Precision of Biomarkers to Define Chronic Inflammation in CKD

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Key Words

Biomarkers, precision • Chronic inflammation • Chronic kidney disease • CKD stage 5D • Inflammatory biomarkers, inpatient variance • Tunneled dialysis catheter

Abstract

Background/Aims: Several inflammatory biomarkers have been found to be associated with cardiovascular disease or all-cause mortality in dialysis patients, but their usefulness in clinical practice or as surrogate endpoints is not certain. The purpose of the present study was to determine the inpatient variation of C-reactive protein, IL-6, fetuin-A and albumin in a population of dialysis patients. **Methods:** Apparently healthy dialysis patients with either a tunneled dialysis catheter or fistula had monthly assessments of these biomarkers for a total of four determinations, and the intraclass correlation coefficients were calculated as measures of intersubject variance. **Results:** Our results showed large within-subject variation relative to the total variation in the measurements (31–46%). Having a tunneled catheter as opposed to a fistula was not significantly associated with mean levels, suggesting that chronic subclinical catheter infection does not explain the variation seen in the biomarkers. In contrast, there was a rapid change in these biomarkers with a clinically apparent acute infection. **Conclusion:** These results suggest that these biomarkers have limitations for use as surrogate endpoints in clinical trials due to wide fluctuations, even in apparently clinically healthy individuals.

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Inflammation has been implicated in the etiology of coronary artery disease in the general population; biomarkers such as C-reactive protein (CRP) have been shown to predict cardiovascular events in both symptomatic and asymptomatic individuals [1]. Inflammatory markers have also been associated with carotid intimal thickness and coronary artery calcification [2, 3] in the general population. Importantly, single measurements of elevated levels of biomarkers such as CRP and interleukin-6 (IL-6), and lower levels of fetuin-A and albumin in patients with CKD stage 5D (on dialysis) are associated with increased mortality [4–7]. These data suggest that dialysis patients may have chronic inflammation, but the cause of inflammation has not been clearly delineated. Several possible etiologies have been implicated including, but not limited to, tunneled dialysis catheters, oxidative stress, non-pure water, and periodontal disease [8–10].

Given the data demonstrating a strong association between biomarkers of inflammation and mortality in CKD stage 5D, the next logical step is to intervene with a therapy to reduce inflammation in order to improve cardiovascular disease and/or all-cause mortality. However, such a study requires a large population. A preferred intermediate step is an interventional trial with an agent to reduce inflammation using a surrogate biomarker as an endpoint. The Food and Drug Administration defines a biomarker as 'a characteristic that is objectively measured and evaluated as an indicator of normal biologic or pathogenic processes or pharmacological responses to a thera-

peutic intervention' [11]. Biomarkers are often used in early drug development to establish proof of concept as a surrogate endpoint in clinical trials. However, the use of a biomarker as a surrogate endpoint requires demonstration that it correlates with the clinical endpoint (accuracy) and that it is reproducibly measured (precision) [11]. An estimate of the within-subject variance of a biomarker could facilitate the design of a clinical study.

The purpose of the present study was to determine the inpatient variance of inflammatory biomarkers in CKD stage 5D patients and to determine if the presence of a tunneled dialysis catheter (compared to fistula) explained some of the variation levels of these biomarkers. A second aim was to observe changes in these markers following a clinically identifiable acute event.

Methods

The primary aim of the study was to determine the inpatient variance of biomarkers of inflammation in patients on dialysis and whether tunneled dialysis catheters (TC) explained the variation. To examine this, we measured CRP, IL-6, fetuin-A, and albumin in patients who were clinically without acute medical complaints at baseline and monthly thereafter for 3 months. Monthly measurements were chosen so as to coincide with other routine blood draws. Patients were interviewed for clinical symptoms weekly by the nurse practitioner and monthly by the investigators to assess acute clinical events. The second aim of the study was to determine the timing and magnitude of change in these biomarkers in subjects who developed a clinical event such as infection or other significant morbidity. For the latter, we measured weekly levels of these biomarkers beginning at the onset of illness and continuing for 2 months.

The inclusion criterion was an AVF or TC as an access (i.e. no grafts) in use for at least 2 months. This inclusion criterion was to clearly distinguish those patients with and without foreign objects that may serve as a nidus for infection. Exclusion criteria included inability to give informed consent, significant liver disease (cirrhosis or liver function tests three times normal), a chronic infectious process (HIV, osteomyelitis) or malignancy; life expectancy <6 months; on immunosuppressants. Subjects were also excluded if they had a significant infection in the previous 2 months defined as requiring hospitalization, positive blood cultures, positive chest x-ray for pneumonia, 50% increase in total WBC count, myocardial infarction, stroke or other event requiring hospitalization including access declogging procedures. The study was approved by the IUPUI/Clarian Institutional Review Board.

All serum was collected before dialysis after a short (2-day) interval at the beginning of each calendar month for 3 months, immediately aliquoted, and frozen at -80°C until analyzed at the end of the study by batch analyses. Fetuin-A levels were measured by ELISA (Epitope Diagnostics, Inc.). IL-6 was measured by ELISA (R&D Laboratories), and high sensitivity (CRP) was analyzed by ELISA (Alpha Diagnostics International). Serum albumin lev-

els were analyzed by bromocresol purple methodology (ALBm, Synchron®, Beckman Coulter). The coefficient of variation (CV) for the kits in our laboratory were 2.2% (albumin), 7.3% (IL-6 and CRP) and 9.6% (fetuin-A).

Statistical Methods

Intraclass correlation coefficients (ICC) were calculated as measures of intersubject variance. The ICC is a measure of the proportion of variation resulting from measurement of different subjects. Since the total variation consists of variation coming from repeated measurements of the same subject and from measurements of different subjects, one minus the ICC ($1 - \text{ICC}$) gives us the proportion of variation that comes from repeatedly measuring the same subject. In other words, $1 - \text{ICC}$ expresses the magnitude of within-subject variance relative to total variance.

Both CRP and IL-6 showed deviation from normality, so each of these was log transformed for modeling purposes. Baseline characteristics, medication usage and laboratory values albumin, fetuin-A, log(CRP), and log(IL-6) levels were compared between groups by use of an analysis of variance (ANOVA) model or χ^2 . Correlations of measurements at each visit were estimated by Spearman's rank. Longitudinal measurements of albumin, fetuin-A, ln(CRP) and ln(IL-6) were modeled using a repeated measures ANOVA with terms for group (AVF vs. TC), visit and the group by visit interaction. For fetuin-A there was one outlier, subject 20 at visit 3. The outlier was removed for the analysis of fetuin-A and the model refit. Other patient characteristics (age, gender, race, duration of dialysis, diabetes, being a smoker) were tested for association with longitudinal measures of the biomarkers by use of a linear model which included a term for the patient characteristic and visit. A random patient effect was also included to incorporate into the model the correlation of measurements from the same patient.

Results

92 subjects were approached from three dialysis units affiliated with Indiana University for the research study; 69 consented. 12 consented subjects were not included in the primary aim (monthly levels) of the study because of a clinical event during the study and thus they were no longer clinically stable: 4 of these subjects had an infection (4 TC, 0 fistula), 3 underwent a kidney transplant, 3 changed to daily dialysis, 1 had a change in dialysis access, and 1 patient had a cerebral vascular accident. Patient demographic and baseline laboratory values are shown in table 1. The only significant differences between fistula and TC patients were dialysis vintage and diabetes. There were significant, but not strong correlations between inflammatory markers at baseline. Fetuin-A correlated with IL-6 ($r = -0.34$, $p = 0.01$), IL-6 correlated with CRP ($r = 0.39$, $p = 0.002$), albumin correlated with IL-6 ($r = -0.45$, $p < 0.001$) but there were no correlations between CRP and albumin or CRP and fetuin-A.

Table 1. Patient characteristics at baseline

	AVF (n = 32)	TC (n = 26)
Age, years	50 ± 15	55 ± 14
Male/female	21/11	11/15*
Race (African-American/Caucasian)	24/8	21/5
Dialysis vintage, months	84.2 ± 92.3	42.5 ± 53.5**
Cause of ESRD: GN/HTN/DM/other	9/13/7/3	3/8/13/2
Diabetes, yes/no	9/23	15/11**
Current smoker, yes/no	10/22	6/20
Hospitalization in last 12 months yes/no	12/20	12/14
Previous cardiac event, yes/no	8/24	6/20
Previous stroke or TIA, yes/no	4/28	6/20
PVD, yes/no	4/28	3/23
Hepatitis C, yes/no	7/25	2/24
Statin, yes/no	17/15	9/17
Average weekly darbepoetin dose, µg	57 ± 52	60 ± 56
Vitamin D, yes/no	20/12	13/13
Hct, %	37.4 ± 4.3	36.4 ± 6.6
WBC, cells/mm ³	7.2 ± 2.3	7.7 ± 2.6
Ferritin, ng/ml	677 ± 265	610 ± 399
PTH, pg/ml	563 ± 508	554 ± 683
Calcium, mg/dl	8.9 ± 0.8	9.2 ± 0.6
Phosphorus, mg/dl	5.6 ± 1.5	5.3 ± 1.6
Albumin, g/dl	3.8 ± 0.6	3.6 ± 0.7
Fetuin-A, µg/ml	335 ± 92 (172 – 548)	378 ± 99 (142 – 562)
High-sensitivity CRP, ng/ml	12.32 ± 15.92 (0.6 – 65.5)	21.94 ± 30.4 (0.7 – 91.1)
ln(CRP)	1.80 ± 1.25	2.18 ± 1.40
IL-6, pg/ml	14.9 ± 19.1 (1.8 – 92.9)	8.6 ± 8.0 (1.1 – 29.5)
ln(IL-6)	2.17 ± 0.99	1.78 ± 0.87

Data presented as mean ± SD and ranges for those values that were subsequently log transformed.

AVF = Arteriovenous fistula; TC = tunneled dialysis catheter. Cause of ESRD: GN = glomerulonephritis, HTN = hypertension, DM = diabetes.

* p = 0.08; ** p < 0.05.

Table 2. Variation of inflammatory biomarkers

	Between-subject variation	Within-subject variation	Within-subject SD	ICC	1 – ICC
Fetuin-A	7,759	6,722	82	0.54	0.46
CRP	376	1,224	35	0.24	0.76
ln(CRP)	1.27	0.57	0.75	0.69	0.31
IL-6	108	326	18	0.25	0.75
ln(IL-6)	0.69	0.33	0.57	0.68	0.32
Albumin	0.11	0.20	0.45	0.36	0.64

CRP = C-reactive protein; IL-6 = interleukin-6; ICC = intra-class correlation coefficients.

When patients were examined longitudinally, the inflammatory markers changed frequently despite no clinically apparent illness. There was large month-to-month fluctuation for all biomarkers. To determine if some of this variation seen in clinically stable patients was explained by the presence of TC, we compared these biomarkers at baseline between patients with an AVF to those with a TC, and found no difference (table 1). We then analyzed all monthly measurements and found a significant difference between mean fetuin-A for patients with AVF as compared to those with TC at visit 3 only, but this was due to an outlier value from a single patient. Once this patient's value was removed, the difference became non-significant. There were no significant differences in ln(CRP), ln(IL-6), or albumin between patients with AVF and those with TC at any visit.

The 1 – ICC calculation (table 2) demonstrated that 46% of total variation in fetuin-A levels comes from within-patient variance or each patient's fluctuations in monthly levels. Corresponding 1 – ICC was 31% for ln(CRP), 32% for ln(IL-6), and 64% for albumin. 30% of subjects changed from one tertile of ln(CRP) to another during the 3-month study. Other patient characteristics were tested for association with biomarker levels. Smoking was the only patient characteristic that was significantly associated with any of the biomarkers. It was negatively associated with fetuin-A levels and positively associated with ln(IL-6).

Despite the large inpatient variance of these biomarkers seen in these clinically stable patients, there was a predictable response to acute inflammation. Four subjects with infection during the study were followed weekly – all with TC. All of these patients had a sudden rise in CRP and IL-6, and drop in fetuin-A within 1 week of onset of the infection, and a return to baseline values within 3 weeks following the onset of the clinical symptoms of infection. The most serious infection was endocarditis, and the changes in inflammatory biomarkers for this subject are shown in figure 1. No data for these patients were included in Aim 1 of the study (monthly) as Aim 1 sought to determine variation in clinically stable patients.

Discussion

Dialysis patients have a large burden of cardiovascular disease, with both traditional Framingham and many 'non-traditional' risk factors identified. One such non-traditional risk factor is inflammation. A syndrome of

Fig. 1. Change in inflammatory markers in patient with endocarditis: a patient was diagnosed with acute infectious endocarditis 7 days after baseline (week 0) laboratory values. The patient had weekly inflammatory biomarkers measured to assess the change with antibiotic treatment and mitral valve replacement. To show the trends for all biomarkers on the same graph, the albumin levels were multiplied by 10, and the fetuin-A was multiplied by 100.

malnutrition, inflammation and atherosclerosis has been identified in dialysis patients and implicated in the pathogenesis of cachexia and cardiovascular disease [9]. Pathologically, inflammation is a known contributor to intimal atherosclerotic disease in which macrophages and other inflammatory cells are thought to be critical in the pathogenesis of plaques [12]. Inflammation has been shown to lead to reduced fetuin-A levels in serum, likely making patients more vulnerable to extraskeletal calcification when this serum calcium-binding protein is decreased [13]. The advent of the high-sensitivity CRP has led to a plethora of studies in the general population and in dialysis patients – nearly all showing a strong association of elevated levels with cardiovascular and all-cause mortality. However, inflammation is an end event of numerous inciting factors and mediators, and many of the biomarkers utilized to define or diagnose inflammation are part of the acute phase response, which is a non-specific diffuse response initiated by multiple factors [14].

In the present study, we documented that these biomarkers change with acute events such as infection and stroke, with recovery as the event recovers. Thus the accuracy of the biomarkers for acute inflammation is not questioned. However, we found large variances in patients who were clinically stable, and this variance was

not explained by having a TC as opposed to an AVF. TC have been hypothesized to contribute to intermittent inflammation and elevated biomarkers of inflammation [8]. In the present study, patients with TC were more likely to get infected, but in patients without clinically apparent infections, the presence of TC did not explain the variance. Between 30 and 46% of the total variation was due to within-patient variation despite assay performance (CV) that was within manufacturer recommendations.

Similar estimates of variance have been previously reported in a smaller study in dialysis patients. A study of 29 patients, excluding subjects with temporary catheters, measured levels weekly for 16 months and found intra-subject variance even higher, with 71.3 and 86.7% of total variation for CRP and IL-6, respectively [15]. An equally poor variance was found for serum amyloid A protein, another inflammatory biomarker [15]. In contrast, estimates of variation in the general population are much lower. In one study, the intraindividual variation for $\ln(\text{CRP})$ for healthy adults was 14% compared to 9% for those with angina [16]. Thus, this variance is substantially higher in dialysis patients than in the healthy adults, even in those with native fistulas. The reason for the variation could not be assessed from this study as we did not perform random blood cultures, dental examinations, tagged white cell scans or other searches for clinically hidden causes of inflammation.

Unfortunately, the lack of stability of these biomarkers makes practical use problematic. Clinical use of the biomarkers to identify patients at risk or who have smoldering infections is not likely valid given that, for example, over one-third of subjects changed from one tertile of CRP to another in the course of just 3 months. This is consistent with the study of van Tellingen et al. [17], who found patients frequently changed tertiles of CRP and concluded that clinical judgment was as predictive of infection as was a rise in CRP levels. The inpatient variation will need to be taken into account if any of these biomarkers are used as a surrogate endpoint in research studies that employ specific interventions aimed at reducing inflammation. Certainly, sample size calculations for studies using these biomarkers in dialysis patients should take into account this variation and study designs should include repeated measurements to be certain that the observed effect is stable over time. Our data support the need to validate that a change in these biomarkers prospectively predicts a change in clinical endpoints prior to their use as a valid surrogate endpoint. Lastly, authors should report their own laboratories' CV when using these assays. Nearly all samples analyzed in the pres-

ent study required dilution of the sample to remain within the manufacturer's standard curve, which may create increased assay error.

There are several limitations to our study. First, this is a relatively small sample size although the largest reported to date. We only followed patients for 4 months, and perhaps longer follow-up, or more frequent follow-up would have altered our findings, although Tsirpanlis et al. [15], measuring weekly values found similar variability. We also only measured four potential inflammatory biomarkers, all of which have limited specificity for inflammation. Lastly, there may have been some selection bias in those patients enrolled. Our patients were predominately African-American, had a high percentage of glomerulonephritis and were younger than the average US dialysis population. However, these apparently clinically stable patients may be enrolled in clinical trials and thus the results provide important information on the limitations of using these biomarkers in clinical trials.

In summary, there is considerable inpatient variation in biomarkers CRP, IL-6, fetuin-A and albumin.

Whether this phenomenon is a problem with the assays, a problem with our patients or both is a question that remains to be answered. Given problems with circulating fragments in other peptides such as PTH [18], interference in assay measurements by inactive fragment or other uremic toxins is a possibility. However, a more likely explanation for the high and fluctuating levels in dialysis patients is that these biomarkers can be elevated by a number of processes, and thus may simply reflect the multiple co-morbid illnesses present in our patients. Unfortunately, this means further studies are needed to refine the precision of these biomarkers before they can be considered true surrogate markers for cardiovascular disease in dialysis patients. Until that time, clinical studies of anti-inflammatory measures should evaluate clinically meaningful endpoints to prove efficacy.

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