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## Abstract

Cytokines levels in blood are not yet fully considered as a biomarker for disease even if some significant progresses have been made in linking certain cytokines to some diseases. The aim of this present study was to look for the stability of some cytokines in blood collected in two different days separated by one month. Fifteen healthy young men aged 20 to 30 years were selected for this study. Each subject participated in two 24-hour sessions spaced a month apart. Blood sample was taken at 11:00, 17:00, 22:00, 01:00, 04:00, 06:00, and 08:00. Concentrations of interleukin-6, interleukin-1-receptor antagonist, soluble IL-2 receptor, interleukin-1beta, and interleukin-2 were measured in serum. The circadian pattern of each variable was compared between the two days. The results show that there is no reliability for the measured cytokines. This study shows that cytokine levels measured in blood is far being the reliable variables or to be considered as stable markers in healthy subjects.

## Introduction

Cytokines mediate and regulate immunity, inflammation, and hematopoiesis (Thomson and Lotze, 2003). They are produced *de novo* in response to an immune stimulus when facing a major physiological challenge. The complexity of these small proteins resides in their pleiotropic character which means that different cell types secrete the same cytokine or that a single cytokine acts on several different cell types. Additionally, cytokines are also redundant in their activity, i.e., several cytokines may share the same activity. All these properties render the comprehension of the physiological and pathophysiological role of cytokines less well understood. Another aspect of difficulties is that cytokines are produced within tissues and this makes their evaluation somewhat difficult in human. Thus measurements of cytokines in blood are more common. But the question aroused is whether cytokine levels measured in blood are reliable from day to day in the same subject. It should be noted that cytokines levels in blood are not yet fully considered as a biomarker for disease even if some significant progresses have been made in linking certain cytokines to some

diseases. This is why the study of the stability of cytokine production may help in providing information concerning cytokines and their clinical usefulness in measuring cytokines levels in serum.

### Subjects and protocol

This work is part of the broad study on the effect of ELF electromagnetic field on the immune system (Selmaoui et al. 2011). Data from control group who were not exposed were processed for this study. Healthy young men (12-15), aged 20 to 30 years, were selected after routine clinical and laboratory examination. Selection criteria included regular sleep habits, no chronic disease or disability, no recent acute illness; none were taking drugs or medications, no night work, no travel to another time zone within the preceding 2 months, and no smoking.

Each subject participated in two 24-hour sessions (D1 and D2) spaced a month apart. Catheters were placed in the antecubital vein for the 24-hour period, and the first blood sample was taken at 11:00, 17:00, 22:00, 01:00, 04:00, 06:00 and 08:00. The local ethics committee of the Pitié Salpêtrière approved this protocol.

### Cytokine assay

IL-1RA (R&D System, Minneapolis, MN, USA), IL-2R (Genzyme Corporation, Cambridge, MA, USA), IL-1β, IL-2, and IL-6 (Biosource, Europe S.A., Fleurus Belgium), were assayed by ELISA in duplicate using serum samples drawn during the two 24-h study days. Sampling occurred at 11:00, 17:00, 22:00, 01:00, 04:00, 06:00, and 08:00. Inter- and intra-assay coefficients of variation vary between 4 and 20.5 % (see Selmaoui et al 2011).

### Statistical analysis

The reliability of the measures of serum cytokines was analyzed by the test retest reliability intraclass correlation coefficient (ICC). The ICC is usually relevant in assessing inter-rater reliability or to evaluate the stability of a measure over time (Shrout and Fleiss, 1979). For each subject, the ICC was calculated to determine the reliability between the 2 days for the 7 recording points on a daily basis. Significance was tested when the 95% confidence interval of the coefficient did not contain 0.

In addition, the data were analyzed as a group for circadian rhythm by a computerized inferential statistical method (mean population cosinor) involving the fit of a 24-hour cosine curve by the method of least squares, rhythm detection considered statistically significant when *P* is 0.05 or less.

*Results and Discussion*

The ICC has shown no reliability for the measured cytokines (IL-6, IL1β, IL2, IL-1RA, sol IL-2R) (Table 1). This shows a large variability in secretion in the young and healthy subjects between day 1 and day 2 spaced a month apart.

With regard to the circadian variations, the serum mean concentrations of IL6 showed a significant day night variation during the first session of sampling (Table 2). However this variation was not significantly observed in the second session of sampling (day 2). No other cytokines measured (IL-1β; IL-1RA; IL-2, and sol IL-2R) changed significantly (Table2). The serum mean concentrations of IL6 began to rise in the afternoon, and reached a peak during the evening hours.

Thus, this study shows that cytokine levels measured in blood is far being the reliable variables or to be considered as stable markers in healthy subjects, unlike cortisol and melatonin (Selmaoui and Touitou, 2003). This variation in the secretion may be a result of the higher sensitivity of these proteins since they are produced when body is facing infections, injuries, or tissue trauma. The fact that cytokines are involved in the immune system, their alterations in the production, and their levels in serum, plasma and body fluids reflect the changes in the equilibrium of the immune system. Thus, measuring cytokine levels in blood is somewhat indicative of the severity degree of

infection in human being, but in our study, subjects are healthy and the intra-individual variations can be ascribed to a response to environmental aggressor such as stress which is thought to affect immune system and increases susceptibility to infection (Dhabhar, 2003; Rohleder et al. 2012). It is possible that stress could be one among other factors that may lead to the significant variation in cytokine production. Additionally, some studies have investigated the effect of partial or total sleep deprivation on cytokine levels in healthy subjects and found increased levels of IL-6 (Irwin et al., 2006; Vgontzas et al., 2004; Shearer et al., 2001; Gundersen et al., 2006; Voderholzer et al., 2012), and IL-1RA (Frey et al., 2007).

The other factors that could induce a variation in the cytokine production seem to be related to diet. Actually, previous studies have shown an association between the intake of n–3 fatty acids and lower concentrations of inflammatory markers (Brown et al., 2001). Other studies have also shown changes in CRP and IL-6, when subjects were supplemented with $\alpha$-linolenic acid or oils rich in long-chain n–3 fatty acids (Ciubotaru et al., 2003; Thies et al., 2001). On the other hand, diets high in *trans* fat or saturated fat were found to be associated with higher concentrations of CRP and IL-6, in a randomized crossover study in men (Baer et al., 2004). In another study, consumption of fish oil and vitamin E inhibited the production of IL-1β and IL-6 (Wu et al., 2004) while high fat diet affects the 24-hour pattern of circulating adipocytokines in rats (Cano et al., 2009). Taking into account of the above results, it is plausible that significant variations of cytokine production observed in healthy men could be related to specific dietary intake which was not controlled in our study.

In conclusion, our study shows that a great variation in cytokine production exists in the same subject from day-to-day sampling. Thus, this study is in accordance with the previous one (Cava et al., 2000) in which serum IL-6 and sIL-2R have shown a much higher variability within subjects. The cause of these intra-individual variations remains unclear and raises question about the relevance of cytokines assays in peripheral blood and their significance in clinical study. However, it remains worthwhile to examine theses biomarkers in relation to diseases and other inflammation-

related chronic diseases even with a single measurement since the large variation in theses cytokines between patients and their respective control group may exceed the intra-individual variation.

Finally, this intra-individual variability added to the weakness in the cytokine assays which is not performed as routine clinical laboratory procedures (Cava et al., 2000; Whiteside, 2002) makes cytokine monitoring much complex.
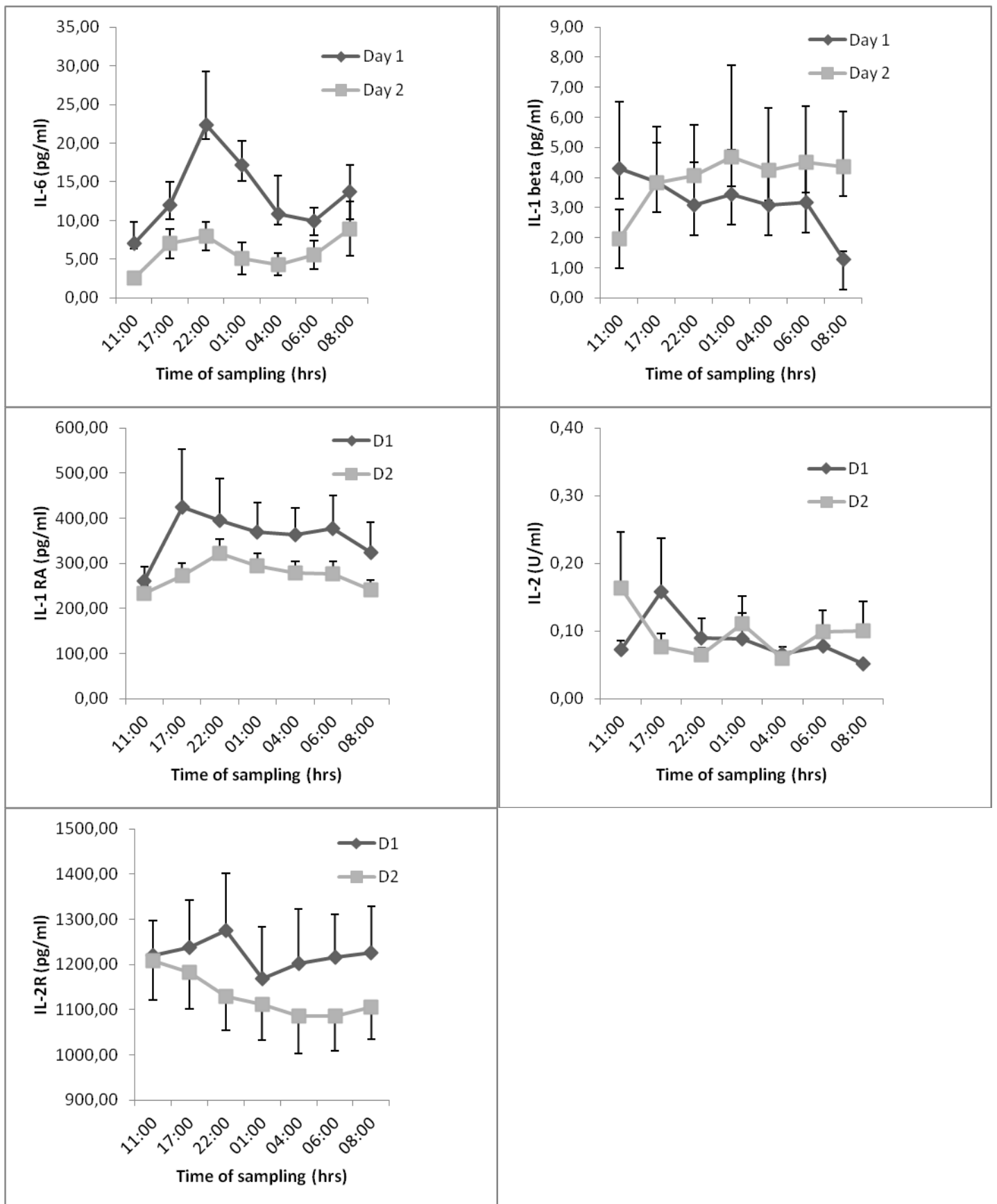
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**Figure 1: The pattern of serum cytokine (IL-6, IL-1β, IL-1RA, IL-2 and IL-2R) variation in healthy volunteers sampled in two different days (D1 and D2) spaced a month apart.**

**Table 1:** The ICC compared the similarity of the 7 time-of-day measures between the 2 days for each variable.

| | ICC | 95%CI | | ICC | 95%CI | | ICC | 95%CI | | ICC | 95%CI | | ICC | 95%CI | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Subjects | **IL-6** | Lower | Upper | **IL-1 beta** | Lower | Upper | **IL-1 RA** | Lower | Upper | **IL-2** | Lower | Upper | **Sol IL-2 R** | Lower | Upper |
| S1 | 0,2 | -0,53 | 0,80 | 0,0 | -0,60 | 0,67 | 0,0 | -0,53 | 0,67 | - | - | - | 0,3 | -0,14 | 0,78 |
| S2 | -0,2 | -0,90 | 0,60 | 0,7 | -0,04 | 0,96 | -0,1 | -0,28 | 0,46 | 0,6 | -0,17 | 0,91 | 0,1 | -0,88 | 0,77 |
| S3 | 0,5 | -0,17 | 0,89 | 0,0 | -0,53 | 0,67 | -0,1 | -0,65 | 0,64 | -0,3 | -1,00 | 0,58 | 0,4 | -0,18 | 0,84 |
| S4 | -0,1 | -0,51 | 0,60 | -0,1 | -0,69 | 0,67 | -1,1 | -0,34 | 0,46 | 0,0 | -0,75 | 0,75 | -0,2 | -0,45 | 0,48 |
| S5 | -0,2 | -0,86 | 0,72 | -0,3 | -1,59 | 0,93 | 0,0 | -0,05 | 0,27 | 0,8 | 0,08 | 0,98 | -0,1 | -0,47 | 0,72 |
| S6 | 0,5 | -0,41 | 0,90 | - | - | - | 0,7 | -0,07 | 0,94 | 0,0 | -0,69 | 0,70 | 0,4 | -0,14 | 0,84 |
| S7 | 0,3 | -0,74 | 0,83 | 0,0 | -0,71 | 0,71 | 0,0 | -0,22 | 0,49 | 0,0 | -0,75 | 0,75 | 0,1 | -0,22 | 0,62 |
| S8 | -0,1 | -0,70 | 0,67 | -0,1 | -0,74 | 0,80 | 0,1 | -0,09 | 0,52 | 0,0 | -0,71 | 0,71 | 0,4 | -0,62 | 0,86 |
| S9 | 0,3 | -0,30 | 0,83 | -0,6 | -1,16 | 0,33 | -0,1 | -0,96 | 0,71 | -0,3 | -1,01 | 0,59 | -0,2 | -0,59 | 0,55 |
| S10 | 0,0 | -0,54 | 0,68 | -0,2 | -0,51 | 0,45 | 0,0 | -0,13 | 0,49 | 0,0 | -0,56 | 0,68 | -0,1 | -0,12 | 0,28 |
| S11 | -0,1 | -0,65 | 0,63 | 0,7 | 0,04 | 0,94 | 0,3 | -0,16 | 0,81 | -0,1 | -0,69 | 0,63 | 0,1 | -0,87 | 0,77 |
| S12 | 0,0 | -0,13 | 0,46 | 0,2 | -0,76 | 0,80 | 0,1 | -0,51 | 0,74 | -0,1 | -0,62 | 0,60 | 0,0 | -0,11 | 0,27 |
| S13 | -0,1 | -0,25 | 0,45 | - | - | - | 0,4 | -0,569 | 0,87 | - | - | - | -0,1 | -0,563 | 0,62 |
| S14 | -0,2 | -0,86 | 0,61 | 0,0 | -0,71 | 0,71 | 0,3 | -0,62 | 0,84 | 0,0 | -0,75 | 0,75 | 0,3 | -0,51 | 0,81 |
| S15 | 0,2 | -0,70 | 0,83 | 0,0 | -0,55 | 0,67 | -0,2 | -0,81 | 0,60 | 0,0 | -7,22 | 0,71 | -0,1 | -0,24 | 0,43 |

**Table 2**: The cosinor method was used to characterize the circadian rhythm of the variables. Rhythm detection considered statistically significant when $P$ is 0.05 or less.

| Cosine | D1 | D2 |
|---|---|---|
| IL-6 | **< .05** | > .05 |
| IL-1 beta | > .05 | > .05 |
| IL-1 RA | > .05 | > .05 |
| IL-2 | > .05 | > .05 |
| Sol IL-2 R | > .05 | > .05 |