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The variability and accurate assessment of microinflammation in haemodialysis patients

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Abstract

Background. Systemic microinflammation is correlated with atherosclerosis. It needs a reliable assessment. This study explores the temporal variations of three inflammatory indexes [C-reactive protein (CRP), serum amyloid A (SAA) and interleukin-6 (IL-6)] in a period free of clinical events and tests the reliability of their multiple measurements for the assessment of microinflammation in haemodialysis (HD) patients, a population at high risk of atherosclerotic cardiovascular disease.

Methods. For 4 months, serum CRP, SAA and IL-6 were measured in 29 HD patients during the weeks they were free of inflammatory clinical events ($\geq$12 measurements for each index in every patient). The components of the variance as well as the reliability of two to five measurements for each index, aimed at assessing microinflammation precisely, were computed.

Results. The median (interquartile range) of CRP was 2.3 (0.9–4.9) mg/l, of SAA 3.7 (2.1–9.3) mg/l and of IL-6 4.4 (2.2–7.7) pg/ml. Patients were approximately equally distributed between three groups of low, intermediate and high variability for each index. The contribution of intraindividual (biological) variation to the total of variance was 71.3%, 69.3% and 86.7% for CRP, SAA and IL-6, respectively (higher than in all other similar studies in healthy populations). Using two measurements, the estimated reliability was 57–68% for CRP in two-thirds of the patients (comparable with that found in healthy subjects) and 57% for SAA and IL-6 in only one-third of the patients. Increasing the number of measurements up to five did not change the reliability.

Conclusions. Individual factors significantly influence the levels of inflammatory indexes in HD patients in periods free of inflammatory clinical events. The mean of two weekly CRP measurements, but not of SAA or IL-6, seems to assess microinflammation in most patients with a sufficient reliability.

Keywords: cardiovascular disease; C-reactive protein; inflammation; interleukin-6; longitudinal study; serum amyloid A

Introduction

The investigation of low-level systemic inflammation in the general population has been intensive in recent years, as it has been associated with atherosclerosis [1]. High-sensitivity C-reactive protein (CRP) is used for the long-term prognosis of cardiovascular disease (CVD) in individuals with as well as without renal failure [2,3]. Taking into consideration this new use of inflammatory markers, the accurate assessment of microinflammation is important.

The variability over time of the inflammatory indexes is an obstacle to the precise assessment of microinflammation with a single measurement at a random point of time [2]. A few studies with a longitudinal design have investigated CRP variance in healthy populations or patients without renal failure [4,5]. A longitudinal study of haemodialysis (HD) patients also has shown a significant variability over time in inflammatory indexes [6], but it did not investigate them during a period free of inflammatory clinical events. The study of the variation of microinflammation over time and, especially, its accurate assessment might help to better understand this process and, more importantly, to precisely correlate a low-level, persistent

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inflammation with the increased CVD morbidity and mortality that is observed in HD patients.

In this longitudinal study we explore the variance characteristics of three positive inflammatory indexes [CRP, serum amyloid A (SAA) and interleukin-6 (IL-6)] in a period free of inflammatory clinical events and test the reliability of multiple measurements in the precise assessment of microinflammation in HD patients.

Subjects and methods

Study design

From a group of 38 stable HD patients we selected 29 patients for inclusion in this study. For 16 consecutive weeks in a morning mid-week dialysis session, a blood sample was taken from every patient and high-sensitivity CRP, SAA and IL-6 were quantified in those samples.

On every HD day, patients were asked about medical problems that may have developed. In cases where responses were positive for an inflammatory condition or if the responsible physician detected such a condition, the patients were clinically examined and, if necessary, confirmatory laboratory tests were ordered. On the day of weekly blood sampling, the same investigator recorded a medical history for the week immediately preceding based on the data that were collected during the last three HD sessions. This history included any clinical event that could induce inflammation (such as infections, operations, trauma, cardiovascular events, diabetic or vascular foot conditions, diagnosis of neoplasia, antibiotic use, dental treatment and non-specific fever), as well as relevant laboratory or clinical confirmation (e.g. positive urine culture for urinary tract infection, clinical documentation of acute bronchitis or a vascular foot ulcer in a diabetic patient, etc.) and the treatment given. White blood cell (WBC) counts were also determined at the beginning of the study and every month thereafter and in patients with leukocytosis (WBC $>10\,000$/l) at any point, every week after its discovery.

For a patient's data to be included in the study, they had to be free of an inflammatory clinical event in the week before blood collection and have had at least 12 such measurements in the 4 months of the study. Twenty-nine out of 38 initially investigated HD patients fulfilled the above criteria and were included in the study.

From the 464 patient-weeks (29 patients $\times$ 16 weeks), 23 were excluded because of the presence of inflammatory clinical events, which included leukocytosis in two patients for 4 and 1 weeks, respectively, influenza in two patients for 4 weeks, urinary tract infections in two patients (one with polycystic disease and one with chronic interstitial nephritis) for 4 weeks, vascular foot ulcers in two diabetic patients for 4 weeks, acute bronchitis in two patients for 3 weeks, sinusitis in one patient for 2 weeks and in one patient a dental abscess recorded in 1 week of the study. Means $\pm$ SD for CRP, SAA and IL-6 in the excluded 23 patient-weeks were 25.90 ± 30.81 mg/l, 85.52 ± 144.61 mg/l and 19.53 ± 25.78 pg/ml, respectively.

An additional 17 patient-weeks were excluded because of missing measurements (blood samples missing or insufficient in quantity for all tests to be performed). The total number of measurements, for each inflammatory index, that finally were accepted for the interpretation of results was 424.

Clinical events were recorded and interpreted for the duration of the study. All laboratory assays were performed after the end of the 4 months of study.

The study was performed after informed consent was obtained from all patients.

Patient selection: characteristics

Patients with hepatic dysfunction (including those with hepatitis B or C), chronic heart failure (ejection fraction $<55\%$ in an echocardiogram, during the preceding year), active collagen disease, on HD <6 months and those with temporary vascular accesses were excluded. All patients were white, from the same outpatient renal unit (Dragini Clinic), on conventional HD and being dialysed with bicarbonate dialysate and the same type of dialyser (EVAL; polyethylene-vinyl-alcohol membrane, Kawasumi Laboratories, Inc.), which did not change during the study. Water processing (central reverse osmosis water treatment system) and the type of concentrate were also common for the entire group and duration of study.

The 29 patients (14 female) were (mean $\pm$ SD) 63.96 ± 13.12 years old (range 34–85 years) and on HD for 45.93 ± 49.86 months (range 6–211 months). Six were diabetics, eight had hypertension and only one was a smoker. Atherosclerosis (coronary, cardiovascular or peripheral vascular disease) of any grade was present in seven out of 29 patients. Atherosclerotic CVD profiles in each patient were evaluated using the CVD portion of the index of co-existing disease, as applied by Cheung *et al*. [7] to patients in the HEMO study. The body mass index (BMI) was $25.43 \pm 4.25\,\mathrm{kg/m^2}$ and did not change significantly during the course of the study. Causes of end-stage renal failure (ESRF) were chronic glomerulonephritis in 10 patients, diabetic nephropathy in six, hypertensive nephrosclerosis in five, polycystic disease in four, chronic interstitial nephritis in two and unknown in two. Of the cohort, 22 had an arteriovenous fistula, seven a vascular graft and six had one or more non-functioning vascular accesses. The dialyser effective surface area was $1\,\mathrm{m^2}$ for eight patients, $1.3\,\mathrm{m^2}$ for 14 and $1.6\,\mathrm{m^2}$ for seven, while dialyser ultrafiltration coefficient was >10 ml/h mmHg in nine patients and <10 ml/h mmHg in the remaining 20 patients. Of the subjects, 27 were being treated with erythropoietin, 13 with intravenous ferrum (maintenance treatment, 100 mg once per week, for the duration of the study), 15 with acetylsalicylic acid (100 mg/day), five with angiotensin-converting enzyme inhibitors (ACEI) and none with statins.

Laboratory methods

Blood samples of 3 ml were collected in serum separator tubes before dialysis from the vascular access. Serum was separated from the coagulated blood within 60 min of collection by centrifugation. The serum was then immediately transferred to sterile tubes and stored at -20°C until use. IL-6 was determined on two consecutive days by enzyme-linked immunosorbent assay (R&D System Europe Ltd, Oxon, UK) for all patients. The coefficients of variation (CVs) for intra-assay precision were 1.6–4.2% for IL-6

concentrations 16.8–186 pg/ml and for interassay precision were 3.3–6.4% for IL-6 concentrations 17.2–191 pg/ml for the method used. The lowest detection limit was 0.1 pg/ml. The CRP and SAA in serum samples were assayed by particle-enhanced immunonephelometry on a Behring Nephelometer 2. The high sensitivity CRP assay was designed to measure CRP concentrations within an overall range of ~0.175–1100 mg/l and the SAA within a range of ~0.5–1000 mg/l. The respective CVs for CRP concentrations of 0.5, 1.1, 2.1, 15, 26 and 62 mg/l were 3.1%, 3.8%, 3.4%, 4.0%, 2.3% and 4.4% for the intra-assay precision of the method used and 2.5%, 3.8%, 2.1%, 2.6%, 3.9% and 5.9% when CRP concentrations of 0.5, 1.3, 2.1, 14, 24 and 56 mg/l were used to determine the interassay reproducibility. The interassay CVs for SAA were 2.8–4.7% while the intra-assay CVs were 5.4–6.4% for the method used. All samples and standards were assayed in duplicate.

Statistical analysis

Partitioning of the patients into three groups was done according to the percentiles of the extreme value distribution (EVD) [8]. The overall fit of the patient's maximum values was sufficient (data not shown), suggesting that this was a reasonable decision.

We conducted a random effects one-way analysis of variance (ANOVA), considering the 29 subjects as if randomly selected from a population of such patients. From the resulting ANOVA table we computed the components of variance [9], the between-subject variation (the variation between patients of the average response, denoted by σ_b^2) and the within-subject variation (the variation in the same individual, denoted by σ_w^2). The calculations were performed for both the original values of all molecules and their natural logarithm.

Subsequently we computed the ratio:

$$\rho = \frac{\sigma_b^2}{\sigma_b^2 + \sigma_w^2}$$

$$= \frac{MS(\text{between subjects}) - MS(\text{within subjects})}{MS(\text{between subjects}) + (n-1)MS(\text{within subjects})}$$

which is usually referred to as the intraclass (intraindividual) correlation coefficient and measures the percentage of the total variance attributable to the between subjects variance. MS is the mean square.

Finally, we computed the reliability index, which is the ratio:

$$r = \frac{MS(\text{between subjects}) - MS(\text{within subjects})}{MS(\text{between subjects})}$$

and measures the reliability of the average of the repeated measurements in each patient. This reliability index (r) is sometimes referred to as the intraclass correlation coefficient ICC (1,n), where n is the average number of measurements per subject. That is, this quantity is a measure of the average reliability over n measurements. The ICC (1,1) (the quantity ρ mentioned above) is an intraclass correlation coefficient that measures the reliability of a single measurement and both are referred within the context of the random effects one-way ANOVA. Further discussion on intraclass correlation coefficients can be found in two reports [10] and [11].

To investigate the influence of the number of repeated measurements on the reliability for a given group of patients, we conducted a bootstrap analysis [12], choosing randomly a group of 2, 3, 4 and 5 weeks from the data and repeating the calculations for the random effects one-way ANOVA. All bootstrap resamplings were with 500 repetitions and the analysis was conducted separately for each measured substance and each group of patients.

To compare the demographic characteristics of the patients between the groups (low, intermediate and high for each of the three molecules), we applied the Kruskall–Wallis non-parametric analysis of variance to continuous variables and Pearson's chi-square test for the comparisons of categorical variables; the latter was also used for assessing the correlation of the three grouping variables (CRP, SAA and Il-6) with each other. In all cases, significant results were declared to be those with a P-value of < 0.05. All analyses were performed using Stata 7.0 (Stata Corp.) and various PERL (Practical Extraction and Report Language) programs written by the authors.

Results

The median (interquartile range) of CRP was 2.3 (0.9–4.9) mg/l, of SAA 3.7 (2.1–9.3) mg/l and of IL-6 4.4 (2.2–7.7) pg/ml during the 16 weeks of study in the 29 patients. The mean (and minimum and maximum) values of each index in the 16 weeks of study are shown for every patient in Figure 1.

Not all patients manifested the same degree of variation in the inflammatory indexes examined. When patients were categorized according to the maximum value for each index, they were approximately equally distributed between one group of low, one of intermediate and one of high variability for each of CRP, SAA and IL-6 (Tables 1–3).

The components of variability for the three inflammatory indexes calculated are shown in Table 4. The intraindividual variation of CRP contributed 71.2%, 84.2% and 90.1%, respectively, to the total CRP variance in the group of patients with low, intermediate and high variability for this index. The contribution of intraindividual variation to the totals of SAA and IL-6 variances was 62.6%, 94.8% and 90.3% for the former and 76.1%, 93.8% and 97.7% for the latter index in the group of patients with low, intermediate and high variability, respectively.

The overall reliability index based on two random weekly measurements for the three inflammatory indexes in the 16 weeks of study were (means ± SD) 0.53 ± 0.21 for CRP, 0.43 ± 0.21 for SAA and 0.45 ± 0.23 for IL-6 (irrespective of variability grouping). The estimated reliability in the three groups (with low, intermediate or high variability in each inflammatory index) is shown in Table 5.

Patients' characteristics (sex, BMI, diabetes, hypertension, atherosclerosis, cause of ESRF, time on HD, type of vascular access and presence of an non-functioning vascular access), dialysis conditions (dialyser effective surface area and ultrafiltration coef-

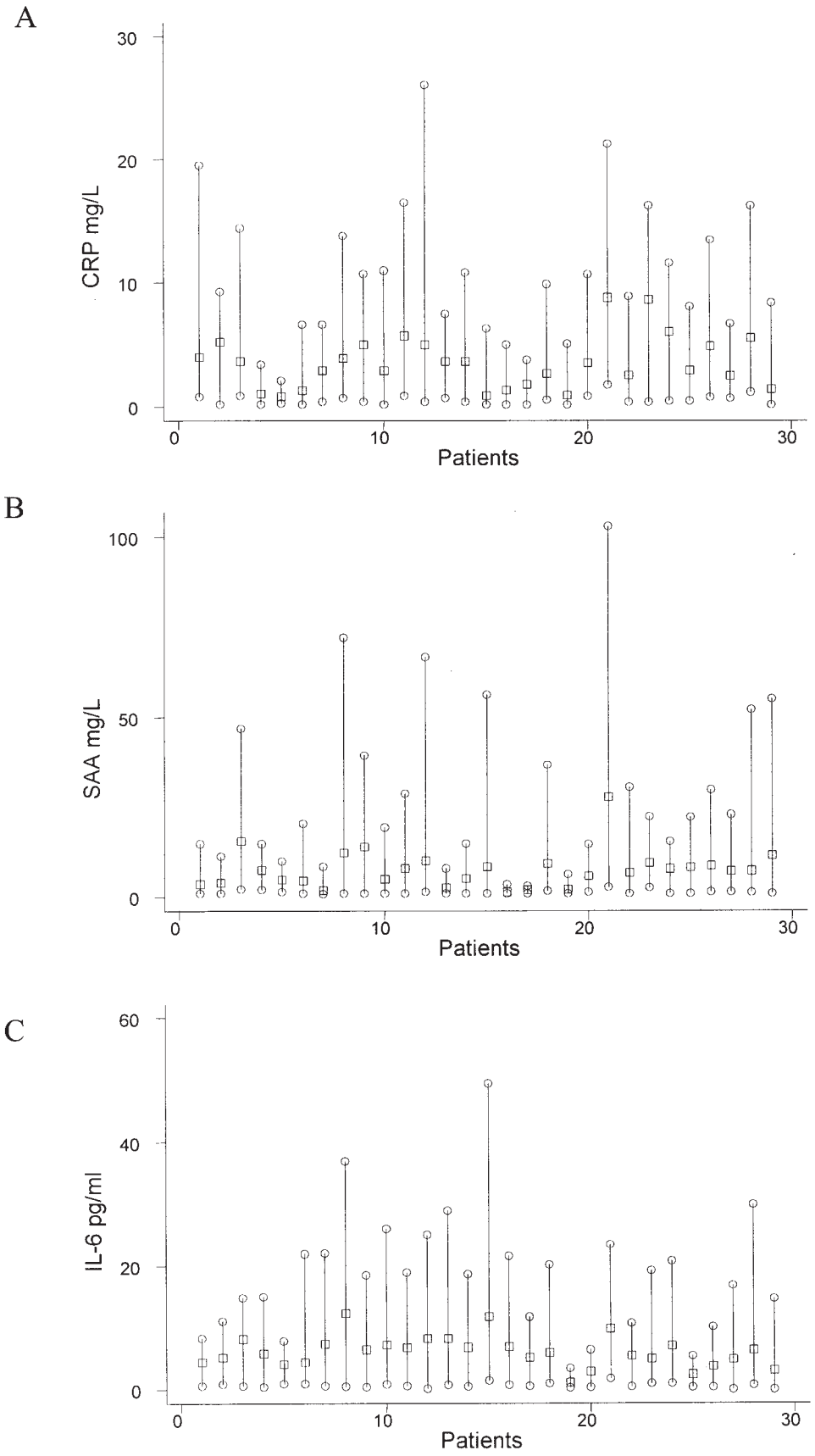

Fig. 1. CRP (**A**), SAA (**B**) and IL-6 (**C**) variability in the 16 weeks of the study for each of the 29 HD patients. Open circles, minimum and maximum values; open squares, means.

Table 1. Variability of CRP and SAA (29 patients, 424 measurements for each index in the 16 weeks of study)

	SAA variability (mg/l)			Total patients *n* (%)
	Low 1.3–4.4 (2.4)[a]	Intermediate 2.3–9.6 (4.1)	High 2.9–13.4 (6.7)	
CRP variability (mg/l)				
Low, 0.6–2.4 (1.0)	7[b]	2	1	10 (34.5%)
Intermediate, 1.2–4.7 (2.8)	2	5	3	10 (34.5%)
High, 2.15–7.5 (4.0)	1	3	5	9 (31%)
Total patients	10	10	9	29 (100%)
		$P = 0.038$		

[a]Interquartile range (median). [b]Number of patients.

Table 2. Variability of SAA and IL-6 (29 patients, 424 measurements for each index in the 16 weeks of study)

	IL-6 variability (pg/ml)			Total patients *n* (%)
	Low 1.4–5.5 (3.3)[a]	Intermediate 2.6–7.6 (4.8)	High 2.5–10.8 (5.8)	
SAA variability (mg/l)				
Low, 1.3–4.4 (2.4)	6[b]	1	3	10 (34.5%)
Intermediate, 2.3–9.6 (4.1)	3	5	2	100 (34.5%)
High, 2.9–13.4 (6.7)	0	4	5	9 (31%)
Total patients	9	10	10	29 (100%)
		$P = 0.040$		

[a]Interquartile range (median). [b]Number of patients.

Table 3. Variability of IL-6 and CRP (29 patients, 424 measurements for each index in the 16 weeks of study)

	CRP variability (mg/l)			Total patients *n* (%)
	Low 0.6–2.4 (1.0)[a]	Intermediate 1.2–4.7 (2.8)	High 2.15–7.5 (4.0)	
IL-6 variability (mg/l)				
Low, 1.4–5.5 (3.3)	3[b]	4	2	9 (31%)
Intermediate, 2.6–7.6 (4.8)	2	5	3	10 (34.5%)
High, 2.5–10.8 (5.8)	5	1	4	10 (34.5%)
Total patients	10	10	9	29 (100%)
		$P = 0.342$		

[a]Interquartile range (median). [b]Number of patients.

Table 4. Variance components for CRP, SAA and IL-6[a]

Parameter		Interindividual variation (σ_b^2) (% total variance)	Intraindividual variation (σ_w^2) (% total variance)
CRP (mg/l)	2.3 (0.9–4.9)[b]	3.87 (28.7%)	9.63 (71.3%)
Log CRP	0.75 (−1.61–3.26)[c]	0.49 (41.2%)	0.70 (58.8%)
SAA (mg/l)	3.7 (2.13–9.10)	0.32 (30.5%)	0.73 (69.5%)
Log SAA	1.47 (−0.22–4.63)	21.88 (19.6%)	89.53 (80.4%)
IL-6 (mg/l)	4.4 (2.2–7.7)	0.16 (13.3%)	1.04 (86.7%)
Log IL-6	1.34 (−4.60–3.90)	4.40 (11.8%)	32.99 (88.2%)

[a]Twenty-nine patients, 424 measurements for each index. [b]Median (interquartile range). [c]Mean (range).

Table 5. Estimated reliability (%) of the multiple random CRP, SAA and IL-6 measurements in the groups of patients with low, intermediate or high value variability for each index

	Estimated reliability of the multiple measurements (number of measurements)				
	2	3	4	5	16
CRP variability					
Low (0.2–7.5 mg/l)[a]	68%	71%	74%	77%	89%
Intermediate (0.2–11.6 mg/l)	57%	60%	64%	66%	80%
High (0.4–26.1 mg/l)	50%	52%	54%	54%	64%
SAA variability					
Low (0.8–14.7 mg/l)	57%	69%	74%	78%	91%
Intermediate (0.8–29.7 mg/l)	47%	49%	50%	51%	64%
High (0.8–100.3 mg/l)	45%	50%	54%	55%	70%
IL-6 variability					
Low (0.3–11.7 pg/ml)	57%	66%	71%	71%	82%
Intermediate (0.1–20.9 pg/ml)	43%	44%	45%	45%	48%
High (0.1–49.5 pg/ml)	22%	23%	25%	25%	27%

[a]Range.

ficient) and pharmaceutical regimen (erythropoietin, ferrum, acetylsalicylic acid and ACEIs) did not differ significantly between the patients with low, intermediate or high variability (data not shown). Patients in the high variability groups were older than those in groups with intermediate and low variability in CRP values (mean age 69.3, 64.7 and 58.4 years respectively), in SAA values (73, 62.3 and 57.5 years, respectively) and in IL-6 values (65.6, 65.1 and 59.8 years, respectively), but these differences were of statistical significance only for the SAA variability groups ($P = 0.02$).

Discussion

This longitudinal study showed that in HD patients there is significant variability over time of the inflammatory indexes, in a period free of inflammatory clinical events. The contributions to this variability of intraindividual variations were much higher than those of interindividual variation.

Variation is usually separated into two parts: intraindividual or biological, the variation in an individual that is adjusted also for analytic variation (the variation attributable to measurement error), and interindividual, the variation between patients in the average response [5]. The analytic variation for the three inflammatory indexes determined was small and did not influence our results.

The finding that intraindividual variability was higher than interindividual variability stands in contrast to the results of other longitudinal studies in healthy populations or patients. De Maat *et al.* [4] measured CRP every 3 weeks for 6 months in 20 healthy young individuals and 26 patients with stable angina pectoris and found that the contribution of the intraindividual variation to the variance of the total value for log CRP was 14% and 9% (the corresponding finding in our study was 58.8%). Ockene *et al.* [5], with three to five quarterly measurements of CRP in 113 healthy subjects, found a contribution of the intraindividual variation to the total variance equal to 44.2% and 21.7% for CRP and log CRP (in our study this percentage was 71.3% for the non-transformed CRP). In every case, intraindividual variation of CRP in the group of HD patients we studied was much higher than the average of 30% reported in a recent review [13] of studies most similar to ours. Finally, in the single longitudinal study of SAA the intraindividual variance was 25% [14].

The higher intraindividual component of the variance of inflammatory indexes in HD patients could indicate that, in this patient category, some factors influence the inflammatory response fluctuations. These factors could be shared by all patients with ESRF on HD or they might be specific to some groups or some patients in this category. In this study, three groups with distinctly different variabilities (low, intermediate or high) were detected. Older patients seem to have a more 'labile' inflammatory status. The accumulation of medical conditions (advanced glycation product generation, degenerative diseases or others) might account for this labile state. The effect of low-dose aspirin (100 mg/day) does not seem to influence the variability grade. Atherosclerosis was present in only seven patients in the group examined and it also did not influence variability level. The absence of other correlations permits some hypotheses to explain this variance. Genetic factors might influence individual fluctuations of the inflammatory response. Pankow *et al.* [15] showed that CRP levels are determined, at least partially, by genetic factors. Girndt *et al.* [16] recently showed that patients with a specific polymorphism of IL-10 gene – a 'low producer' genotype for this anti-inflammatory cytokine – had higher levels and more fluctuating patterns of inflammatory responses. Although the weeks with clinically evident infections were excluded from this study, subclinical infections, such as periodontal disease [17] or chronic *Chlamydia*

pneumoniae infections [18], could not be ruled out. Finally, if oxidative stress is correlated with inflammation in these patients, its fluctuations (due to uraemia, dialysis or other factors) might explain this phenomenon [19]. Future studies with larger numbers of patients are awaited to resolve these points.

The practical consequence of this large intraindividual variation of the inflammatory molecules in HD patients is that measuring them once at a randomly chosen point of time is not sufficient for the accurate assessment of microinflammation in these patients; multiple measurements are needed to accomplish that.

The estimated reliability using the average of two CRP measurements was high enough (57–68%) in the group of patients with low and intermediate variability for this index, however, this was valid (57% estimated reliability) only for the group with low variability for SAA and IL-6. This means, as verified by bootstrap analysis, that the average of two weekly measurements, in two randomly selected weeks in the course of the study, is close enough to the average of the 12–16 weekly measurements for each inflammatory index in each group of patients. An estimated reliability close to 60%, but for entire groups of subjects examined, was also found (using a different method of reliability assessment applicable for three to five consecutive measurements, but not to the 12–16 measurements of our study) in longitudinal studies in healthy populations for CRP, cholesterol and SAA [4,5].

From the above-mentioned data, it seems that the average of two weekly measurements of CRP (using ln CRP) provides a reliable indication of microinflammation in ~70% of the HD patients (with low and intermediate variability). A higher number (five) of measurements improves this assessment, but not by very much (this latter result is in agreement with the studies in healthy populations [5]). For the remaining 30% of patients (with high variability), the assessment had a lower reliability. If the cut-off point for CRP that was recently proposed [2] as indicative of an inflammation unrelated to cardiovascular aetiologies in the general population (10 mg/l) is applied to HD patients as well and CRP values up to that cut-off point are repeated, we believe that the reliability of the assessment of microinflammation (that has newly been proposed to estimate the risk of CVD) could be satisfactory for the vast majority of HD patients.

The assessment of microinflammation is more difficult using SAA. Only in one-third of the patients (with low variability) did the average of two measurements offer a reliable assessment (close to 60%). These results contrast with those from the unique longitudinal study of SAA, which estimated the reliability of multiple measurements for this index and found it similar to the one for the CRP [14]. Our different results are difficult to explain. The wider range of values for SAA, compared with CRP, could be an explanation [20].

Finally, the reliability of microinflammation assessment is even lower with IL-6. In the majority of patients (20 out of 29) the average of the two to five measurements for this index did not provide a reliable indication. The absence of similar longitudinal studies of this cytokine in healthy populations makes comparisons impossible. The possibility that serum levels of IL-6 poorly reflect the activity of this cytokine and probably better correlate with its local (cellular) or regional (e.g. hepatic) concentrations might be an explanation.

A major limitation and a strong point of this study that need to be highlighted are, respectively, the small number of patients and the large number of measurements (double or triple compared with similar longitudinal studies). Our results regarding variability characterization and assessment are partially corrected by the large number of measurements we had of inflammatory indexes. On the other hand, the investigation of the causes that could provoke this variability is limited by the small number of patients. Finally, the absence of a healthy control group is another limitation of this study, but that is difficult to overcome in a longitudinal study with so many samplings of blood.

In conclusion, a large intraindividual variation in inflammatory indexes is observed in HD patients. For the investigation of the factors influencing this variation, more longitudinal studies, including more patients and eventually more parameters (e.g. pro-inflammatory molecule gene polymorphisms) are needed. A relatively accurate assessment of microinflammation can be achieved in most HD patients by two weekly CRP measurements during a period free of inflammatory clinical events. SAA and IL-6 seem to have a lower reliability for microinflammation assessment in these patients.

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Conflict of interest statement. None declared.

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