Plasma Mitochondrial DNA Level is a Prognostic Marker in Peritoneal Dialysis Patients

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Key Words
Renal failure • Survival • Cardiovascular disease

Abstract

Background/Aims: Circulating bacterial DNA fragment is related to systemic inflammatory state in peritoneal dialysis (PD) patients. We hypothesize that circulating mitochondrial DNA, which has a similar structure with bacterial DNA, correlates with systemic inflammatory state and predicts cardiovascular event in new PD patients. Methods: We measured plasma mitochondrial DNA level by quantitative polymerase chain reaction (PCR) in 197 new PD patients and 150 patients with chronic kidney disease. PD patients were followed for 24 months for the development of cardiovascular event, hospitalization, and patient survival. Results: There was a stepwise increase in plasma mitochondrial DNA level with worsening renal function. The average plasma mitochondrial DNA level was 18.0 ± 1.2 PCR cycles. Plasma mitochondrial DNA level correlated with serum CRP level (r = -0.538, p < 0.0001). At 24 months, the event-free survival was 67.4%, 66.4%, 63.4% and 44.2% for plasma mitochondrial DNA level quartiles I, II, III and IV, respectively (p = 0.049). After adjusting for confounders, plasma mitochondrial DNA level, malnutrition-inflammation score, and baseline arterial pulse wave velocity were independent predictors of composite cardiovascular end-point; each doubling in plasma mitochondrial DNA level confers 16.0% (95% confidence interval, 2.5 – 31.3%, p = 0.001) excess in risk. Plasma mitochondrial DNA also correlated with the number of hospital admission (r = -0.218, p = 0.002) and duration of hospitalization for cardiovascular reasons (r = -0.232, p = 0.001). Conclusion: Plasma mitochondrial DNA level significantly correlates with systemic inflammatory state, and is a strong predictor of cardiovascular event as well as the need of hospitalization in new PD patients.

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Published by S. Karger AG, Basel
Introduction

Cardiovascular disease (CVD) is the most common cause of mortality in chronic kidney disease (CKD) patients [1, 2]. In CKD patients, the risk of dying from CVD is higher than the risk of progressing to become dialysis-dependent [1, 2]. Similarly, CVD is an important cause of mortality and morbidity of dialysis patients [3-5]. Although CVD and CKD share a number of common risk factors [6], CKD per se contributes to the pathogenesis of CVD [7].

Systemic inflammation is often present in CKD patients and plays a central role in the pathogenesis of atherosclerosis [8-12]. However, the cause of systemic inflammation in CKD is not completely understood. There is emerging evidence that bacterial fragments are present in the circulation of CKD patients and contributes to the systemic inflammatory state [13, 14]. For example, plasma endotoxin level correlates with the degree of systemic inflammation and carotid intimal thickness in peritoneal dialysis (PD) patients [15, 16], while plasma bacterial DNA fragment level is a strong predictor of cardiovascular event and hospitalization [17].

Along this line, the role circulating mitochondrial DNA in the pathogenesis of systemic inflammation in CKD has not been explored. Mitochondrial DNA is structurally similar to bacterial DNA as both contain pro-inflammatory unmethylated CpG motifs [18-22]. Damaged mitochondria are degraded by autophagy, followed by an inflammatory cascade similar to that triggered by bacterial DNA fragments [23]. Previous studies showed that pressure overload of the heart induces a change of mitochondrial morphology, which may lead to cardiac dysfunction [24-26]. Mitochondrial DNA that escapes autophagy activates the toll-like receptor system, which results in acute cardiac damage [27, 28]. However, the role of circulating mitochondrial DNA fragment has not been examined in CKD patients.

Patients and Methods

Patient Selection

The study was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong; written informed consent was obtained from all participants and all procedures are in adherence to the Declaration of Helsinki. We studied 197 consecutive new PD patients in our center. Patients who plan to have elective living donor transplant or transfer to other renal center within 6 months were excluded. Baseline clinical data were recorded by chart review. Plasma mitochondrial DNA fragment levels, peritoneal equilibration test, and dialysis adequacy assessment were performed around 4 weeks after starting dialysis. We also studied 10 healthy subjects with normal renal function, 150 patients with pre-dialysis CKD as controls.

Plasma mitochondrial DNA level

The method of quantification of plasma mitochondrial DNA has been reported [29]. In essence, plasma DNA was prepared by a commercial kit. Real-time quantitative polymerase chain reaction (RT-QPCR) standard curves were created. Since plasma was directly used as the template and there is no intrinsic housekeeping gene for comparison, the number of polymerase chain reaction (PCR) cycles at which bacterial DNA could be detected is reported. Samples that produced no PCR products after 40 cycles were considered undetectable and the threshold cycle (CT) number was set to 40 for statistical computation.

Study of peritoneal transport

Standard peritoneal permeability test (PET) was performed by the method of Twardowski and has been described previously [30]. Briefly, a 4-hour dwell study was carried out with 2 liters of dextrose 2.5% dialysis fluid (Dianeal, Baxter-Travenol, Deerfield, IL). Dialysate creatinine and glucose levels at 0, 2 and 4 hours, plasma creatinine and glucose levels at 2 hour are measured. Dialysate-to-plasma ratios of creatinine (D/P) at 4 hours was calculated after correction of glucose interference. Mass transfer area coefficients of
creatinine (MTAC) normalized for body surface area (BSA) is calculated by a standard formula [31]. Body surface area (BSA) is determined from body weight and height by the formula of Gehan and George [32].

Dialysis adequacy, nutrition and inflammation markers

The method of dialysis adequacy assessment has been described previously [33]. Briefly, 24-hour 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 nitrogen appearance (NPNA). For SGA, the 4-item 7-point scoring system validated in PD patients was used [34]. The calculation of MIS was described previously [35]. NPNA was calculated by the modified Bergström’s formula [36]. Serum C-reactive protein (CRP) was measured by a commercially available ultra-sensitive assay (Roche Diagnostics GmbH, Mannheim, Germany).

Pulse wave velocity study

Arterial pulse wave velocity (PWV) was measured by an automatic computerized recorder and analyzed using the Complior® SP program (Artech Medical, France). The method of PWV measurement has been described previously [37]. In this study, we measured only the carotid-femoral PWV.

Clinical follow up

All patients were followed for 24 months. The clinical management was decided by individual clinician and not affected by the study. The primary end point was a composite one that consists of cardiovascular death, non-fatal myocardial infarction or stroke, hospital admission for unstable angina, coronary intervention, congestive heart failure, transient ischemic attack, cerebrovascular accident, or peripheral vascular disease that require surgical reconstruction or amputation. For the event-free survival analysis, non-cardiovascular deaths, transfer to hemodialysis, and transplantation were censored. Secondary end points include number of hospital admission, and duration of hospitalization during the study period, patient, technique survival, and peritonitis-free survival. Technique failure was defined as transfer to long-term hemodialysis. Censoring events for technique survival include kidney transplant, recovery of renal function, loss to follow up, and transfer to other dialysis centers.

Statistical analysis

Statistical analysis was performed by SPSS for Windows software version 18.0 (SPSS Inc., Chicago, IL). Data were expressed as means ± SD unless otherwise specified. Data were compared by Student’s t test, Chi square test or Pearson’s correlation coefficient as appropriate. The relationship between plasma mitochondrial DNA levels and the primary composite end point or survival was analyzed by stratifying patients into quartiles according to the mitochondrial DNA level. Survival rates were analyzed using Kaplan–Meier survival curves. The Cox proportional hazards model was used to adjust for potential confounders and identify independent predictors of the composite cardiovascular end-point, patient survival, and technique survival. In addition to baseline plasma mitochondrial DNA level, the Cox models were constructed by age, Charlson’s comorbidity score, CF-PWV, serum CRP, serum albumin, malnutrition inflammation score, SGA score, total Kt/V, NPNA and residual GFR. These parameters were selected for the construction of the Cox model because of their importance in determining the survival of PD patients. The assumption of proportional hazard was tested and confirmed by graphical methods. All variables were added independently into the Cox model. Kidney transplant and conversion to long term hemodialysis were treated as competing events in the Cox regression analysis. Backward stepwise elimination was applied to remove insignificant variables. Interaction between variables was excluded as correlation matrix shows only modest internal correlations and additional direct testing in the final model.

The number of hospital admission and duration of hospitalization are compared between plasma mitochondrial DNA level quartiles after adjusted for the duration of follow up because the data were significantly skewed. Since plasma mitochondrial DNA level is a continuous variable, the log-linear model was then used to analyze hospitalization [38, 39]. The clinical variables used for analysis were similar to those for survival analysis. A value of p < 0.05 was considered statistically significant. All probabilities were two-tailed.
Results

We studied 197 consecutive new PD patients. The demographic, baseline clinical and biochemical information are summarized in Tables 1 and 2, respectively. The average level of plasma mitochondrial DNA was 18.0 ± 1.2 cycles; serum CRP level was 4.48 ± 2.77 mg/L. The distribution of plasma mitochondrial DNA amongst PD patients is summarized in Figure 1. There is a stepwise decrease in plasma mitochondrial DNA PCR cycle number with worsening renal function (Figure 2). Post hoc subgroup analysis showed that PD patients had significantly higher plasma mitochondrial DNA levels than normal control, patients with CKD stage 2 to 3, and those with CKD stage 4 to 5 (p < 0.0001 for all comparisons).

Relation with baseline clinical data

Plasma mitochondrial DNA level significantly correlate with serum CRP level (unadjusted Pearson’s correlation coefficient, r = -0.538, p < 0.0001). There was also a modest but significant correlation between plasma mitochondrial DNA level and total cholesterol (r = -0.208, p = 0.006) as well as LDL cholesterol level (r = -0.209, p = 0.006). There was also a trend of correlation between plasma mitochondrial DNA level and residual GFR, but the result did not reach statistical significance (see Table 2). Plasma mitochondrial DNA level had no significant correlation with the Charlson’s comorbidity score, peritoneal transport status, arterial PWV, nutritional or dialysis adequacy indices (details not shown).

Relation with cardiovascular end point

The average follow up was 21.7 ± 5.2 months. During this period, 70 patients (35.5%)...
developed cardiovascular events as defined by the primary composite end point. These include hospital admission for heart failure (43 cases), non-fatal stroke (12 cases), non-fatal myocardial infarction or acute coronary syndrome (10 cases), elective admission for

Table 2. Baseline biochemical data and dialysis prescription

<table>
<thead>
<tr>
<th>Plasma mitochondrial DNA quartile</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>49</td>
<td>49</td>
<td>49</td>
<td>50</td>
<td></td>
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<tr>
<td>Malnutrition inflammation score</td>
<td>6.47±2.86</td>
<td>5.77±3.86</td>
<td>6.82±4.67</td>
<td>7.03±3.51</td>
<td>p = 0.5a</td>
</tr>
<tr>
<td>Subjective Global Assessment</td>
<td>5.43±0.63</td>
<td>5.51±0.82</td>
<td>5.41±0.96</td>
<td>5.24±1.03</td>
<td>p = 0.6a</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>9.5±2.0</td>
<td>9.2±1.2</td>
<td>9.6±1.3</td>
<td>9.3±1.6</td>
<td>p = 0.6a</td>
</tr>
<tr>
<td>Serum albumin (g/L)</td>
<td>34.7±4.3</td>
<td>34.5±4.0</td>
<td>34.3±5.0</td>
<td>34.0±5.7</td>
<td>p = 0.9a</td>
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</table>

Lipid profile

<table>
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<tr>
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<th>Total cholesterol (mmol/l)</th>
<th>Triglyceride (mmol/l)</th>
<th>LDL cholesterol (mmol/l)</th>
<th>HDL cholesterol (mmol/l)</th>
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</thead>
<tbody>
<tr>
<td>No. of patients</td>
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<td>49</td>
<td>49</td>
<td>50</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td></td>
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</table>

Peritoneal transport

<table>
<thead>
<tr>
<th></th>
<th>Ultrafiltration volume (L)</th>
<th>D/P creatinine at 4 hour</th>
<th>MTAC creatinine (ml/min/1.73 m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>49</td>
<td>49</td>
<td>49</td>
</tr>
<tr>
<td>p value</td>
<td></td>
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</table>

Dialysis adequacy

<table>
<thead>
<tr>
<th></th>
<th>Weekly total Kt/V</th>
<th>Residual GFR (ml/min/1.73 m²)</th>
<th>NPNA (g/kg/day)</th>
<th>FEBM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>49</td>
<td>49</td>
<td>49</td>
<td>50</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td></td>
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</tbody>
</table>

HDL, high density lipoprotein; LDL, low density lipoprotein; D/P, dialysate-to-plasma concentration ratio of creatinine; MTAC, mass transfer area coefficient; GFR, glomerular filtration rate; NPNA, normalized protein nitrogen appearance; FEBM, fat-free edema-free body mass by creatinine kinetics. Patients were divided to quartiles of plasma mitochondrial DNA. Quartile I had the lowest while quartile IV the highest plasma mitochondrial DNA level. Data are expressed as mean ± standard deviation or *median (inter-quartile range), and compared by one way analysis of variance (ANOVA) or Kruskal Wallis test.
coronary interventions (3 cases), and limb amputation for peripheral vascular disease (2 cases); 12 patients had cardiovascular deaths but all developed other cardiovascular events as end point before they died. At 24 months, the event-free survival was 67.4%, 66.4%, 63.4% and 44.2% for plasma mitochondrial DNA level quartiles I, II, III and IV, respectively (log rank test, p = 0.049) (Figure 3). By multivariable analysis with the Cox proportional hazard model to adjust for confounders, plasma mitochondrial DNA level, malnutrition-inflammation score, and PWV were the independent predictors of the composite cardiovascular end-point (Table 3). In this model, one fewer PCR cycle of plasma mitochondrial DNA level (approximately equivalent to doubling in plasma mitochondrial DNA level) confers a 16.0% (95% confidence interval [CI], 2.5 – 31.3%) excess in risk of developing the composite cardiovascular end point.

### Relation with survival

During the study period, 27 patients (13.7%) died. The causes of death were cardiac arrest (6 cases), coronary artery disease (2 cases), stroke (4 cases), peritonitis (5 cases), non-peritonitis infection (6 cases), cancer (1 case), and other specific causes (3 cases). During this period, another 10 patients had kidney transplant, and 6 were changed to long term hemodialysis. At 24 months, the overall patient survival was 93.3%, 89.2%, 83.1% and 75.7% for plasma mitochondrial DNA level quartiles I, II, III and IV, respectively (p = 0.11) (Figure 4), while technique survival was 83.3%, 81.6%, 79.6% and 67.5%, respectively (p = 0.25). There was no relation between plasma mitochondrial DNA level and peritonitis rate or peritonitis-free survival (details not shown).

### Relation with hospitalization

During the study period, there were altogether 548 hospital admissions, of which 163 admissions were for cardiovascular reasons; 54 patients (27.4%) did not require any hospital admission. The total duration of hospitalization was 3841 days, with 1158 days for cardiovascular reasons. The number of hospital admission and duration of hospitalization for cardiovascular reasons are compared between plasma mitochondrial DNA level quartiles and summarized in Figure 5. In short, plasma mitochondrial DNA significantly correlated with the

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**Table 3.** Cox proportional hazards model of composite cardiovascular end-point*

<table>
<thead>
<tr>
<th>variable</th>
<th>AHR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>plasma mitochondrial DNA (1 PCR cycle)</td>
<td>0.862</td>
<td>0.761 – 0.976</td>
<td>p = 0.001</td>
</tr>
<tr>
<td>MIS (1 point)</td>
<td>1.133</td>
<td>1.013 – 1.269</td>
<td>p = 0.029</td>
</tr>
<tr>
<td>PWV (1 m/sec)</td>
<td>1.174</td>
<td>1.033 – 1.333</td>
<td>p = 0.014</td>
</tr>
</tbody>
</table>

AHR, adjusted hazard ratio; CI, confidence interval; PCR, polymerase chain reaction; MIS, malnutrition inflammation score; PWV, pulse wave velocity.

*See Patients and Methods for the definition of composite cardiovascular end-point; covariates used for model construction but excluded by backward stepwise analysis: age, Charlson’s score, serum C-reactive protein, subjective global assessment score, serum albumin, total Kt/V, residual glomerular filtration rate, and normalized protein nitrogen appearance.

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**Fig. 3.** Kaplan-Meier plot of event-free survival. Patients were divided to quartiles of plasma mitochondrial DNA. Quartile I had the lowest while quartile IV the highest plasma mitochondrial DNA level. Data are compared by the log rank test.
total number of hospital admission for cardiovascular reasons \((r = -0.218, p = 0.002)\) and duration of hospitalization for cardiovascular reasons \((r = -0.232, p = 0.001)\). The correlations between plasma mitochondrial DNA level and total number of hospital admission \((r = -0.217, p = 0.002)\) as well as total duration of hospitalization \((r = -0.214, p = 0.002)\) were also significant but less strong. By multivariable analysis with the log-linear regression model to adjust for confounders, plasma mitochondrial DNA level, malnutrition inflammation score, and PWV were the independent predictors of the duration of hospitalization for cardiovascular reasons (Table 4). The result of log-linear regression model remained similar when number of hospital admission was used instead of duration of hospitalization as the dependent variable (details not shown).

**Discussion**

In the present study, we found that plasma mitochondrial DNA level increases with the severity of kidney failure. More importantly, plasma mitochondrial DNA level significantly correlates with serum CRP level, and is a strong predictor of cardiovascular event as well as the need of hospitalization in new PD patients.

![Fig. 4. Kaplan-Meier plot of patient survival. Patients were divided to quartiles of plasma mitochondrial DNA. Quartile I had the lowest while quartile IV the highest plasma mitochondrial DNA level. Data are compared by the log rank test.](image)

![Fig. 5. Comparison of (A) number of hospital admission; and (B) duration of hospitalization between quartiles of plasma mitochondrial DNA level. Quartile I had the lowest while quartile IV the highest plasma mitochondrial DNA level. Overall comparison between quartiles by Kruskal Wallis test. CVD, cardiovascular disease.](image)
The cause of elevated plasma mitochondrial DNA level in CKD and PD patients remains unclear. Nonetheless, our result is in line with the recent report by Cao et al [40], who showed that circulatory mitochondrial DNA level is elevated in patients on maintenance hemodialysis, and its level is closely correlated with chronic inflammation. To the best of our knowledge, there is no data on the role of renal clearance for circulating mitochondrial DNA fragments. Although there is a statistically significant correlation between glomerular filtrate rate and plasma mitochondrial DNA level, the latter is highly variable amongst patients with the same degree of renal failure (see Figure 2), suggesting that other factors are equally, if not more, important. Previous studies showed that in hemodynamic stress and pressure overload-induced heart failure, there is a substantial defect in cardiac oxidative capacity due to mitochondrial damage [25], up-regulation of autophagy to remove the damaged mitochondria is an important adaptive response [26], and mitochondrial DNA that escapes from autophagy further aggravates systemic inflammation and heart failure [27]. Since occult fluid overload is common amongst CKD and PD patients [41], it seems possible that plasma mitochondrial DNA level may originate from the myocardium or other edematous tissue and represent the severity of hemodynamic stress or fluid overload. Unfortunately, we did not have any formal assessment of body fluid status or cardiac function in our cohort, and the hypothesis needs to be tested by further studies.

The mechanism of circulating mitochondrial DNA induced inflammatory state and atherosclerosis is also incompletely understood. Mitochondrial DNA is structurally similar to bacterial DNA and contains unmethylated CpG motifs that binds to toll-like receptor-9 (TLR-9) [18-22], which results in downstream activation of an inflammatory cascade and potentially acute cardiac damage [27, 28]. Our previous studies showed that plasma level of bacterial DNA fragment, which activates the same TLR-9 pathway, correlates with the severity of systemic inflammation and is a strong predictor of cardiovascular event, need of hospitalization, as well as the progressive change in arterial stiffness in new PD patients [14, 17]. Taken together, these studies suggest that both endogenous (mitochondrial) and exogenous (bacterial) DNA fragments are important uremic toxins that contribute to the pathogenesis of systemic inflammation and cardiovascular disease in PD and probably CKD patients.

Although both are important, the relative contribution of endogenous and exogenous DNA fragments to the systemic inflammatory state of PD patients, however, is unknown. Based on our studies, both plasma mitochondrial and bacterial DNA levels correlate with serum CRP level and predict cardiovascular events [14, 17], but the plasma levels of the two DNA species have no internal correlation (our unpublished data). It could be argued that since the PCR cycle number of plasma mitochondrial DNA level is generally around 15 lower than bacterial DNA level, the absolute level of the former is at least 1000-fold higher than the latter. However, TLR-9 probably has a higher binding affinity to bacterial than mitochondrial DNA.

### Table 4. Independent predictors of the duration of hospitalization for cardiovascular reasons by log-linear model

<table>
<thead>
<tr>
<th>variable</th>
<th>e^{COEF}</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>plasma mitochondrial DNA level* (2-fold)</td>
<td>-0.384</td>
<td>-0.219 to -0.549</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>MIS (1 point)</td>
<td>0.104</td>
<td>0.027 to 0.181</td>
<td>p = 0.008</td>
</tr>
<tr>
<td>PWV (1 m/sec)</td>
<td>-0.090</td>
<td>-0.011 to -0.169</td>
<td>p = 0.025</td>
</tr>
</tbody>
</table>

CI, confidence interval; MIS, malnutrition inflammation score; PWV, pulse wave velocity.

NB. e^{COEF} was the exponential coefficient indicating the relative duration of hospitalization (days per year of follow up) compared to the 2-fold lower of plasma mitochondrial DNA level (i.e. one extra threshold cycle of polymerase chain reaction), 1 point less for MIS, and 1 m/sec smaller for PWV.
There are a number of inadequacies of our present study. First, the sample size estimation was based on the primary composite end point and is therefore not enough to determine the effect of plasma mitochondrial DNA on patient survival. The negative result of survival analysis may represent type 2 statistical error. As mentioned above, we do not have any information on body fluid status (for example, as measured by bioimpedance spectroscopy) or cardiac function (for example, by echocardiogram). Because of the limitation of our study design, we also do not have serial monitoring of PWV to determine the effect of circulating mitochondrial DNA on the progression of arterial stiffness.

In the present study, we report plasma mitochondrial DNA level as PCR cycles. 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. Recently, free DNA fragment level in body fluid could be quantified (as copy number per ml) by digital PCR system [42], but the high cost of such technology may preclude its use for routine clinical care.

Based on our result, it is tempting to hypothesize therapeutic measures that lower plasma mitochondrial DNA levels may reduce cardiovascular event in PD patients. However, we are not aware of any treatment that could increase the clearance of circulating DNA fragments. We have previously showed that using ultrapure dialysate for hemodialysis effectively reduces circulating endotoxin but not bacterial DNA level in hemodialysis patients [43]. Further clinical trials in this area are much needed.

Conclusion

In summary, we found that plasma mitochondrial DNA level is a strong predictor of cardiovascular event and need of hospitalization in new PD patients. Further studies are needed to determine whether therapeutic interventions that lower circulating mitochondrial DNA fragments could prevent cardiovascular disease in PD patients.

Disclosure Statement

Dr CC Szeto receives research grant and consultancy from Baxter Healthcare. The authors declare no other conflict of interest.

Acknowledgement

This study was supported by the Richard Yu Chinese University of Hong Kong (CUHK) PD Research Fund and CUHK research account 6901031. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The results presented in this paper have not been published previously in whole or part, except in abstract format.

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