen prior to inclusion and did not experience any signs or symptoms of disease. At the 3-year follow-up nine of the 30 women were not included due to the patient's request (Jensen et al 1997).

Exercise. Serum IL-6 was determined before physical exercise, immediately after exercise, and again 1 and 3 h post-exercise in: (1) healthy subjects ($n=14$); (2) patients with early, untreated RA (i.e. disease duration <6 months, $n=10$); and (3) patients with long-term erosive RA ($n=10$). The participants underwent a biphasic exercise programme using an ergometer bike (Monarch Vikterergometer model 90814 E). At first the participants were allowed a 5-min warming up session aiming to reach a submaximal level at 70–80% of their maximal pulse capacity. This level was maintained during the exercise session. Progressively higher work rates were attained by increasing load (0.5, 0.7, 0.9, 1.1, 1.3 kg) every 4 min for 20 min. Serum samples were allowed to clot at RT followed by centrifugation at 4000*g* for 10 min.

Ethics. All participants gave their written informed consent. The local ethics committees approved the studies and the protocols were conducted in accordance with the Declaration of Helsinki II (J. No: M-2359-02, KL 01-090/04).

Statistical analyses

Descriptive statistics for IL-6 levels are presented by the median or the geometric mean, and range. The variation over time is given by the CV. The CVs of IL-6 levels analyzed over time (variability over 3 weeks, 6 months, 12 months and 3 years) were compared with the intra-assay CV. The variance components within subject, between subjects and between rounds have been estimated assuming a random effects model with IL-6 log transformed and presented by the coefficient of variation of the

geometric means for the within subject (Kirkwood 1979). The relative homogeneity between subjects compared to the total variation was estimated by the intraclass correlation coefficient.

Serum and plasma samples of IL-6 were compared using Wilcoxon's signed rank test and Spearman's rank correlation. Association of IL-6 to sex and age was carried out using Wilcoxon's rank sum test and Spearman's rank correlation. The relations between IL-6 levels and time to processing at either 4°C or RT were analyzed using linear regression with log-transformed IL-6 values and taking repeated measures into account and adjusting for subject/patient. IL-6 levels in the analysis of circadian variation and physical activity were studied using a general linear model with repeated measures. Estimates were obtained by generalized estimating equations assuming a normal distribution on the log scale. p-Values less than 5% were considered significant. All statistical calculations were performed using SAS (Statistical analysis system, version 9.1, SAS Institute, Cary, NC, USA).

Results

Methodological variations

Detection limit. The limit of the blank was 0.01 pg ml^{-1} (mean +3 SD). The manufacturer's specification of a minimal detectable dose was 0.04 pg ml^{-1} (mean + 2 SD).

Intra- and interassay CV. The intra-assay CV for a serum sample with a low IL-6 level (mean serum IL-6 0.95 pg ml^{-1}, $n=37$) was 6.3%, CV for a medium serum IL-6 level (1.57 pg ml^{-1}, $n=37$) was 10.5%, and CV for a high serum IL-6 level (6.24 pg ml^{-1}, $n=37$) was 6.6%. The intra-assay CVs for three controls (not included in the ELISA kit, but provided separately by R&D) with low, medium or high IL-6 concentrations were 8.4% (low: 0.46 pg ml^{-1}, $n=37$), 6.2% (medium: 2.35 pg ml^{-1}, $n=37$), and 7.9% (high: 4.62 pg ml^{-1}, $n=37$), respectively. Interassay CVs over a period of 22 months using five different ELISA batches for three control samples provided by R&D with low, medium or high IL-6 concentrations were 17.7% (0.55 pg ml^{-1}, $n=125$), 10.9% (3.13 pg ml^{-1}, $n=125$) and 8.9% (5.82 pg ml^{-1}, $n=120$), respectively.

Recovery. The observed final concentration as a proportion of the expected final concentration was 99% (range 84–111) for EDTA plasma IL-6. This is in accordance with the manufacturer's specifications with a mean recovery between 94% and 97% (range 84–113) for serum and EDTA plasma samples.

Different types of blood samples (anticoagulation) and temperature. Table I gives the IL-6 concentrations in serum and different types of plasma in healthy subjects and RA patients. Circulating levels of IL-6 were significantly elevated in RA patients compared with healthy subjects ($p=0.005$). The IL-6 concentrations did not change significantly, either in the group of healthy subjects (Kruskal–Wallis, $p=0.97$) or in RA patients ($p=0.94$), or when all samples from both groups were pooled ($p=0.97$).

Delay and temperature before centrifugation. Table II shows the changes in serum and EDTA plasma concentrations of IL-6 after delays of processing from 0.5 to 24 h

Table I. Interleukin (IL)-6 concentrations (pg ml^{-1}) in different types of plasma and serum from ten healthy subjects and ten patients with rheumatoid arthritis (RA). Values are median (range).

	Healthy subjects	RA patients	All samples
Serum IL-6	0.76 (0.47–3.17)	2.55 (0.78–21)	1.35 (0.47–21)
EDTA plasma IL-6	0.83 (0.39–3.39)	2.45 (0.69–20)	1.24 (0.39–20)
EDTA ice plasma IL-6	0.81 (0.45–4.13)	2.70 (0.76–20)	1.50 (0.45–20)
Heparin plasma IL-6	0.85 (0.41–3.54)	2.53 (0.74–22)	1.26 (0.41–22)
Citrate[a] plasma IL-6	0.89 (0.44–3.53)	2.63 (0.76–20)	1.33 (0.44–20)

[a]The citrate plasma levels are corrected with a factor 1.11.

compared with baseline values in healthy subjects and RA patients. Circulating levels of IL-6 were significantly elevated in the RA patients compared to healthy subjects ($p = 0.032$).

A small but significant decrease were seen in healthy subjects for EDTA at RT from 30 to 45 min and a similar drop was seen for serum at RT. Analysis was done using linear regression with log-transformed IL-6 values and taking repeated measures into account and adjusting for subject/patient and data pooled for both healthy subjects and RA patients. IL-6 levels were stable during the first 3 h in serum and EDTA plasma at RT and 4°C. The *p*-values were 0.44 (serum at RT), 0.13 (EDTA plasma at RT), 0.50 (serum at 4°C) and 0.30 (EDTA plasma at 4°C).

Comparing the 3-h time point to the 4-h time point showed a significant decrease (13%, $p = 0.0005$) for EDTA plasma at RT. Comparing the 3-h time point with the 6-h time point showed a significant increase for serum at RT (5%, $p = 0.03$), a significant decrease for serum at 4°C (12%, $p < 0.0001$), and a significant decrease for EDTA plasma at RT (8%, $p = 0.001$). EDTA plasma at 4°C did not show a significant difference ($p = 0.83$). Finally, comparing 24-h delay to the 3-h time point resulted in a significant increase for serum at RT (3.7 times, $p < 0.0001$), a significant decrease for serum at 4°C (11%, $p = 0.0001$) and a significant decrease for EDTA plasma at RT (12%, $p = 0.002$). EDTA plasma at 4°C did not show significant changes ($p = 0.36$).

Repeated freeze–thaw cycles. The plasma IL-6 concentration did not change significantly on repeated freeze–thaw treatment in the group of healthy subjects ($p = 1.0$ to $p = 0.429$), in the group of RA patients ($p = 1.0$ to $p = 0.352$) or when data were pooled ($p = 1.0$ to $p = 0.670$) (Wilcoxon rank sum test).

Biological variation

Reference interval (normal range). Figure 1 illustrates the individual serum IL-6 (A) and plasma IL-6 (B) in 318 healthy blood donors according to age and sex. The median serum IL-6 was 1.4 pg ml^{-1} (range: 0.25–22.5; 5th–95th: 0.51–4.92; 10th–90th: 0.68–3.4) and the median plasma IL-6 was 1.3 pg ml^{-1} (range: 0.33–26; 5th–95th: 0.63–4.50; 10th–90th: 0.75–3.30). There was no significant difference between the median values of serum and plasma IL-6 ($p = 0.813$) (Mann–Whitney Rank sum test). Significant correlation was found in the healthy blood donors between serum and plasma IL-6 (Spearman rho = 0.90, $p < 0.0001$) (Figure 1C). Figure 1D gives a Bland–Altman plot of the difference between serum and plasma IL-6 and the mean value of corresponding serum and plasma samples. There was no difference between the median values of serum and plasma IL-6 in men ($p = 0.615$) and women ($p = 0.775$). There was no difference between men and women in serum IL-6

Table II. Changes in serum and EDTA plasma interleukin (IL)-6 concentrations in healthy subjects ($n = 10$) and rheumatoid arthritis (RA) patients ($n = 5$) according to temperature and time delay between blood sampling and processing. Values are median (range).

	Time delay between blood sampling and processing							
	30 min	45 min	60 min	2 h	3 h	4 h	6 h	24 h
Healthy persons								
Serum, RT	1.1 (0.4–2.0)	1.0* (0.3–1.9)	1.0* (0.3–1.9)	0.9 (0.3–2.0)	1.0 (0.3–1.9)	1.2 (0.5–2.0)	1.7**(0.6–26)	6.5**(1.5–21)
Serum, 4°C	1.1 (0.3–1.9)	1.1 (0.4–1.9)	1.3 (0.4–2.5)	1.0 (0.3–1.8)	1.2 (0.3–1.7)	1.1 (0.3–1.8)	1.1*(0.3–1.7)	1.0 (0.3–1.7)
EDTA, RT	1.1 (0.3–1.8)	0.9**(0.3–1.9)	0.9**(0.2–1.8)	0.9**(0.2–1.8)	0.8**(0.2–1.7)	0.9**(0.2–1.7)	0.9**(0.2–1.9)	0.8*(0.2–1.6)
EDTA, 4°C	1.0 (0.3–1.9)	1.1 (0.3–1.7)	1.2 (0.3–1.7)	1.1 (0.2–1.7)	1.1 (0.3–1.8)	1.0 (0.3–2.0)	1.1 (0.3–1.5)	0.9 (0.2–1.5)
RA patients								
Serum, RT	18 (0.9–20)	20 (0.8–28)	20 (0.9–23)	21 (0.9–27)	19 (0.8–21)	12 (0.8–20)	17 (0.8–22)	17 (6–19)
Serum, 4°C	16 (0.8–24)	19 (0.8–24)	20 (0.9–25)	22 (0.9–24)	21 (0.9–23)	23 (0.8–24)	17 (0.8–22)	17 (0.9–19)
EDTA, RT	18 (0.8–25)	19 (0.8–33)	16 (1.1–21)	17 (0.9–32)	18 (1.1–30)	17 (0.7–29)	17 (0.9–19)	16 (1.1–27)
EDTA, 4°C	20 (0.8–35)	19 (0.8–27)	20 (0.8–32)	20 (1.0–25)	19 (1.0–28)	21 (1.1–28)	17 (0.8–29)	18 (0.8–29)

RA, rheumatoid arthritis; RT, room temperature. Wilcoxon signed rank test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with 30-min value.

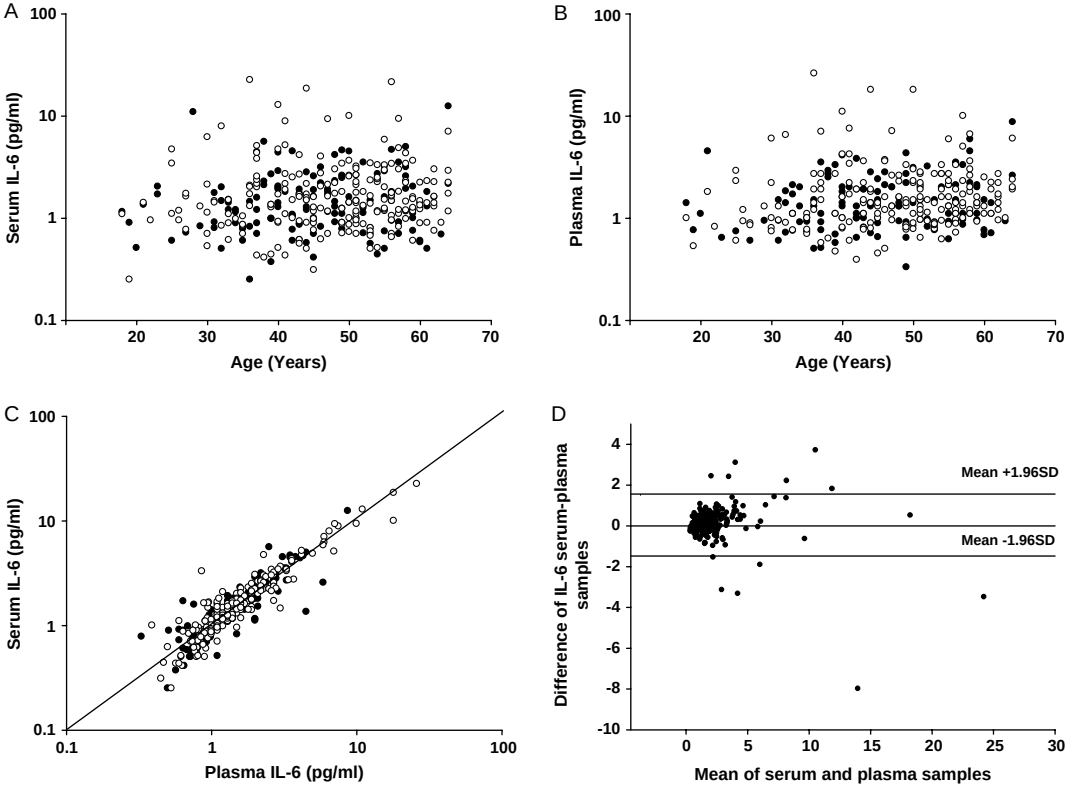

Figure 1. Individual interleukin (IL)-6 concentrations in serum (A) and EDTA plasma (B) in 318 healthy subjects according to age and sex. Women (open circles) and men (closed circles). (C) The correlation between serum and plasma IL-6 levels in healthy women and men. The *y*-axis is a log scale. (D) Bland–Altman plot of corresponding serum and plasma samples. Reference lines are 1.96 ± SD.

($p = 0.059$) and plasma IL-6 ($p = 0.173$). Low correlations were found between age and serum IL-6 (rho = 0.11, $p = 0.035$) and plasma IL-6 (rho = 0.18, $p = 0.0016$).

Diurnal variations. Figure 2 shows the changes in serum IL-6 at seven time points during 24 h in healthy subjects (A), in early RA patients (B), and in patients with long-term, erosive RA (C). Circulating levels of IL-6 were significant elevated in RA patients compared with healthy subjects ($p < 0.001$). We found no changes between serum IL-6 at 10 AM day 1 and 2 in healthy subjects or in RA patients. Serum IL-6 at 10 PM was 80% higher ($p < 0.0001$) compared with 4 PM, and 92% higher ($p < 0.0001$) compared with 10 AM in healthy subjects (Wilcoxon signed rank test). In the early RA patients serum IL-6 reached a minimum at 4 PM (42% lower, $p = 0.025$) compared with 10 PM. In patients with erosive RA serum IL-6 at 10 PM was significantly higher compared with 4 PM (62%, $p = 0.0026$) and 7 PM (34%, $p = 0.046$), and serum IL-6 was lower at 10 AM (10 AM vs. 1 PM, $p = 0.004$; 10 AM vs. 4 PM, $p = 0.004$ and 10 AM vs. 7 PM, $p = 0.043$).

Variation during 3 weeks and 12 months. The median day-to-day CV of plasma IL-6 for each subject (all samples taken at 8 AM) was 27% (range 7–99%, first quartile 13%, third quartile 43%) for the 1st round, 31% (range 5–99%, first quartile 14%, third quartile 52%) 6 months later for the 2nd round, and 26% (range 7–67%, first quartile 17%, third quartile 39%) 12 months later for the 3rd round. The CVs adjusted for assay variation were approximately 2% lower. Comparing the sample taken at 2 PM with the sample taken at 8 AM on the same day for each subject did not demonstrate a systematic difference (1st round: $p = 0.17$; 2nd round: $p = 0.43$; 3rd round: $p = 0.56$, Wilcoxons signed rank test). Figure 3A illustrates the individual weekly changes in plasma IL-6 in the 27 subjects during the 3rd round. Figure 3B illustrates the variation in plasma IL-6 in 27 subjects collected at five time points during a month and five times again after 6 and 12 months. Comparing plasma IL-6 between the three periods using a linear model showed significantly higher plasma IL-6 in first two rounds (27%, $p = 0.04$; and 28%, $p = 0.01$) compared with the 3rd round. There was no significant difference between the two first rounds ($p = 0.81$). It should be noted that plasma IL-6 levels were low and the CVs were therefore relatively high. The estimates of the variance components using the random effects model resulted in a within subject CV of 41.5% and a CV over the 12 months of 11.5%. The within-subject variation including the variation over time and interassay variation was 44.5%. The intraclass correlation coefficient over the 12 months was 61%.

Variation during 3 years. Figure 4 illustrates the weekly changes in serum IL-6 in healthy women at five time points during a month and subsequently again after 3 years. There were 30 women in the first set and the median CV was 25% (range 4–140%). Using a repeated measure analysis of serum IL-6 in the 21 healthy women included in both study periods over the 3-year period did not demonstrate a significant difference (14% lower after 3 years, $p = 0.33$). The median CV of the individual variation in the first period was 23% (range 4–140%) and 26% for the second period (range 9–137%) in the 21 women included in both study periods. The estimates of the variance components using the random effects model result in a within-subject CV of 38.9% and a variation over the 3 years of 7.3%. The within-

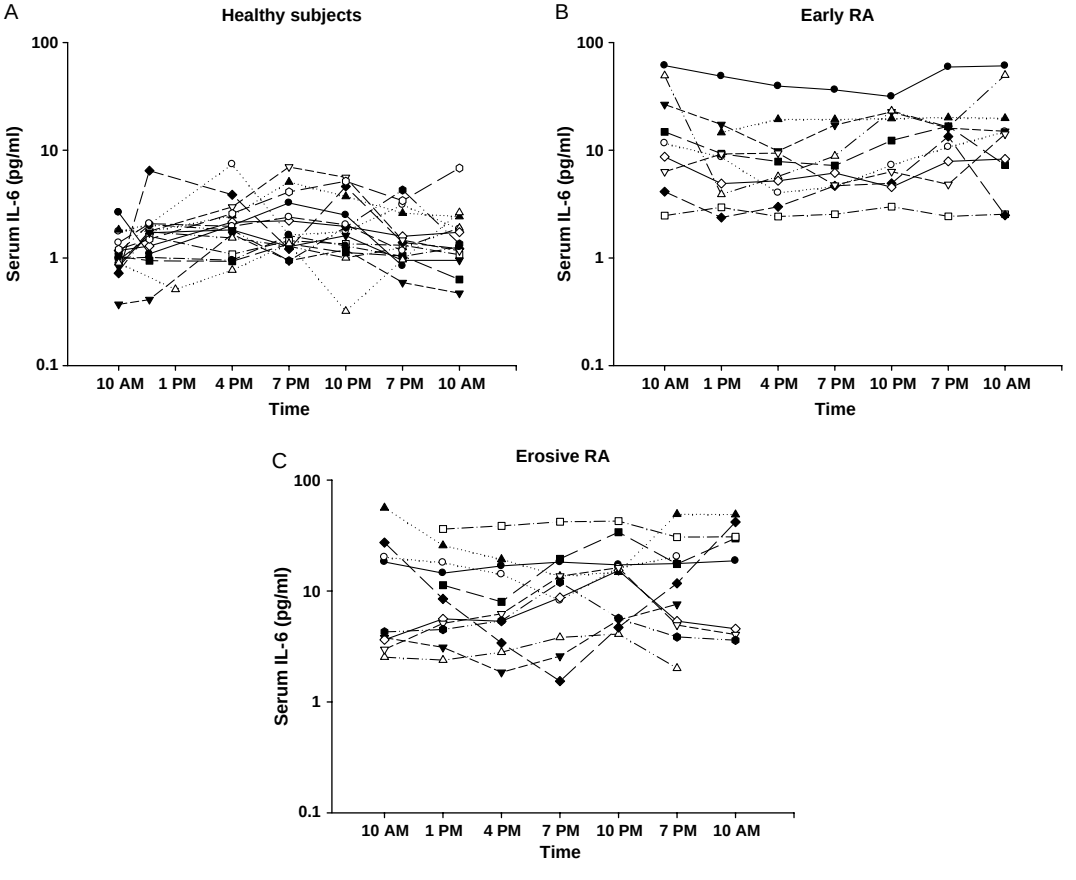

Figure 2. The diurnal changes in serum concentrations of interleukin (IL)-6 in healthy subjects (A), and in patients with early rheumatoid arthritis (RA) (B) and long-term erosive RA (C). The individual fluctuations are shown. The *y*-axis is a log scale.

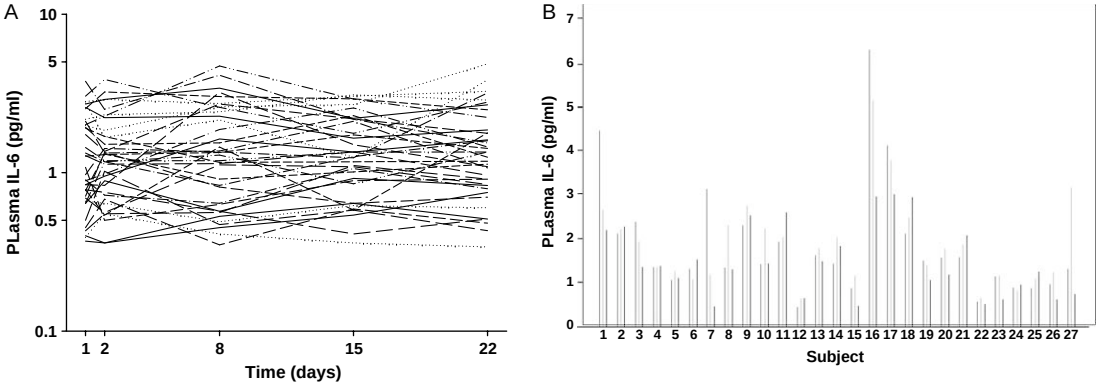

Figure 3. The short- and long-time variations in plasma interleukin (IL)-6 concentrations. (A) The individual fluctuations during a month. (B) The median plasma IL-6 concentration in each subject during 1 month (1. column) and repeated again after 6 months (2. column) and 12 months (3. column).

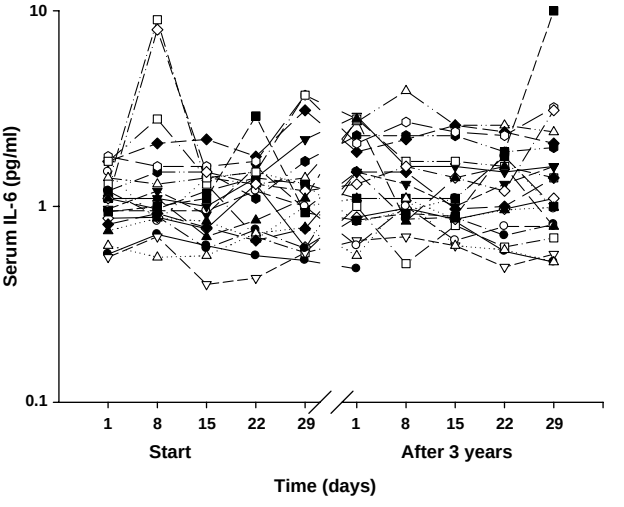

Figure 4. The long-term variation in serum interleukin (IL)-6 in healthy women during 1 month and repeated again after 3 years. The individual fluctuations are shown. The *y*-axis is a log scale.

subject CV including the variation over time and interassay variation was 40.9%. The intraclass correlation coefficient over the 3 years was 51%.

Exercise. Figure 5 illustrates the changes in serum IL-6 before and after 25 min of cycling in healthy subjects (A), in patients with early RA (B) and in patients with long-term erosive RA (C). Serum IL-6 was significantly elevated in RA patients compared with healthy subjects ($p < 0.001$). In healthy subjects serum IL-6 increased immediately after exercise (mean 21% increase, $p = 0.0006$), 1 h later (mean 53% increase, $p = 0.002$) and 3 h after exercise (mean 55% increase, $p = 0.027$). There was no difference in serum IL-6 between 1 h and 3 h after exercise ($p = 0.91$). In patients with RA no changes in serum IL-6 were found after exercise (early RA: immediately after, 1 h after and 3 h after exercise compared with start: $p = 0.63$, $p = 0.21$, $p = 0.55$, respectively; long-term RA: immediately after, 1 h after and 3 h after exercise compared with start: $p = 0.56$, $p = 0.60$, $p = 0.14$, respectively).

Discussion

This study evaluated pre-analytical and biological variations of circulating IL-6 in healthy subjects and in patients with RA. The circulating levels of IL-6 were stable regarding sample handling and showed little variation over time in healthy subjects.

Some blood collecting tubes can be contaminated with endotoxin, which may stimulate the white blood cells to produce significant quantities of cytokines. Since EDTA inhibits such induction, it has been proposed that EDTA plasma is the preferred anticoagulant for assessment of cytokines (Riches et al. 1992, Thavasu et al. 1992). In the present study no significant differences were found in IL-6 concentrations between serum, EDTA plasma, EDTA plasma on ice, heparin or citrate samples. This is in accordance with another study (De Jongh et al. 1997), but it has also been reported that EDTA and serum gave comparable results but different from heparin and citrate plasma (Riches et al. 1992).

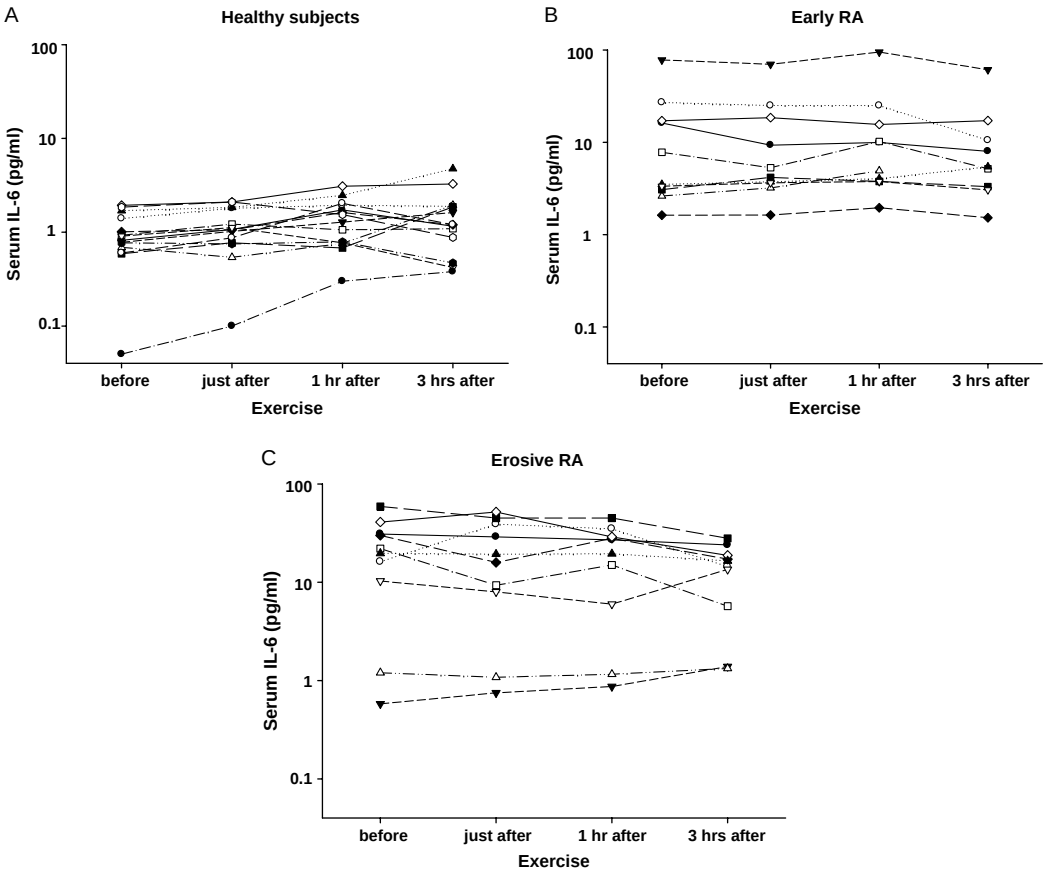

Figure 5. The changes in serum interleukin (IL)-6 after 25 min of cycling in healthy subjects (A), and in patients with early rheumatoid arthritis (RA) (B) and long-term erosive RA (C). The individual fluctuations are shown. The *y*-axis is a log scale.

The importance of time delay between blood sampling and processing of the sample was also examined. The time interval between drawing of blood and centrifugation of blood stored at RT or 4°C must be 3 h or less for serum samples and 3 h or less for tubes with EDTA stored at RT. IL-6 concentrations in tubes with EDTA stored at 4°C were stable for 24 h. Otherwise significant and not disease-related elevations of IL-6 were found in the serum and EDTA plasma samples left to clot for a longer time when compared with IL-6 concentrations in serum and EDTA plasma samples centrifuged within 3 h after venipuncture. IL-6 concentrations in serum and EDTA plasma were stable in up to ten repeated freeze–thaw cycles. Others found no significant changes in serum IL-6 stored at 4°C or RT for up to 24 h (De Jongh et al. 1997, Kenis et al. 2002), or a decrease in plasma IL-6 when stored at both 4°C and RT and no change in plasma for up to six repeated freeze–thaw cycles (Thavasu et al. 1992).

Over the course of 1 day significant differences were seen in serum IL-6 in healthy subjects and RA patients. In general, serum IL-6 was highest at 10 PM compared with earlier in the evening, the afternoon, and morning. Serum IL-6 was not determined during the night in the present study. Others have found that serum IL-6 in healthy subjects either change in a biphasic circadian pattern with two minimums at 8 AM and 9 PM and two maximums at 5 AM and 7 PM (Vgontzas et al. 2004, 2005) or has only one minimum in the day time and not a day time maximum (Bauer et al 1994, Sothern et al. 1995b). In patients with RA a rise in serum IL-6 in the late evening and during the early morning has also been reported (Arvidson et al. 1994, Crofford et al. 1997, Cutolo et al. 2005).

In the present study, the intra-individual variations in healthy subjects of plasma IL-6 collected at five time points during a month and again after 6 and 12 months were investigated. The CVs in the three rounds were between 26% and 31% with significantly higher plasma IL-6 in the first two rounds compared with the last round. The intra-individual variation of serum IL-6 in healthy women at five time points during a month and again after 3 years showed CVs of 23% and 26% and no significant difference in serum IL-6 in the 3-year period. Few studies have examined the variation of serum or plasma IL-6 over time. One study evaluated the variation of serum IL-6 over a 6-month period and found an overall within-subject CV of approximately 30% (Cava et al. 2000). Another study evaluated the long-term variation of serum IL-6 over a period of 2 years and found an intraclass correlation coefficient of 0.48 (Ho et al. 2005). We found an intraclass correlation coefficient of 0.61 over a period of 1 year and an intraclass correlation coefficient of 0.51 over a period of 3 years suggesting a reasonable reproducibility.

The normal range for IL-6 concentrations in serum and EDTA plasma in the present study are in accordance with the normal range reported by others using the same ELISA (Hager et al. 1994, Sothern et al. 1995a, Pantsulaia et al. 2002, Hong et al. 2004, Haddy et al. 2005). There was no difference between the normal range of IL-6 in serum and EDTA plasma, and a weak association between age and IL-6 levels. In the present study the elderly in the general population are missing, since our reference intervals for IL-6 are measured in healthy blood donors with an age between 18 and 64 years. This might explain why there was not a stronger correlation between age and the IL-6 concentration as reported by others and no difference in IL-6 concentrations between men and women (Wei et al. 1992, Hager et al. 1994, Harris et al. 1999, Maggio et al. 2006).

In order to evaluate the effect of light exercise an examination of the changes in serum IL-6 before and after 25 min of cycling in healthy subjects and RA patients were studied. In healthy subjects, serum IL-6 increased immediately after exercise, and was still increased compared with baseline levels 1 h and 3 h after stopping the exercise. In patients with RA no changes in serum IL-6 were found. This could be due to the less strenuous exercise performed by the RA patients because of their widespread joint pain. Several studies have found that plasma IL-6 in healthy subjects increases in an exponential manner and peaks at the end of exercise. The magnitude of increased plasma IL-6 is probably related to the duration and intensity of the muscle work, the mass of muscle recruited and the subject's endurance capacity (Pedersen & Hoffman-Goetz 2000, Febbraio & Pedersen 2002, Penkowa et al. 2003).

The present study was not designed to evaluate the differences in the plasma or serum concentrations of IL-6 between healthy subjects and RA patients, but about one-third of the RA patients had plasma or serum concentrations of IL-6 within the range of healthy subjects (e.g. concentrations below the 95% confidence limit of healthy subjects). This is in accordance with a previous report (Manicourt et al. 1993). Others have found that only 10–21% of RA patients had normal serum or plasma IL-6 (Dasgupta et al. 1992, Knudsen et al. 2006). In contrast it has been reported that approximately 66% of RA patients had normal serum IL-6 (Houssiau et al. 1988). Some of these differences are probably due to different assay used and different RA populations with different disease activity.

In conclusion pre-analytical and biological factors should be accounted for when serum or plasma IL-6 concentrations are used as a biomarker of disease activity in RA patients, patients with other inflammatory diseases or healthy subjects. Stadardized and uniform handling of blood samples are important to minimize changes in analytical variations in serum and plasma IL-6 that are not related to disease processes. The intra- and interassay CVs of the IL-6 ELISA from R&D used in this study are $<10.5\%$ and 17.7%, respectively. It is not known if automated testing of serum or plasma IL-6 or another ELISA method has a lower analytical variation and detection limit. The normal range of circulation IL-6 levels may also change according to the method used. Based on the present study the anticoagulant used could be either serum or EDTA plasma. The blood sample should be processed and stored at $-80^{\circ}C$ within 3 h after blood tapping, and sampling should not be done in the early morning hours. Serum and plasma IL-6 changed in healthy subjects during a day, a month, a year and tested up to 3 years with a CV $<31\%$. The biological variations, defined as the variation over time minus the analytical variation, were between 23% and 31%. We therefore suggest that changes in serum or plasma IL-6 concentrations greater than 60% (2 times the biological variation) in healthy subjects or patients are likely to reflect significant changes in disease activity and not only pre-analytical conditions, methodological and normal biological variability. Large, prospective, longitudinal studies of patients with RA, other inflammatory diseases or cancer are needed to clarify if the changes in plasma or serum IL-6 concentrations in these patients before and during different treatment regimens are greater than 60% and related to disease activity and prognosis.

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