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Research Article

Effect of a Hemodialysis Session on Markers of Inflammation and Endotoxin

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Background. People receiving hemodialysis (HD) treatment have higher cardiovascular morbidity and mortality, ascribed to an increased prevalence of traditional cardiovascular risk factors. However, the role of nontraditional risk factors, such as inflammation, has become increasingly recognized. The origin of this inflammation remains elusive and one putative cause is elevated levels of circulating bacterial endotoxin. *Methods*. In this study, serum concentrations of endotoxin and inflammatory biomarkers, including high-sensitivity C-reactive protein (hsCRP), interleukin-6 (IL-6), interleukin-1β (IL1β), ferritin and tumor necrosis factor (TNF), were measured in 30 adults receiving HD and 10 healthy individuals without kidney disease. In people receiving HD, samples were collected immediately before dialysis (preHD), after dialysis (postHD), and 48 hours after (postHD$_{48hrs}$). *Results*. Endotoxin was detectable in only 1 of 90 samples analyzed. There were no significant differences in serum hsCRP, IL1β, and IL6 levels, before and after dialysis. Serum TNF levels decreased significantly from 30.9 (8.0, 39.5) pg/mL preHD to 13.9 (8.5, 17.3) pg/mL post-HD ($p = 0.002$) and then increased back to 27.37 (14.5, 35) pg/mL 2 days later ($p < 0.001$). Ferritin increased from 1153 ng/mL (782, 1458) preHD to 1313 ng/mL (657, 1638) post HD ($p < 0.001$) and then decreased back to 1186 ng/mL (754, 1597) ($p = 0.66$) postHD$_{48hrs}$. Compared to controls, people receiving HD had significantly elevated levels of hsCRP [6.16 mg/L (2.1, 16.8) vs. 1.1 mg/L (0.81, 3.63) $p = 0.015$], IL1β [1.5 pg/mL (0.05, 2.51) vs. 0.5 pg/mL (1.81, 2.95) $p \le 0.001$], and ferritin [1153 (782, 1458) vs. 132.9 (111, 257) ng/mL $p \le 0.001$], but comparable levels of in IL6 [6.15 pg/mL (4.82, 9.12) vs. 7.49 pg/mL (4.56, 10.39), $p = 0.77$] and TNF [27.35 pg/mL ± 17.48 vs. 17.87 pg/mL ± 12.28, $p < 0.12$]. In *conclusion*, people on HD have elevated levels of inflammatory biomarkers, which are not associated with endotoxemia (which is rare) or the dialysis procedure.

1. Introduction

People with kidney failure experience high rates of cardiovascular morbidity and mortality despite receiving hemodialysis (HD) therapy [1]. Various factors contribute to these adverse outcomes, including hypertension, diabetes, and obesity [2, 3]. However, these traditional cardiovascular risk factors only partially explain the increased incidence of cardiovascular disease, thereby prompting evaluation of the role of nontraditional risk factors, such as inflammation [3, 4]. Inflammation is highly prevalent in people receiving HD and has been linked to malnutrition, anemia, and accelerated vascular disease [5, 6]. The cause of the heightened level of inflammation repeatedly demonstrated in the dialysis population is likely to be multifactorial. These include the following: (i) Exogenous factors, such as dialysis membranes, tunnelled catheters, vascular grafts, and impure dialysate water; (ii) Cellular factors, such as oxidative stress and cellular senescence; (iii) Tissue factors, such as hypoxia, fluid and solute shifts, or temperature changes; (iv) Microbial factors, such as immune dysfunction and gut dysbiosis; and (v) Retention of uremic toxins [7, 8]. These

factors are directly affected by the actual process of hemodialysis where a typical session lasts 4–5 hours. Therefore, it is plausible that elevated levels of inflammation in the dialysis population may be related to the actual process of dialysis. Another potential cause of inflammation is the finding of circulating levels of endotoxin in the dialysis population [9, 10]. Pre-existing arterial disease, together with splanchnic vasoconstriction and/or hypotension during dialysis is thought to compromise the blood gut barrier thereby facilitating the egress of gut bacteria and endotoxin into the circulation and inciting inflammation. In addition, gut dysbiosis present in CKD may also be an additional risk factor.

The aim of this study was to use a multiple cytokine approach to evaluate the effect of a HD session on levels of inflammation and endotoxin.

2. Methods

2.1. Study Population. This single centre, cross-sectional observational cohort study evaluated consecutive, prevalent, adult HD patients receiving dialysis treatment at Cairns Hospital between 1 June 2017 and 31 July 2017 (see Figure 1). There were 120 prevalent patients receiving outpatient dialysis. Inclusion criteria were age >18 yrs and receiving HD treatment >3 months. Exclusion criteria included (a) Recent (<4 weeks) infection, hospital admission, or surgery or (b) the use of a tunnelled HD catheter. Informed consent was obtained from all participants prior to study involvement. The study was approved by the Cairns and Hinterland Human Research Ethics Committee (reference number HREC/17/QCH/15–1118). Healthy adult staff volunteers without disease, exclusion criteria, or chronic kidney diseases were selected as controls.

All participants received dialysis therapy with Fresenius system 4008 or 5008 model dialysis machines with a FxCor Diax® filter using a standard dialysate solution containing 140 mmol/L of sodium. Data on patient demographics, comorbidities, blood pressure, dialysis prescription, body mass index, kidney replacement therapy time, hemoglobin, and serum albumin concentration were recorded. This dialysis centre subscribed to the International Organisation for Standardisation (ISO) standard for dialysate water quality [11–14]. Briefly, the facility used a central reverse osmosis system. This was disinfected weekly and the "loop" supply was disinfected daily. Bacterial culture and endotoxin batch testing were performed monthly.

2.2. Sample Collection. After insertion of dialysis cannulae, 15 mL of whole blood was collected into ethylene diamine triacetic acid, lithium-heparin, and gel clotted tubes. The first blood collection was timed on the first day after the longest dialysis break but prior to commencing dialysis and prior to heparinization (preHD). A second sample was then collected at the end of the dialysis session (postHD) before the cannulae were removed. A third sample was collected at the beginning of the next dialysis session 48 hours later (postHD$_{48hrs}$). The whole blood was stored in ice and then processed within 15–20 min of collection. Samples were centrifuged at 1900xg in an Eppendorf 5702 AG centrifuge. The plasma was separated and collected in endotoxin-free cryosafe tubes and stored at −40°C immediately after separation.

2.3. Endotoxin Measurement. Endotoxin levels were measured using a Charles River Laboratories (CRL) Kinetic Chromogenic assay, Endochrome-K. A more detailed description of the methodology used has been presented in a separate report where the effect of assay type, buffers, dilutions, and collection factors was investigated [15]. Samples were thawed and diluted to 1 : 20 with a bio-dispersing agent (CRL, BD100) and heated to 75°C for 15 minutes. A standard curve (0.005 EU/mL and 5 EU/mL) was prepared using control standard endotoxin supplied by CRL. If endotoxin was detected, the samples were then reconstituted with an endotoxin-specific buffer (Charles River Endosafe BG-120) to avoid any β-glucan-related false positive reaction. A positive product control was included for each sample to eliminate the possibility of enhancement or inhibition of endotoxin measurement. The absorbance at 405 nm was measured at 30 second intervals for a total of 3600 seconds using a Biotek ELx808 plate reader. The assay reactions were held at 37°C throughout the assay period. Charles River Endosafe, EndoScan V software was used to generate a standard curve and analyze data using an onset time of 0.1 absorbance unit.

2.4. Inflammatory Biomarker Measurement. High-sensitivity C-reactive protein (hsCRP), interleukin 6 (IL6), interleukin 1β (IL1β), and tumour necrosis factor (TNF) were measured using a Merck Millipore Milliplex High sensitivity T cell panel. Diazyme hsCRP immunoturbidometric assay was performed on Cobas Mira. Serum ferritin was measured using Roche ferritin CS Elecsys V2 assay.

2.5. Statistical Analysis. The primary outcome was the change in endotoxin and inflammatory marker concentrations preHD, postHD, and postHD$_{48hrs}$. Data are presented as number (percent) for categorical variables and median 7 for non-parametric or mean ± SD for continuous, normally distributed variables. Normality was assessed using the Shapiro–Wilk index and by calculating the percentage of standard deviation to mean. Between-group differences were assessed using the paired T test for normally distributed data and the Wilcoxon signed rank test for nonparametric data.

3. Results

Thirty consecutive patients receiving HD were included in the study. Their baseline characteristics are shown in Table 1.

3.1. Endotoxin Levels. Endotoxin was initially detectable in six samples from three participants (participants 7, 20, and 24) out of a total of 90 samples from 30 participants
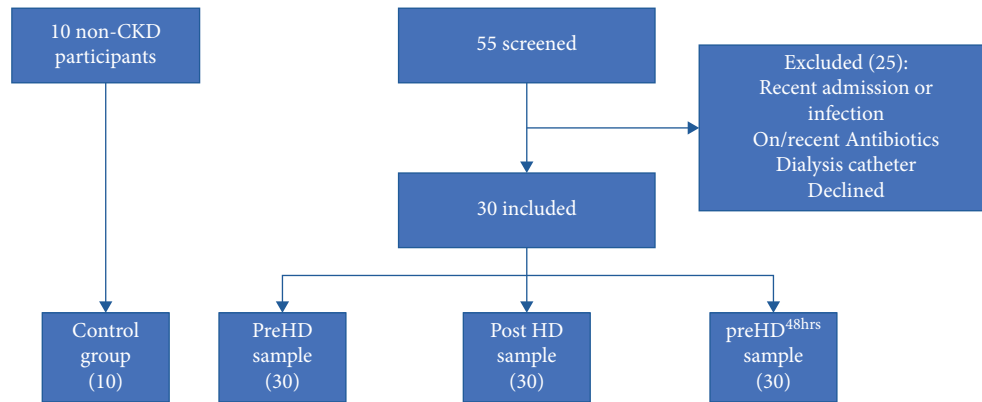
FIGURE 1: Flow chart of study population.

TABLE 1: Characteristics of HD patients.

Characteristic	Value ($n = 30$)
Age (yrs)	58.5 ± 13.9
Male sex	16 (53%)
KRT vintage (yrs)	6.1 ± 4.6
Dialysis access	
AVF	29 (97%)
AVG	1 (3%)
BMI (kg/m^2)	32 ± 7
Ethnicity:	
First nations	18 (60%)
Caucasian	10 (33%)
Other	2 (7%)
Diabetes	18 (60%)
IHD	11 (36%)
PVD	4 (13%)
CVD	2 (7%)
HD modality	
High flux	19 (63%)
HDF	11 (37%)
Serum albumin (g/L)	36 ± 3
PreHD SBP (mmHg)	150 ± 25
PostHD SBP (mmHg)	137 ± 21
Interdialytic weight gain (kg)	3.1 ± 1.2

Results are presented as mean with SD. AVF, arteriovenous fistula; AVG, arteriovenous graft; BMI, body mass index; CVD, cerebrovascular disease; HD, hemodialysis; HDF, hemodiafiltration; IHD, ischemic heart disease; KRT, kidney replacement therapy; PVD, peripheral vascular disease.

(Table 2). However, 5 of the samples tested negative after the addition of endotoxin-specific (ES) buffer leaving only 1 (1.1%) out of 90 with detectable endotoxin (0.28 EU/ml). This was a postHD sample.

3.2. Inflammatory Biomarkers.

Table 3 shows the results of serum inflammatory marker measurements. A between-groups paired comparison of serum hsCRP, IL1β, and IL6 levels revealed no statistically significant differences for each of the 3 paired time points (i.e., preHD vs. postHD, preHD vs. postHD$_{48hrs}$, and postHD vs. postHD$_{48hrs}$) (Table 3). Serum TNF levels decreased significantly from 30.86 (8.0^{25th}, 39.5^{75th}) pg/mL preHD to 13.9 (8.5, 17.3) pg/mL postHD ($p = 0.002$) and then increased back to 27.37 (14.5, 35) pg/ml

2 days later ($p < 0.001$). Ferritin increased from 1153 ng/ml (782, 1458) preHD to 1313 ng/ml (657, 1638) post HD ($p < 0.001$) and then decreased back to 1186 ng/ml (754, 1597) ($p = 0.66$) postHD$_{48hrs}$.

3.3. Comparison with Controls.

Endotoxin was not able to be detected in the serum of 10 control subjects (all values less than 0.1 EU/mL, recovery $100 \pm 34\%$). Serum levels of hsCRP, IL1β, and ferritin were all significantly lower in controls compared to patients receiving HD, whilst serum IL6 and TNF concentrations were comparable between control and dialysis samples (Table 3).

4. Discussion

In this cross-sectional observational study of 30 patients receiving hemodialysis, circulating endotoxin was only detected in 1% of serum samples tested, whilst serum concentrations of inflammatory biomarkers (hsCRP, IL1β, and IL6) were significantly elevated compared with those of healthy controls. There was no compelling evidence of an association between serum endotoxin and inflammatory biomarker measurements or between these measurements and the dialysis procedure itself.

A potential mechanism for circulating endotoxaemia is a reduction in the cellular integrity of the gut blood barrier. Specific risk factors in the dialysis population include oedema and hypoperfusion of the gut wall, thereby potentiating the translocation of gut derived endotoxin [16]. In addition, perturbations in secreted alkaline phosphatase or antibacterial peptides, mucus production, and the intestinal mononuclear phagocyte system could further undermine the barrier integrity [17]. Some studies have demonstrated endotoxemia in the dialysis population [9, 18] whilst others have not [19, 20]. The present study was also unable to demonstrate any appreciable degree of endotoxaemia in the dialysis population, either before or after dialysis. The reasons for these apparently disparate findings possibly relate to methodological issues, including collection, storage, processing, and assay used [15]. Specifically, some studies reporting appreciable endotoxemia did not use glucan blocker to mitigate the risk of false positive results [21]. The

Table 2: Serum endotoxin levels measured in 30 people with kidney failure before HD (preHD), after HD (postHD), and 2 days later (postHD[48hrs]).

Pt no:	PreHD endotoxin (EU/ml)	Recovery %	Post HD endotoxin (EU/ml)	Recovery %	PostHD[48hrs] endotoxin (EU/ml)	Recovery %
1	<0.1	127	<0.1	96	<0.1	96
2	<0.1	98	<0.1	149	<0.1	116
3	<0.1	111	<0.1	98	<0.1	97
4	<0.1	98	<0.1	88	<0.1	118
5	<0.1	99	<0.1	101	<0.1	105
6	<0.1	126	<0.1	105	<0.1	119
7	<0.1	61	<0.1	149	<0.1	54
8	<0.1	128	<0.1	180	<0.1	176
9	<0.1	131	<0.1	114	<0.1	182
10	<0.1	148	<0.1	145	<0.1	189
11	<0.1	134	<0.1	312	<0.1	160
12	<0.1	68	<0.1	44	<0.1	55
13	<0.1	59	<0.1	49	<0.1	69
14	<0.1	58	<0.1	56	<0.1	106
15	<0.1	52	<0.1	64	<0.1	102
16	<0.1	64	<0.1	66	<0.1	80
17	<0.1	64	<0.1	51	<0.1	92
18	<0.1	59	<0.1	54	<0.1	66
19	<0.1	110	<0.1	56	<0.1	102
20	<0.1	81	**0.28**	76	<0.1	47
21	<0.1	105	<0.1	125	<0.1	121
22	<0.1	77	<0.1	83	<0.1	98
23	<0.1	113	<0.1	97	<0.1	111
24	<0.1	47	<0.1	88	<0.1	71
25	<0.1	94	<0.1	77	<0.1	89
26	<0.1	101	<0.1	82	<0.1	76
27	<0.1	165	<0.1	103	<0.1	86
28	<0.1	132	<0.1	102	<0.1	138
29	<0.1	130	<0.1	169	<0.1	133
30	<0.1	48	<0.1	133	<0.1	126

Table 3: Inflammatory markers before HD (preHD), after HD (postHD), and 2 days later (postHD48 hrs).

Pair analyzed	Pre HD vs. post HD	Post HD vs. postHD48 hrs	Pre HD vs. postHD48 hrs	Control vs. pre HD
HsCRP	6.16 (2.1, 16.8) vs. 6.79 (2.0, 17.4) ($p = 0.58$)	6.79 (2.0, 17.4) vs. 6.05 (1.3, 17.3) ($p = 0.78$)	6.16 (2.1, 16.8) vs. 6.05 (1.3, 17.3) ($p = 0.61$)	1.1 (0.81, 3.63) vs. 6.16 (2.1, 16.8) ($p = 0.015$)
IL1b	1.5 (0.05, 2.51) vs. 1.67 (0.76,2.51) ($p = 0.93$)	1.67 (0.76,2.51) vs. 1.42 (0.05, 2.28) ($p = 0.97$)	1.5 (0.05, 2.51) vs. 1.42 (0.05, 2.28) ($p = 0.88$)	0.5 (1.81, 2.95) vs. 1.5 (0.05, 2.51) $p = 0.001$
IL6	7.49 (4.56, 10.39) vs. 7.46 (5.31, 14.59) ($p = 0.31$)	7.46 (5.31, 14.59) vs. 6.38 (3.47, 10.86) ($p = 0.11$)	7.49 (IQR 5.8) vs. 6.38 (IQR 7.39) ($p = 0.1$)	6.15 (4.82, 9.12) vs. 7.49 (4.56, 10.39) $p = 0.77$
TNF	30.86 (8.0, 39.5) vs. 13.9 (8.5, 17.3) ($p = 0.002$)	13.9 (8.5, 17.3) vs. 27.37 (14.5, 35) ($p < 0.001$)	30.86 (8.0, 39.5) vs. 27.37 (14.5, 35) ($p = 0.51$)	15.37 (7.24, 23.91) vs. 30.86 (8.0, 39.5) $p = 0.16$
Ferritin	1153 (782, 1458) vs. 1313 (657, 1638) ($p < 0.001$)	1313 (657, 1638) vs. 1186 (754, 1597) ($p = 0.09$)	1153 (782, 1458) vs. 1186 (754, 1597) ($p = 0.66$)	132.9 (111, 257) vs. 1153 (782, 1458) $p < 0.001$

Median values presented with 25th and 75th centile in brackets. Data sets are available on request from the corresponding author.

methodology employed in this study has been validated and reported in another study by our group [15]. In the present study, 5 serum samples tested positive for endotoxin but were extinguished when a glucan blocker was used. Furthermore, previous studies have varied with respect to excluding underlying infection, presence of tunnelled catheters, and using ultrapure water, as in this study [12]. Another potential reason that endotoxin may be present but undetectable in plasma is its rapid protein binding effectively "hiding" it from the LAL assay. In one study, mice injected with LPS had almost undetectable levels of endotoxin at 4 hrs (>85% reduction even with high dose inoculation) but levels measured by mass spectrometry remained elevated post-innoculation [22].

Serum endotoxin has been demonstrated even in healthy volunteers and at concentrations far in excess of the minimum dose required to incite severe inflammation [23, 24]. This casts further doubt as to the accuracy of the assay when used on plasma. Lastly, only a single participant tested positive for endotoxin post-dialysis. There was no history of

hepatic or gastrointestinal disease. In this participant, the level of hsCRP, IL1b, IL6, and ferritin, pre-dialysis was lower than the median and TNF was within 10% of the median further reducing any association with endotoxin.

It is plausible that a hemodialysis session may be proinflammatory. One mechanism may be related to the large-scale immune activation on blood exposure to foreign antigen, such as dialyser membrane or tubing. Although uncommon, severe hypersensitivity type reactions are observed on dialysis. They may represent exposure to constituents of the equipment used (e.g., polyvinylpyrrolidone or bisphenol A) or direct immune activation (e.g., by complement) [25]. The effect of dialysis on inflammatory markers has yielded conflicting results, with some studies reporting no change in CRP levels [26, 27] whilst others observed significant increases [28–30]. In a post-hoc analysis of the large Netherlands Cooperative Study on the Adequacy of Dialysis (NECOSAD), only 25% of patients showed an increase in serum CRP levels during a dialysis session, which in turn was correlated with increased mortality [28]. However, there was no change in the mean CRP before and after dialysis following correcting for hemoconcentration [27]. In the current study, a hemoconcentration correction factor was not applied but would only have served to correct the postHD inflammatory value to a lower concentration, further bolstering the absence of an appreciable rise.

The present study found no elevation in serum concentrations of hsCRP, IL1β, or IL6 following dialysis. IL6 induces liver production of CRP and therefore the absence of a change in either of the cytokines before and after a session of hemodialysis is congruent. Furthermore, the biological plausibility of CRP rising within 4 hours after starting HD is questionable. CRP typically starts increasing 4–6 hours after the onset of inflammation and peaks 36–50 hours later [31]. Therefore, this may explain the absence of any difference before and after dialysis. However, in this study, there was also no appreciable increase in inflammatory markers from baseline to forty-eight hours later. Serum TNF levels actually decreased immediately post-dialysis representing a net decrease in inflammation The reduction in TNF levels most likely reflected dialytic clearance of TNF, which is comparatively smaller in size at 17.3 kDa compared to CRP (23 kDa), IL6 (26 kDa), and IL1β (30.9 KDa) [32]. Ferritin is an acute phase reactant and typically rises in response to inflammation. In this study, the observed rise in serum ferritin post-dialysis was not congruent with the other inflammatory markers. None of the study population received intravenous iron during the dialysis session. This may represent a type 1 error related to the smaller sample size.

Finally, another potential reason for the disparate inflammatory marker results with comparative studies may relate to patient selection with respect to occult infection, tunnelled catheters, or grafts.

The strengths of this study include the multiple time point sampling and the multicytokine approach. The pre-dialysis samples 48 hrs later at the next dialysis session allowed validation of the baseline day one pre-dialysis samples, which were comparable in terms of both inflammatory markers and endotoxin and also allowed examination of the potential delayed time course of inflammatory markers. In addition, the methodology used for endotoxin detection had been previously validated [15]. However, this study was limited by its small sample size and the possibility that minor changes in inflammatory markers may have been missed. However, this is unlikely to explain the lack of demonstrable difference in endotoxin at any time point. The single centre design also limited the generalisability of the study's findings.

5. Conclusion

Endotoxemia is rarely present in people receiving HD and therefore does not account for the heightened inflammation observed in this population. HD per se does not contribute to changes in serum concentrations of either endotoxin or inflammatory biomarkers. Prospective studies to further explore these findings is crucial to elucidate the origin of inflammation in the dialysis population.

Abbreviations

EU: Endotoxin units
HD: Hemodialysis
KCA: Kinetic chromogenic assay
KTA: Kinetic turbidimetric assay
LAL: Limulus amoebocyte lysate
LRW: LAL reagent water
OD: Optical density
Tris: Trisaminomethane.

Data Availability

Data sets used are available upon request to the corresponding author.

Ethical Approval

The study was approved by the Cairns and Hinterland Research Ethics Committee HREC/17/QCH/15–1118.

Consent

Written, informed consent was obtained prior to study participation. Written informed consent was obtained from the patient(s) for their anonymized information to be published in this article.

Disclosure

The funding source did not play any role in study design, conduct, analysis, interpretation, or decision to publish.

Conflicts of Interest

DJ has received consultancy fees, research funds, speaker's honoraria, and travel sponsorships from Baxter Healthcare and Fresenius Medical Care, as well as consultancy fees from Astra Zeneca, Bayer, and AWAK, speaker's honoraria from Ono and BI & Lilly, and travel sponsorships from Amgen.

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Authors' Contributions

SD researched literature and conceived the study. SD and MF were involved in protocol development, gaining ethical approval, patient recruitment, and data analysis. SD wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

Acknowledgments

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