Overnight variations in IL-6 in synovial fluid and plasma in patients with active rheumatoid arthritis

Aim: To investigate for the first time overnight variations in absolute values and patterns of synovial fluid IL-6, and other proinflammatory cytokines including IL-1β and TNF, and to relate them to blood concentrations of cytokines and cortisol in rheumatoid arthritis. **Patients & methods:** Six people with active rheumatoid arthritis and large knee effusions who had received no recent glucocorticoids were admitted overnight. Blood and synovial fluid samples were obtained at 13 time points between 21:00 and 10:00. **Results:** The geometric mean synovial fluid IL-6 concentration increased significantly from 1483 pg/ml at 21:00 to 5084 pg/ml at 08:00 (repeated measures ANOVA $p < 0.001$). The geometric mean plasma IL-6 and cortisol concentrations increased significantly overnight. In a random coefficient model including data from all participants, the estimated mean synovial fluid IL-6 value began to rise approximately 6 h before the estimated mean plasma IL-6, which in turn began to rise approximately 3 h before plasma cortisol. In the modeled results the three peaks occurred at approximately 09:00, 09:00 and 11:00, respectively. **Conclusion:** In people with active rheumatoid arthritis of varying disease duration there are high concentrations of IL-6 in synovial fluid that rise significantly during the night, rise before plasma IL-6 and are higher than plasma concentrations at all time points. This suggests that intrinsic synovial fluid IL-6 production is the main driving force for the overnight blood IL-6 changes and future investigation into the source of the IL-6 should focus on the underlying synovial pathology.

KEYWORDS: cortisol ■ cytokines ■ IL-1β ■ IL-6 ■ pathogenesis ■ rheumatoid arthritis ■ synovial fluid ■ TNF

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Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease that primarily affects the synovial joints. Disease activity in RA has been shown to have a circadian rhythm with maximum activity around 03:00 and minimum activity in the early afternoon [1]. In RA the synovitis is driven in part by deregulation of the inflammatory process and production of proinflammatory cytokines such as IL-6 and TNF [2]. These inflammatory cytokines are found at abnormally high concentrations in RA in both synovial fluid (SF) [3–5] and blood [6–8]. When single paired samples of blood and SF have been investigated, the IL-6 concentration found in the SF has been consistently higher than that seen in the blood [8–13]. In contrast to IL-6, similar paired concentrations of other cytokines such as TNF and IL-1β are rarely reported. Published reports do not clearly identify paired samples so that the comparisons can be corrupted by the large interindividual variations in concentrations [14–16] and do not show a consistently raised concentration in SF or plasma [6,11,15,17]. Nevertheless, it has been assumed that all inflammatory cytokines are produced in the synovium and diffuse into the blood [6–9,18,19]. The concentration of inflammatory cytokines, including IL-6, appears to correlate with disease activity and is reduced when RA is controlled with systemic treatments [20,21].

More recently, investigators have focussed on the possibility of circadian variations in relevant cytokines. A circadian pattern was suggested in the blood IL-6 concentration in patients with RA for varying durations, with samples taken every 3 h between 07:30 and 22:30 [22]. The maximum value was at the start of the study period, suggesting that the peak in IL-6 concentration may have occurred prior to 07:30. This observation was subsequently confirmed in five people with newly diagnosed RA over 24 h with a peak in IL-6 occurring at 07:00 [23] and thereafter in a variety of disease durations [24]. Further evidence of the overnight variation in IL-6 (but not other cytokines measured) derives from our own work [25] in which plasma IL-6 rose to a peak much higher than normal and persisted well into the morning. The conclusion that cytokines diffuse from the synovium to the blood has been based on paired sampling at single time points, but the discovery of circadian variations in blood IL-6 raises the possibility that there may be circadian variation in SF IL-6 production, or in the mechanism by which IL-6 diffuses into the blood, or

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the possibility that blood IL-6 does not arise from SF after all. This can be resolved only by measurements of SF and blood IL-6 during the night, when the main circadian changes in blood IL-6 occur. We therefore developed in a pilot study a synovial catheter method for obtaining repeated SF samples [26] and report here the use of this method to take repeated paired blood and SF samples during the night.

Patients & methods

Patients

We recruited six patient volunteers with RA according to the criteria of the American College of Rheumatology [27] following the approval of the United Bristol Healthcare Trust Research Ethics Committee. The study was performed in compliance with the Helsinki Declaration and written consent was obtained from each participant. Each patient had active disease (≥3 swollen and ≥3 tender joints) and a large knee effusion. No participants had received glucocorticoids by any route in the previous 93 days, or biologic therapy at any time. All female participants were postmenopausal. The blood concentrations of IL-6 for these patients are included in a previous report of a larger group of patients concentrating on blood variations in a variety of cytokines and cortisol [25]. Patient numbers (11–16) are the same as those used in the previous publication. The study size of at least six patients was determined from a consideration of the previously published pilot study, where three patients provided statistically significant data, and a recognition that the analysis would be principally based on changes within patients.

Procedure

Participants attended at 19:00 and remained on bed rest throughout the study. An intravenous cannula was inserted and flushed immediately with 10 ml 0.9% saline (repeated before and after each blood sample) and left for at least 1 h. With the patient on the bed, we used an aseptic nontouch technique including sterile gown and gloves to insert a synovial catheter as previously described [26]. The synovial catheter was not flushed with 0.9% saline and nothing else was injected into the synovial catheter throughout the study. The first 2 ml of each SF sample and the first 3 ml of each blood sample taken from the intravenous cannula were discarded. The blood samples were placed in a plastic blood tube containing ethylenediaminetetraacetic acid and the SF samples were placed directly into a plain plastic tube. Paired SF and blood samples were taken at 21:00, 22:00, 23:00, 01:00, 03:00, 04:00, 05:00, 05:45, 06:30, 07:15, 08:00, 09:00 and 10:00. Low-level lighting was used when participants wanted to sleep.

Measurements

After each sample was taken, the plasma was separated and the plasma and SF stored in aliquots at -80°C within 1 h. C-reactive protein and plasma viscosity analysis were performed through the routine clinical laboratories at the United Bristol Healthcare Trust (Bristol, UK). Total plasma cortisol and plasma TNF, IL-1β and IL-6 concentrations were measured by in-house radioimmunoassay and Luminex® 100 ELISA, respectively, as previously reported [26]. Values below the limit of detection were set at the limit for statistical analysis. In contrast to the measurements in plasma, the viscosity of the SF precluded accurate measurements of cytokines using the Luminex 100 ELISA and we therefore used Quantikine® ELISA kits (R&D Systems) with interassay variability of less than 13% and intra-assay variability of less than 10%. Owing to the limited SF volume at each time point we first measured IL-6 in the SF and then measured TNF and IL-1β in the remaining SF samples. SF IL-6 was measured at all time points, but owing to the small size of the remaining samples, we were not able to use exactly the same time point for measuring SF TNF and IL-1β in each participant. Therefore, for each participant we chose one 'early' SF sample (21:00 or 22:00), one 'middle' sample (between 04:00 and 05:45, and on one occasion 03:00) and one 'late' sample (09:00–10:00, and on one occasion 08:00). These times were chosen to give an indication of whether there was an overnight variation that mirrored any changes in SF IL-6. All plasma assays for cytokines were performed within 8 months, and SF cytokine assays within 23 months of collection.

Statistical analysis

IL-6 and TNF concentrations were log-transformed (base 10) prior to analysis to remove skewness. Repeated measures analyses of variance (ANOVA) were first used to determine whether there were changes in mean blood and mean SF concentrations for each of the constituents over time in the patient group as a whole ('proc MIXED', SAS version 8.2: SAS Inst. Inc., 1999–2001, Cary, NC, USA). Individual patient's patterns of IL-6 were explored using a cubic regression model ($y = at^3 + bt^2 + ct + d$, where t = time in h) and the values and timings of troughs (minima) and peaks (maxima) estimated from the

model coefficients. In an analysis of the patients as a group, a random coefficient cubic regression model was fitted to all the patient data using proc MIXED. Each of the coefficients was assumed to vary across patients and their mean variances and covariances estimated. In addition, the ratio of SF to blood concentration of IL-6 was calculated for each patient at each time point and that of TNF at the early, middle and late time points.

Results

Clinical characteristics of the patients included are shown in summary in Table 1. (Data for individual patients are given in Supplementary Table 1.) There were five men and one woman, mean age 61 years (range: 55–70 years) and mean disease duration was 6 years (range: 0.2–18 years), four (66%) were taking nonsteroidal anti-inflammatory drugs and two (33%) were taking disease-modifying antirheumatic drugs (one each methotrexate and sulfasalazine).

Table 1. Summary of clinical characteristics of patients.

Characteristic	Median (range)
Gender M:F†	5:1
Age (years)	60 (55–70)
Disease duration (years)	3.25 (0.2–18)
Erosive disease (%)	50
Hemoglobin (mg/dl)	11.85 (10.5–14)
PV (mPa)	1.805 (1.6–2.17)
C-reactive protein (mg/l)	50 (10–159)
Rheumatoid factor +ve (%)	50
Weight (kg)	75.2 (62.1–100)
Height (m)	1.645 (1.6–1.7)
BMI (kg/m^2)	27.5 (22–38)
Tender joint count	6.5 (2–18)
Swollen joint count	5.5 (3–18)
Early morning stiffness (min)	45 (30–120)
Patient global opinion (cm)	4.3 (2.4–5.3)
Pain (cm)	3.15 (1.5–8.8)
Clinician global opinion (cm)	6 (3.3–7.5)
DAS-28	4.42 (4.14–6.17)
HAD (A)	6 (3–8)
HAD (D)	4.5 (2–8)

†Number.
DAS: Disease Activity Score; HAD: Hospital Anxiety and Depression; F: Female; M: Male; PV: Plasma viscosity.

Laboratory results

Plasma cortisol and cytokine results in these six patients followed the same pattern as the larger group of patients previously reported [15] in which they were included. Table 2 shows the cortisol and cytokine geometric means and ranges, and data for individual patients are given in Supplementary Table 2. The concentrations of TNF and IL-1β showed no significant overnight change (ANOVA mixed model repeated measures analysis of variance).

Synovial fluid cytokine concentrations are shown in Table 3 as the geometric means and ranges at each time point for IL-6 (including two interpolated values for time point 04:00 and one each for time points 05:45 and 06:30, which were not included in the modeling procedures and the geometric means for the 'early', 'middle' and 'late' times for TNF and IL-1β). (Details for individual patients are given in Supplementary Table 3.) Over the course of measurements, mean IL-6 concentrations were 34–72-times greater in SF than plasma. The ratio differed between patients but within an individual the ratios were relatively consistent throughout the night time

Table 2. Plasma cortisol, IL-6 and TNF geometric means and ranges at each time point for patients 11–16.

Sample time	Cortisol		IL-6		TNF	
	Geometric mean	*Range*	*Geometric mean*	*Range*	*Geometric mean*	*Range*
21.00	84	11–204	43	13–109	98	25–521
22.00	65	18–136	38	12–105	91	24–471
23.00	49	10–117	43	13–135	98	24–508
01.00	49	13–107	50	17–104	93	24–501
03.00	41	11–108	54	16–127	93	24–476
04.00	63	11–277	49	19–122	102	25–528
05.00	97	32–251	63	19–151	100	23–532
05.45	123	27–446	75	25–144	95	23–548
06.30	113	31–374	66	18–158	91	22–537
07.15	145	33–342	69	14–260	85	22–403
08.00	157	32–349	76	19–307	90	24–455
09.00	159	38–497	87	25–332	91	24–517
10.00	196	65–336	74	21–336	91	24–544

Table 3. Cytokine concentrations (pg/ml) in synovial fluid of six participants with rheumatoid arthritis.

Time	IL-6 geometric mean	IL-6 range	Time	TNF geometric mean	IL-1β geometric mean
21:00	1483.0	114–26789	21:00–22:00	12.0	5.0
22:00	1685.5	128–27183			
23:00	1916.0	177–30352			
01:00	2421.2	228–35832			
03:00	3028.9	706–46249	03:00–05:45	16.7	5.0
04:00	3380.1†	827–49529†			
05:00	3772.0	790–52810			
05:27	4474.4	722–47884			
06:18	4771.7	675–52986			
07:09	4991.8	727–53104			
08:00	5083.6	662–45666	08:00–10:00	16.9	9.5
09:00	3832.8	724–37499			
10:00	4018.9	920–32457			

†*After interpolation for missing values in patients 11 and 16. Without interpolation the values are 1843.5 and 821–10268.*

samples (Figure 1). (Patient 12 had a steadily rising ratio, although there were no other clinical or laboratory features to distinguish this patient from the other patients.) The mean (standard deviation) ratios for patients 11–16 were 387 (96), 73 (69), 80 (15), 78 (15), 12 (3) and 26 (6), respectively. There was no correlation between the size of the effusion and any of the individual patient's mean, minimum or maximum SF IL-6 concentration. TNF concentrations were up to 40-times lower in SF than in plasma and the mean (standard deviation) ratios for patients 11–16 were: 0.17 (0.05), 0.21 (0.09), 0.07 (0.02), 0.89 (0.23), 0.26 (0.04) and 0.04 (0.02), respectively. For IL-1β there were only six occasions (in three patients) at which paired values for SF and plasma were available because in the majority of plasma samples IL-1β was below the limit of detection. The mean IL-1β SF:plasma ratio was 0.82 (standard deviation: 0.58).

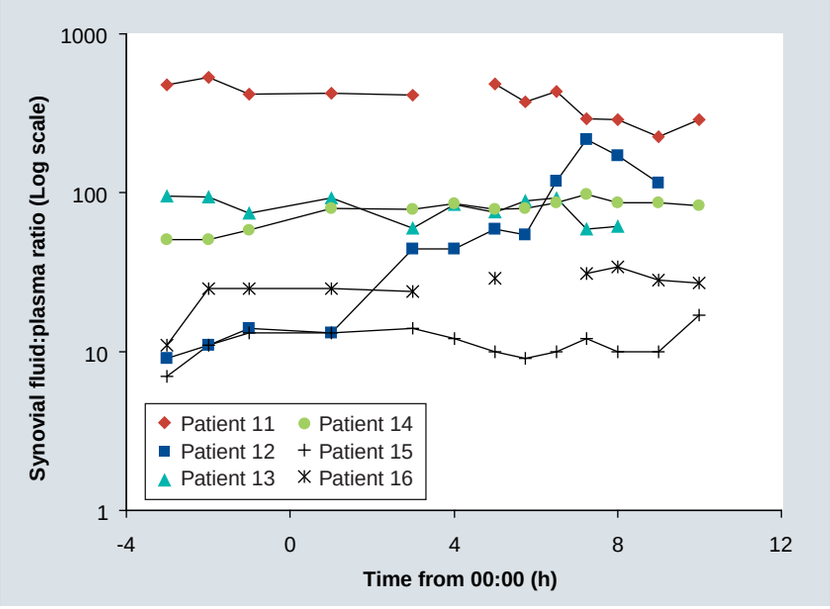

Figure 1. IL-6 synovial fluid:plasma ratios in paired samples from patients 11, 12, 13, 14, 15 and 16.

Statistical modeling

The random effects coefficient models including all (log transformed) data from all patients for plasma cortisol, plasma IL-6 and SF IL-6 are shown in Figure 2 and represent the model equations: value = $at^3 + bt^2 + ct + d$. These findings are illustrated using the back-transformed model results in Figure 3. By calculating the trough of each mean curve, we estimated that the rise in mean SF IL-6 concentration began 341 min prior to plasma IL-6, which in turn began to rise 172 min prior to cortisol.

The subsequent fall in blood IL-6 began to occur 5 min prior to cortisol, which in turn occurred 79 min prior to the SF IL-6. It is important to note that the minimum SF IL-6 concentration occurred just over 5 h prior to the study period making this time estimate less reliable. However, the SF concentration clearly begins to rise prior to 21:00 and the plasma IL-6 after 21:00, making it likely that the SF IL-6 begins to rise prior to the plasma IL-6.

Discussion

By using a novel synovial catheter method we have been able to compare IL-6 (and other cytokine) concentrations in paired SF and

blood samples obtained repeatedly overnight from people with active RA. SF IL-6 concentrations showed evidence of circadian variation, but were always higher (sometimes several hundred-fold higher) than plasma concentrations. This finding is in agreement with the single time point comparisons in the literature, which show that SF IL-6 is generally higher than plasma IL-6 [8–13], but circadian variation in SF IL-6 has not been previously reported. The SF IL-6 concentration began to rise approximately 6 h before plasma IL-6, and this is consistent with the theory that IL-6 is produced in the inflamed synovium and diffuses into the blood at a constant rate overnight. Although this assumption has been widely accepted, these are the first sequential data that document overnight variation in SF IL-6 concentrations that match changes in plasma IL-6. Despite wide variations in SF and plasma IL-6 concentration between individuals, the SF:plasma ratio was generally constant for each individual.

We chose cubic polynomial modeling for our final analysis as it fitted our overnight data better than sinusoidal (circadian) modeling that is suited to 24 h data collection. Cortisol variations in the normal population are not sinusoidal [23,28], and plasma IL-6 concentrations in RA are unlikely to be sinusoidal [23–25]. Thus, it was thought a cubic polynomial model, including a trough and a peak during the observation period, would be more likely to reflect physiological and pathological variations. In addition, a polynomial model allows the time between peak and trough to vary, rather than the fixed period found in circadian rhythms.

Our study has a number of limitations. The number of participants was small, but this type of study demands a large commitment from all concerned, and in particular patient participants. We did not measure how much the sampling disturbed the participants, although we did collect some informal estimates and formed the impression that there was a low level of disturbance. However, such measurements should be included in future studies. Some authors have suggested that age [29] and sleep deprivation [30,31] alter the IL-6 concentration, but even with conservative calculations our values of IL-6 and TNF are 6–20-times and 40-times higher, respectively, than those reported in these normal volunteers. It is therefore unlikely that the small variations reported in the healthy population would have a significant effect on our data. The current study includes mostly men with only one woman, however, there has not been any previous suggestion in the literature that there is a variation between the sexes in overnight levels of the cytokines we report. A further limitation is that we only have data for 13 h overnight. This period was chosen due to the practical considerations of staff availability and when the relevant changes in cortisol and IL-6 were thought to occur but future investigations should collect 24-h data. It is important to note that within the overall pattern of the observations there was

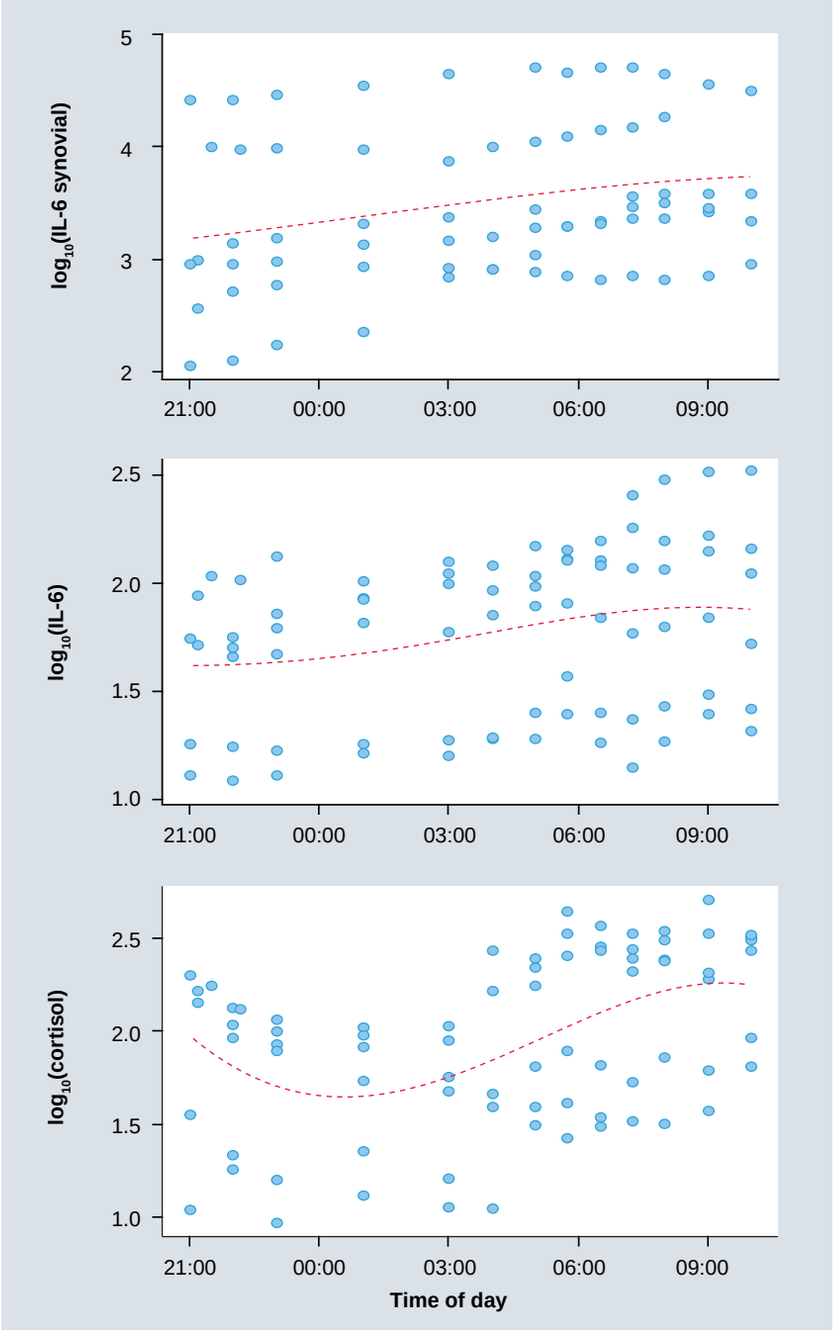

Figure 2. Model best fit lines for cortisol and IL-6 with log transformed values shown for each patient as dots.

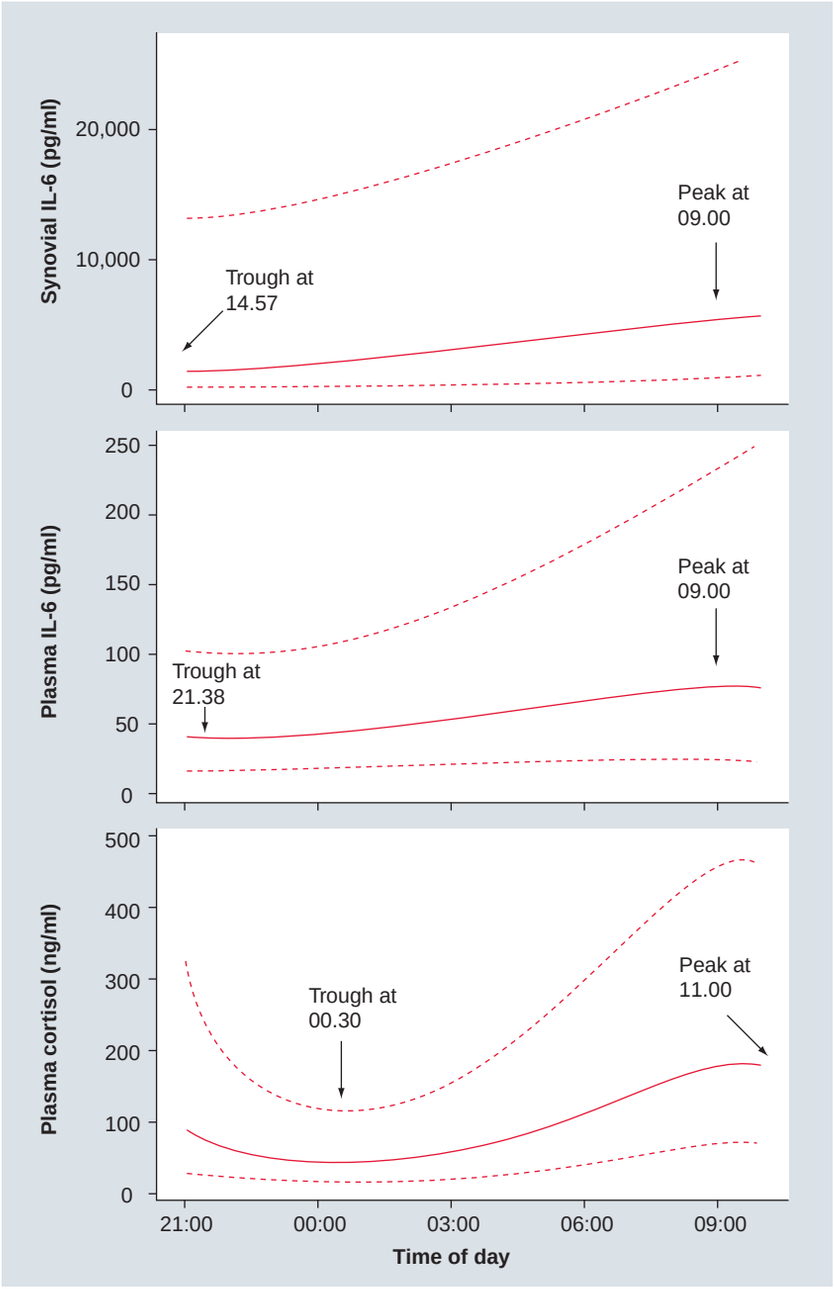

Figure 3. Model estimated mean values and 95% CIs for cortisol and plasma IL-6 and synovial fluid IL-6 with absolute values and minima and maxima indicated.

individual variation in the sequence of rises in cortisol and IL-6 concentrations and we cannot explain this observation.

Although it is widely assumed that all inflammatory cytokines are produced within the synovium and diffuse into the blood, previously reported paired SF and plasma samples only show that this is reproducible for IL-6 [8–13], with the relationship between SF and plasma for TNF and IL-1β being unclear [6,11,15,17]. Many previous studies have not shown the relationship between blood and paired SF concentrations of TNF and IL-1β as many data may be corrupted by large interindividual variations in absolute concentrations (e.g., [14–16]) or are extrapolated from other conditions (e.g., [16,18,32]). Two previous studies give conflicting results reagarding TNF, with one reporting higher concentrations in SF [6] and the other higher in blood [17]. Reports of IL-1β are less clear but the weight of evidence suggests that blood and SF concentrations are similar [11,15]. In the current data SF TNF and IL-1β showed little or no change during the overnight measurements (although we had many fewer samples on which these were measured). Furthermore, in contrast to IL-6, SF TNF concentrations were up to 40-times lower than those found in the blood and, where measurable, IL-1β was similar in SF and plasma. This suggests that intrinsic SF IL-6 production is the main driving force for the overnight blood IL-6 changes and future investigation into the source of the IL-6 should focus on the underlying synovial pathology.

Although it is well-established that IL-6 is produced within rheumatoid synovial tissue, the mechanisms underlying increased IL-6 expression in RA are not clear. Increased IL-6 production is linked with a cascade of pro- and anti-inflammatory cytokines in RA, which leads to a disequilibrium favoring inflammation within the arthritic joint [33]. In addition to this localized inflammatory effect, there is strong evidence that IL-6 is secreted from synovial tissues into the blood in RA [8–11]. Our data are further evidence that intra-articular changes in IL-6 may precede and even be responsible for subsequent plasma increases in plasma IL-6, and it has already been suggested that plasma IL-6 may drive an abnormal cortisol response [23]. Our previous daytime pilot study suggested that plasma and synovial IL-6 may vary independently and challenged the notion that plasma IL-6 is merely a reflection of synovial IL-6 production [25]; however, the current more substantial overnight data set clearly indicate that plasma IL-6 concentrations reflect synovial production. The relationship between SF and plasma TNF and IL-1β concentrations remains less defined, although it is possible that individual local factors or antagonists metabolize these and possibly other cytokines within the synovium, preventing their diffusing into the blood in significant concentrations.

Synovial fluid:plasma ratios of IL-6 seemed to remain reasonably constant during the observation period, supporting the possibility that

intrinsic synovial IL-6 production or metabolism is the driving force for the overnight SF and plasma IL-6 changes.

In conclusion we provide evidence that in people with active RA of varying disease durations there are overnight increases in SF IL-6 that precede increases in plasma IL-6. This finding raises the possibility that treatments that can reduce IL-6 production, such as glucocorticoids, might be more effective if they can be administered during the night before IL-6 increases occur, and recent clinical evidence supports this hypothesis [34].

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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