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Original Article

ORIGINAL ARTICLE

Gut permeability, circulating bacterial fragments and measures of congestion in peritoneal dialysis

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ABSTRACT

Background. Limited data exist on the association between gut permeability, circulating bacterial fragment and volume overload in peritoneal dialysis (PD) patients. We measured circulating bacterial fragments, N-terminal pro B-type natriuretic peptide (NT-proBNP), calprotectin and zonulin levels, and evaluate their association with the clinical outcomes in PD patients.

Methods. This was a single-center prospective study on 108 consecutive incident PD patients. Plasma endotoxin and bacterial DNA, and serum NT-proBNP, calprotectin and zonulin levels were measured. Primary outcomes were technique and patient survival, secondary outcomes were hospitalization data.

Results. There was no significant correlation between plasma endotoxin and bacterial DNA, and serum NT-proBNP, calprotectin and zonulin levels. The Homeostatic Model Assessment for Insulin Resistance (HOMA)-2β index, which represents insulin resistance, positively correlated with plasma bacterial DNA ($r = 0.421$, $P < .001$) and calprotectin levels ($r = 0.362$, $P = .003$), while serum NT-proBNP level correlated with the severity of volume overload and residual renal function. Serum NT-proBNP level was associated with technique survival even after adjusting for confounding factors [adjusted hazard ratio (aHR) 1.030, 95% confidence interval 1.009–1.051]. NT-proBNP level was also associated with patient survival by univariate analysis, but the association became insignificant after adjusting for confounding factors (aHR 1.010, $P = .073$). Similarly, NT-proBNP correlated with the number of hospitalizations and duration of hospitalization by univariate analysis, but the association became insignificant after adjusting for confounding factors.

Conclusion. There was no correlation between markers of gut permeability, circulating bacterial fragments and measures of congestion in PD patients. Bacterial fragments levels and gut permeability are both associated with insulin resistance. Serum NT-proBNP level is associated with the severity of volume overload and technique survival. Further studies are required to delineate the mechanism of high circulating bacterial fragment levels in PD patients.

Keywords: cardiovascular disease, inflammation, metabolism

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KEY LEARNING POINTS

What was known:

- Intestinal dysbiosis and changes in intestinal epithelial barrier function may contribute to the development of cardiovascular disease in dialysis patients.

This study adds:

- There was no correlation between markers of gut permeability, circulating bacterial fragments and left ventricular strain in peritoneal dialysis patients.
- Bacterial fragments levels and gut permeability are both associated with insulin resistance.

Potential impact:

- Contrary to the usual belief, the elevated circulating bacterial fragment level in dialysis may not be caused by intestinal epithelial barrier dysfunction.

INTRODUCTION

Dialysis is a life-saving treatment for patients with end-stage kidney failure (ESKF). However, mortality among dialysis patients remains 6.1–7.8 times higher than that of the age-matched general population, with cardiovascular disease being the leading cause of death in dialysis patients [1]. The increased cardiovascular mortality in dialysis patients cannot be fully explained by traditional cardiovascular risk factors, and despite advances in dialysis facilities, cardiovascular risk in ESKF patients has not significantly improved. As a result, attention has turned to nontraditional cardiovascular risk factors, including anemia, inflammation, and abnormal bone and mineral metabolism [2].

Inflammation is a well-recognized non-traditional cardiovascular risk factor in patients undergoing peritoneal dialysis (PD). The estimated prevalence of systemic inflammation in PD patients ranges from 12% to 65% [3]. Systemic inflammation in PD patients is associated with cardiovascular, metabolic and nutritional consequences. These consequences can present clinically as accelerated atherosclerosis, vascular calcification, muscle atrophy, anorexia and erythropoietin resistance [4, 5]. The causes of inflammation in PD patients can be broadly categorized into factors related to decreased renal function and factors related to dialysis. These factors include residual renal function and uremic toxins, endotoxemia, fluid volume overload, unphysiological peritoneal dialysis fluid and peritonitis [4, 6].

In recent years, increasing attention has been given to intestinal dysbiosis and changes in intestinal epithelial barrier function in patients with chronic kidney disease (CKD). One major consequence of intestinal dysbiosis and disruption of the intestinal barrier is the transfer of bacterial fragments from the intestinal lumen into the systemic circulation [7]. Numerous studies have demonstrated that translocated bacterial fragments play a significant role in the pathogenesis of uremic toxicity, inflammation, insulin resistance, protein energy depletion and CKD progression [8–10].

In recent years, fluid overload has been increasingly recognized as a possible cause of gut permeability barrier dysfunction in CKD [11]. Edematous heart failure patients have been found to have higher plasma endotoxin levels compared with non-edematous patients and healthy volunteers [12]. In CKD, there is a correlation between small intestinal water content and plasma lipopolysaccharide level, suggesting that bowel wall edema contributes to gut permeability barrier dysfunction [13, 14]. This dysfunction is postulated to promote the translocation of bacterial fragments into the systemic circulation, resulting in systemic inflammation and cardiovascular disease. This creates a hypothetical vicious cycle, as fluid overload worsens [2].

Studies in this area were few because the assessment of gut permeability was difficult and not standardized, but zonulin and calprotectin have been proposed as potential surrogate markers [15–19]. Zonulin is the major physiological modulator of intercellular tight junctions that is involved in trafficking of macromolecules across the gut [15]. Transient zonulin release to the systemic circulation happens following an increase in intestinal permeability [16, 17]. Calprotectin is mainly found within neutrophils and is released as a consequence of neutrophil migration through the gastrointestinal epithelium due to an inflammatory process [18]. Plasma calprotectin is a systemic inflammation marker that represents the inflammatory burden of the gastrointestinal tract [19]. The objective of our present study is to determine the relation between fluid overload [as represented by N-terminal pro B-type natriuretic peptide (NT-proBNP) level], gut permeability barrier dysfunction (as represented by zonulin and calprotectin levels) and circulating bacterial fragment (i.e. plasma endotoxin and bacterial DNA fragment) levels in ESKF patients who are treated with PD, and to investigate their relationship with the clinical outcome of PD patients.

MATERIALS AND METHODS

This is a prospective observational study approved by the Joint Chinese University of Hong Kong—New Territories East Cluster Clinical Research Ethics Committee (approval number CREC-2021.740). All study procedures were in compliance with the Declaration of Helsinki.

Case selection and overall arrangement

We recruited 108 consecutive adult incident PD patients from July 2018 to December 2019 in our center. Patients who were unlikely to survive or had a plan of living donor kidney transplantation in the coming 6 months were excluded. After written informed consent, blood levels for endotoxin, bacterial DNA, NT-proBNP, zonulin and calprotectin, as well as peritoneal transport study, anthropometric measurement, dialysis adequacy and nutritional status assessment, multi-frequency bioimpedance spectroscopy study, assessment of insulin resistance and arterial pulse wave velocity study were performed around 4–6 weeks after the patient was stable on PD. Baseline clinical and laboratory data were obtained by chart review.

Comorbidity load was measured by Charlson comorbidity index as described previously [20].

Circulating bacterial fragment levels

Circulating bacterial fragment was represented by plasma endotoxin and bacterial DNA levels. Plasma endotoxin level was measured by a commercially available Limulus Amebocyte Lysate assay (Cambrex, Verviers, Belgium) as described previously [21]. All samples were diluted to 20% with endotoxin-free water and then heated to 70°C for 10 min to inactivate plasma proteins. The detection limit of the assay was 0.01 EU/mL. Plasma bacterial DNA level was measured by the QuantStudio 3D Digital Polymerase Chain Reaction (PCR) System (Life Technologies, Carlsbad, CA, USA) as described previously [22]. In essence, PCR amplification was performed by the ProFlex µPCR system, the result captured by the QuantStudio 3D Digital PCR Instrument, and analyzed by the QuantStudio Analysis Suite Software (all from Life Technologies).

Markers of gut permeability

Blood calprotectin and zonulin levels were taken as markers of gut permeability [15–18]. Serum calprotectin level was measured by the Calprotectin Human Enzyme-Linked Immunosorbent Assay Kit (Invitrogen, Waltham, MA, USA). Zonulin level was measured by the Zonulin Human Enzyme-Linked Immunosorbent Assay Kit (Cusabio Technology LLC, Houston, TX, USA). All assays were performed in duplicate. The inter-assay coefficient of variation meets the requirements given in the manufacturer's instructions.

Markers of fluid overload

The severity of fluid overload was quantified by serum NT-proBNP level and multi-frequency bioimpedance spectroscopy. Serum NT-proBNP level is regarded as a marker of left ventricular strain and intravascular fluid overload, and was measured by a commercial ELISA kit (Biomedica Medizinprodukte GmbH, catalog number SK-1204, Vienna, Austria). For multi-frequency bioimpedance spectroscopy, we used the device Body Composition Monitor® (BCM, Fresenius Medical Care, Germany). Briefly, electrodes were attached to one hand and one foot with the patient in a supine position. Extracellular water (ECW), intracellular water (ICW), ECW-to-ICW ratio (E:I ratio), lean tissue mass, adipose tissue mass, extracellular-to-intracellular volume ratio and volume of overhydration were computed in this study.

Dialysis adequacy and nutritional status

The method of dialysis adequacy assessment has been described previously [23]. In essence, 24-h urine and dialysate collection were performed for the calculation of the total Kt/V. Residual kidney function was represented by the residual glomerular filtration rate (GFR), which was calculated as the average of 24-h urinary urea and creatinine clearances [24]. Nutritional status was represented by serum albumin level, subjective global assessment score, comprehensive malnutrition-inflammation score, normalized protein nitrogen appearance and fat-free edema-free body mass. For subjective global assessment, the 4-item 7-point scoring system validated in PD patients was used. Normalized protein nitrogen appearance was

Table 1: Baseline clinical characteristics of the study population.

No. of patients	108
Age (years)	60.1 ± 12.3
Male, no. of cases (%)	58 (53.7)
Body weight (kg)	68.90 ± 28.66
Body height (cm)	162.41 ± 8.06
Body mass index (kg/m^2)	25.78 ± 8.64
Blood pressure (mmHg)	
Systolic	149.06 ± 17.72
Diastolic	79.91 ± 11.24
Primary renal disease, no. of cases (%)	
Diabetes mellitus	58 (53.7)
Hypertension	7 (6.5)
Glomerulonephritis	27 (25.0)
Polycystic kidney disease	3 (2.8)
Urological	1 (0.9)
Others	2 (1.9)
Unknown	10 (9.3)
Coexisting comorbidities, no. of cases (%)	
Diabetes	60 (55.6)
Ischemic heart disease	17 (15.7)
Previous stroke	9 (8.3)
Charlson comorbidity index	5.53 ± 2.30
Machine-assisted peritoneal dialysis, no. of cases (%)	19 (17.6)

Data are presented as mean ± SD unless otherwise indicated.

Table 2: Baseline biochemical characteristics of the study population.

No. of patients	108
Endotoxin level (EU/mL)	0.75 ± 0.54
Bacterial DNA level (copy/μL)	1.25 ± 0.60
NT-proBNP level (pmol/L)	36.58 ± 35.13
Calprotectin level (μg/mL)	1.11 ± 1.91
Zonulin level (ng/mL)	2.52 ± 2.44
Albumin (g/L)	27.91 ± 4.34
Hemoglobin (g/dL)	9.96 ± 1.49
C-reactive protein (mg/L)	2.52 ± 0.93
Glycemic profile	
Fasting plasma glucose	7.47 ± 2.46
Serum C-peptide level	9.03 ± 6.58
Fasting insulin level	23.31 ± 27.60
HOMA-IR index	7.63 ± 10.68
HOMA-2β index	66.53 ± 80.23
Body composition by multi-frequency bioimpedance	
Volume of overhydration (L)	4.60 ± 4.25
E:I ratio	1.03 ± 0.19
Lean tissue mass (kg)	39.31 ± 10.19
Adipose tissue mass (kg)	21.86 ± 12.12
Peritoneal transport	
D/P4	0.65 ± 0.14
MTAC creatinine (mL/min/1.73 m^2)	9.84 ± 6.50
Total weekly Kt/V	2.22 ± 0.81
Residual GFR (mL/min/1.73 m^2)	4.30 ± 3.48
NPNA (g/kg/day)	1.15 ± 0.25
FEBM (kg)	37.08 ± 10.15
Arterial pulse wave velocity (cm/s)	
Carotid-radial	10.45 ± 1.74
Carotid-femoral	11.00 ± 1.82

Data are presented as mean ± SD unless otherwise indicated.
D/P4, dialysate-to-plasma creatinine concentration at 4 h; MTAC, mass transfer area coefficient; NPNA, normalized protein nitrogen appearance; FEBM, fat-free edema-free body mass.

Table 3: Relation with clinical and biochemical parameters[a].

	Endotoxin	Bacterial DNA	NT-proBNP	Calprotectin	Zonulin
Body height	r = –0.055, P = .788	r = –0.127, P = .544	r = 0.048, P = .824	r = 0.002, P = .989	r = –0.061, P = .833
Body weight	r = 0.029, P = .916	r = –0.076, P = .727	r = 0.063, P = .788	r = 0.101, P = .680	r = 0.009, P = .983
Body mass index	r = 0.052, P = .797	r = –0.018, P = .962	r = 0.113, P = .575	r = 0.135, P = .544	r = 0.073, P = .788
Systolic blood pressure	r = –0.148, P = .471	r = –0.060, P = .788	r = 0.084, P = .703	r = 0.087, P = .727	r = 0.153, P = .577
Diastolic blood pressure	r = –0.003, P = .983	r = –0.080, P = .727	r = –0.071, P = .764	r = 0.068, P = .788	r = 0.118, P = .680
Charlson comorbidity index	r = –0.142, P = .680	r = –0.054, P = .792	r = 0.198, P = .193	r = –0.010, P = .983	r = 0.181, P = .471
Albumin	r = 0.210, P = .172	r = –0.021, P = .955	**r = –0.527, P = .001**	r = –0.135, P = .548	r = 0.082, P = .788
Hemoglobin	r = 0.092, P = .680	r = 0.114, P = .575	r = –0.208, P = .172	**r = –0.318, P = .026**	r = 0.006, P = .983
C-reactive protein	**r = 0.374, P = .0013**	**r = 0.457, P = .001**	r = 0.153, P = .408	r = –0.061, P = .788	r = 0.119, P = .680
Fasting plasma glucose	r = –0.086, P = 0.680	r = –0.155, P = .408	r = –0.028, P = .916	r = –0.122, P = .575	r = –0.106, P = .697
Serum C-peptide level	r = 0.123, P = .544	r = 0.059, P = .788	r = 0.010, P = .983	r = –0.080, P = .729	r = –0.115, P = .680
Fasting insulin level	r = 0.210, P = .170	**r = 0.412, P = .0013**	r = 0.173, P = .288	**r = 0.298, P = .031**	**r = 0.397, P = .012**
HOMA-IR index	r = 0.179, P = .264	**r = 0.301, P = .022**	r = 0.152, P = .408	r = 0.258, P = .087	**r = 0.342, P = .041**
HOMA-2β index	r = 0.201, P = .185	**r = 0.421, P = .0013**	r = 0.179, P = .264	**r = 0.295, P = .036**	**r = 0.362, P = .026**
Volume of overhydration	r = –0.117, P = .577	r = –0.005, P = .983	**r = 0.545, P = .001**	r = 0.106, P = .680	r = –0.005, P = .983
E:I ratio	r=-0.243, P = .106	r = 0.044, P = .863	**r = 0.619, P < .0001**	r = 0.109, P = .680	r = 0.143, P = .654
Lean tissue mass	r = 0.139, P = .532	r = –0.092, P = .680	r = –0.028, P = .928	r = 0.086, P = .729	r = –0.083, P = .552
Adipose tissue mass	r = –0.015, P = .983	r = –0.009, P = .983	r = 0.133, P = .544	r = –0.043, P = .878	r = 0.166, P = .788
D/P4	r = –0.089, P = .680	r = 0.120, P = .548	**r = 0.277, P = .031**	**r = 0.281, P = .046**	r = –0.015, P = .983
MTAC	r = –0.112, P = .575	r = 0.154, P = .408	r = 0.181, P = .215	r = 0.209, P = .215	r = –0.013, P = .983
Total weekly Kt/V	r = 0.142, P = .471	r = 0.181, P = .264	**r = –0.414, P = .0013**	r = –0.094, P = .680	r = –0.050, P = .878
Residual GFR	r = 0.022, P = .955	r = 0.072, P = .739	**r = –0.457, P = .001**	r = –0.138, P = .544	r = –0.070, P = .797
NPNA	r = 0.066, P = .783	**r = 0.255, P = .031**	r = –0.152, P = .421	r = –0.040, P = .878	r = –0.113, P = .680
FEBM	r = 0.007, P = .983	r = 0.020, P = .955	**r = 0.284, P = .026**	r = 0.146, P = .532	r = –0.048, P = .878
Carotid-radial	r = –0.012, P = .983	r = –0.123, P = .575	r = –0.047, P = .841	r = 0.012, P = .983	r = 0.123, P = .680
Carotid-femoral	r = –0.155, P = .437	r = 0.035, P = .889	r = –0.034, P = .892	r = –0.052, P = .840	r = 0.079, P = .788

[a]Spearman's rank correlation coefficients are depicted; P-values are adjusted for multiple testing by the Benjamini–Hochberg method.
D/P4, dialysate-to-plasma creatinine concentration at 4 h; MTAC, mass transfer area coefficient; NPNA, normalized protein nitrogen appearance; FEBM, fat-free edema-free body mass. Results in BOLD indicate statistical significance.

calculated by the modified Bergstrom's formula [25]. Fat-free edema-free body mass was determined by the creatinine kinetic method according to the formula described by Forbes and Bruining [26].

Assessment of insulin resistance

We measured serum insulin level and C-peptide levels. The insulin resistance was represented by the Homeostatic Model Assessment for Insulin Resistance (HOMA) of insulin resistance (IR) index [27]:

$$\text{HOMA-IR} = \text{fasting glucose (in mmol/L)} \times \text{fasting insulin (in } \mu\text{U/mL)}/22.5$$

The beta cell function was represented by the Homeostatic Model Assessment-2 beta cell function (HOMA-2β) index [27]:

$$\text{HOMA-2}\beta = 20 \times \text{fasting insulin (in } \mu\text{U/mL)} \,/\, \text{fasting glucose (in mmol/mL)} - 3.5$$

Arterial pulse wave velocity study

Arterial pulse wave velocity was measured with an automatic computerized recorder and analyzed using the Complior @ SP program (Artech Medical, France) as previously described [28]. In the present report, we computed the carotid-radial and carotid-femoral pulse wave velocity.

Clinical outcome

The overall clinical management was decided by the attending clinician and was not affected by the study. The primary outcome was patient survival and technique survival. For patient survival analysis, transferal to hemodialysis for more than 30 days, kidney transplantation, loss to follow-up, recovery from kidney function and transfer to another dialysis center were censored. For technique survival analysis, death and transferal to hemodialysis for more than 30 days were counted as events, while kidney transplantation, loss to follow-up, recovery from kidney function and transfer to another dialysis center were censored. Secondary outcome measures of this study included total number of hospital admissions and total duration of hospital stay, both adjusted for the duration of follow-up.

Statistical analysis

Statistical analysis was performed by SPSS for Windows software version 27 (SPSS Inc., Chicago, IL, USA). Descriptive data were presented as mean ± standard deviation (SD) if normally distributed and median [interquartile range (IQR)] otherwise. Baseline clinical parameters were compared by Student's *t*-test, chi-square test and one-way analysis of variance, while the correlation was analyzed by Spearman's rank correlation as appropriate. *P*-values were adjusted for multiple testing by the Benjamini–Hochberg method. The data were log-transformed before analysis if they were highly skewed. Kaplan–Meier plots were constructed for patient survival and technique survival. The NT-proBNP and calprotectin levels were divided in tertiles for analysis. Log-rank test was used to compare between the

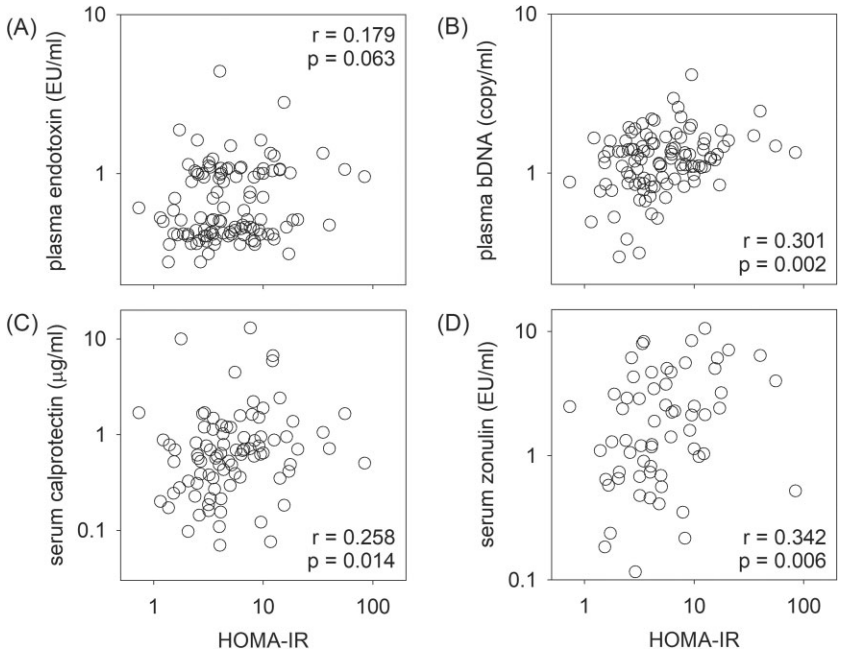

Figure 1: Correlations between HOMA-IR and plasma (**A**) endotoxin; (**B**) bacterial DNA (bDNA); and serum (**C**) calprotectin and (**D**) zonulin levels. All axis are depicted in log scale. Data are compared by Spearman's rank correlation coefficient.

curves. Univariate and multivariate Cox proportional hazards models were constructed to further identify independent predictors of patient survival and technique survival after adjustment of potential confounders. In this analysis, we included age, sex, diabetes mellitus, Charlson comorbidity index, ECW, serum albumin and C-reactive protein level for the Cox models because factors were known to be predictors for the outcome of PD patients. The final $P < .05$ was considered as statistically significant. All probabilities were two-tailed.

RESULTS

We studied 108 adult incident PD patients. Their demographic, clinical and biochemical characteristics are summarized in Tables 1 and 2. Their average plasma endotoxin level was 0.75 ± 0.54 EU/mL, bacterial DNA level 1.25 ± 0.60 copy/μL, NT-proBNP level 36.58 ± 35.13 pmol/L, calprotectin level 1.11 ± 1.91 μg/mL and zonulin level 2.52 ± 2.44 ng/mL. The internal correlations between circulating bacterial fragment, NT proBNP and gut permeability markers are summarized in Supplementary data, Table S1. In essence, there was no significant internal correlation between these parameters.

Relation with clinical and biochemical characteristics

The correlation between plasma endotoxin and bacterial DNA, and serum NT-proBNP, calprotectin and zonulin levels and other clinical and biochemical parameters are summarized in Table 3. Notably, serum C-reactive protein level had significant correlation with both endotoxin ($r = 0.374$, $P = .0013$) and bacterial DNA level ($r = 0.457$, $P = .001$). NT-proBNP level had a modest but significant correlation with serum albumin ($r = –0.527$, $P = .001$), volume of overhydration ($r = 0.545$, $P = .001$) and extracellular-to-intracellular volume ratio ($r = 0.619$, $P < .0001$), but not lean tissue mass or adipose tissue mass. Both NT-proBNP and calprotectin levels correlated significantly with peritoneal transport characteristics, while only NT-proBNP inversely correlated with total weekly Kt/V ($r = –0.414$, $P = .0013$) and residual GFR ($r = –0.457$, $P = .001$). Plasma bacterial DNA, calprotectin, and zonulin levels had significant correlation with glycemic profile including fasting insulin level, HOMA-IR index and HOMA-2β indices (see Table 3). Further subgroup analysis showed that the correlation between calprotectin and zonulin levels with HOMA-IR ($r = 0.314$, $P = .026$ and $r = 0.396$, $P = .020$, respectively) and HOMA-2 ($r = 0.326$, $P = .021$ and $r = 0.415$, $P = .015$) was significant only in patients with diabetes, but not in non-diabetic ones. In addition, plasma endotoxin level only had a modest correlation with fasting insulin level and HOMA-2β indices (Figs 1 and 2).

Relation with clinical outcome

The patients were follow for a median of 40.6 months (IQR 26.3–46.5 months). During the study period, 28 patients (25.9%) died. Their cause of death were ischemic heart disease (6 cases), sudden cardiac arrest (3 cases), stroke (2 cases), peritonitis (3 cases), non-peritonitis infection (11 cases) and other specific causes (3 cases). During this period, 17 patients were converted to hemodialysis, 3 received kidney transplantation, 1 was transferred to another dialysis center and 1 had recovery of kidney function.

The relationship between clinical and biochemical characteristics, and patient and technique survival by univariate Cox analysis is summarized in Table 4. In essence, serum NT-proBNP level, as well as Charlson comorbidity index, body weight, body mass index, volume of overhydration, E:I ratio and serum albumin, were associated with patient survival by univariate analysis. The patient survival rate at 24 months for patients with serum NT-proBNP level tertiles I to III were 97.0%, 91.0% and 84.6%, respectively (log rank test, $P = .004$) (Fig. 3). However, after adjusting for the confounders by the multivariable Cox proportional hazard model, only Charlson comorbidity index ($P = .003$) and patients' age ($P = .020$) remained as independent predictors

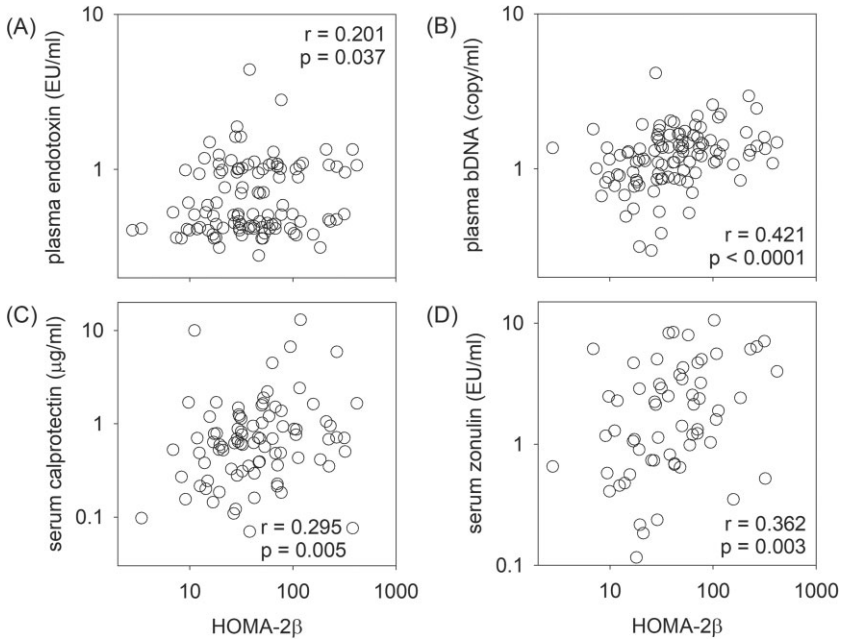

Figure 2: Correlations between HOMA-2β and plasma (**A**) endotoxin; (**B**) bacterial DNA (bDNA); and serum (**C**) calprotectin and (**D**) zonulin levels. All axis are depicted in log scale. Data are compared by Spearman's rank correlation coefficient.

of patient survival, while NT-proBNP just fell short of statistical significance [adjusted hazard ratio (aHR) 1.010, 95% confidence interval (CI) 0.999–1.021, $P = .073$] (see Table 4A).

For technique survival, serum NT-proBNP and calprotectin levels, as well as sex, diabetic status, Charlson comorbidity index, body mass index, overhydration volume, E:I ratio and adipose tissue mass, had significant association by univariate analysis (Table 4). The 24-month technique survival rates for serum NT-proBNP level tertiles I to III were 88.9%, 85.7% and 69.0%, respectively ($P < .0001$), and those for serum calprotectin level tertiles I to III were 86.4%, 79.4% and 70.0%, respectively ($P = .024$) (Fig. 3). After adjusting for other clinical confounders by the multivariate Cox regression model, NT-proBNP but not calprotectin was an independent predictor of technique survival (aHR 1.030, 95% CI 1.009–1.051, $P = .005$) (see Table 4B).

During the follow-up period, there were 345 hospital admissions for a total of 3336 days. The overall median rate of hospital admission was 0.81 (IQR 0.24–1.83) episodes per year, and the median duration of hospital stay was 4.48 (IQR 0.81–17.89) days per year. The relation between endotoxin, bacterial DNA, NT-proBNP, zonulin and calprotectin levels with adjusted hospitalization data by univariate analysis are summarized in Table 5. In essence, NT-proBNP level had a significant but modest correlation with the number of hospital admission per year ($P = .004$) and the duration of hospital stay per year ($P = .012$). However, the correlations became insignificant after adjusting for the confounders by the multiple linear regression analysis. There was no significant correlations between endotoxin, bacterial DNA, calprotectin, or zonulin levels and hospitalization parameters.

DISCUSSION

In this study, we found that there is no correlation between markers of gut permeability, circulating bacterial fragments and NT-proBNP level in PD patients. In essence, NT-proBNP level was associated with the severity of volume overload, patient survival and hospitalization with univariate analysis. However, the association of NT-proBNP level was only significant with technique survival after adjusting for clinical confounding factors by multivariable analysis. Although circulating bacterial fragment levels and gut permeability markers did not correlate with each other, they both seemed to be associated with insulin resistance.

Blood endotoxin levels are approximately 6 times higher in PD patients than in pre-dialysis CKD patients [29], indicating that PD itself aggravates endotoxemia. Endotoxin has been shown to promote the systemic inflammatory state in PD patients and is an independent predictor of patient mortality [30]. However, we did not observe any significant association between circulating bacterial fragment levels and the clinical outcome of PD patients in the present study. Previous studies reported consistent relation between circulating bacterial fragment levels and the severity of systemic inflammation [11, 28, 31], but the association with the clinical outcome of dialysis patients was controversial [21, 22, 28, 29]. Similarly, we found significant correlations between plasma endotoxin and bacterial DNA levels with serum CRP but not with the clinical outcome of PD patients in the present study.

Evidence suggests that the gastrointestinal tract is the major source of endotoxin in the circulation [31]. Alteration of gut permeability, commonly observed in CKD patients, is believed to facilitate the translocation of endotoxin and other bacterial fragments to the bloodstream, resulting in systemic inflammation and adverse cardiovascular consequences [6]. Contrary to theoretical predictions, we did not find any relationship between plasma endotoxin or bacterial DNA levels and markers of gut permeability or the clinical outcome of PD patients. Our results suggest that increased gut permeability may not be the direct cause of the increase in plasma bacterial fragment levels in CKD patients, but rather may be caused by changes in gut flora or bacterial overgrowth. On the other hand, we found a modest but significant correlation between plasma bacterial fragment levels and fasting insulin levels and insulin resistance indices. This

Table 4: Cox models of patient and technique survival.

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*-value	aHR (95% CI)	*P*-value
(A) Patient survival				
Endotoxin	1.083 (0.680–1.725)	.736		
Bacterial DNA	0.928 (0.577–1.493)	.759		
NT-proBNP	2.141 (1.300–3.527)	.003	1.010 (0.999–1.021)	.073
Calprotectin	1.359 (0.816–2.264)	.239		
Zonulin	1.085 (0.583–2.018)	.798		
Sex	1.813 (0.834–3.940)	.133	1.361 (0.528–3.505)	.523
Age	1.026 (0.989–1.064)	.168	0.948 (0.906–0.991)	.020
Diabetes mellitus	5.094 (1.766–14.696)	.003	0.450 (0.152–1.333)	.150
Charlson comorbidity index	1.311 (1.135–1.515)	.000	1.465 (1.135–1.891)	.003
Body height	1.008 (0.962–1.057)	.725		
Body weight	1.023 (1.005–1.042)	.011	0.973 (0.916–1.035)	.387
Body mass index	1.111 (1.042–1.185)	.001	1.151 (0.950–1.395)	.150
Systolic blood pressure	1.016 (0.992–1.040)	.204		
Diastolic blood pressure	0.985 (0.948–1.023)	.433		
C-reactive protein	0.873 (0.571–1.335)	.530		
Volume of overhydration	1.110 (1.036–1.190)	.003	0.972 (0.883–1.071)	.566
E:I ratio	10.800 (2.005–58.172)	.006		
Lean tissue mass	1.020 (0.981–1.062)	.316		
Adipose tissue mass	1.028 (0.994–1.063)	.102		
Carotid-radial PWV	1.156 (0.927–1.443)	.198		
Carotid-femoral PWV	1.062 (0.849–1.329)	.596		
D/P4	0.918 (0.052–16.244)	.954		
MTAC	1.032 (0.963–1.105)	.375		
Hemoglobin	0.834 (0.649–1.071)	.155		
Albumin	0.856 (0.786–0.932)	.000	0.976 (0.883–1.080)	.639
Total weekly Kt/V	0.617 (0.318–1.196)	.152		
Residual GFR	0.929 (0.814–1.061)	.276		
NPNA	2.321 (0.445–12.105)	.318		
FEBM	1.006 (0.970–1.043)	.747		
Fasting plasma glucose	1.028 (0.887–1.191)	.713		
Serum C-peptide level	0.938 (0.857–1.027)	.167		
Fasting insulin level	0.996 (0.980–1.012)	.595		
HOMA-IR index	0.994 (0.956–1.032)	.745		
HOMA-2β index	0.998 (0.992–1.003)	.413		
(B) Technique survival				
Endotoxin	1.005 (0.704–1.433)	.980		
Bacterial DNA	1.216 (0.846–1.748)	.290		
NT-proBNP	1.559 (1.089–2.232)	.015	1.030 (1.009–1.051)	.005
Calprotectin	1.651 (1.099–2.481)	.016	0.725 (0.355–1.479)	.377
Zonulin	1209 (0.774–1.889)	.404		
Sex	1.862 (1.026–3.378)	.041	0.273 (0.074–1.011)	.052
Age	0.988 (0.965–1.012)	.337	1.073 (0.953–1.210)	.245
Diabetes mellitus	1.864 (1.012–3.436)	.046	3.555 (0.345–36.589)	.286
Charlson comorbidity index	1.136 (1.012–1.276)	.031	1.209 (0.756–1.933)	.428
Body height	1.196 (0.988–1.060)	.196		
Body weight	1.019 (1.011–1.027)	.000		
Body mass index	1.067 (1.038–1.096)	.000	1.259 (0.946–1.675)	.114
Systolic blood pressure	0.977 (0.980–1.015)	.745		
Diastolic blood pressure	1.007 (0.978–1.036)	.652		
C-reactive protein	0.960 (0.705–1.305)	.792		
Overhydration volume	1.073 (1.011–1.138)	.020	0.940 (0.797–1.108)	.459
E:I ratio	4.662 (1.175–18.496)	.029		
Lean tissue mass	1.015 (0.985–1.046)	.332		
Adipose tissue mass	1.039 (1.016–1.062)	.001	0.961 (0.867–1.065)	.452
Carotid-radial PWV	1.074 (0.899–1.284)	.431		
Carotid-femoral PWV	0.929 (0.769–1.121)	.442		
D/P4	1.122 (0.126–10.027)	.918		
MTAC	1.005 (0.950–1.063)	.867		
Hemoglobin	0.926 (0.760–1.129)	.448		

Table 4: Continued.

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*-value	aHR (95% CI)	*P*-value
Albumin	0.932 (0.873–0.995)	.036		
Total weekly Kt/V	0.909 (0.608–1.358)	.640		
Residual GFR	1.000 (0.916–1.092)	.995		
NPNA	2.769 (0.806–9.520)	.106		
FEBM	1.006 (0.978–1.035)	.696		
Fasting plasma glucose	1.022 (0.912–1.146)	.703		
Serum C-peptide level	0.974 (0.923–1.029)	.351		
Fasting insulin level	1.000 (0.990–1.010)	.981		
HOMA-IR index	1.000 (0.976–1.024)	.994		
HOMA-2β index	1.000 (0.996–1.003)	.799		

D/P4, dialysate-to-plasma creatinine concentration at 4 h; MTAC, mass transfer area coefficient; NPNA, normalized protein nitrogen appearance; FEBM, fat-free edema-free body mass; PWV, pulse wave velocity.

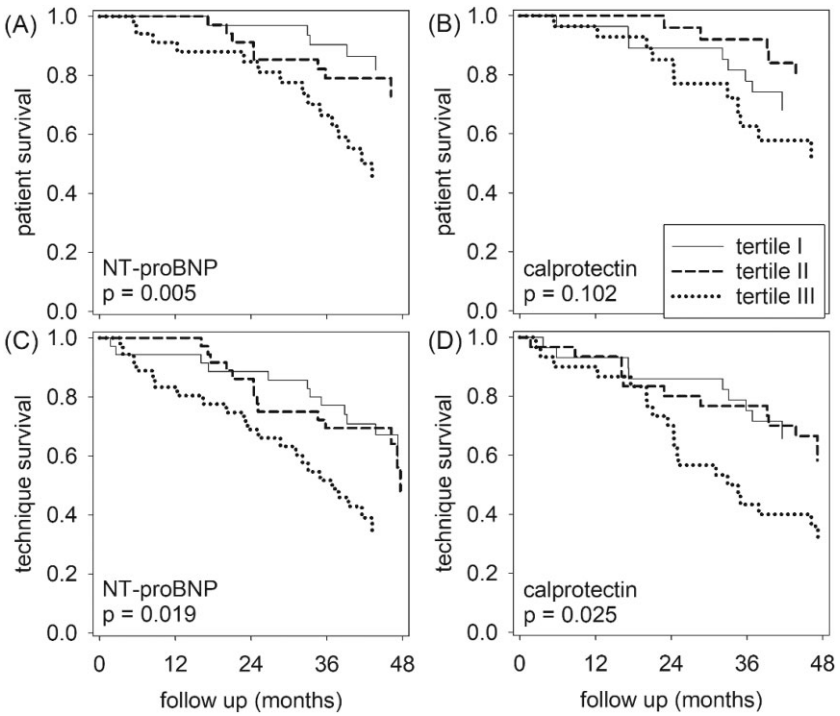

Figure 3: Kaplan–Meier plot of patient survival by the tertiles of (**A**) serum NT-proBNP; (**B**) serum calprotectin levels; and technique survival by the tertiles of (**C**) serum NT-proBNP and (**D**) serum calprotectin levels. Tertile I indicates the lowest levels. Data were compared by log-rank test.

result is consistent with previous reports on elevated plasma endotoxin levels in patients with diabetes or insulin resistance [32], which are believed to result from alterations in gut transit time and bacterial overgrowth in the small intestine [33, 34]. In fact, the correlation between insulin resistance index is more robust with plasma bacterial DNA than endotoxin level, which is in line with the notion that the bacterial DNA fragment is a more representative parameter for the circulating microbial load because it is derived from both Gram-positive and Gram-negative bacteria.

We found that serum NT-proBNP level is a predictor for technique survival and probably patient survival. Our result is consistent with previous reports [35, 36]. Its predictive power appears to be stronger than measures of left ventricular mass and systolic function, possibly due to its relationship with extracellular volume expansion [35, 36]. We found that serum NT-proBNP levels correlated with the severity of volume overload and inversely with residual renal function, which is consistent with the notion that NT-proBNP levels reflect extracellular volume expansion. Contrary to previous reports [36–38], we did not find any relation between serum NT-proBNP level and markers of systemic inflammation, suggesting that it represents the hemodynamic burden but the inflammatory load of the patient.

An unexpected finding in our present study is the correlation between insulin resistance and markers of gut permeability, i.e. serum calprotectin and zonulin levels. The mechanism of this correlation is not clear and the results need to be validated by further studies and preferably multivariable analysis to exclude the confounding effect of other clinical factor. Based on published literature, calprotectin is a marker of local intestinal

Table 5: Relation between bacterial DNA, endotoxin, NT-proBNP, zonulin and calprotectin levels with adjusted hospitalization data by univariate analysis.

	Number of hospital admission per year		Duration of hospital stay per year	
	Unstandardized B (95% CI)	P-value	Unstandardized B (95% CI)	P-value
Endotoxin	0.106 (–0.094 to 0.307)	.295	0.387 (–0.095 to 0.870)	.114
Bacterial DNA	–0.049 (–0.230 to 0.133)	.595	–0.183 (–0.622 to 0.256)	.410
NT-proBNP	0.004 (0.001 to 0.007)	.004	0.009 (0.002 to 0.017)	.012
Calprotectin	–0.040 (–0.103 to 0.023)	.209	–0.134 (–0.286 to 0.018)	.084
Zonulin	0.010 (–0.046 to 0.065)	.722	0.016 (–0.126 to 0.157)	.824

inflammatory activity and is unaffected by conditions that cause systemic inflammation [18, 39], while zonulin is a tight junction protein involved in gut permeability regulation [15–17]. Previous studies showed that calprotectin is increased in subjects with insulin resistance, obesity, type 2 diabetes and cardiovascular disease [40], whereas increase in zonulin levels were observed in patients with type 2 diabetes [41, 42]. In our present study, there was no difference in serum calprotectin or zonulin levels in patients with and without diabetes. Our finding that the correlations between calprotectin and zonulin levels with insulin resistance indices were significant in diabetic patients is consistent with the previous study by Ortgea *et al.* [40]. In addition, we also observe a trend of association between serum calprotectin, but not zonulin, level and hospitalization as well as technique survival in univariate analysis. The mechanism of these observations are not clear. Further studies would be required to explore the relation between serum calprotectin level and the risk of peritonitis.

In the present study, we did not determine the bacterial fragments, zonulin and calprotectin levels in PD effluent, which may provide additional insights on their relation with cardiovascular disease and clinical outcome. Previous studies have reported that PD effluent endotoxin and bacterial DNA levels are prognostic indicators of PD-related peritonitis [43, 44], but their relation to systemic inflammation or patient survival were not stated in these reports. To the best of our knowledge, the clinical significance of PDE zonulin and calprotectin levels had not been explored.

Our study has several limitations. Serum NT-proBNP levels are affected by cardiovascular diseases such as chronic heart failure, acute coronary syndrome and previous myocardial infarction. In our study, the underlying cardiovascular status was not thoroughly assessed. Second, we do not have the data on dietary fluid intake. Although many patients in this study had considerable residual renal function, their hydration status may be substantially affected by the amount of dietary sodium and water intake, which would inevitably have an impact on their clinical outcome. Ideally, dietary compliance should be assessed in the clinical study of volume overload in patients with kidney disease. In addition, we do not have the complete data on the peritoneal glucose load in our patients, which may have important impact on the development of insulin resistance and fluid accumulation. Finally, our present study is observational, and it only reveals correlation rather than causation.

In summary, we found no correlation between markers of gut permeability, circulating bacterial fragments and fluid overload in incident PD patients. By multivariable analysis, serum NT-proBNP level was associated with the technique survival. Plasma bacterial fragments levels and markers of gut permeability, especially bacterial DNA and calprotectin levels, respectively, are both associated with insulin resistance. Further studies are required to delineate the mechanism of high circulating bacterial fragment levels in PD patients, as well as the relation between gut permeability and insulin resistance.

SUPPLEMENTARY DATA

Supplementary data are available at *ckj* online.

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AUTHORS' CONTRIBUTIONS

C.L. performed part of the laboratory assay, analyzed the data and wrote the first draft of the manuscript. J.K.-C.N., G.C.-K.C. and W.W.-S.F collected and validated the clinical data. K.-B.L., P.Y.-K.P. and C.C.-W.L. performed additional laboratory tests. K.-M.C. was responsible for database maintenance and project administration. C.-C.S. was responsible for the original idea, overall supervision and writing the final version of the manuscript.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

CONFLICT OF INTEREST STATEMENT

C.-C.S. is member of the *CKJ* Editorial Board. The other authors have no conflicts of interest to declare.

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