

# Circulating VEGF as a biological marker in patients with rheumatoid arthritis? Preanalytical and biological variability in healthy persons and in patients

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**Abstract.** *Background:* Soluble vascular endothelial growth factor (VEGF) is a promising biomarker in monitoring rheumatoid arthritis (RA), but studies of pre-analytical and biologic variability are few.

*Methods:* VEGF was measured by ELISA methods in serum and plasma from healthy persons and RA patients. Pre-analytical factors were investigated. A reference interval for VEGF was established in serum and plasma from 306 healthy persons. Diurnal, day-to-day, week-to-week, long-term variability, and impact of exercise were evaluated.

*Results:* Delayed processing time, room temperature, low centrifugal force and contamination of plasma with cellular elements lead to significant increases in VEGF levels, whereas storage for up to 2 years at  $-80^{\circ}\text{C}$  or up to 10 freeze/thaw cycles did not affect VEGF levels. Serum VEGF levels were 7–10 fold higher than plasma VEGF levels. Reference intervals for VEGF (plasma: 45 pg/ml (range: non-detectable to 352); serum: 328 pg/ml (53–1791)) were independent of gender and age. Short- and long-term biologic variability included diurnal variation (sampling should take place after 7 AM) and impact of exercise (increased VEGF immediately after bicycling normalised within 1 hour).

*Conclusions:* Pre-analytical factors and biologic variability including diurnal variation and impact of exercise should be accounted for in future studies that include circulating VEGF as a biological marker.

**Keywords:** Rheumatoid arthritis, vascular endothelial growth factor, exercise, biological marker

## 1. Introduction

Rheumatoid arthritis (RA) is an inflammatory disease of unknown aetiology. Angiogenesis appears to

be an important factor in the pathogenesis of RA [6, 12, 19, 25]. The inflamed synovium in active RA is histologically characterized by angiogenesis with loss of the normal, vascular structure. The protein vascular endothelial growth factor (VEGF) plays a critical role in angiogenesis. It stimulates proliferation and migration of endothelial cells to form new blood vessels and increases the permeability of the vessels [1, 5, 11, 20]. There is increased expression of VEGF in macrophages and fibroblasts in the synovial membrane from RA pa-

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tients [6,13,21] and VEGF is present in the synovial fluid of RA patients [6,9,13,18].

The serum VEGF concentration has been reported to be elevated in patients with active RA compared to healthy persons and to be correlated to serum C-reactive protein, the number of swollen joints and disease activity score [2,9,16,18,24]. Serum VEGF is decreased in RA patients, who respond to medical treatment [2,9,18], and *in vitro* studies have shown decreased VEGF synthesis in synovial cells from RA patients after stimulation with steroid [18]. In one study serum VEGF levels in patients with early RA was predictive of progression of joint destruction after one year [2].

Based on these observations, serum VEGF has been suggested as a potential biomarker in monitoring of disease activity, treatment response, erosive disease and extra-articular involvement in RA patients [2,9,15,17,22]. Circulating biomarkers are, however, subject to variability arising from sampling procedures and biologic variation, and this variability must be determined and adjusted for in the interpretation of laboratory results.

The aims of the present study were to determine the methodological variability, short- and long-term biological variability in healthy persons and RA patients, to establish a reference interval in healthy persons, and to investigate differences in serum and plasma concentrations of VEGF.

## 2. Materials and methods

### 2.1. VEGF ELISA

Serum and plasma concentrations of VEGF were determined by a commercially available human VEGF quantitative enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, Oxon, UK) in accordance with the manufacturer's instructions. The monoclonal antibodies in the ELISA are raised in mice against recombinant human VEGF<sub>165</sub>.

100  $\mu$ l serum or plasma was used for each analysis. All samples and standards were analysed in duplicate, and the mean value was used for statistical calculations. All samples from one subject were analyzed on one ELISA plate. Reanalysis was done if the coefficient of variation (CV) of the mean value was > 10%. Samples with values higher than the highest standard (2000 pg/ml) were diluted. Optical densities were determined using a microplate reader (Spectra II, Wallac, Austria) at 450 nm. A standard curve was estimated using a smoothed spline function. The calibration on each microplate used recombinant human VEGF standards and three internal standards from our laboratory.

### 2.2. Methodological variability

#### 2.2.1. Pre-analytical handling

Serum and EDTA plasma samples were obtained at room temperature. The samples were centrifuged after 30 minutes at 1700 g for 10 minutes at 4°C and stored at -80°C until analysis unless otherwise stated below.

#### 2.2.2. Detection limit

The detection limit was assessed 1) by calculating *the limit of the blank* (a signal 3SD above the mean for a calibrator that is free of analyte) ( $n = 20$ ); 2) by the 15.6 pg/ml standard ( $n = 20$ ); and 3) by a pooled patient sample with a plasma VEGF level of 16 pg/ml ( $n = 20$ ).

#### 2.2.3. Intra- and interassay CV

Three standards from two patient plasma pools were included on each plate: Mean VEGF concentration 374 pg/ml; 487 pg/ml and 216 pg/ml (diluted 1:2).

#### 2.2.4. Recovery

VEGF standard (2000 pg/ml) (dilution 1:2, 1:4 and 1:8) was added to three plasma pools (30, 311, and 425 pg/ml) and corresponding serum pools (346, 913, and 1050 pg/ml). Original and added pools were measured 3 times in duplicate.

#### 2.2.5. Delay and temperature before centrifugation

Corresponding serum and plasma samples were collected from 10 healthy persons and 5 RA patients. From each individual, 8 serum and 8 EDTA plasma tubes were kept at room temperature, and a similar number was kept at 4°C. After 30, 45, 60 min, 2, 3, 4, 6 and 24 hours, one serum and one EDTA tube were centrifuged at 2000 g for 10 minutes at room temperature and the serum and plasma were stored at -80°C until analysis.

#### 2.2.6. Centrifugation and strata

Corresponding samples of plasma and serum from 12 healthy persons and 12 RA patients were centrifuged at different centrifugal forces (g). From each tube the upper, medium, lower and the lowest 0.5 cm of the supernatant was aliquoted into separate tubes.

#### 2.2.7. Buffy coat contamination

In 18 healthy persons, plasma was collected on ice and centrifuged at 2500 g. The supernatant (except the 5 mm above the buffy coat) was mixed and aliquoted into 4 tubes. The remaining 5 mm of the supernatant was added to tube 1. Contamination with cellular elements could be disclosed in tube 1 compared with the 3 non-contaminated tubes.

### 2.2.8. Different types of blood samples and temperature

Corresponding samples of serum, heparin plasma, citrate plasma, EDTA plasma (all stored at room temperature, the latter also stored on ice for 30 minutes,) were collected from 10 healthy persons and 10 RA patients. After centrifugation the serum and plasma were aliquoted (except the 5 mm stratum above the buffy coat). VEGF concentrations in citrate plasma were corrected for buffer volume.

### 2.2.9. Repeated thawing

Corresponding serum and EDTA plasma samples were collected from 10 healthy persons and 10 RA patients. The serum and EDTA plasma samples were stored at  $-80^{\circ}\text{C}$  and thawed/refrozen from 0 to 10 times before analysis.

### 2.2.10. Storage

Plasma VEGF in 80 RA patients was measured shortly after blood collection. 2 years later, the samples were thawed and reanalysed.

## 2.3. Biological variability

### 2.3.1. Preanalytical handling

Serum and EDTA plasma samples were obtained at room temperature, centrifuged within 2 hours at 2500 g for 10 minutes at  $4^{\circ}\text{C}$  and stored at  $-80^{\circ}\text{C}$  until analysis, unless otherwise stated below.

### 2.3.2. Reference interval (normal range)

VEGF was determined in serum and plasma from 306 healthy, volunteer blood donors, who were well characterized: A health questionnaire was filled out and approved before every blood donation, and donation was not allowed during disease periods.

### 2.3.3. Short-term (diurnal) variability

Diurnal variability was determined in three populations: 1) Early, untreated RA ( $n = 11$ ); 2) Long-term erosive RA ( $n = 10$ ); 3) Healthy persons ( $n = 16$ ). All study subjects were hospitalized and had uniform schedules for meals and sleep. They were not permitted to exercise. Plasma was collected at seven time points during a 24 h period (10 AM, 1 PM, 4 PM, 7 PM, 10 PM, 7 AM and 10 AM) and centrifuged at 1200 g.

### 2.3.4. Variability during 3 weeks

VEGF was determined in plasma samples collected at 8 AM on day 1, 2, 8, 15, 22 in thirty-two persons recruited among hospital staff. In addition, VEGF was determined in plasma collected at 2 PM at day 8.

### 2.3.5. Long-term variability

The 3 weeks variability study was repeated after 6 (2<sup>nd</sup> round) and 12 months (3<sup>rd</sup> round).

### 2.3.6. Exercise

Plasma VEGF was collected *before* physical exercise, *immediately after* exercise (25 minutes bicycling with incremental work load every 4 minutes), and again *1 and 3 hours post-exercise* in: 1) Patients with early, untreated RA ( $n = 10$ ); 2) Patients with long-term erosive RA ( $n = 10$ ); and 3) Healthy persons ( $n = 14$ ).

## 2.4. Ethics

All participants gave their written informed consent. The local ethics committees approved the studies, which were carried out in accordance with the Declaration of Helsinki.

## 2.5. Statistical analyses

Descriptive statistics for VEGF levels are presented by the median or the geometric mean, and range. The variation over time is given by the CV. The CV's of VEGF levels analyzed over time (variability over 3 weeks and long-term variability studies) were compared to the intra-assay CV. Serum and plasma samples of VEGF were compared using Wilcoxon's signed rank test and Spearman's rank correlation. Association of VEGF to gender and age were done using Wilcoxon's rank sum test and Spearman's rank correlation. The relation between VEGF levels and time to processing at either  $4^{\circ}\text{C}$  or room temperature was analysed using linear regression with VEGF level and time log transformed. VEGF 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 done using SAS (Statistical analysis system, version 9.1, SAS Institute, Cary, NC, USA).

## 3. Results

### 3.1. Methodological variability

#### 3.1.1. Detection limit

The limit of the blank was 11.6 pg/ml. The detection limit based on the 15.6 pg/ml standard was mean 10.9 pg/ml (CV: 14.4%), and based on the patient sample mean 12.7 pg/ml (CV: 20.6%). These corresponded satisfactory with the manufacturer's specifications of a sensitivity of 9 pg/ml and a detection limit of 16 pg/ml.

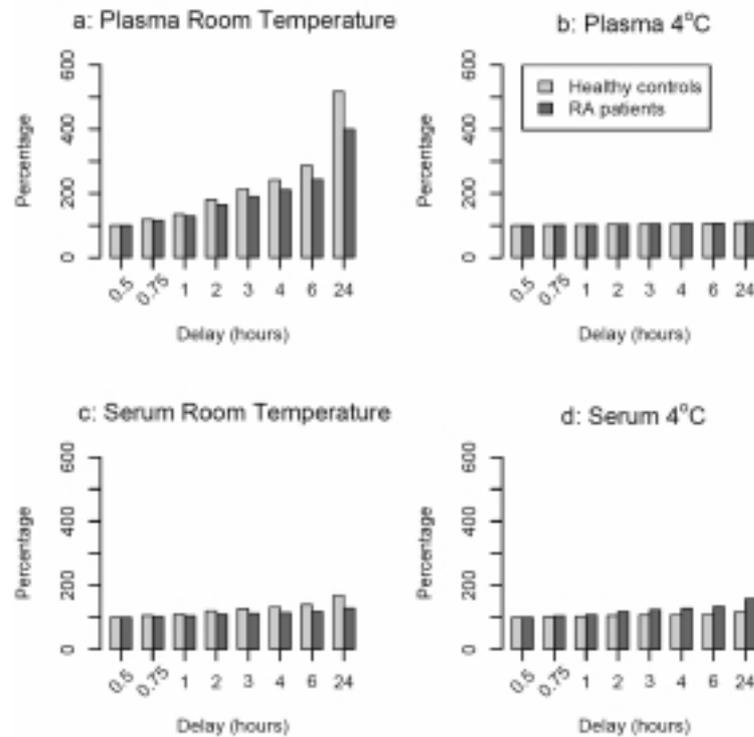


Fig. 1. Impact of delay of centrifugation. The percent changes in plasma VEGF (from 0.5 hours) at room temperature (a) and 4°C (b) and in serum VEGF at room temperature (c) and 4°C (d) in healthy persons (light columns) and patients with rheumatoid arthritis (dark columns) are shown.

### 3.1.2. Intra- and interassay CV

The intra-assay CVs for plasma samples with VEGF levels around the detection limit was 17.4% (mean plasma VEGF 14.1 pg/ml,  $n = 34$ ). In plasma control samples with high VEGF concentration it was 4.0% (169 pg/ml,  $n = 35$ ). The intra-assay CVs for two serum samples with low or high VEGF concentrations were 11.3% (223 pg/ml,  $n = 36$ ) and 4.1% (764 pg/ml,  $n = 34$ ), respectively. Inter-assay CV over a period of 21 months using 5 different ELISA batches was 9.1% (mean VEGF 216 pg/ml,  $n = 378$ ), 9.7% (374 pg/ml,  $n = 66$ ) and 12.8% (487 pg/ml,  $n = 416$ ).

### 3.1.3. Recovery

The observed final concentration as a proportion of the expected final concentration was 1.03 for serum VEGF and 1.02 for EDTA plasma VEGF, in accordance with the manufacturer's specifications.

### 3.1.4. Delay and temperature before centrifugation

Figure 1 shows the mean percent changes in VEGF after delays of processing from 0.5 to 24 hours com-

pared to baseline values. Plasma VEGF stored at 4°C from healthy persons and patients did not increase significantly over time for up to 24 hours ( $p = 0.79$  and  $p = 0.50$ , respectively). For the same samples stored at room temperature, plasma VEGF was significantly associated to processing time ( $p < 0.0001$ ) for both healthy persons and patients, and had increased by 34% (after 1 hour) and 80% (after 2 hours) in healthy persons; in RA patients it was 28% and 64%, respectively. Serum VEGF was significantly associated to processing time at both temperatures. At 4°C, the increase was 3% (after 1 hour) and 6% (after 2 hours) in healthy persons ( $p = 0.04$ ); 9% and 18% in RA patients ( $p = 0.0006$ ); at room temperature it was 10% and 21% in healthy persons ( $p < 0.0001$ ), and 5% and 10% in RA patients ( $p = 0.0004$ ).

### 3.1.5. Centrifugation and strata

In healthy persons significantly lower plasma VEGF was found for samples prepared at 3000 g (geometric mean 31 pg/ml (non-detectable (ND)-105)) compared to 2000 g (40 pg/ml (ND-261) (22% lower),  $p = 0.007$ ).

Table 1

VEGF concentrations (pg/ml) in different strata of EDTA plasma and serum according to different centrifugation procedures in patients with rheumatoid arthritis

	N	Centrifugation (g)	EDTA plasma Upper stratum	EDTA plasma Medium stratum	EDTA plasma Lower stratum	EDTA plasma Lowest 0.5 cm stratum	Serum Upper stratum	Serum Medium stratum	Serum Lower stratum	Serum Lowest 0.5 cm stratum
Rheumatoid arthritis	12	2000	64 (ND-1108)	85 (16-1032)	66 (ND-1073)	85 (ND-1069)	582 (106-2622)	604 (118-2669)	641 (112-3089)	663 (181-2951)
		2500	82 (ND-1013)	81 (ND-1073)	79 (ND-1096)	92 (17-1077)	607 (101-3025)	611 (98-2901)	623 (112-3023)	652 (150-2986)
		3000	49 (ND-1109)	65 (ND-1089)	66 (ND-1106)	79 (ND-1097)	615 (83-3025)	620 (83-2806)	648 (87-3064)	626 (93-2927)

VEGF: Vascular endothelial growth factor. Values are geometrical means (range). ND = not detectable (<16 pg/ml).

Table 2

VEGF concentrations (pg/ml) in different types of plasma and serum in healthy persons and patients with rheumatoid arthritis

	N	EDTA plasma	EDTA plasma on ice	Heparin plasma	Citrate plasma	Serum
Healthy persons	10	ND (ND-69)*	ND (ND-85)	32 (ND-64)	17 (17-54)	313 (96-494)
Rheumatoid arthritis par	10	40 (ND-144)*	16 (ND-78)	34 (ND-157)	18 (17-47)	492 (113-924)

VEGF: Vascular endothelial growth factor. Values are medians (range). Kruskal-Wallis rank sum test for differences between groups.

\*\*\* :  $p < 0.001$  for difference between serum and each of the plasma groups.

\* $p = 0.01$  for difference between healthy persons and RA patients.

Centrifugation at 1200 g resulted in significantly higher plasma VEGF (56–67 pg/ml) (61–91% higher) than at 2200 g (35–48 pg/ml),  $p = 0.03$  with intermediate values at 1700 g (43–51 pg/ml). Plasma VEGF was significantly higher in the supernatant closest to the buffy coat (48–67 pg/ml) compared to the top stratum (43–57 pg/ml),  $p = 0.01$ . In RA patients, centrifugation at 2500 g resulted in 31% higher concentration of plasma VEGF than at 3000 g ( $p = 0.01$ ), with highest concentration in the lower and the lowest stratum ( $p < 0.0001$ ), Table 1. No significant interactions between centrifugation speed and plasma stratum could be detected. Serum VEGF was not significantly related to centrifugal speed or strata.

### 3.1.6. Different types of blood samples and temperature

Table 2 shows the VEGF concentrations in different types of blood samples in healthy persons and RA patients. In both groups serum VEGF was several times higher than in the different types of plasma. Heparin plasma and EDTA plasma (room temperature) had similar VEGF concentration, whereas significantly lower levels were found in citrate plasma. Collection of EDTA plasma on ice resulted in lower levels of VEGF than collection at room temperature.

### 3.1.7. Buffy coat contamination

Plasma VEGF was 33% higher in the samples that contained the supernatant closest to the buffy coat compared to the samples without ( $p = 0.01$ ).

### 3.1.8. Repeated thawing

Plasma VEGF was significantly higher in a sample that had only been thawed once compared to thawing 2 to 10 times (mean difference 20%,  $p = 0.005$ , GLM repeated measures adjusted for normal/patient). No difference could be demonstrated for plasma samples thawed from 2 to 9 times, whereas a significantly lower level was found for the 10<sup>th</sup> thaw (mean difference 8%),  $p = 0.003$ . Serum VEGF levels were not significantly different if thawed from 1 to 9 times whereas the concentration was lower after 10 thaws (mean difference 18%),  $p = 0.003$ .

### 3.1.9. Storage

In the initial analyses, the plasma VEGF was median 88 pg/ml (range ND-537), and in the reanalyses after 2 years it was 85 pg/ml (ND-601). The median difference was 1 pg/ml (–85–24),  $p = 0.27$ .

## 3.2. Biological variability

### 3.2.1. Reference interval (normal range)

Figure 2 illustrates the individual VEGF concentrations in plasma (a) and serum (b) in 306 healthy persons according to age and gender. The median plasma VEGF was 45 pg/ml (range: ND-351; 5–95% interval: ND-137). Serum VEGF was 328 pg/ml (range: 53–1791; 5–95% interval: 118–990). The correlation between VEGF concentrations in plasma and serum was

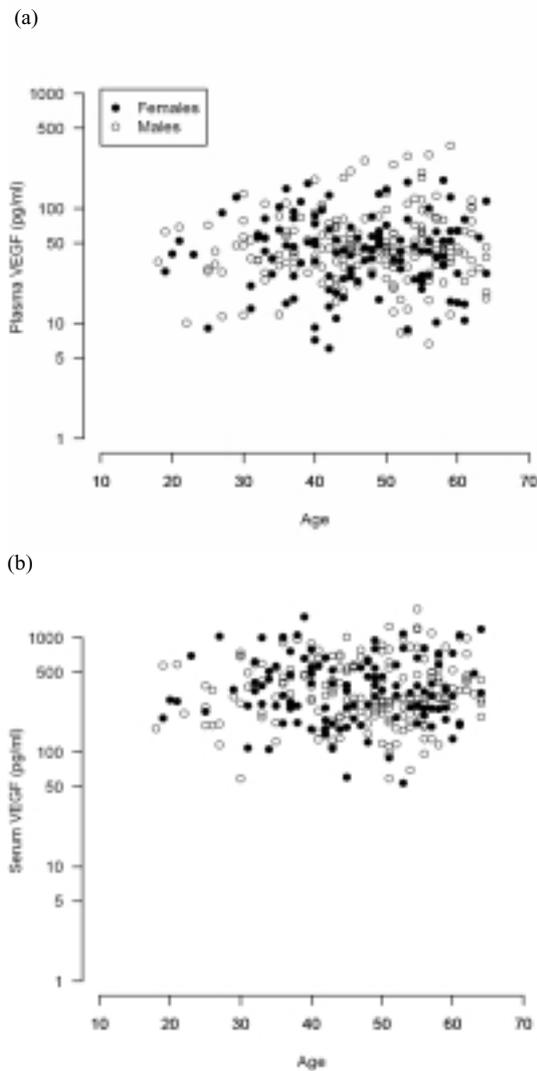


Fig. 2. Individual VEGF concentrations in EDTA plasma (a) and serum (b) in 306 healthy persons according to age and sex. The y-axis is a log scale.

$r = 0.67$ ,  $p < 0.0001$ . There was no difference between men and women (plasma:  $p = 0.25$ ; serum:  $p = 0.92$ ) and no correlation to age (plasma:  $r = 0.001$ ,  $p = 0.99$ ; serum:  $r = -0.01$ ,  $p = 0.88$ ).

### 3.2.2. Diurnal variability

Figure 3 shows the diurnal changes in plasma VEGF in healthy persons (a), in patients with early, untreated RA (b), and in patients with established, erosive RA (c). Comparing plasma VEGF in the three groups did not demonstrate statistical significance (healthy versus early RA,  $p = 0.26$ ; healthy versus established RA,

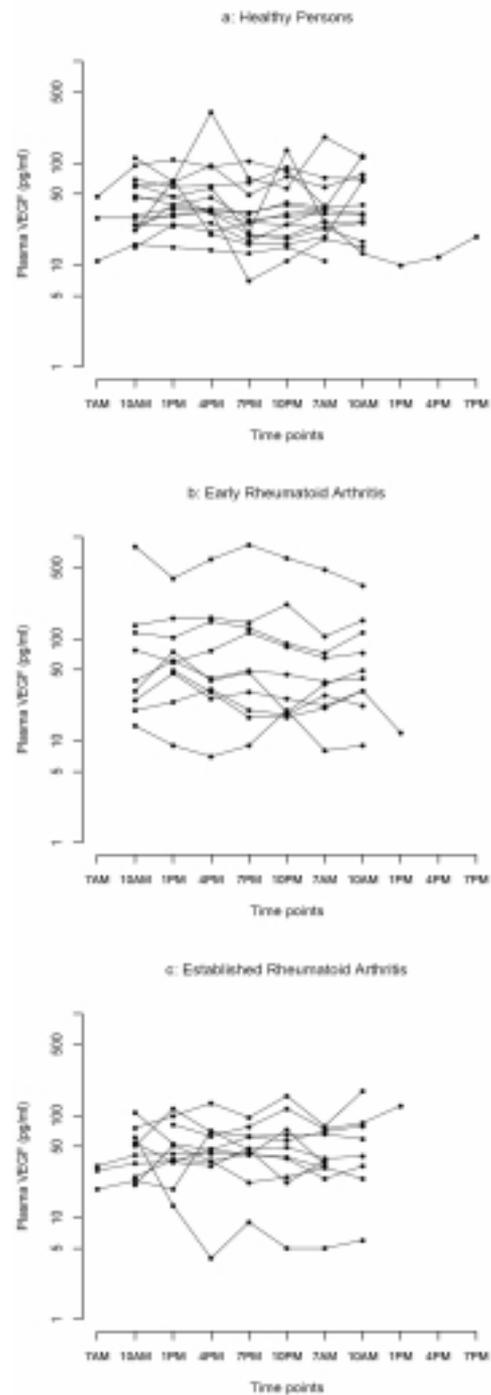


Fig. 3. The diurnal changes in plasma VEGF in healthy persons (a), and in patients with early rheumatoid arthritis (b) and established, erosive rheumatoid arthritis (c). The y-axis is a log scale.

$p = 0.82$ ; and early RA versus established RA,  $p = 0.42$ ). A linear model adjusting for group showed that

plasma VEGF at 7 AM was significantly lower than the samples at 1 PM (27% higher,  $p = 0.007$ ) and 4 PM (18% higher,  $p = 0.006$ ).

### 3.2.3. Variability during 3 weeks

The median day to day CV of plasma VEGF for each subject (all samples taken at 8 PM) was 48% (23–126%) for the 1<sup>st</sup> round, 56% (22–122%) six months later (2<sup>nd</sup>), and 39% (11–149%) 12 months later (3<sup>rd</sup>). Comparing the sample taken at 2 PM to the sample taken at 8 PM on the same day for each subject could not demonstrate a systematic difference: 1<sup>st</sup> round:  $p = 0.09$ ; 2<sup>nd</sup> round:  $p = 0.84$ ; 3<sup>rd</sup> round:  $p = 0.13$ .

### 3.2.4. Long-term variability

The plasma VEGF levels were significantly different between the three rounds ( $p < 0.0001$ ): The median plasma VEGF for each person initially, 6 months later and 1 year later were 21 pg/ml (16–131), 16 pg/ml (16–43) and 51 pg/ml (21–309), respectively.

### 3.2.5. Exercise

Figure 4 shows the individual changes in plasma VEGF before and after 25 minutes of bicycling in healthy persons (a), in patients with early RA (b) and in patients with established, erosive RA (c). Plasma VEGF was increased immediately after bicycling in the healthy persons (16% higher,  $p = 0.04$ ) and in the patients with erosive RA (64%,  $p = 0.0002$ ), but not in early RA.

## 4. Discussion

Circulating VEGF has been suggested as a possible biomarker for the monitoring of disease activity, treatment response and erosive progression in patients with RA. Thus, serum VEGF is elevated in patients with active RA compared to healthy subjects, and it has been reported to correlate to serum CRP, the number of swollen joints and disease activity score, and to decrease in RA patients, who respond to disease-modifying treatment and tumour-necrosis factor alpha inhibitors [2,9,16,18,24]. One study reported that serum VEGF level in patients with early RA was correlated with the progression of joint destruction after one year [2].

The lack of methodological studies of circulating VEGF in RA patients prompted us to perform the present studies. The results show that the contribution of pre-analytical factors such as delayed process-

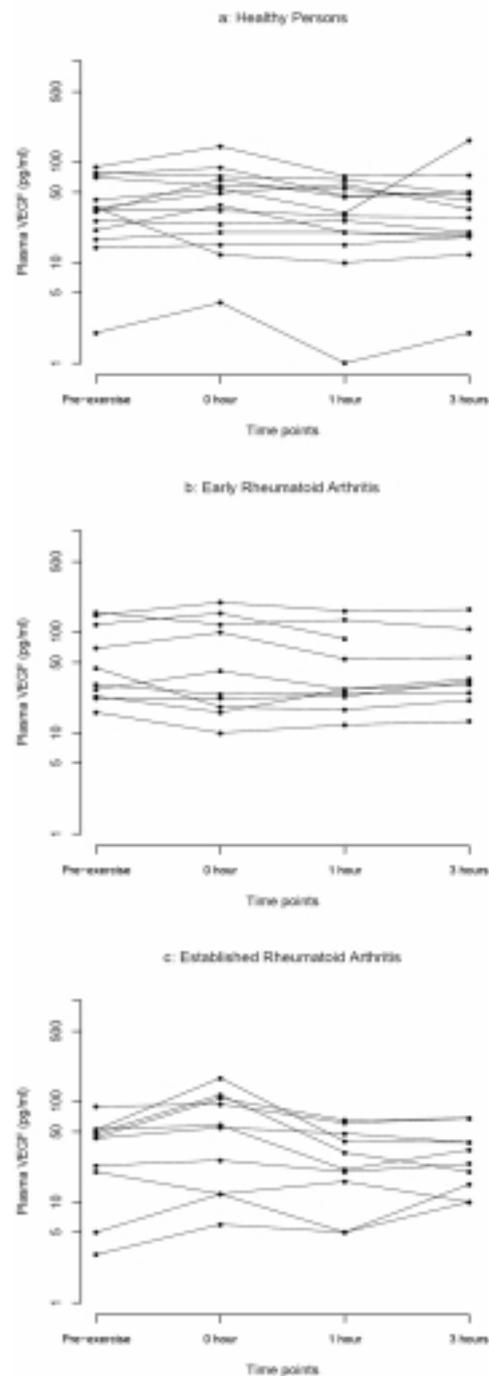


Fig. 4. The changes after 12 min of bicycling in plasma VEGF in healthy persons (a), and in patients with early rheumatoid arthritis (b) and established, erosive rheumatoid arthritis (c). The individual fluctuations are shown. The y-axis is a log scale.

ing time, low centrifugal force, contamination of plasma with cellular content and high temperature before

centrifugation, all leading to increased in vitro concentrations of VEGF, should be taken into account in the interpretation of data in RA studies. It confirms and expands previous findings in healthy persons [10]. VEGF in serum was 7 to 10 fold higher than in plasma, which put a question mark against the suitability of *serum* VEGF as a biomarker.

Low centrifugal force and sampling from the supernatant close to the buffy coat resulted in higher plasma VEGF. This is probably caused by a higher cellular content of the sample, and is in accordance with a previous study [10]. The variation in plasma VEGF in the different strata of the supernatant was large enough to confound the measurement considerably and should be taken into account when VEGF is analysed in clinical studies. The findings stress the significance of standardized sampling procedures (e.g. leaving 5 mm of the supernatant above the buffy coat untouched, aliquot the supernatant in one pipetting procedure, apply uniform centrifugal force with a least 1700 g across laboratories).

It has not previously been discussed whether serum or plasma should be recommended for analysis of VEGF as a potential biomarker of angiogenesis in studies of patients with rheumatoid arthritis. In vivo, the synovium is perfused by whole blood, thus being exposed to growth factors present in the plasma [10]. In contrast, growth factors in serum reflect concentrations after coagulation, which rarely occurs in vivo. Thus, during blood clotting, a time-dependant efflux of VEGF from platelets and neutrophils take place, leading to increased serum concentrations compared to plasma [3, 8,26,27]. In accordance with this, we found that serum VEGF was several fold higher than the corresponding plasma samples both in patients with RA (approximately 10 times higher) and in healthy persons (7 times).

VEGF levels in EDTA and heparin plasma were similar. The lowest VEGF levels were seen in citrated plasma, indicating that prevention of the release of VEGF from platelets was most efficient there. The difference was almost eliminated when the collection of EDTA plasma was done on ice. In contrast, others have found that cold temperatures tend to elevate plasma VEGF [10].

In agreement with others, we found that serum VEGF was stable for up to 9 freeze-thaw cycles [23,26], and that plasma VEGF was equally stable for 2 to 9 cycles. This should be taken into account, when samples are frozen and thawed several times before analysis. Storage of plasma for 2 years at  $-80^{\circ}\text{C}$  did not result in any systematic change in VEGF levels.

We established a reference interval of corresponding serum and plasma VEGF concentrations in 306 blood donors, and found that VEGF levels were unrelated to gender and age. Our reference interval for plasma VEGF is similar to that found in a recent cohort study [4]. In contrast, our reference interval for serum VEGF is higher than what was found in 136 healthy persons in a colo-rectal cancer study [14] (median 328 vs. 174 pg/ml). It is not stated how the healthy persons were recruited into this study. In another colo-rectal cancer study [7], plasma and serum VEGF were 9 pg/ml and 152 pg/ml, respectively in 116 persons with benign colon disease. In this study, citrated plasma was used, which may in part explain the lower plasma values. In the cohort study [4] there was a trend for higher plasma VEGF in women than in men, which is in contrast to our findings. The assay variation was, as expected, high at low VEGF concentrations due to the shape of the standard curve. This should be taken into account in plasma samples in normal individuals, who have low VEGF concentrations.

In both RA and in healthy persons no systematic diurnal variation could be documented, except that 7 AM samples were significantly lower than afternoon samples. This has the practical consequence that the sampling may take place at any time during the day after 7 AM.

Changes in plasma VEGF during one month were measured at 5 time points and were repeated again after 6 and 12 months. During the first and second period of sampling, some individuals showed large fluctuations over time. The fluctuations were not systematic and gave suspicion to pre-analytic variability. The sampling procedure was therefore altered during the third period of sampling to avoid the VEGF concentration gradient in plasma: The whole supernatant was aliquoted in one pipette, leaving the 5 mm stratum closest to the buffy coat untouched. Afterwards the supernatant was divided into four tubes. This reduced, but did not eliminate the longterm variability, which indicates that also biological variability contributed to the fluctuations.

We found increased levels of plasma VEGF in healthy persons and in patients with established RA right after bicycling. This did not apply to patients with early RA, probably because their widespread joint pain resulted in less vigorous exercise. The impact of physical activity may have contributed to the long-term variability, in which the study conditions were less standardised, allowing some light exercise prior to blood sampling like bi-cycling to work and brisk walking while at work.

In conclusion, pre-analytical factors should be accounted for and reported in studies regarding circulating VEGF. We established a reference interval of serum and plasma VEGF in healthy persons. Measurement of VEGF in serum lead to several fold higher concentrations than in plasma, probably reflecting that serum VEGF is a combination of both the VEGF from platelet release on coagulation and the circulating VEGF. Significant short- and long-term biologic variability included diurnal variation and impact of physical activity, which should be standardized in future clinical studies.

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