Original Paper

Kidney & Blood Pressure Research

Kidney Blood Press Res 2004;27:63–70
DOI: 10.1159/000075809

Accepted: October 7, 2003
Published online: December 23, 2003

Exploring Inflammation in Hemodialysis Patients: Persistent and Superimposed Inflammation

A Longitudinal Study

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Key Words

C-reactive protein · Serum amyloid A · Interleukin-6 · Cardiovascular risk · Microinflammation

Abstract

Background: Inflammation is frequently elevated, and seems to be episodic in hemodialysis (HD) patients. Whether, its episodic character is due to the temporal variability, in periods free of clinical events, of the inflammatory indices or due, to the acute phase response induced by common inflammatory stimuli, has not been investigated yet in a longitudinal study. This study explores inflammation forms, characteristics and causes which are probably related to the high cardiovascular disease (CVD) morbidity in HD patients. ***Methods:*** In 37 HD patients, high-sensitivity C-reactive protein (hs-CRP), serum amyloid A (SAA) and interleukin-6 (IL-6) were weekly measured for 16 consecutive weeks. Inflammatory clinical events, in the week before every measurement, were recorded. Repeated measures ANOVA were applied for statistical analysis. ***Results:*** Fifty-one of 533 patient-weeks were positive for a clinical event. Mean ± SD (range) hs-CRP was 7.01 ± 16.06 (0.2–169) mg/l for all the weeks of the study, 38.25 ± 39.35 (2.1–169) mg/l for the weeks with clinical events and 3.70 ± 3.86 (0.2–26.1) mg/l for the weeks free of events. Variations for SAA and IL-6 were similar. 'Clinical events' strongly influenced acute-phase proteins and IL-6 levels. The effect of the factor 'time' (as assessed by inflammatory indices variation in weekly repeated measurements) was significant for all the 3 indices measured, independently of the factor 'clinical events'. ***Conclusions:*** In periods free of clinical events, microinflammation characterizes HD patients and fluctuates in time. Inflammation due to common clinical events is added, periodically, to this microinflammation. The high level persistent microinflammation as well as the superimposed – due to clinical events – inflammation could be related to the CVD in these patients.

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Introduction

Inflammatory indices are frequently elevated in end-stage renal failure (ESRF) patients. Recent studies showed that CRP [1, 2] and interleukin-6 (IL-6) [3] are independent predictors of all cause and cardiovascular mortality in this population. Inflammation is episodic in HD patients [4]. This characteristic may reflect persistent inflammation – mainly due to uremia and HD-related factors [5] – that varies in time or, a superimposed inflammation due to other acute-phase response stimuli.

The majority of cross-sectional studies correlating inflammation to cardiovascular disease (CVD) morbidity and mortality in HD patients, measures inflammatory indices once, at a random time point [6, 7]. Variability in time as well as distinction between persistent and superimposed inflammation are difficult to be assessed in these studies. A longitudinal study showed a significant variability in time of the inflammatory indices, but it did not examine the causes of this variability [4]. Other recent studies [8, 9] have shown that common clinical events are significant inflammation-inducing factors in HD patients, but they have not distinguished persistent (in a period free of inflammatory clinical events) from the superimposed (induced by clinical events) inflammation.

Exploration of the inflammatory causes, distinction between persistent and superimposed – probably modifiable – inflammation, as well as investigation of the episodic character of this process in HD patients are of importance. Inflammation characteristics might be related to the high CVD mortality in these patients.

This study explores inflammation forms in HD patients. It has a longitudinal design for temporal investigation of this process. It assesses baseline inflammation – taking into consideration variability in time – measuring 3 commonly used inflammatory indices (high-sensitivity C-reactive protein, hs-CRP, serum amyloid A, SAA and interleukin-6, IL-6) in periods free of clinical events and by recording common inflammatory clinical events it evaluates, at the same time, the superimposed inflammation due to these events, in patients included.

Methods

Study Design

For a time period of 4 months (every week for 16 consecutive weeks) in a morning mid-week dialysis session, a blood sample was collected from each of 37 HD patients included in this study. In this blood sample hs-CRP, SAA and IL-6 were measured.

Every HD day, patients were asked for any medical problem that was eventually presented and in case that this report was positive for an inflammatory condition or if the responsible physician detected such a condition, the patients were clinically examined and – if necessary – a laboratory confirmation was ordered. On the day of blood collection the same investigator summarized the medical history, based on the data of the last 3 HD sessions before measurement. This recording included all clinical events, possibly related to inflammation induction (infections, operations, trauma, cardiovascular events, diabetic or vascular foot events, diagnosis of neoplasia, antibiotic use, dental treatment, non-specific fever, etc.), as well as their laboratory or clinical confirmation and their treatment.

White blood cells were also determined at the beginning of the study, every month thereafter and, in the cases of patients with leukocytosis (white blood cells $>10{,}000/\mu l$) at one time point, every week thereafter. Leukocytosis, albeit a laboratory finding, was included in 'clinical events', because these patients frequently present inflammatory events without some expected signs of inflammation, like fever.

Clinical events were recorded and interpreted during study duration. All laboratory measurements were performed after the end of the 4 months' duration of the study.

The study was performed after informed patients' consent.

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 last year), active collagen disease, on HD <6 months and those with a temporary vascular access were excluded. All patients were whites, from the same outpatient renal unit (Dragini Clinic), on conventional HD with bicarbonate dialysate and were dialyzed with the same type of dialyzer (EVAL, – polyethylene-vinyl-alcohol – membrane), which did not change during the whole study period. Water processing (central reverse osmosis water treatment system) and the type of concentrate were also common for the whole group of patients and during the whole study period.

Thirty-seven patients – 17 females – were included in the study. Mean ± SD, age 65.78 ± 12.57 (34–85) years, and Body Mass Index (BMI) was 25.69 ± 4.30 kg/m^2. Eight patients were diabetics, 9 had hypertension and only 1 was a smoker. Atherosclerosis (coronary, cardiovascular or peripheral vascular disease) of any grade was presented in 9 of 37 patients. Atherosclerotic CVD profiles in each patient were evaluated using the CVD portion of the Index of Co-Existing Disease as applied elsewhere [10].

Patients included were on HD for 44.00 ± 46.75 (6–211) months. Causes of ESRF were chronic glomerulonephritis in 12 patients, diabetic nephropathy in 8, hypertensive nephrosclerosis in 7, polycystic disease in 6, chronic interstitial nephritis in 2 and in 2 of them the cause was unknown. Twenty-five of the patients had an arteriovenous fistula, 12 a vascular graft and 8 of them had one or more non-functioning vascular accesses. Thirty-two of the patients were on treatment with erythropoietin, 14 on intravenous iron (maintenance treatment, 100 mg once per week, for the whole study period), 17 on acetyl-salicylic acid (100 mg/day), 6 on ACEIs and no one on statins.

Laboratory Methods

Blood samples of 3 ml were taken before dialysis from vascular access. Sera were separated from the coagulated blood within 60 min after collection by centrifugation. Then serum was immediately transported to sterile tubes and stored at $-20\,^{\circ}C$ until use. IL-6 was

measured in two consecutive days for all patients, after the end of the 4 months' duration of the study, by ELISA (R&D System Europe Ltd, Oxon, UK). Coefficients of variation (CV) for the intra-assay precision were 1.6–4.2% for IL-6 concentrations 16.8–186 pg/ml and 3.3–6.4% for IL-6 concentrations 17.2–191 pg/ml for the interassay precision for the method used, and the lower detection limit 0.1 pg/ml. Hs-CRP and SAA in serum samples were assayed by particle-enhanced immunonephelometry on Behring Nephelometer 2. The hs-CRP assay was designed to measure CRP concentrations within an overall range of approximately 0.175 to 1,100 mg/l and the SAA within a range of about 0.5 to 1,000 mg/l. The CV for CRP concentrations of 0.5–62 mg/l was 2.3–4.4% for the intra-assay precision of the method used and 2.1 –5.9% when concentrations of CRP 0.5–56 mg/l were used to determine the interassay reproducibility. The interassay CV for SAA were 2.8–4.7% while the intra-assay CV were 5.4–6.4% for the method used. All samples and standards were assayed in duplicate.

Statistics

The components of variation (between and within-person variances) were computed by assuming a one-way analysis of variance model, for the original values of all molecules examined, using as independent the variable corresponding to each patient [11]. For measuring the correlation between the 4 molecules examined (hs-CRP, SAA, IL-6) we used the nonparametric correlation coefficient of Spearman.

To investigate the effect of the multiple measurements, and the effect of the patient's clinical condition associated with that particular week, we performed a three-way analysis of variance. In the statistical model we used for the repeated measures ANOVA [12] we used as the dependent variable the measurement on the ith (i = 1,2,...,37) patient, on the jth (j = 1,2,...,16) week under the kth clinical condition (k = 0,1), and as independent variables the effects associated with each patient, week and clinical condition. In this kind of analyses the fact that measurements are correlated (and hence not independent) introduces the need to adjust the degrees of freedom of the numerator and the denominator of the F-distribution against which we have to compare the F-statistic derived from the analysis of variance (regarding the within-subject effects). Both numerator and denominator have to be multiplied with a number denoted by ε (epsilon) [13]. We used the most commonly used estimates of epsilon, the Greenhouse-Geisser (G-G) estimate [14], the Huynh-Feldt (H-F) estimate [15], and finally the conservative estimate of Box that uses the lower bound for epsilon [13, 14]. The finding of a significant p value using Box's lower bound for ε should make one feel fairly safe about the validity of the results [12].

We performed logistic regression analysis using the clinical events (1 = yes, 0 = no) as the dependent variable and the 3 molecules (one at a time) as the predictors. With the appropriate sensitivity analyses on the above logistic regressions, we determined the cut-off values for the three molecules predicting the presence of the clinical events. We computed the ROC (receiver's operator characteristics) curve and the area under the curve to estimate the value that better predicts an inflammatory clinical event for each index (CRP, SAA and IL-6) determined. The above analysis ignores the longitudinal nature of the data. In all analyses statistically significant results were considered those with $p < 0.05$.

Table 1. Clinical inflammatory events in the study period (533 patient-weeks)

Inflammatory events	Number of patient-weeks
Vascular access infection	11
Leukocytosis	10
Bronchitis	6
Urinary tract infections	7
Influenza	6
Diabetic foot/vascular ulcer	4
Biliary tract infections	3
Sinusitis	2
Dental abscess	1
Vascular graft creation	1
Total	51

Results

In 16 patients, 16 measurements for each molecule (hs-CRP, SAA and IL-6) were completed, at the end of the study. In 11 patients 15 measurements, in 2 of them 14, in another 2, 13 measurements, in 2 other patients 12 measurements, in 1 patient 11 measurements, in 1 patient 10 measurements, in 1 patient 7 measurements and in 1 patient 6 measurements for every molecule were completed at the end of the study. The missing measurements (fewer than 16) in the rest of the patients were due to the missing blood collections and some of them due to insufficient sample quantity for further tests. The total number of values that entered in the interpretation of the results was 533 for CRP, 533 for SAA and 531 for IL-6.

Inflammation-inducing clinical events during the study are shown in table 1. Vascular access infections were observed in 3 patients with a vascular graft. One of them suffered from an infection resistant to antibiotics for 6 weeks, he was hospitalized (at this time the study was terminated for him) and 2 weeks later he died from sepsis. The patient with the new vascular graft creation also presented a 2-week infection of this new graft (he was dialyzed 4 weeks with a temporary catheter) and a third patient also had an uncomplicated vascular access infection, treated with antibiotics, for 3 weeks. Leukocytosis has been observed in 3 patients (for 5, 4 and 1 week, respectively), without an obvious inflammation-inducing focus. Urinary tract infections were recorded in 3 patients (in 2 with polycystic disease for 2 and 3 weeks, respectively, and in 1 with chronic interstitial nephritis for 2 weeks). Vascular foot ulcers, in 2 diabetic patients (for 2 weeks in

Table 2. Mean (min-max) and SD (overall, between patients and within patients), for hs-CRP and SAA, in all the weeks of the study, and in weeks with and without clinical events

	C-reactive protein			Serum amyloid A		
	mean mg/ml	SD mg/ml	min-max mg/ml	mean mg/ml	SD mg/ml	min-max mg/ml
All the weeks[a]						
Overall	7.01	16.06	0.2–169	20.10	64.59	0.39–710
Between patients		8.44			33.45	
Within patients		13.67			55.26	
Weeks with c.e.[b]						
Overall	38.25	39.35	2.1–169	134.40	167.89	1.56–710
Between patients		28.28			97.19	
Within patients		27.37			139.61	
Weeks free of c.e.[c]						
Overall	3.70	3.86	0.2–26.1	8.00	11.04	0.39–103
Between patients		2.02			4.94	
Within patients		3.29			9.87	

[a] 533 measurements, 37 patients.
[b] With clinical events: 51 measurements, 17 patients.
[c] Free of clinical events: 482 measurements, 37 patients.

Table 3. Mean (min-max) and SD (overall, between patients and within patients), for IL-6, in all the weeks of the study, and in weeks with and without clinical events

	Interleukin-6		
	mean pg/ml	SD pg/ml	min-max pg/ml
All the weeks[a]			
Overall	7.99	10.98	0.1–97.6
Between patients		6.23	
Within patients		9.04	
Weeks with c.e.[b]			
Overall	23.18	24.83	0.5–97.6
Between patients		17.65	
Within patients		17.47	
Weeks free of c.e.[c]			
Overall	6.41	6.73	0.1–52.1
Between patients		3.29	
Within patients		5.87	

[a] 531 measurements for IL-6, 37 patients.
[b] With clinical events: 50 measurements for IL-6, 17 patients.
[c] Free of clinical events: 481 measurements for IL-6, 37 patients.

each one), were resolved with conservative treatment without hospitalizations. The patient with biliary tract infection was hospitalized for 7 days (she suffered from cholelithiasis, but she did not undergo operation). The rest of infections were usual and of mild severity. Totally, 17 out of 37 patients presented one or more inflammatory clinical events, for 1 or more weeks, and 2 were hospitalized (1 having been excluded from the study). The 8 diabetic patients presented significantly more clinical events in the course of the study than the 29 nondiabetic patients (χ^2 test, $p = 0.028$).

The mean (from all measurements) of each inflammatory index and SD for all measurements, for between patients measurements (inter-individual SD) and for within patients measurements (intra-individual SD) for all the weeks of the study, for the weeks with clinical events and for the weeks free of events are shown in tables 2 and 3. Hs-CRP values (natural logarithms) for the 37 patients in the 16 weeks of the study are shown in figure 1.

Hs-CRP was strongly correlated with SAA ($r = 0.9132$, $p < 0.0001$) and IL-6 (0.4758, $p < 0.0001$), and SAA with IL-6 ($r = 0.5078$, $p < 0.0001$) during all the weeks of the study, as well as during the weeks with or without clinical events (data not shown).

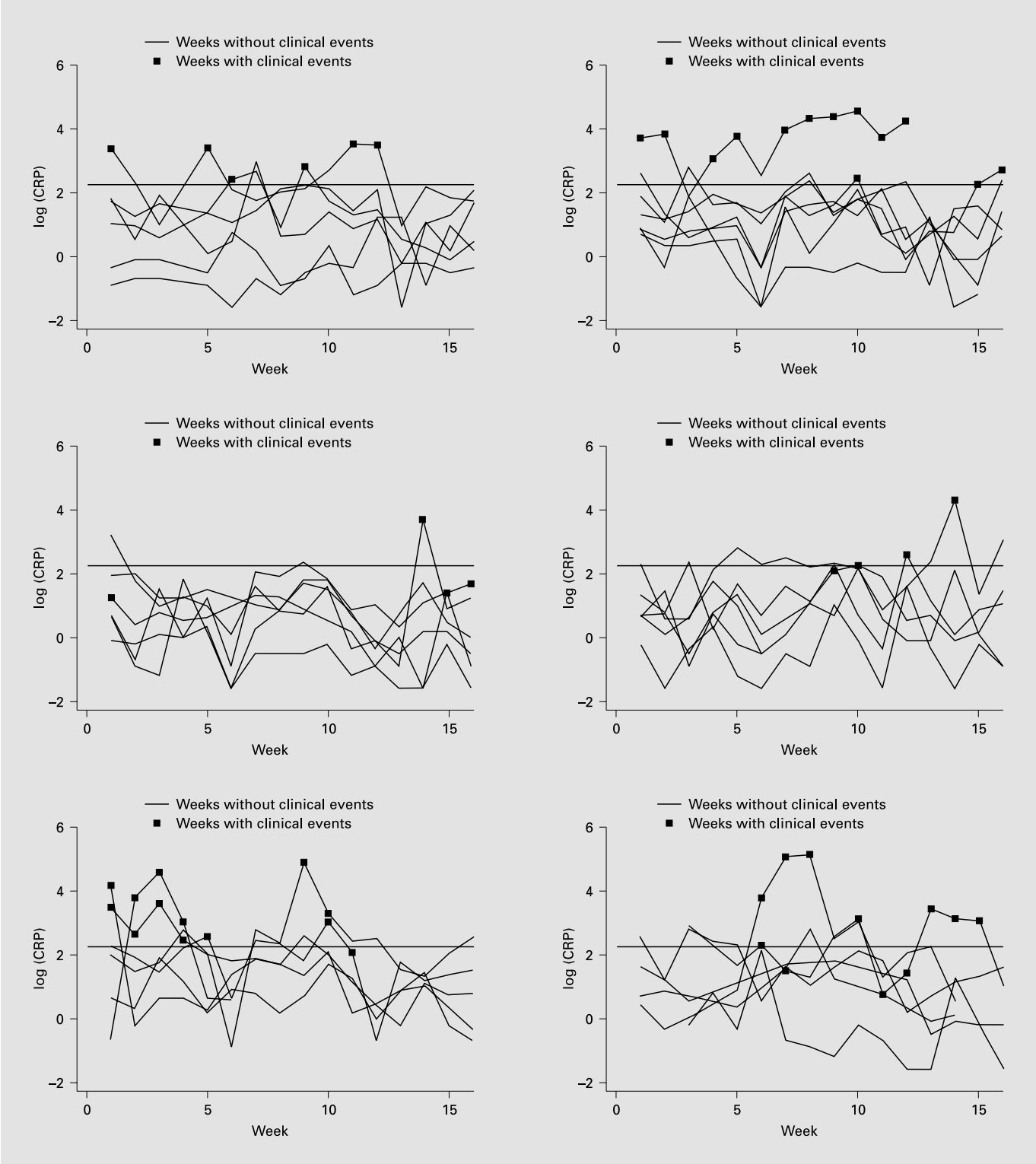

Fig. 1. log hs-CRP in the 37 hemodialysis patients (randomly divided into 6 groups) in the 16 weeks of the study (with or without clinical events).

Table 4. Effect of the 'clinical events' on the inflammatory index levels (repeated measures ANOVA)

	N[a]	F	p value
CRP	533	134.41	<0.0001
SAA	533	166.33	<0.0001
IL-6	531	14.63	0.0001

[a] The number of measurements.

Table 6. Cut-off points of CRP, SAA and IL-6 that better predict an inflammatory clinical event [results from the receiver operating characteristics (ROC) computed for each inflammatory index]

	Cut-off point	Sensitivity %	Specificity %
CRP	9.5 mg/l	84.3	91.5
SAA	20 mg/l	74.5	91.9
IL-6	9.3 pg/ml	68.0	78.8

Table 5. Effect of 'time' (repeated weekly measurements) on the inflammatory index levels

	Effect of 'clinical events' included		Effect of 'clinical events' excluded	
	F	p value	F	p value
CRP	5.95	0.0204	5.40	0.0266
SAA	15.20	0.0005	12.03	0.0015
IL-6	11.56	0.0018	11.24	0.0021

Repeated measures ANOVA – Box's conservative estimation.

In the repeated measures ANOVA that we conducted, the effect of the independent variable 'clinical events' was highly significant for hs-CRP, SAA and IL-6 levels (table 4). With the same type of statistical analysis, the effect of factor 'time' (repeated weekly measurements of the inflammatory indexes) was found highly significant for all inflammatory indices determined in this study. All the p-values from the appropriate F-tests were found <0.0001, using either the G-G or the H-F adjustments for the lack of sphericity among the residuals. By using the most conservative estimate of Box (the lowest bound for ε), the effect of the independent variable 'time' was also significant, whether or not the significant effect of 'clinical events' was included in the model (table 5).

Finally, we determined the cut-off values for the 3 positive inflammatory indexes measured, that better predict the presence of an inflammatory clinical event computing the ROC for each of the 3 markers. The area under the ROC curve, the standard error and the 95% confidence interval for the ROC computed for hs-CRP were 0.9445, 0.016 and 0.9118–0.9770, for the ROC computed for SAA 0.8901, 0.0296 and 0.8220–0.9482 and for the ROC computed for IL-6 0.7569, 0.0434 and 0.6717–0.8420, respectively. The cut-off points, their sensitivity and specificity for each marker are shown in table 6.

Discussion

Main Findings of the Study

Inflammatory indices are increased in HD patients. Inflammation in these patients is partially caused by inflammatory clinical events. Some cut-off points of the inflammatory indices could predict a clinical event. In periods free of events microinflammation persists and fluctuates in time.

Clinical Events – Cut-off Points of the Inflammatory Indexes

Inflammation-inducing events were observed in about 9% of the 533 patient-weeks of the study. The great majority of these events were of infectious origin with vascular access infections being the first cause. Leukocytosis of unknown origin was detected as the second cause. Although unspecific, this is a frequent finding in acute phase response [16]. The cut-off point for the CRP (9.5 mg/l) that better predicted a clinical event was almost identical to the one (10 mg/l) that has been established as suspicious for an inflammatory, unrelated to the cardiovascular etiologies, cause of CRP elevation in general population [17]. Furthermore, the cut-off point for CRP detected in this study is in agreement with another longi-

tudinal study in healthy population [18] and it is also in accordance with the results of two other studies in HD patients [9, 19].

Persistent and Superimposed Inflammation in HD Patients

This study is, to our knowledge, the first longitudinal one that explores inflammation forms (persistent microinflammation and superimposed inflammation due to clinical events) in time, in HD patients. Our results showed that persistent microinflammation in periods free of clinical events in these patients, is higher than in general population (mean, median, range hs-CRP 2.82, 1.42, 0.1–16.1 mg/l in 113 healthy subjects in a similar longitudinal study [18] and 3.70, 2.40, 0.2–26.1 mg/l in this study). The discrepancy between persistent inflammation level found in the present study and the similar one found in the majority of cross-sectional studies in HD patients (mean CRP > 8 mg/l in about half of the patients [6, 7]) is apparently large, but explicable mainly for two reasons. First, the majority of cross-sectional studies determine once, at a random time point, inflammatory indices ignoring their temporal variability; in the present longitudinal study a mean or median value, the result of multiple measurements which takes into consideration the significant temporal variability [20] of these indices, were calculated; second, in many of the above mentioned studies, non-high sensitivity methods for CRP were applied [6, 7]. Additionally, lower prevalence of hypertension (24.3%), atherosclerosis (24.3%) and smoking (only 1 patient), in patients included in the present study compared with other studies in HD patients, might have had an impact on our results also. We consider that, although fewer co-morbidities of patients included, might limit the applicability of our results in other HD patient groups, it also permits a more realistic estimation of the micro-inflammation that exists in all HD patients and is mainly caused by uremia per se and HD procedure. Medications with an anti-inflammatory activity did not influence our results regarding persistent micro-inflammation estimation [20]; furthermore, aspirin in a dose of 100 mg/day –17 of 37 patients were on this regimen – seems to have antiplatelet and not anti-inflammatory activity [21].

Superimposed inflammation in the present study was due to the common clinical events-induced acute-phase response. A part of the episodic character of inflammation recorded (high inflammation waves – fig. 1) was attributed to these events (hs-CRP mean ± SD in weeks with clinical events, 38.25 ± 39.35 mg/l). Another part of the fluctuating inflammation pattern (small inflammation waves – fig. 1) recorded was explicable from significant temporal micro-inflammation variability (hs-CRP mean ± SD in weeks free of clinical events 3.70 ± 3.86 mg/l – the factor 'time' remained significant for inflammatory indice levels after clinical events exclusion). The causes for this variability may be related to genetic factors [22], occult infections [23], oxidative stress influences [24] or other factors.

Inflammation and CVD Risk in HD Patients

Micro-inflammation level assessed in the present study – although lower than in cross-sectional studies – classifies HD patients as a high risk group for CVD [17], according to the criteria proposed in non-renal failure patients. Waveform inflammation pattern – due to the superimposed inflammation from common clinical events or to the temporal persistent inflammation variability – recorded in this longitudinal study, seems to characterize HD patients. This (added to the already existing micro-inflammation) fluctuating inflammation might be a risk factor for atherosclerosis in this population. Although the model of inflammation as risk factor for atherosclerosis is not the predominant one nowadays [17], this model might be valid for populations such as patients with collagen diseases [25], renal-failure or others, in whom repetitive waves of inflammation are observed. This probability is supported by recent basic research studies [26, 27] which support the model of inflammation as a risk factor and not only as a risk index for atherosclerosis.

Limitations of the Study

Certain limitations have to be noticed in the present study. The first one is the relatively small number of patients included. More studies with more patients are needed for our results confirmation. A second limitation is the absence of a control group with healthy subjects, although this is difficult to be realized in a longitudinal study with such a big number of blood samples. Fewer co-morbidities in patients included (although helpful in the distinction of inflammation forms) may have yielded to results not as representative to other HD patient groups. Finally, the missing blood samples might have influenced our results.

Conclusions

Micro-inflammation characterizes, in periods free of clinical events, HD patients and fluctuates significantly in time. Its level classifies these patients as a high risk group

for CVD. Superimposed to this persistent inflammation is the one attributed to the common inflammatory clinical events. Repeated waves of inflammation – due to clinical events or to temporal variability of the inflammatory indices – may be correlated to the atherosclerotic process in these patients.

Acknowledgments

We thank Omicron Medical S.A. for financial support. We thank also Ms Garifalia Kalatzopoulou and Ms Paraskevi Tseke for their valuable technical assistance.

This work was presented in abstract form in the World Congress of Nephrology, Berlin, Germany, 8–12 June 2003.

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