Circulating cell-free DNA in hemodialysis patients predicts mortality

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Abstract

Background. Circulating cell-free DNA (CFD) appears following cell damage and DNA release, and increases in hemodialysis (HD) patients particularly following HD. We hypothesized that CFD is an integrative marker of tissue damage and can be an independent predictor for all-cause mortality in HD patients.

Methods. In a prospective study, CFD levels before and after HD were evaluated in 31 chronic HD patients with no acute disease, using the reported rapid non-cumbersome inexpensive fluorometric assay developed in our laboratory. Follow-up levels were assessed at 18 months in 22 patients. All-cause mortality was a primary endpoint.

Results. During 42 months of follow-up, 13 of the 31 (41.9%) patients died. The decedents were older than the survivors (mean age 69.9 versus 61.5 years, P = 0.06), but did not differ in end-stage renal disease (ESRD) duration, gender, albumin and hemoglobin, diabetes mellitus and weight. Post-dialysis CDF levels were significantly lower in survivors (median 688 versus 880 ng/mL, P = 0.01). The sensitivity and specificity of CDF levels of 850 ng/mL to predict 42 months (3.5 years) mortality were 73 and 75%, respectively, and the area under the receiver-operating characteristic curve was 0.77 [95% confidence interval (CI) 0.60–0.94]. The Cox proportional hazard regression model showed that CDF higher than 850 ng/mL adjusted for age, gender, albumin and dialysis duration predicted 42 months mortality.

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ESRD duration, weight and creatinine (stepwise model) was highly predictive of all-cause death with a hazard ratio of 8.0 (95% CI 2.3–28.5, P = 0.001).

**Conclusions.** Post-dialysis CFD level is an independent predictor of all-cause mortality in patients undergoing HD. We propose that CFD detection is an inexpensive applicable tool for identifying patients at risk and their follow-up.

**Keywords:** cell-free DNA; CFD; hemodialysis

**Introduction**

In hemodialysis (HD) patients, various pathological processes occur in most of the body systems leading to their impaired function and to combined tissue damage, with subsequently increased morbidity and mortality. Different clinical conditions are associated with the deteriorating prognosis and an increased mortality risk in patients undergoing HD. These include cardiovascular disease, diabetes mellitus (DM), atherosclerosis, infection, malnutrition, inflammation, oxidative stress, iron deficiency, anemia, calcification, uremia and volume overload [1–5]. Furthermore, various markers can be used for the prediction of short- and long-term outcomes in the HD population. These include residual renal function [6], decreased erythropoiesis-stimulating agent (ESA) responsiveness [5], low serum levels of albumin, low ferritin, high levels of fibroblast growth factor (FGF)-23, parathyroid hormone (PTH), phosphate, alkaline phosphatase, C-reactive protein (CRP), vitamin D and hemoglobin (Hg) [2, 7–13].

However, the sum effect of the numerous risk factors in HD patients is cumulative, additive, interrelated, complex and not infrequently unpredicted or completely unknown. Thus, it is not surprising that the accuracy of the mortality and morbidity prediction based on any particular marker or clinical condition is not optimal. Therefore, a parameter that assesses combined tissue damage may be helpful in addition to the specific clinical conditions and biochemical processes markers.

Circulating cell-free DNA (CFD) appears following cell damage and DNA release. CFD is increased in various pathologic processes including DM and inflammation [14], which are common and significant risk factors in HD patients. Previous studies in HD patients have demonstrated elevated serum levels of CFD in HD and an additional increase during the HD session [15]. The prognostic significance of CFD in HD patients is uncertain. Moreover, available data were obtained by time-consuming expensive polymerase chain reaction (PCR), techniques which are not practical for routine clinical laboratory use. Recently, we developed a novel rapid and direct fluorescent assay for CFD quantification which was shown to be inexpensive, accurate and reproducible [16]. Using this method, in previous studies, we found the elevation of CFD in acute ST-elevation myocardial infarction patients, in patients with primary colorectal cancer and in a rat stroke experimental model [17–19].

We hypothesized that CFD is an integrative marker of tissue damage and can be an independent predictor for all-cause mortality in HD patients.

**Materials and methods**

**Population**

This prospective study was conducted in the Soroka University Medical Center, a tertiary 1000-bed hospital. We enrolled 31 unselected HD patients with end-stage renal disease (ESRD), with no acute disease such as infection, cardiovascular event or thrombosis and no hepatitis B or C, who agreed to participate and signed an informed consent form. Enrolled patients were assessed once before and once after going through HD at the baseline and 18 months later. Survival status was assessed at 42 months (3.5 years) following the enrollment. The patients were treated on HD thrice weekly, with a mean dialysis time of 3.8 ± 0.4 h and a dialysis dose of 1.32 ± 0.39 (double pool Kt/V). Twenty-one patients were on the high-efficiency dialyzer-F8 HPS (polysulfone, Fresenius Medical Care, Bad Hamburg, Germany), nine patients were treated with the high-flux dialyzer-Fx80 (polysulfone, Fresenius Medical Care) and two patients were on the high-efficiency dialyzer Sureflux-150E (cellulose triacetate, Nipro Medical Industries, Gunma, Japan) due to hypersensitivity to polysulfone. The water of the HD setup in the Soroka Medical Center is regularly monitored for endotoxin levels <1 EU/mL and general bacterial count of <100 colonies/mL with no pseudomonas or Escherichia coli contamination. The dialyzate contained 1.38 mEq/L sodium, 2 mEq/L potassium, 34 mEq/L bicarbonate and 2.5 or 3 mEq/L calcium. For control of chronic kidney disease (CKD)-mineral bone disease, patients were treated with cinacalcet, sevelamer hydrochloride and alfacalcidol. For the treatment of anemia, patients were treated with ESAs including erythropoietin and novel erythropoiesis-stimulating protein and intravenous iron saccharate. At baseline, we assessed demographic and clinical data, and the patients were tested for plasma levels of creatinine, albumin and Hg. CFD levels were measured in all the patients before and after the HD session using the reported rapid non-cumbersome inexpensive fluorometric assay developed in our laboratory [16].

**CFD measurements**

Blood samples were collected in commercial gel tubes using the BD Vacutainer (Becton, Dickinson and Company, Plymouth, UK). Sera were separated and kept at −20°C until assayed. CFD was quantified by a novel rapid fluorometric assay, the fluorochrome SYBR® Gold, which does not require prior processing of samples, i.e. DNA extraction and amplification. The method was tested in comparison with the gold standard, QPCR, and was found to be in good correlation of 0.9987 (P < 0.0001) as described previously [16]. Briefly, SYBR® Gold Nucleic Acid Gel Stain (Invitrogen, Paisley, UK) was diluted 1:1000 in dimethyl sulfoxide and then 1:8 in phosphate-buffered saline. Ten microliters of DNA standard or sample were applied to a 96-well plate and 40 μl of the diluted SYBR® Gold were applied to each well. Fluorescence was measured with a 96-well fluorometer at an emission wavelength of 535 nm and an excitation wavelength of 485 nm. The intra-day coefficient of variation was 16, 7.9 and 4.8% in the low (383 ng/mL), elevated (1152 ng/mL) and high DNA range (2735 ng/mL), respectively. The day-to-day coefficient of variation was 31, 6.7 and 8% in the low, elevated and high DNA range, respectively.

**Interleukin-6 determination**

Interleukin-6 (IL-6) in plasma concentrations was quantified by Duoset ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s protocol.

**Statistical analysis**

All-cause mortality at 42 months was a primary endpoint. The results are presented as the mean (SD) for continuous variables and as the total patients (percentage of total patients) for categorical data. The t-test was used for the comparison of the continuous variables or χ² test for categorical data with the use of Fisher’s exact test if needed. We used the Mann–Whitney U-test for the comparison of variables with non-normal distribution that were presented as median with inter-quartile range.
Results

Thirty-one chronic dialysis patients were enrolled into the study. During the 42 months of follow-up, 13 (41.9%) patients died.

Table 1 depicts the clinical and demographic characteristics of the patient population. The majority of patients were male, with the mean age being 65 (±12.07) years. The median duration of ESRD in months was numerically, but not statistically higher among the survivors (24.5 versus 15.0, P = 0.98). Survivors were younger than decedents (61.5 ± 10.8 versus 69.9 ± 12.5 years, P = 0.06). There were no differences between the two groups in gender, weight, DM prevalence, creatinine, albumin or Hg levels at baseline.

Table 2 presents a univariate comparison between survivors and decedents in CFD parameters at the baseline. Following dialysis, levels of CFD increased from a median of 592 to 758 ng/mL, P < 0.001, at baseline (Figure 1), but less so at 18 months (second dialysis) from a median of 960 to 1195 ng/mL, P = 0.09. Pre-dialysis CFD negatively correlated with creatinine both at the baseline and at 18 months (Spearman’s ρ = 0.394, P = 0.03 and ρ = −0.595, P = 0.006, respectively). At baseline post-dialysis, CFD positively correlated with IL-6 (Spearman’s ρ = 0.51, P = 0.02) and negatively with creatinine (ρ = −0.45, P = 0.01).

We found no difference in the CFD levels between patients treated with the high-efficiency dialyzer-F8 HPS (n = 21) and the high-flux dialyzer-Fx80 (n = 9). The median pre-HD CFD levels were 595 versus 592 (P = 0.44) and the post-HD CFD levels were 823 versus 735 (P = 0.19) accordingly. Eleven patients with DM had higher median post-dialysis CFD levels when compared with patients without DM: 1045 versus 729 ng/mL, P = 0.018. The median post-dialysis CFD levels were significantly lower in survivors (688 versus 880 ng/mL, P = 0.01).

In 18 survivors, there was an increase in both pre- and post-CFD levels from the baseline to 18 months: from a median of 479 to 787 ng/mL and from a median of 688 to 1266 ng/mL, respectively, and P = 0.005 and 0.002, respectively.

Figure 2 shows an ROC curve representing the discriminative ability of post-dialysis CFD measurement to predict a 42-month all-cause death. The AUC is 0.77 [95% confidence interval (CI) 0.60–0.94]. None of the other tested parameters was significant in ROC curve analysis (creatinine, albumin, uric acid, weight, age etc.).

The post-dialysis level of 850 ng/mL emerges as a point with the highest Youden’s index (Figure 3). At this level, the sensitivity and specificity of CFD to predict 42-month mortality were 73 and 75%, respectively. Out of 11 patients with initial post-dialysis CFD ≥850 ng/mL, 8 (72.7%) patients died compared with 5 out of 20 (25.0%) with low levels of CFD. The Kaplan–Meier survival analysis (Figure 4) showed statistically significant lower survival rates for patients who had CFD levels higher than 850 ng/mL (log-rank test, P = 0.02).

In an unadjusted Cox proportional hazard regression model, CFD levels higher than 850 ng/mL were associated with all-cause mortality at 42 months with a hazard ratio of 4.9 (95% CI 1.6–15.3, P = 0.006). The CFD level higher than 850 ng/mL adjusted for age, duration of ESRD, weight and creatinine level (stepwise model) is predictive of all-cause death, with a hazard ratio of 8.0 (95% CI 2.3–28.5, P = 0.001).

Discussion

The main finding of our study is that the post-dialysis CFD level is an independent predictor of all-cause mortality in patients undergoing HD. The CFD level of 850 ng/mL emerges as the most accurate cutoff point for the prediction of 42-month mortality.

Various markers were found to be associated with the increased mortality risk in HD patients. A recent study demonstrated that small-sized high-density lipoprotein particles alone and combined with elevated high-sensitivity CRP concentrations are independent predictors of reduced survival in HD patients [20]. These assessments are difficult and expensive to perform when compared with CFD. The prognostic significance of high P, FGF-23, PTH and low 25 vitamin D and 1,25 vitamin D has been reported in HD patients [2, 8, 10–12]. Pre-HD and post-HD CRP levels were found to be associated with increased mortality [9, 13].
Table 2. Univariate comparison between survivors and decedents in CFD parameters at baseline

<table>
<thead>
<tr>
<th></th>
<th>Survivors (n = 18)</th>
<th>Decedents (n = 13)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFD pre-dialysis, median (IQR)</td>
<td>479 (323–687)</td>
<td>651 (409–811)</td>
<td>0.21</td>
</tr>
<tr>
<td>CFD post-dialysis, median (IQR)</td>
<td>688 (495–838)</td>
<td>880 (743–1262)</td>
<td>0.01</td>
</tr>
<tr>
<td>Absolute change, median (IQR)</td>
<td>225 (61–339)</td>
<td>332 (147–514)</td>
<td>0.14</td>
</tr>
<tr>
<td>Relative change (%), median (IQR)</td>
<td>49 (13–79)</td>
<td>50 (18–90)</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Fig. 1. CFD levels before and after HD.

Fig. 2. The ROC curve of CFD levels post-HD as a predictor of survival in 42 months. The AUC of 0.77 (95% CI 0.60–0.94).
CFD concentrations increased after 18 months. Since CFD is mainly removed by the liver (reviewed in Fleischhacker and Schmidt [21]), we believe that the elevated levels reflect the general deterioration in health and not just the decrease in residual renal function. Unfortunately, we have no data on residual renal function. However, we found no correlation between CFD and patients’ ESRD duration, a parameter that strongly affects residual renal function.

**Fig. 3.** Post-dialysis CFD level stratified by the survival status. Horizontal line represents Youden’s index (point of maximal accuracy based on $Y = \text{sensitivity} + \text{specificity} - 1$).

**Fig. 4.** The Kaplan–Meier survival curve for CFD levels stratified by the CFD level higher or lower than 850 ng/mL.
We decided to measure IL-6 as a marker of inflammation since it was shown by Panichi et al. [22] to be a better outcome predictor to HD patients than CRP. We have found that CFD levels correlate with IL-6 and are especially elevated in patients with pro-inflammatory conditions such as DM. The association of HD treatment to inflammation was previously reported; Korevaar et al. [9] have shown that an increase in CRP levels following the HD session was observed in 25% of the HD patients and was associated with increased mortality independent of the pre-HD CRP level. The association between CFD and IL-6 and diabetes are not surprising since both inflammation and diabetes are destructive conditions which increase the cell death. As expected by our hypothesis, we found a negative correlation between CFD and elevated creatinine levels which reflect muscle mass and are considered positive markers of good health for HD patients [23]. In addition, an increase in CFD levels in the population of survivors is probably indicative of the expected deterioration of their health.

Previous studies have shown that plasma DNA levels significantly increase after HD [15, 24, 25], an effect that was not affected by the type of dialysis membrane [15]. Similarly, we found a significant increase in CFD levels following the HD session. This elevation could be explained by direct mechanical damage caused by the dialysis filter to blood cells during dialysis and by oxidative stress to leukocytes or other tissue (reviewed in Morena et al. [26]). Various dialysis membranes were shown to prime and activate leukocytes [27]. Furthermore, causes for HD-related inflammation include oxidative stress and uremic toxins and intravenous iron administration [28, 29], contaminated dialysate and colonization of dialysate lines with bacteria as well as biocompatibility of dialyzers [4, 27]. Twenty-one of our patients were on the polysulfone high-efficiency dialyzer, eight patients on the polysulfone high-flux dialyzer and only two patients were on the cellulose triacetate dialyzer. Similar to the findings of Garcia Moreira et al. [15], we found no correlation between the dialyzer type and CFD levels (pre- or post-HD). Since only two were on cellulose triacetate, it is impossible to evaluate this less biocompatible membrane in relation to CFD levels. Unfortunately, we have not collected hemoconcentration data and have not assessed this effect on CFD levels. However, the mean CFD increase during HD was around 30%, which is clearly above the expected increase from hemoconcentration alone. Yet, we cannot rule out that hemoconcentration might have an additional contribution to post-HD CFD levels or to their association with outcome.

In their study, Garcia Moreira et al. [15] showed that CFD returned to pre-HD levels by 30 min after HD and therefore concluded that CFD can be considered a reliable diagnostic tool when measured at least 30 min after an HD session. We believe that this recommendation is not valuable for the evaluation of patient outcome with our CFD assay. Using our new method, we found that post-HD CFD levels are in better correlation with outcome than pre-CFD levels. Possibly, the destructive effect of HD which is reflected in the transient post-HD elevation of CFD is a significant risk factor that affects patient outcome.

Our study has several limitations. We performed a single-center study on a relatively small patient cohort. A small sample size with relatively few outcomes might result in data over-fitting, making conclusions somewhat inconclusive. Especially, due to the limited number of outcomes, we could not fit the survival model inclusive of the multiple predictors. Therefore, potential confounding cannot be excluded. The current study design did not allow us to fully assess the dynamics of CFD, i.e. how the temporal change in the CFD level is related to mortality or morbidity. Nevertheless, this hypothesis-generating study has shown that post-HD levels of CFD are reliable and independent predictors of all-cause mortality.

In conclusion, post-dialysis CFD is an inexpensive and easy to perform assay that could be used as an integrative parameter to follow in populations of patients undergoing HD.

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Conflict of interest statement. None declared.

References
Intradialytic hypotension (IDH) is still a major clinical problem for haemodialysis (HD) patients. Haemodiafiltration (HDF) has been shown to be able to reduce the incidence of IDH.

HFR-Aequilibrium international multicentric study (AIMS)

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Effect of a plasma sodium biofeedback system applied to HFR on the intradialytic cardiovascular stability. Results from a randomized controlled study

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