Effect of the anticoagulant, storage time and temperature of blood samples on the concentrations of 27 multiplex assayed cytokines -consequences for defining reference values in healthy humans.

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Running title: Sampling conditions and reference ranges for plasma cytokine levels

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Abbreviations: EDTA, ethylenediamine tetraacetic acid; IL, interleukin; RT, room temperature

Abstract

Cytokines are potentially useful biomarkers of sepsis and other inflammatory conditions. Many cytokines can be released by leukocytes and platelets after sampling. The sampling and processing techniques are consequently critically important to measure the *in vivo* levels. We therefore examined the effects of four different anticoagulants, lepirudin, heparin, citrate and EDTA, compared to serum, on the levels of 27 different cytokines. The effects of storage temperature, freezing and thawing on the plasma cytokines were examined. Cytokines were analysed using a multiplex immunoassay. The cytokine levels in serum were significantly higher compared with plasma, consistent with release of cytokines *in vitro* during coagulation. In general, the lowest values for all cytokines were found in EDTA samples, stored on crushed ice, centrifuged within 4 h and thereafter store at -80°C. MCP-1 and MIP-1β levels were highest in heparin plasma and storage for up to 4 h at room temperature significantly increased the IL-2, IL-6, IL-8, IFN-γ and GM-CSF levels in EDTA plasma, indicating post-sampling release. In contrast, the IL-10 and IP-10 levels in EDTA plasma were unaffected by sample storage at both temperatures. Our results indicate that the cytokines were more stable in plasma than in whole blood after sampling. Thus, cytokines should be analysed in EDTA plasma samples stored on ice and centrifuged within 4 h. Based on these data, the reference ranges of 27 cytokines in EDTA plasma in 162 healthy human donors were calculated.

1. Introduction

Cytokines, chemokines and growth factors are potential biomarkers of sepsis and other inflammatory diseases [1-3]. These compounds are extensively used as biomarkers of inflammation in a number of human diseases and inflammatory conditions [4-10]. In the case of many analytes in general, much attention has been focussed on the preanalytical sampling conditions [11, 11-13]. Although poorly studied, the sampling and processing techniques used for analysis of the cytokine levels in plasma and/or serum samples might also be important. To obtain cytokine values representative of the *in vivo* conditions, the sampling techniques must prevent cytokines from being released by blood leukocytes after sampling. The time and the storage temperature between the sampling and centrifugation steps might therefore be important.

Human blood leukocytes, including monocyte-macrophages and neutrophils, synthesize a number of cytokines after stimulation with an endotoxin or bacteria through Toll-like receptor activation [14]. A number of other stimuli, including bacterial products [15], complement fragment C5a [16], immune complexes, complement activation, C5a [17] and calcium ionophores also stimulate cytokine synthesis [18]. Coagulation activation itself and thrombin can also enhance the release of IL-1β, IL-6 and TNF by monocytes through activation of protease-activated receptors [19]. Thrombin can activate protease-activated receptor (PAR)-1 and PAR-4, leading to leukocyte activation and cytokine release [19].

When blood is drawn into plastic tubes, complement and coagulation activation by the plastic surface leads to the activation of immune-competent cells and platelets, in part due to their recognition of this foreign surface [20]. This phenomenon of activation by foreign surfaces is termed bioincompatibility and opposite biocompatibility [20]. Complement is rapidly activated by plastic and other foreign surfaces and participates in the activation of platelets and leukocytes [21]. Coating plastic tubing with heparin effectively reduces complement activation and cytokine release [22]. The release of the anaphylatoxin C5a

is involved in cytokine release triggered by several different stimuli [17, 23]. In addition, coagulation activation and thrombin can also stimulate immune-competent cells, including leukocytes, leading to cytokine release [24].

Previously, most studies used serum for cytokine measurements [25]. The manufacturer of many ELISA and immunoassay kits for cytokine analyses also recommends using serum samples for the analyses. However, several studies indicated that coagulation activation is linked to inflammation in a process called immunothrombosis [26]. The potential role of thrombin in coagulation activation leading to cytokine release in human whole blood samples is yet to be examined. Several anticoagulants, such as EDTA and citrate, bind calcium and thereby inhibit both coagulation and the activation of the complement system. However, newer anticoagulants, such as hirudin and its recombinant analogue lepirudin, which specifically inhibit thrombin, have not been evaluated as anticoagulants for use in cytokine analyses. In contrast to heparin and calcium-binding anticoagulants, such as EDTA and citrate, lepirudin has no adverse effects on complement activation [27]. Lepirudin has therefore been considered the preferred anticoagulant for whole blood when the role of complement activation *in vitro* is examined [27]. The levels of 27 different cytokines, interleukins and growth factors in EDTA, citrate, heparin and lepirudin plasma and serum obtained from ten healthy blood donors were therefore examined. The influence of storage time and temperature before and after centrifugation on cytokine levels were also studied.

2. Materials and methods

2.1 Study population

To examine the effects of temperature and storage time on cytokine levels, venous blood was collected from ten healthy blood donors (five females and five males). The donors were without any medication and did not experience fever or and other symptoms of infectious or inflammatory illness during the two weeks prior to blood sampling. The blood donors provided written informed consent. The study was approved by the regional ethics committee of the northern Health Region of Norway. To determine the reference ranges, EDTA plasma samples from the following groups of healthy donors were included: 1) 49 healthy blood donors from the Blood Bank of Nordlandssykehuset Bodø, 2) 42 healthy controls in a clinical epidemiological study performed in Nordland County, Norway, 3) and 4) 71 heathy persons from a local study on health care workers. ; giving a total of 162 healthy Norwegian persons (107 females and 55 males, aged range 18-85). The samples were obtained by venipuncture after an overnight fast, between 8 and 9 a.m. and were immediately placed on ice, centrifuged within 15 min at +4°C and stored at -80°C until analysis.

2.2 Blood sampling and sample tubes

To prevent contamination by tissue thromboplastin during sampling, blood was first drawn into one dry 5-mL Vacutainer® tube (Becton Dickinson (BD), Plymouth, UK), which was discarded. Winged blood collection sets (BD Vactuainer® Safety-Lok™, needle gauge 21, needle length 0.75 inches, tubing length 7 inches, with luer locks (Franklin Lakes, NJ, U.S.A.) were used to collect the blood. Tubes were filled in the following order: blood collected in lepirudin-containing tubes (Refludan®, 50 µg/mL) was added to 4.5-mL Nunc™ polypropylene CryoTubes™ (NUNC A/S, Roskilde, Denmark) using lids from dry 4.5-mL BD Vacutainer® tubes, blood for serum samples was collected in tubes without an additive (Vacuette®, 2 mL), in 3.2% sodium citrate Vacuette® tubes (2 mL), in lithium heparin tubes (Vacuette®, 4 mL), in K3EDTA tubes (Vacuette®, 2 mL), and in serum sep clot activator tubes (Vacuette®, 4 mL). All Vacuette® tubes were purchased from Greiner Bio-One GmbH (Kremsmünster, Austria). All of the tubes were held vertically during sampling to prevent contamination by anticoagulants between using the different tubes. All tubes were gently rotated eight times by hand after sampling.

2.3 Whole blood storage conditions

After the blood samples were collected, one tube (T0) was immediately centrifuged at 3220 x *g* for 15 min at 4°C and stored at -80°C. Two tubes were stored at +4°C and two tubes were stored at room temperature (RT), one for one hour (T1h) and one for four h (T4h). In the case of the two serum sampling tubes, the same procedure was initiated after one h of coagulation at room temperature.

2.4 Treatment of plasma samples

Four blood donors were selected using the same exclusion criteria applied in the first experiment and using the same blood sampling technique. We chose four different plasma collection tubes, as follows: 1) lepirudin-containing tubes, to which EDTA was added immediately before centrifugation to prevent further complement activation, 2) citrate-containing tubes (3.2% sodium citrate), 3) EDTA-containing tubes (4.43 mM final concentration) and 4) EDTA containing tubes (10 mM final concentration).

To obtain higher levels of cytokines for testing cytokine stability, some of the lepirudin anticoagulated whole blood samples were incubated with *E. coli* (1 x 10^7/mL) in Nunc™ cryotubes. The tubes were incubated for 2 h at 37°C before centrifugation. Control samples were obtained by immediate centrifugation ($T0_p$). To examine the stability of cytokines in the plasma samples, the samples were stored in Nunc tubes in the dark at either room temperature (RT) or at +4°C for two, four and 24 h. The *E. coli* strain LE392 (ATCC 33572) was obtained from American Type Culture Collection (Manassas, VA, U.S.A.). *E. coli* was grown overnight on a lactose dish, after which 5-10 colonies were transferred to LB-medium (1% tryptone, 0.5% (w/v) yeast extract, 1% (v/v) NaCl) purchased from Becton Dickinson (Sparks, MD) and grown overnight. The bacteria were harvested and washed once with Dulbecco's PBS without Ca^{2+} and Mg^{2+} by centrifugation at 3220 x *g* for 10 min at 4°C. The bacteria were aliquoted, heat inactivated for 1 h at 60°C and stored at -80°C. A frozen ampoule was thawed at ambient temperature and the cells were washed six times with PBS by centrifugation at 3220 x *g* for 10 min at 4°C.

2.5 Freezing and thawing of plasma

The effect of freezing and thawing on the cytokines was examined using the $T0_p$ plasma samples (the lepirudin, sodium citrate, 4.43 mM and 10 mM EDTA samples) The samples were frozen (-80°C) and then thawed on ice (0°C) for 20 min, followed by one h of storage on ice to simulate standard sample handling. The samples were then maintained in a

-80°C freezer for a minimum of 2 h to ensure freezing. The samples were then frozen and thawed 1, 3 and 6 times.

2.6 Cytokine analyses

The cytokines in the plasma and serum samples were analysed using a Bio-Plex Human Cytokine 27-Plex Panel kit purchased from Bio-Rad Laboratories Inc. (Hercules, CA, U.S.A.). The analyses was performed using high-sensitivity detection according to the manufacturer`s instructions. Estimated values below the lowest standard were used. The following cytokines were analysed: interleukin (IL)-1 beta (IL-1β), IL-1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (C-X-C motif chemokine ligand 8; CXCL8), IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin-1 (C-C motif ligand 11; CCL11), basic fibroblast growth factor (FGF basic), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), IP-10, monocyte chemoattractant protein 1 (MCP-1 or CCL2), macrophage inflammatory protein-1-alpha (MIP-1α or CCL3), macrophage inflammatory protein-1-beta (MIP-1β or CCL4), platelet-derived growth factor-BB (PDGF-BB), RANTES (CCL5), tumour necrosis factor (TNF) and vascular endothelial growth factor (VEGF).

2.7 Statistical analyses

GraphPad Prism 5 (GraphPad Software, CA) was used for the statistical calculations. A non-parametric, one-way repeated measurement ANOVA with Dunn's post-test was used to compare the pre-analytical sampling conditions. To analyse the effects of whole blood incubation, all of the T0 samples were compared to the T0 tube with the highest median concentration, which was the control. The dilution of the plasma due to the volume of citrate added to the citrate-containing tubes was corrected for and each donor's haematocrit was corrected according to the method of Van Beaumont [28]. The data regarding storage of whole blood and plasma at different temperatures (4°C and RT) in each type of sample tube were analysed separately, leaving time as the only variable, by comparing the T1h and T4h samples to the T0 sample in the same series. $P<0.05$ was considered to indicate significant differences. The Excel program (Microsoft Corp., Redmond, WA) replaced immeasurable cytokine levels with random numbers between 0.001 and the lower detection limit value. Measurable cytokine levels that were lower than that of LDL were accepted as valid numbers. Some of the levels of the cytokine RANTES were greater than the highest detection limit (HDL). Excel replaced these values with random numbers between the level of HDL and the highest measured concentration of RANTES that we measured. The data for the MIP-1β, IL-1β and IP-10 levels included one extreme outlier that exceeded the mean value +/- 3 SD of the other data, which were therefore excluded from the T0 figures without affecting the results of the statistical analyses. The distribution of cytokines in females and males were compared using Mann-Whitney U-test.

3. Results

We first aimed to investigate the optimal conditions for sampling, handling and storage of samples to be analysed for the 27 cytokines. Ten blood donors were selected for these experiments.

3.1 Effect of plasma anticoagulants on cytokine levels in immediately cooled and centrifuged blood samples compared with serum samples

The cytokine levels in EDTA, citrate, lepirudin and heparin-plasma obtained from immediately cooled and centrifuged blood samples and compared them with the levels in serum samples were studied. Two different serum-collection tubes were used, one was a plain tube lacking a gel and the other contained a gel and a clot activator. Of the 27 cytokines analysed, all were detected above the lower detection limit in > 25% (see below for details). . Twelve of these were representative for the group and are presented in detail (Figures 1 and 2). In general serum samples showed substantionaly and significantly ($P<0.05$) higher levels than plasma samples (Figure 1), , whereas IP-10 and MCP-1 did not show these major difference between plasma and serum (Figure 2). Notable, two of the cytokines, MIP1-β and MCP-1, were markedly and significantly increased in heparin plasma (Figure 2). Two of the cytokines, RANTES and PDGF-BB, were markedly higher in serum than in plasma samples (Figure 1), consistent with *in vitro* release during coagulation.

Upon correcting for the time during which the serum samples coagulated at RT, reached by comparing the T0 serum with plasma that had been stored at RT for 1 h, it was found that the differences in all of the cytokine levels, except for those of IL-5 and MIP-1α, were significant.

All together, these results indicate that cytokines should be analysed in EDTA blood samples, immediately cooled and rapidly centrifuged to obtain EDTA plasma to be stored.

3.2 Effect of storage time and temperature of whole blood samples upon plasma separation for detection of cytokine levels

The effect of storage time and temperature on the cytokine levels in EDTA-, citrate- and lepirudin-anticoagulated whole blood was investigated (Table 1). The here listed 15 cytokines were selected based on their robust measurable amount in the samples from tubes that were stored for 1 and 4 h at RT or 4°C. Stable cytokine levels were observed in whole blood anticoagulated with EDTA, citrate and lepirudin when the samples were stored at 4°C for one h. However, when EDTA-, citrate- or lepirudin-anticoagulated whole blood was stored at RT, a significant increase in some of cytokine levels were observed after only one h of storage (Table 1). An even more pronounced increase in the levels of several cytokines was observed after 4 h of incubation at RT, indication an *in vitro* cytokine release during storage at RT.

3.3 Effect on the cytokines of temperature.

We next examined the stability of the cytokines in plasma during storage. Plasma obtained from *E. coli*-incubated whole blood contained high levels of 13 different cytokines (Table 2). The cytokine levels were stable for up to four h at 4°C in the lepirudin- and EDTA-plasma, but after 24 h, the levels of some of the cytokines decreased. Upon incubation at RT, a decrease in some of the cytokine levels was observed after only 4 h and the levels of some cytokines even increased with increasing incubation time. The highest concentration of EDTA examined (10 mM) did not influence the stability of any of the cytokines in plasma . The results indicate that EDTA plasma samples can be stored for up to 4 h at 4°C after centrifugation and before freezing.

3.4 The effect of freezing and thawing on plasma cytokine levels

The effect of freezing and thawing on cytokine stability were then examined in EDTA and citrate plasma (Table 3). There was no significant change in the cytokine levels in plasma frozen and thawed up to three times. After freezing and thawing 6 times, a slight but significant decrease in the IL-1β and RANTES levels was observed in EDTA plasma, suggesting a maximum of three freezing and thawing in the recommendations.

We second aimed to define a reference range in a healthy human population. Based upon the above findings, we decided to test EDTA plasma, immediately stored cold, centrifuged within one h and stored at -80°C.

3.5 Reference ranges of the cytokine levels in EDTA plasma obtained from 162 healthy controls

The reference ranges for the cytokines in EDTA plasma obtained from 162 healthy blood donors were then established (Table 4). Both the 2.5-97.5th percentile and the < 95th percentile levels of the cytokines are shown. Some of the cytokines were detected in samples obtained from all of the blood donors, implying that both the 2.5-97.5th percentile values could be calculated. The frequency distribution of these cytokines indicated that the upper limit was similar in females and males (Figure 3, Table 4, Supplementary Table 1, and supplementary Figure 1). However, The frequency distribution and statistical analyses of these 27 biomarkers indicated that none of these inflammatory biomarkers were normally distributed (Supplementary Figure The number of individuals (%) with detectable cytokines (n) were: >75%: n=16; 50-75%: n=6; 25-50%: n=5; <25%=n=0.

4. Discussion

The findings of this study indicate that the sampling conditions greatly affect the cytokine levels. In general, serum showed considerably higher levels in samples taken from both types of serum collection tubes as compared with those in plasma. After centrifugation and separation, the cytokine levels were stable in plasma samples. In contrast, storage of whole blood samples, particularly when the latter were stored at room temperature, lead to increase in many of the cytokines, consistent with *in vitro* release from the cells. EDTA-plasma, obtained from blood stored on slushed ice < one h, consistently contained the lowest levels for the cytokines and could be recommended as guidelines for sampling. Thus, the tentative reference ranges for the cytokines were calculated using EDTA plasma obtained according to these guidelines from samples obtained from 162 healthy controls.

The higher cytokine levels observed in serum compared with those in plasma suggest that the coagulation process and thrombin activation enhances cytokine release. This result is consistent with those of previous studies [29, 30]. The cytokine levels in samples taken from both types of serum collection tubes after one h of coagulation at RT were significantly higher than the levels in the baseline anticoagulated samples (T0) and anticoagulated whole blood that was stored for one h at RT. When the levels of the soluble terminal complement complex (sTCC) as a measure of complement activation were analysed using an ELISA, we found that complement was activated only in the serum collection tube containing a gel (data not shown). This result indicates that complement activation is not responsible for the high concentration of cytokines in serum. Thus, we suggest that coagulation itself induce cytokine release from whole blood leukocytes. This hypothesis is in accordance with a previous observation that thrombin activates leukocytes and cytokine release through the activation of PAR-1 and PAR-4 [31].

In anticoagulated whole blood, the levels of many cytokines were increased when the samples were stored at RT, even in the presence of EDTA. These results are likely due to the *in vitro* synthesis or release of pre-synthesized cytokines by leukocytes EDTA itself [32], a process that is prevented at low temperature. Interestingly, in the lepirudin-anticoagulated whole blood in which thrombin activity was specifically inhibited, only a small increase in the release of PDGF-BB, IL-2, IL-4 and IL-5 was observed, indicating that thrombin is involved in the release of these cytokines. Further studies are needed to explain these findings. Due to the temperature effect, we recommend that whole blood samples be placed on ice (0°C) after collection and be immediately centrifuged. If centrifugation is delayed, we recommend storage for up to one h and a maximal storage of 4 h at +4°C before centrifugation.

The use of heparin plasma should be avoided because the levels of several cytokines, in particular MIP-1β and MCP-1, were elevated in such samples. While the reason for this phenomenon remains to be elucidated, we speculate that heparin may activate monocytes and cytokine release in whole blood, as indicated in a previous report [33]. Furthermore, heparin and citrate, in contrast to EDTA, significantly affected the upregulation of tissue factor and platelet activation in fresh human whole blood [34]. This result supports the observation that the anticoagulant used significantly affects coagulation, platelet activation, and the cytokine levels in fresh human whole blood and it is reasonable to suggest that among the available anticoagulants, EDTA is the one to be preferred for cytokine analysis.

The levels of cytokines in plasma were more stable than were those in whole blood. Therefore, plasma should be separated from the cellular fraction optimally within one h and maximally four h after blood sampling, After separation of plasma, the cytokines were relatively stable although time and temperature influenced the levels. Storage at RT for up to 24 hours lead to a decrease in some cytokines and increase in others. A decrease may be related to the lower level of stability of some cytokines in plasma, their degradation by proteases or the binding of the cytokines to their respective soluble cellular receptors. Changes in

this binding over time could also possibly explain the changes in cytokine levels that occur during sample storage. Notably, none of the cytokines changed during storage of EDTA plasma for up to 4 hours at 4°C.

Finally, the reference ranges of the cytokines in 162 healthy controls were estimated using the upper 95th percentile and the 2.5-97.5th percentile values from EDTA plasma samples, obtained in the morning and treated according to the condition described above. We suggest the upper 95th percentile values to be the best estimate of the reference range instead of 2.5th and 97.5th for two reasons. First, from a pathophysiological point of view an increased value of a cytokine would be more frequent and reflect a disease state than a low value. Second, for most of the cytokines there were some individuals with undetectable levels, precluding an exact 2.5th percentile to be calculated. Although the distribution of cytokine values were statistically different in males and females for eight of the cytokines, most of them had very similar upper 95th percentile values. Thus, the significant difference was due to a different distribution pattern, not influencing the 95th percentile. We therefore suggest that a common reference range can be used for males and females.

In conclusion, our data indicate that the cytokine levels in several plasma samples, including EDTA plasma samples, are significantly lower than those in serum. Previeous studies using serum should be interpreted with some caution. The mechanisms underlying the greater cytokine levels in serum compared with those in plasma remain to be elucidated, but this study indicates that thrombin-induced cytokine release might be involved. Recommended guidelines for cytokine analyses based on our data would be: EDTA blood immediately cooled on crushed ice, centrifuged within one h, plasma stored maximum 4 h at 4°C and stored at -80°C. The samples can be thawed and frozen up to three times.

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Figure legends

Figure 1.

Cytokine levels in serum compared with those in immediately centrifuged and frozen EDTA, citrate, lepirudin and heparin plasma samples. The levels of (a) IL-1β, (b) IL-1ra, (c) IL-6, (d) IL-8 or C-X-C motif chemokine ligand 8, (e) TNF, (f) IL-17, (g) RANTES and (h) PDGF-BB were analysed using Multiplex technology and expressed as pg/mL on the Y axis. The data are given as median values with whiskers (10 and 90 percentiles, n=10). Serum +; serum tubes containing a gel and a clot activator. $^{*}P<0.05$ compared with the tube in which the highest median concentration of cytokine was detected (H). $^{\#}P<0.05$ compared with that detected in the tube with lowest median cytokine concentration (L).

Figure 2.

Cytokines detected at similar levels in serum and the immediately centrifuged and frozen EDTA, citrate, lepirudin and heparin plasma samples (a, b), and those who were elevated in heparin plasma (c,d). The levels of (a) Eotaxin-1, (b) IP-10, (c) MCP-1 and (b) MIP-1β were analysed using Multiplex technology and expressed as pg/mL on the Y axis. The data are given as median values with whiskers (10 and 90 percentiles, n=10). Serum +: serum tubes containing a gel and a clot activator. $^{*}P<0.05$ compared with the tube in which the highest median concentration of cytokine was detected (H). $^{\#}P<0.05$ compared with that detected in the tube with lowest median cytokine concentration (L)

Figure 3.

Figure 3.

Frequency histograms of selected cytokines in 162 healthy individuals. (a) Interleukin (IL)-1β, (b) IL-5, (c) C-X-C motif chemokine ligand 8 or CXCL8, (d) C-C motif ligand 2 or CCL2, (e) CCL3, (f) CCL4, (g) GM-CSF and (h) CXCL10 were measured using a multiplex cytokine assay. The histogram contains grey bar graphs showing the frequency distribution of the cytokines. On the x-axis is the value of the cytokine in pg/mL. On the y-axis is the number of persons with these values. The data were analyzed using the Prism 6 for Mac OS X.

Legends to supplementary figure 1:

Suppl. Figure 1.

Frequency histograms of some cytokines from 107 females (grey bars) and 55 males (black bars). (a) Interleukin (IL)-1β, (b) IL-%, (c) C-X-C motif chemokine ligand 8 or CXCL8, (d) CCL2, (e) CCL3, (f) CCL4, (g) GM-CSF and (h) CXCL10 were measured using a multiplex cytokine assay. The histogram contains grey bar graphs showing the frequency distribution of the cytokines. On the x-axis is the value of the cytokine in pg/mL. On the y-axis is the number of persons with these values. The data were analyzed using the Prism 6 for Mac OS X.

Table 1. Cytokine levels in plasma obtained from whole blood containing different anticoagulants that was stored at 4°C and room temperature before centrifugation.

Cytokine[a]	**EDTA**[b]				**Citrate**[b]				**Lepirudin**[b]			
	4°C		**RT**[c]		**4°C**		**RT**[c]		**4°C**		**RT**[c]	
	T1h[d]	T4h[d]	T1h[d]	T4h[d]	T1h[d]	T4h[d]	T1h[d]	T4h[d]	T1h[d]	T4h[d]	T1h[d]	T4h[d]
IL-1β	0.84	1.1	**3.8***	**5.1***	1.0	1.2	**1.4***	**1.7***	1.1	1.6	**1.8***	**1.9***
	(0.28-2.87)	(0.3-22)	(1.2-32)	(1.2-73)	(0.9-2.3)	(0.9-15)	(1.2-12)	(1.1-13)	(0.7-3.0)	(0.5-2.2)	(1.1-3.0)	(1.0-4.3)
IL-1ra	0.8	1.4	**6.6***	**16***	1.0	1.17	**2.0***	**3.9***	1.1	1.1	**2.8***	**3.2***
	(0.4-4.8)	(0.3-4.4)	(3.8-96)	(1.8-215)	(0.3-2.7)	(0.36-2.17)	(1.5-5.9)	(0.6-29)	(0.2-73)	(0.8-29)	(1.4-106)	(1.1-180)
IL-4	1.0	1.0	**54***	**50***	1.7	**1.7***	**57***	**29***	1.4	2.0	**3.1***	**3.6***
	(0.2-2.2)	(0.04-121)	(2.1-257)	(1.2-392)	(1.0-26)	(1.0-97)	(1.9-87)	(1.0-128)	(0.8-25)	(0.7-15)	(1.8-24)	(1.1-27)
IL-5	1.2	0.9	**9.6***	**12***	1.0	0.9	1.7	**2.1***	1.4	1.3	**2.7***	**3.1***
	(0.8-3.8)	(0.5-66)	(2.5-210)	(1.2-344)	(0.8-1.6)	(0.1-96)	(1.0-43)	(1.0-19)	(0.5-4.8)	(0.4-61)	(1.5-20)	(1.2-25)
IL-6	1.0	1.2	6.5	**11***	1.0	1.1	**2.7***	**2.5***	1.0	1.5	**1.8***	1.8
	(0.5-3.9)	(0.14-4.0)	(1.5-210)	(0.9-290)	(0.8-2.3)	(0.6-1.8)	(1.4-4.8)	(0.9-5.8)	(0.5-3.4)	(0.3-3.1)	(0.8-4.1)	(0.4-3.3)
IL-7	1.0	1.2	18	**27***	1.3	0.9	1.6	1.2	**1.7***	1.5	3.3	2.1
	(0.07-2.7)	(0.1-7.9)	(0.8-33)	(2.1-131)	(0.08-3.1)	(0.08-3.0)	(0.8-22)	(0.9-6.5)	(0.5-34)	(0.2-29)	(1.0-75)	(0.6-31)
IL-8	0.7	0.9	7.6	**11***	1.1	0.8	1.9	1.2	1.2	1.0	**4.1***	1.5
	(0.08-12)	(0.05-20)	(0.4-121)	(0.36-189)	(0.1-28)	(0.01-2.5)	(0.5-23)	(0.03-39)	(0.5-50)	(0.24-1.9)	(1.0-99)	(0.6-132)
IL-9	0.8	1.1	5.1	21	1.0	1.0	2.1	2.1	1.4	1.1	**2.6***	2.4
	(0.08-1.4)	(0.08-3.3)	(0.17-225)	(0.17->1000)	(0.6-4.0)	(0.02-4.3)	(0.5-9.6)	(0.7-39)	(0.3-171)	(0.5-22)	(0.9-323)	(0.8-144)
IL-13	1.1	1.1	3.7	**12***	1.4	0.8	1.9	1.1	0.34	0.6	1.5	1.2
	(0.18-2.1)	(0.34-9.8)	(0.7-126)	(1.3-380)	(0.09-27)	(0.02-2.8)	(0.06-29)	(0.08-19)	(0.12-4.3)	(0.06-3.4)	(0.1-3.4)	(0.06-38)
CCL11	**0.8***	0.9	1.3	**1.5***	**0.9***	0.9	0.9	0.9	0.7	**0.7***	0.9	0.7
	(0.7-1.1)	(0.7-1.2)	(0.8-1.7)	(1.0-2.1)	(0.8-1.1)	(0.6-1.5)	(0.7-1.1)	(0.6-1.3)	(0.5-1.4)	(0.3-1.4)	(0.6-2.2)	(0.5-1.7)
G-CSF	1.0	1.0	**6.9***	**7.0***	1.1	1.0	**7.4***	**3.7***	1.7	1.47	**2.8***	2.5

	(0.01-1.3)	(0.06-15)	(1.4-465)	(2.0-787)	(0.6-36)	(0.9-21)	(1.3-362)	(0.7-432)	(0.5-483)	(0.36-257)	(0.9-586)	(0.4-265)
CXCL10	1.0	1.1	1.0	0.9	0.9	0.9	0.8	0.8	0.9	**0.9***	0.9	0.8
	(0.9-1.2)	(0.8-1.4)	(0.8-1.3)	(0.8-1.1)	(0.8-1.2)	(0.7-1.2)	(0.8-1.6)	(0.5-2.0)	(0.8-1.2)	(0.8-1.1)	(0.8-1.3)	(0.6-1.0)
CCL2	0.8	0.9	0.6	**0.4***	0.9	0.8	**0.7***	**0.36***	0.8	**0.5***	0.8	**0.6***
	(0.5-2.8)	(0.3-1.1)	(0.4-2.9)	(0.13-2.9)	(0.6-2.3)	(0.14-1.2)	(0.1-0.9)	(0.1-0.7)	(0.03-1.1)	(0.04-1.2)	(0.35-1.3)	(0.02-0.9)
PDGF-BB	1.7	2.6	743	**760***	4.1	**6.1***	**84***	**203***	2.8	4.4	**7.3***	**8.1***
	(0.36-18)	(0.05->1000)	(3.6->1000)	(1.7->1000)	(0.3-506)	(1.8->1000)	(3.9->1000)	(2.3->1000)	(0.4-13)	(0.13-11)	(2.3-30)	(1.4-42)
CCL5	1.2	0.9	**16***	**14***	**4.0***	**5.9***	**20***	**18***	2.4	2.8	**3.3***	1.5
	(0.7-2.1)	(0.1-70)	(1.3-92)	(1.5-206)	(2.1-10)	(1.2-83)	(2.3-75)	(2.19-241)	(0.7-5.3)	(0.4-3.8)	(1.1-8.5)	(0.6-3.5)

[a] The cytokine levels are given as the median fold change (10-90 percentile) of their respective T0 values.

[b] The effect on cytokine levels of storing EDTA-, citrate- and lepirudin-anticoagulated whole blood at 4°C and room temperature for one and four h. One sample of each anticoagulant was immediately centrifuged and stored at -80°C. Two samples containing each anticoagulant were stored at 4°C and two samples were stored at room temperature, one for one h and one for four h.

[c] RT= room temperature.

[d] T1h: one h of storage before centrifugation. T4h: four h of storage before centrifugation.

*P<0.05 compared to its respective T0 basal sample.

Table 2. Stability of cytokines in plasma obtained from *E. coli*-stimulated whole blood stored at 4°C or room temperature after centrifugation.

Cytokine[a]	**EDTA**[b]						**Citrate**[b]					
	4°C			**RT**[c]			**4°C**			**RT**[c]		
	T2h[d]	T4h[d]	T24h[d]	T2h[d]	T4h[d]	T24h[d]	T2h[d]	T4h[d]	T24h[d]	T2h[d]	T4h[d]	T24h[d]
IL-1β	1.0 (0.9-1.2)	0.9 (0.7-0.9)	**0.7*** (0.6-1.0)	1.0 (0.9-1.1)	0.9 (0.8-1.0)	0.9 (0.8-1.0)	1.1 (0.9-1.2)	1.0 (0.8-1.1)	0.8 (0.6-0.9)	1.1 (0.9-1.2)	1.0 (0.8-1.2)	**0.8*** (0.6-0.9)
IL-1ra	1.3 (1.0-1.6)	0.7 (0.6-0.8)	0.6 (0.4-1.5)	1.0 (0.9-1.1)	0.8 (0.7-0.9)	0.7 (0.6-1.0)	1.0 (0.7-1.3)	0.9 (0.8-1.2)	0.8 (0.6-1.1)	1.1 (0.6-1.4)	0.9 (0.8-0.9)	0.8 (0.6-0.9)
IL-6	1.1 (1.1-1.3)	1.0 (0.8-1.1)	1.1 (0.9-1.2)	1.1 (1.0-1.2)	1.0 (0.9-1.2)	1.0 (0.9-1.1)	1.1 (1.0-1.2)	1.1 (1.0-1.2)	1.2 (1.0-1.4)	1.2 (0.9-1.4)	1.1 (1.1-1.2)	1.1 (1.0-1.1)
IL-8	1.1 (1.0-1.8)	0.9 (0.6-1.0)	0.7 (0.3-0.9)	1.1 (0.9-1.2)	0.8 (0.5-1.1)	1.1 (0.8-1.4)	1.1 (1.0-1.1)	1.1 (1.0-1.2)	1.1 (0.9-1.2)	1.2 (1.0-1.4)	1.1 (1.1-1.2)	1.0 (0.9-1.2)
IL-9	1.2 (0.8-1.5)	0.6 (0.5-1.1)	0.8 (0.7-1.2)	0.8 (0.6-1.1)	0.7 (0.44-1.2)	0.8 (0.7-1.0)	1.2 (1.0-1.2)	0.9 (0.8-1.1)	0.8 (0.55-0.9)	0.9 (0.8-1.3)	0.9 (0.8-1.2)	0.8 (0.8-0.9)
CCL11	0.9 (0.5-1.1)	0.7 (0.6-0.9)	0.9 (0.8-1.1)	0.9 (0.5-1.0)	**0.8*** (0.4-1.0)	0.9 (0.8-1.0)	1.2 (1.0-1.3)	1.0 (0.8-1.2)	0.9 (0.8-1.1)	1.0 (0.9-1.3)	0.9 (0.9-1.1)	0.9 (0.8-1.0)
IFN-γ	0.8 (0.7-1.8)	0.8 (0.45-1.3)	0.9 (0.6-1.3)	0.9 (0.6-1.3)	0.7 (0.5-1.3)	1.0 (0.9-1.2)	1.0 (1.0-1.2)	0.9 (0.8-1.1)	0.8 (0.6-1.1)	0.9 (0.7-1.2)	0.8 (0.7-1.1)	**0.7*** (0.7- 0.8)
CXCL10	1.0 (0.8-1.2)	0.9 (0.8-1.1)	1.0 (0.9-1.9)	1.0 (0.9-1.0)	0.8 (0.7-1.0)	1.1 (0.9-1.2)	1.0 (1.0-1.1)	1.0 (0.8-1.1)	0.9 (0.8-1.1)	0.9 (0.9-1.2)	0.9 (0.8-1.0)	**0.8*** (0.7-0.9)
CCL3	1.0 (0.9-1.4)	1.0 (0.8-1.3)	1.1 (0.9-1.5)	1.0 (0.9-1.2)	1.0 (0.8-1.2)	1.2 (1.0-1.4)	**1.1*** (1.1-1.2)	1.0 (1.0-1.2)	1.1 (0.8-1.1)	1.1 (1.0-1.3)	1.0 (1.0-1.2)	1.0 (0.8-1.1)
CCL4	1.1 (1.0-1.2)	1.0 (0.9-1.1)	1.1 (0.9-1.2)	1.2 (1.1-1.2)	1.1 (1.0-1.2)	**1.3*** (1.2-1.5)	1.3 (0.7-1.6)	1.1 (0.8-1.7)	1.1 (0.7-1.7)	1.3 (0.8-1.6)	1.2 (1.0-1.3)	1.2 (0.9-1.4)
PDGF-BB	1.0 (0.7-1.1)	0.9 (0.8-1.0)	1.0 (0.5-1.3)	1.0 (0.8-1.2)	0.7 (0.6-1.1)	1.1 (0.9-1.1)	**1.2*** (1.2-1.3)	1.2 (0.8-1.2)	1.0 (1.0-1.2)	1.1 (1.0-1.3)	1.0 (0.9-1.3)	1.1 (1.1-1.1)
CCL5	1.1 (1.1-1.1)	1.0 (0.9-1.1)	1.1 (1.0-1.3)	1.3 (1.1-1.4)	1.1 (1.1-1.5)	**1.3*** (1.2-1.9)	1.2 (0.9-1.3)	1.2 (1.0-1.3)	**1.4*** (1.0-1.4)	1.4 (1.0-1.4)	1.2 (1.1-1.7)	1.3 (1.2-2.2)
TNF	1.0 (0.9-1.2)	0.8 (0.7-1.1)	0.7 (0.6-1.0)	1.0 (0.8-1.2)	0.8 (0.6-1.2)	0.9 (0.6-1.0)	1.0 (1.0-1.1)	1.0 (0.9-1.3)	1.0 (0.7-1.3)	1.2 (0.8-1.4)	1.0 (1.0-1.0)	0.7 (0.7-1.0)

[a] The cytokine levels are given as the median fold change (10-90 percentiles) of their respective T0 values.

[b] The effect on cytokine levels of storing EDTA-, citrate- and lepirudin-anticoagulated whole blood at 4°C and room temperature for one and four h. One sample of each anticoagulant was immediately centrifuged and stored at -80°C. Two samples containing each anticoagulant were stored at 4°C and two samples were stored at room temperature, one for one h and one for four h.

[c] RT= room temperature.

[d] T1h: one h of storage before centrifugation. T4h: four h of storage before centrifugation.

*$P<0.05$ compared to its respective T0 basal sample.

Table 3. The effect of freezing and thawing on cytokine stability

Cytokine[a]	EDTA[b]		Citrate[b]	
	3 times[c]	6 times[c]	3 times[c]	6 times[c]
IL-1β	0.9 (0.9-0.9)	**0.8*** (0.7-0.8)	1.0 (0.9-1.0)	0.9 (0.8-1.1)
IL-1ra	1.1 (0.9-1.1)	0.9 (0.9-1.0)	1.0 (0.9-1.1)	0.9 (0.8-1.2)
IL-6	1.0 (1.0-1.1)	1.0 (1.0-1.1)	1.0 (0.9-1.1)	1.0 (0.9-1.3)
IL-8	1.1 (0.9-1.2)	1.0 (1.0-1.0)	1.0 (0.9-1.1)	1.1 (0.9-1.3)
IL-9	1.1 (0.7-1.5)	1.0 (1.0-1.2)	1.1 (0.8-1.1)	0.9 (0.8-1.1)
CCL11	1.1 (0.9-1.2)	1.1 (1.0-1.2)	1.0 (1.0-1.1)	1.1 (0.8-1.1)
IFN-γ	1.0 (1.0-1.6)	1.0 (0.6-1.6)	0.9 (0.9-1.1)	0.9 (0.8-1.0)
CXCL10	0.9 (0.8-1.0)	0.9 (0.8-1.0)	1.0 (0.9-1.2)	1.0 (0.8-1.2)
CCL3	1.1 (1.0-1.2)	1.0 (0.9-1.0)	1.0 (0.9-1.1)	1.0 (0.9-1.2)
CCL4	1.3 (0.8-1.7)	1.1 (0.7-1.2)	0.9 (0.4-1.1)	1.4 (0.9-2.5)
PDGF-BB	1.2 (0.7-1.2)	1.0 (0.8-1.1)	1.0 (0.8-1.2)	1.0 (0.8-1.3)
RANTES	1.3 (1.1-1.6)	**1.4*** (1.4-1.7)	1.1 (1.0-1.2)	**1.4*** (1.3-1.5)
TNF	1.0 (0.84-1.04)	1.0 (0.88-1.02)	1.1 (0.9-1.2)	0.9 (0.7-1.1)

[a] The cytokine levels are given as the median fold change (10-90 percentiles) of their respective T0 values.

[b] The effect of freezing and thawing on cytokines in EDTA or citrate plasma.

[c] Samples were frozen and thawed three or six times before cytokine analyses.

*P<0.05 compared to its respective T0 value.

Table 4 Reference ranges of cytokines in EDTA plasma

	Median	Reference ranges[d] 2.5-97.5 percentiles	<95 perc.	% values <LDL[e]	LDL[e] (pg/mL)
[a]IL-1β	0.7	0.1-2.0	<1.6	0	0.03
IL-1RA	26	0-352	<147	5.6	1.2
IL-2	0	0-10	<7.2	44	0.2
IL-4	0.5	0-2.0	<1.5	23	0.03
IL-5	0.7	0-2.2	<1.6	29	0.02
IL-6	1.5	0-9.3	<5.4	15	0.02
IL-7	0.5	0-7.6	<6.0	60	0.06
IL-8	2.8	0-11	<7.9	17	0.01
IL-9	5.1	0-17	<59	17	0.04
IL-10	0.0	0-6.6	<3.0	69	0.03
IL-12(p70)	0.2	0-200	<9.4	63	0.2
IL-13	1.3	0-11	<5.9	9.3	0.03
IL-15	0.0	0-2.9	<2.2	46	0.01
IL-17	0.0	0-69	<31	63	0.01
MCP-1 (CCL2)[b]	12.6	3.1-31	<27	0	3.0
MIP-1α (CCL3)	0.0	0-6	<4.2	77	0.15
MIP-1β (CCL4)	21	0-63	<50	59	3.9
RANTES (CCL5)[c]	824	20-8030	<5379	0	19
Eotaxin-1 (CCL11)	38	0-120	<91	16	3.8
FGF basic	0.0	0-51	<40	65	1.0
G-CSF	5.7	0-47	<43	10	0.37
GM-CSF	2.0	0-37	<24	33	0.19
IFN-γ	11.1	0-121	<115	22	1.62
CXCL10	439	184-1398	<1202	0	159
TNF	6.4	0-52	<44	27	0.08
VEGF	0.4	0-12	<6.0	60	0.31

[a]IL, interleukin.

[b]CCL, chemokine ligand.

[c]All cytokines and biomarkers are expressed as pg/mL.

[d]The cytokine reference ranges were calculated in immediately cooled EDTA plasma samples obtained from 162 healthy individuals.

[e]LDL, Lower detection limit, defined as the lowest measurable concentration of each cytokine, including estimated values that were less than that of the lowest standard value.

Supplementary Table 1 Female and male reference ranges of cytokines in EDTA plasma

	Female					**Male**				
	Median	**Reference ranges[d] 2.5-97.5 percentiles**	**<95 perc.**	**% values <LDL[e]**	**LDL[e] (pg/mL)**	**Median**	**Reference ranges[d] 2.5-97.5 percentiles**	**<95 perc.**	**% values <LDL[e]**	**LDL[e] (pg/mL)**
[a]IL-1β	0.6	0.1-1.6	<1.4	0		0.9*	0.1-2.0	<2.0	0	
IL-1RA	31	0.0-460	<294	5.6		21	0.0-170	<138	20	
IL-2	0.5	0.0-11	<8.3	44		0.0	0.0-14	<7.3	60	
IL-4	0.4	0.0-2.0	<1.3	23		0.8	0.0-2.0	<2.0	5.5	
IL-5	0.3	0.0-2.5	<1.4	29		0.9*	0.0-3.6	<2.2	7.3	
IL-6	1.7	0.0-9.5	<4.8	15		0.7	0.0-19	<6.2	29	
IL-7	0.0	0.0-8.6	<4.9	60		2.6*	0.0-7.6	<6.8	18	
IL-8	1.6	0.0-13.4	<7.0	17		4.0*	0.0-11	<9.2	7.3	
IL-9	6.1	0.0-163	<58	17		4.0	0.0-560	<154	29	
IL-10	0.0	0.0-4.9	<2.7	69		0.1	0.0-29	<4.4	44	
IL-12(p70)	0.0	0.0-22	<9.3	63		2.1*	0.0-26	<13.5	22	
IL-13	1.1	0-0-8.2	<4.4	9.3		1.4	0.0-34	<8.7	3.6	
IL-15	0.1	0.0-4.4	<2.6	46		0.0	0.0-2.50.0	<1.6	69	
IL-17	0.0	0.0-93	<45	63		0.0	0.0-46	<27	62	
MCP-1 (CCL2)[b]	13.0	3.1-34	<24	0.9		12.0	1.2-31	<29	1.8	
MIP-1α (CCL3)	0.0	0.0-7.1	<3.6	77		0.0	0.0-9.0	<5.2	58	
MIP-1β (CCL4)	0.0	0.0-57	<47	59		32*	0.0-68	<57	22	
RANTES (CCL5)[c]	1788	14-11627	<5814	0		370*	49-4558	<4036	0	
Eotaxin-1 (CCL11)	42	0.0-125	<92	19		34	0.0-140	<89	26	
FGF basic	0.0	0.0-52	<27	65		0.0	0.0-63	<43	58	
G-CSF	7.1	0.0-50	<45	10.3		3.2	0.0-53	<37	18	
GM-CSF	2.4	0.0-31	<24	33		0.0	0.0-71	<39	53	
IFN-γ	13.4	0.0-135	<114	22		9.3	0.0-120	<117	33	
CXCL10	455	187-1601	<1250	0		390	169-1647	<1095	0	
TNF	6.9	0.0-54	<46	27		5.5	0.0-51	<42	20	
VEGF	0.0	0.0-11	<5.5	60		1.7*	0.0-16	<9.4	27	

[a]IL, interleukin.
[b]CCL, chemokine ligand.
[c]All cytokines are expressed as pg/mL,
[d]The cytokine reference ranges were calculated in immediately cooled EDTA plasma samples obtained from 107 females and 55 males.
[e]LDL, Lower detection limit, defined as the lowest measurable concentration of each cytokine, including estimated values that were less than that of the lowest standard value.
*P<0.05 when the distribution of cytokine values in females was compared to those in men analysed the using the Mann-Whitney U-test.