Circulating bacterial-derived DNA fragments as a marker of systemic inflammation in peritoneal dialysis

Bonnie Ching-Ha Kwan,
Kai-Ming Chow,
Chi-Bon Leung,
Man-Ching Law,
Phyllis Mei-Shan Cheng,
Vincent Yu,
Philip Kam-Tao Li
and Cheuk-Chun Szeto

Department of Medicine and Therapeutics, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong, China

Correspondence and offprint requests to: Cheuk-Chun Szeto; E-mail: ccszeto@cuhk.edu.hk

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ABSTRACT

Background. Endotoxemia is common in peritoneal dialysis (PD) patients, and circulating lipopolysaccharide (LPS) level is related to the degree of systemic inflammation and atherosclerosis. We hypothesize that circulating bacterial DNA, another microbial component, correlates with the degree of systemic inflammation and predicts the survival of new PD patients.

Methods. We measured the plasma bacterial DNA level in the archive blood samples of 300 consecutive new PD patients. The result was compared with serum C-reactive protein (CRP) level, patient survival and peritonitis-free survival.

Results. The average age was 57.8 ± 12.1 years, average plasma bacterial DNA level 34.3 ± 1.3 cycles and average follow-up 37.9 ± 22.2 months. The plasma bacterial DNA level correlated with serum CRP (r = 0.565, P < 0.001) and LPS levels (r = 0.224, P = 0.029). At 36 months, the patient survival were 77.5, 78.3, 74.6 and 65.2% for plasma bacterial DNA level quartiles I, II, III and IV, respectively (log-rank test, P = 0.034). By multivariate analysis with the Cox proportional hazard model to adjust for confounders, the plasma bacterial DNA level had no independent effect. Similarly, peritonitis-free survival were 60.6, 59.8, 60.3 and 50.4% for plasma bacterial DNA level quartiles I, II, III and IV, respectively, at 36 months (P = 0.020), and the difference was not significant after adjusting for confounding factors.

Conclusion. We found that the plasma bacterial DNA level correlated with the degree of systemic inflammatory state in PD patients. Although plasma bacterial DNA level seems to predict patient survival and peritonitis-free survival, the association disappears after adjusting for confounding factors. Further prospective studies are needed to delineate the role of plasma bacterial DNA as a prognostic marker of renal failure patients.

INTRODUCTION

Patients with chronic kidney disease (CKD) or on long-term dialysis are at high risk of developing cardiovascular disease (CVD) [1–3]. It is now recognized that systemic inflammation plays a key role in the pathogenesis of atherosclerosis and CVD [4, 5]. Notably, nearly 50% of CKD or dialysis patients have evidence of systemic inflammation [6–8].

Previous studies showed that chronic infections amplify the risk of atherosclerosis development in the carotid arteries [9], and endotoxemia constitutes a strong risk factor of early atherogenesis in subjects with chronic or recurrent bacterial infections [10]. Epidemiological studies also show that even a low-level endotoxemia constitutes a strong risk factor for the development of atherosclerosis [11]. Circulating lipopolysaccharide (LPS), a component of bacterial cell wall, is bioactive in vivo and correlates with the degree of innate and adaptive immune activation [12]. Epidemiological study suggests that the atherogenic potential of endotoxemia is affected by concomitant...
immune activation [13]. It has recently been shown that circulating microbial products, probably derived from the gastrointestinal tract, are a cause of HIV-related systemic immune activation [12]. Another study [14] further shows that infusion of LPS led to a significant decrease in peripheral endothelial progenitor cells, which represents a strong predictor of CVD [15].

There is now evidence that circulating microbial fragment is not uncommon in CKD patients. The intestinal mucosa barrier is impaired, and bacterial translocation occurs in experimental uraemia [16]. Previous studies showed that LPS is detectable in the serum of CKD and dialysis patients, and the serum LPS level correlates with the severity of systemic inflammation and features of atherosclerosis [17–19]. However, another study actually showed that a higher baseline serum LPS level was associated with a better technique survival in new peritoneal dialysis (PD) patients [20]. The exact reason for this paradoxical phenomenon is unknown.

In addition to LPS, which is a bacterial cell wall component, other bacterial fragments could also be detected in human circulation. bacterial-derived DNA fragment is the most easily and consistently detectable component. Because most bacteria contain the highly conserved 16S rRNA gene in the genome, the sequence could be easily detected and discerned from human DNA. A recent study showed that circulating bacterial-derived DNA fragments were present in around 20% of haemodialysis (HD) patients, and that circulating bacterial-derived DNA fragments are associated with higher levels of C-reactive protein (CRP) and interleukin (IL)-6 in these patients [21]. In the present study, we explored the clinical relevance of circulating bacterial-derived DNA fragment in PD patients.

MATERIALS AND METHODS

Patient selection

The study was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong, and written informed consent was obtained from all participants. We studied the archive blood samples at the time of initiation of dialysis of 300 consecutive new PD patients in our centre from 2005 to 2008. We excluded patients who planned to have elective living donor transplant or transfer to another renal centre within 6 months. Baseline clinical data were recorded by chart review. These included age, sex, underlying renal disease and PD regimen. A panel of comorbid conditions, including coronary artery disease, heart failure, peripheral vascular disease, cerebrovascular disease, dementia, chronic pulmonary disease, connective tissue disorder, peptic ulcer disease, liver disease, diabetes with and without complications, hemiplegia, malignancy and acquired immunodeficiency syndrome, were also recorded. The modified Charlson’s comorbidity index, which was validated in PD patients [22], was used to calculate a comorbidity score. The circulating level of bacterial DNA fragment, LPS level, peritoneal equilibration test and dialysis adequacy assessment were all performed 4–6 weeks after initiation of dialysis and when the patient was in a clinically euvoletic state. In addition, we studied 20 patients with immunoglobulin A nephropathy (IgAN) and CKD Stage 1–2, 10 with IgAN and CKD Stage 3–4 and 25 HD as controls. In 25 randomly selected PD patients, the plasma bacterial DNA level was repeated 6 and 12 months later to determine the intra-individual variation.

Plasma bacterial DNA fragment

The method of bacterial DNA amplification has been described previously [21]. Briefly, DNA from a 200 μL aliquot of ethylenediaminetetraacetic acid-treated whole blood was extracted using the EZ1 DNA tissue kit and BioRobot EZ1 with the EZ1 bacteria card (Qiagen), according to the manufacturer’s instructions. Purified DNA was eluted in 50 μL of elution buffer before amplification. Universal primers used for polymerase chain reaction (PCR) amplification of the bacterial 16S rRNA gene were p16SrRNA+ and p16SrRNA−, which are able to amplify DNA from either Gram positive or Gram negative bacteria. Aliquots of 20-μL DNA samples were used for amplification in a 50-μL PCR reaction mixture. All samples were run in triplicates. Since plasma was directly used as the template and there is no intrinsic housekeeping gene for comparison, the number of PCR cycles at which bacterial DNA could be detected is reported.

Plasma LPS level

The method of plasma LPS quantification has been described previously [12]. Briefly, plasma samples were diluted to 20% with endotoxin-free water and then heated to 70°C for 10 min to inactivate plasma proteins. We then quantified plasma LPS with a commercially available Limulus Amebocyte Lysate assay (Cambrex, Verviers, Belgium) according to the manufacturer’s protocol. The detection limit of this assay was 0.01 EU/mL. Samples with the LPS level below the detection limit were taken as 0 EU/mL. All samples were run in duplicate and background subtracted.

Study of peritoneal transport

Standard peritoneal permeability test was performed by the method of Twardowski and has been described previously [23]. Briefly, a 4-h dwell study was carried out with 2 L of dextrose 2.5% dialysate fluid (Dianeal, Baxter-Travenol, Deerfield, IL, USA). Dialysate creatinine and glucose levels at 0, 2 and 4 h, plasma creatinine and glucose levels at 2 h, are measured. Drainage and ultrafiltration volumes at 4 h are documented. Dialysate-to-plasma ratios of creatinine (D/P) at 0, 2 and 4 h are calculated after correction of glucose interference. Mass transfer area coefficients of creatinine normalized for body surface area (BSA) are calculated by the formula described by Krediet et al. [24]. BSA is determined from body weight and height by nomogram [25].

Dialysis adequacy, nutrition and inflammation markers

The method of dialysis adequacy assessment has been described previously [26]. Briefly, 24-h urine and dialysate collection was performed to calculate total Kt/V. Nutritional status was represented by serum albumin level, subjective global assessment (SGA) comprehensive malnutrition-inflammation score (MIS) and normalized protein

2140
plasma bacterial DNA was 34.3 ± 1.3 cycles, and plasma LPS

Clinical follow-up

All patients were followed till June 2011. The clinical management was decided by individual clinician and not affected by the study. Outcome measures include patient survival, technique survival, peritonitis-free survival and peritonitis rate. Censoring events for patient survival include transfer to long-term HD, kidney transplant, recovery of renal function, loss to follow-up and transfer to other dialysis centres. Censoring events for technique survival include kidney transplant, recovery of renal function, loss to follow-up and transfer to other dialysis centres. For peritonitis-free survival, censoring events included transfer to long-term HD, kidney transplant, recovery of renal function, loss to follow-up and transfer to other dialysis centres.

Statistical analysis

Statistical analysis was performed by SPSS for Windows software Version 15.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as means ± SD unless otherwise specified. Data were compared by Student’s t-test, χ2 test or Pearson’s correlation coefficient as appropriate. The relationship between plasma bacterial DNA level and survival was analysed by stratifying patients into quartiles according to the bacterial DNA level. Survival rates were analysed using Kaplan–Meier survival curves. The Cox proportional hazards model was used to adjust for potential confounders and to identify independent predictors of patient, technique and peritonitis-free survival. In addition to the baseline plasma bacterial DNA level, the Cox models were constructed by age, Charlson’s comorbidity score, plasma LPS level, serum CRP, serum albumin, total Kt/V, NPNA and residual glomerular filtration rate (GFR). These parameters were selected for the construction of the Cox model because of their importance in determining the survival of PD patients. Backward stepwise elimination was applied to remove insignificant variables. A value of P < 0.05 was considered statistically significant. All probabilities were two-tailed.

RESULTS

We studied 300 consecutive new PD patients. The demographic, baseline clinical and biochemical information are summarized in Tables 1 and 2, respectively. Of the 300 patients, 180 (60.0%) received angiotensin–converting enzyme or angiotensin receptor blocker therapy, 126 (42.0%) received statins and 119 (39.7%) received aspirin. The average level of plasma bacterial DNA was 34.3 ± 1.3 cycles, and plasma LPS level was 1.95 ± 0.63 EU/mL. When compared with other patient groups, PD patients had significantly higher plasma bacterial DNA levels than those with CKD Stage 1–2 (P < 0.0001), marginally higher level than CKD Stage 3–4 (P = 0.1), while HD and PD patients had similar plasma bacterial DNA levels (P = 0.9; Figure 1).

Plasma bacteria DNA levels remained static over 12 months for the 25 randomly selected PD patients (33.3 ± 1.6, 33.9 ± 2.6 and 33.0 ± 2.5 cycles at 0, 6 and 12 months, respectively).

Relation with baseline clinical data

There was a modest but statistically significant correlation between the plasma bacterial DNA and plasma LPS levels (r = 0.224, P = 0.029). Both plasma bacterial DNA and plasma LPS levels significantly correlated with the serum CRP level (r = 0.565 and r = 0.592, respectively, P < 0.0001 for both). The plasma bacterial DNA level had significant correlation with the MIS (r = 0.163, P = 0.015) as well as an SGA score (r = -0.132, P = 0.044). In contrast, the plasma LPS level also significantly correlated with body weight (r = 0.216, P = 0.035), serum albumin level (r = 0.285, P = 0.006) and residual GFR (r = 0.217, P = 0.040). Neither plasma bacterial DNA nor plasma LPS level correlated with the Charlson’s comorbidity index 5.4 ± 2.5.

Table 1. Baseline demographic and clinical data (N = 300)

<table>
<thead>
<tr>
<th>Table 1. Baseline demographic and clinical data (N = 300)</th>
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<tbody>
<tr>
<td>Sex (M:F)</td>
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<tr>
<td>Age (years)</td>
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<tr>
<td>Body height (cm)</td>
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<tr>
<td>Body weight (kg)</td>
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<tr>
<td>Blood pressure (mmHg)</td>
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<tr>
<td>Systolic</td>
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<tr>
<td>Diastolic</td>
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<tr>
<td>Renal diagnosis, no. of cases (%)</td>
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<tr>
<td>Glomerulonephritis</td>
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<tr>
<td>Diabetic nephropathy</td>
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<tr>
<td>Polycystic kidney</td>
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<td>Hypertensive nephrosclerosis</td>
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<tr>
<td>Obstructive uropathy</td>
</tr>
<tr>
<td>Others/unknown</td>
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<tr>
<td>Pre-existing vascular disease, no. of cases (%)</td>
</tr>
<tr>
<td>Diabetes</td>
</tr>
<tr>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
</tr>
<tr>
<td>Peripheral vascular disease</td>
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<tr>
<td>Charlson’s comorbidity index</td>
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</table>
comorbidity score, peritoneal transport status or dialysis adequacy indices (details not shown).

Relation with survival
The average follow-up was 37.9 ± 22.2 months. During this period, 106 (35.3%) patients died. The causes of death were cardiac arrest (25 cases), coronary artery disease (19 cases), stroke (11 cases), peritonitis (18 cases), non-peritonitis infection (13 cases), cancer (4 cases), liver failure (1 case), termination of dialysis (9 cases), other specific cause (3 cases) and unknown (3 cases). During this period, another 40 patients had a kidney transplant, 34 were changed to long-term HD, 10 were transferred to other centres and 1 had recovery of renal function.

At 36 months, the patient survival were 77.5, 78.3, 74.6 and 65.2% for plasma bacterial DNA level quartiles I, II, III and IV, respectively (log-rank test, P = 0.034; Figure 1). By multivariate analysis with the Cox proportional hazard model to adjust for confounders, only serum albumin level and Charlson’s comorbidity score were the independent predictors of patient survival, whereas plasma bacterial DNA and plasma LPS levels had no independent effect (Table 3). Similarly, the technique survival rates were 63.9, 62.3, 58.2 and 51.5% for plasma bacterial DNA level quartiles I, II, III and IV, respectively (log-rank test, P = 0.079; Figure 2). By multivariate analysis with the Cox proportional hazard model, only serum albumin level was an independent predictor of technique survival (Table 3).

Relation with peritonitis
During the study period, there were 494 episodes of peritonitis. The overall peritonitis rate was 1 episode every 24.1 patient-months of treatment, or 0.50 episode per patient-year on PD. The peritonitis-free survival rates were 60.6, 59.8, 60.3 and 50.4% for plasma bacterial DNA level quartiles I, II, III and IV, respectively (log-rank test, P = 0.020; Figures 3 and 4). By multivariate analysis with the Cox proportional hazard model, only Charlson’s comorbidity score was an independent predictor of technique survival (Table 3). The plasma bacterial DNA level had no significant correlation with the peritonitis rate (details not shown).

DISCUSSION

In the present study, we found that plasma bacterial DNA level correlated with the degree of systemic inflammatory state and, to a lesser extent, the nutritional status of PD patients. Although plasma bacterial DNA level seems to predict patient survival and peritonitis-free survival, the association disappears after adjusting for confounding factors. Our results resemble previous reports by our group [17] as well as the others [18, 19], which showed that circulating endotoxemia is related to the severity of systemic inflammation, markers of malnutrition, degree of atherosclerosis, cardiac injury and survival in CKD patients. Similarly, Bossola et al. [21] showed that circulating bacterial-derived DNA fragments are associated with higher levels of CRP and IL-6 in HD patients [21].
The correlation between the plasma bacterial DNA and serum CRP levels is also consistent with previous reports [17, 18, 21]. It is important to note that most of the previous studies used plasma LPS (i.e. endotoxin) levels as a marker of the circulating bacterial fragment [17–19], while plasma bacterial DNA level is used in our present study. When compared with the endotoxin level, we believe that plasma bacterial DNA level may represent a more accurate measurement of the load of circulating bacterial fragment, because LPS is by and large the cell wall component of Gram negative bacteria, while the bacterial DNA assay we used detects both Gram positive and Gram negative species. In addition, although both LPS and DNA are stable molecules and methods of quantification are relatively simple, quantification of LPS is frequently complicated by the presence of circulating inhibitors [30].

In our present study, bacterial DNA fragments could be detected in almost all PD patients, which appear to be different from the previous report of Bossola et al. [21], which showed that bacterial DNA fragments were present in around 20% of HD patients. However, it is important to note that Bossola et al. used 35 PCR cycles for amplification [21], while we used up to 40 in the present study. In fact, if the threshold was set at 35 cycles, bacterial DNA could be detected in 15% of patients with CKD Stage 1–2, 50% with CKD Stage 3–4, 71.7% PD and 72% HD patients. This proportion remains slightly higher than that reported by Bossola et al. [21]. The reason of this discrepancy is not clear. It is possible that, with 40 amplification cycles, the result may be a false positive due to contamination during venesection or sample processing (especially as the blood samples were stored ones and not collected specifically for this study). However, since almost all samples also had detectable LPS levels, it seems that the low but detectable levels of bacterial DNA would be genuine.

### Table 3. Cox proportional hazards models

<table>
<thead>
<tr>
<th>Variable</th>
<th>AHR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient survival</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum albumin</td>
<td>0.877</td>
<td>0.808–0.952</td>
<td>0.002</td>
</tr>
<tr>
<td>Charlson’s comorbidity index</td>
<td>1.178</td>
<td>1.008–1.378</td>
<td>0.040</td>
</tr>
<tr>
<td>Age</td>
<td>0.994</td>
<td>0.975–1.013</td>
<td>0.5</td>
</tr>
<tr>
<td>Residual GFR</td>
<td>0.907</td>
<td>0.776–1.060</td>
<td>0.11</td>
</tr>
<tr>
<td>Plasma bacterial DNA</td>
<td>0.894</td>
<td>0.744–1.075</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>Technique survival</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum albumin</td>
<td>0.918</td>
<td>0.855–0.987</td>
<td>0.020</td>
</tr>
<tr>
<td>Charlson’s comorbidity index</td>
<td>1.107</td>
<td>0.963–1.273</td>
<td>0.17</td>
</tr>
<tr>
<td>Age</td>
<td>0.973</td>
<td>0.931–1.017</td>
<td>0.11</td>
</tr>
<tr>
<td>Residual GFR</td>
<td>0.910</td>
<td>0.767–1.080</td>
<td>0.07</td>
</tr>
<tr>
<td>Plasma bacterial DNA</td>
<td>0.856</td>
<td>0.658–1.114</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Peritonitis-free survival</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Charlson’s comorbidity index</td>
<td>1.223</td>
<td>1.052–1.422</td>
<td>0.009</td>
</tr>
<tr>
<td>Age</td>
<td>0.999</td>
<td>0.982–1.017</td>
<td>0.9</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>0.978</td>
<td>0.943–1.014</td>
<td>0.2</td>
</tr>
<tr>
<td>Residual GFR</td>
<td>0.965</td>
<td>0.906–1.029</td>
<td>0.3</td>
</tr>
<tr>
<td>Plasma bacterial DNA</td>
<td>0.829</td>
<td>0.783–1.107</td>
<td>0.3</td>
</tr>
</tbody>
</table>

AHR, adjusted hazard ratio; CI, confidence interval; GFR, glomerular filtration rate.

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**FIGURE 2**: Kaplan–Meier plot of patient survival. Patients were divided into quartiles of plasma bacterial DNA level; quartile I had the lowest, while quartile IV the highest plasma bacterial DNA level. Data are compared by the log-rank test.

**FIGURE 3**: Kaplan–Meier plot of technique survival. Patients were divided into quartiles of plasma bacterial DNA level; quartile I had the lowest, while quartile IV the highest plasma bacterial DNA level. Data are compared by the log-rank test.
A practical difficulty of our study is the method of reporting the result of circulating bacterial DNA level. Since plasma was directly used as the template and there is no intrinsic housekeeping gene for comparison, it was impossible to convert the result into the number of DNA copies per volume or per 100,000 copies of the housekeeping gene. In the previous study reported by Bossola et al. [21], a qualitative approach was used, and patients were classified into detectable and undetectable groups based on a threshold at 35 PCR cycles. Although the method of DNA detection was identical, we used a quantitative approach and reported the number of PCR cycles at which bacterial DNA could be detected. In theory, our approach should be superior as it provides information on the relative amount of bacterial DNA and, as a prognostic marker, it may allow more accurate risk stratification. Interestingly, we observed that excessive mortality risk was largely confined to patients with the highest quartile of plasma bacterial DNA level (Figure 1), which approximately corresponds to 34–35 PCR cycles.

We cannot confirm the source of circulating bacterial DNA in our patients. None of the patients in this study, however, had indwelling vascular catheter at the time of blood test or a history of peritonitis (as they were newly started on PD). On the other hand, it is generally believed that circulating bacterial fragments, either LPS or DNA, come from microbial translocation through the gastrointestinal tract [12], which is a process accentuated by uraemia [16], heart failure [31] or immune suppression [12]. Nevertheless, alternative (i.e. non-gastrointestinal) sources of bacterial fragments cannot be excluded. Because of the limitations in our original study design and availability of suitable primers, we did not attempt to characterize the bacterial species. We believe that circulating bacterial fragments do not only represent a surrogate marker of underlying vascular disease or immune dysfunction, but they are a major cause of the systemic inflammatory state. LPS directly stimulates macrophages and dendritic cells via the toll-like receptor-4 (TLR4) and triggers an inflammatory cascade, whereas bacterial DNA is recognized by TLR9 with a similar downstream signalling machinery [32].

There are a number of inadequacies in our study and our result could only be considered preliminary. Although the sample size was reasonable, our study was retrospective and based on the assay of archive blood samples. We have few data on the serial change of plasma bacterial DNA level with time, or any information on the fluid status or the severity of vascular disease for our cohort. Elaborated multivariate analysis to adjust for clinical confounding factors is therefore not possible. Since we hypothesize that circulating bacterial DNA fragment hastens the progression of vascular disease and fluid overload, assessment of vascular stiffness (e.g. by arterial pulse wave velocity), degree of large vessel atherosclerosis (e.g. by carotid intimal thickness), intravascular volume overload (e.g. by vascular pedicle width in chest radiograph [33]) and systemic overhydration (e.g. by bioimpedance spectroscopy) should ideally be performed.

Secondly, because of the limitations in study design, we do not have concurrent PD effluent samples of our patients for comparison. In an independent group of 67 stable prevalent PD patients, the bacterial DNA levels in PD effluent were 34.1 ± 1.8 cycles (Szeto, unpublished data), which is similar to the plasma levels we observed in this study.

More importantly, our result does not prove causality. It is, at least theoretically, equally possible that patients with high CRP levels may have a higher degree of systemic inflammation, which leads to generalized vascular leakage and sepsis, resulting in a higher level of gut permeability and bacterial translocation, so that the presence of bacterial DNA (and LPS) is the result rather than cause of systemic inflammation. Further studies are necessary to determine the causal role of circulating bacterial fragments in the pathogenesis of systemic inflammation.

**ACKNOWLEDGEMENT**

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**CONFLICT OF INTEREST STATEMENT**

None declared.

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