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Circulating Microbial Signatures and Cardiovascular Death in Patients With ESRD

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Introduction: Patients with end-stage renal disease (ESRD) experience disproportionately high cardiovascular morbidity and mortality. Accumulating evidence suggests a role for the circulating microbiome in the pathogenesis of cardiovascular disease; however, little is known about its association with premature cardiovascular mortality in ESRD.

Methods: In a pilot case-control study of 17 hemodialysis patients who died of a cardiovascular event and 17 matched hemodialysis controls who remained alive during a median follow-up of 2.0 years, we compared the levels and composition of circulating microbiome, including Bacteria, Archaea, and Fungi, in serum samples by quantitative polymerase chain reaction and 16S or Internal Transcribed Spacer (ITS) ribosomal RNA (rRNA) sequencing, respectively. Associations of the circulating cell-free microbial signatures with clinical parameters and cardiovascular death were examined using the Spearman rank correlation and multivariable conditional logistic regression, respectively.

Results: Both 16S and ITS rRNA were detectable in all (except 3 for ITS) examined patients' serum samples. Despite no significant difference in 16S rRNA levels and α diversity between cases and controls, taxonomic analysis demonstrated differential community membership between groups, with significantly greater Actinobacteria and less Proteobacteria observed in cases than in controls at the phylum level. Proportions of Actinobacteria and Proteobacteria phyla were significantly correlated with plasma nuclear factor erythroid 2−related factor 2 (Nrf2) levels (rho = −0.41 and 0.42, P = 0.015 and 0.013, respectively) and marginally associated with risk of cardiovascular death (adjusted odds ratios [95% confidence intervals] = 1.12 [0.98−1.29] and 0.88 [0.76−1.02] for 1% increase, respectively).

Conclusion: Alterations of the circulating cell-free microbial signatures may be associated with higher premature cardiovascular mortality in ESRD.

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End-stage renal disease (ESRD) is a condition characterized by an extremely high risk of cardiovascular morbidity and mortality, consuming a disproportionate amount of financial resources.[1,2] Traditional cardiovascular risk factors (e.g., dyslipidemia) have failed to explain the increased cardiovascular risk in ESRD,[3] and interventions targeting these risk factors have been proved largely ineffective.[4–6] A growing body of evidence indicates that chronic inflammation plays an important role as a nontraditional risk factor in the excess risk of premature cardiovascular mortality in ESRD patients.[7–9] Considerable efforts have been made to reduce the inflammatory load primarily by targeting known

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causative factors, including removal of uremic toxins and optimization of dialysis procedures.[9,10] These interventions, however, are limited in their efficacy to effectively alleviate inflammatory responses,[11] and hence the substantial disease burden attributable to uremic inflammation remains a major health problem in the ESRD population.

Microbial translocation into the bloodstream can occur via different routes in patients with ESRD, including contaminated dialysate, dialysis catheter use, and impaired intestinal barrier, and can be a potential cause of chronic inflammation in these patients.[12] Although bacterial endotoxins (i.e., lipopolysaccharide [LPS]) have been extensively studied among several microbial components identifiable in the blood,[13–17] recent advances in microbial DNA sequencing have allowed the identification of highly diverse microbial communities in the systemic circulation, which is often referred to as "circulating microbiome."[18] Although the detection of microorganisms in the blood is traditionally interpreted as an indication of infection, evidence of the circulating microbial signatures is steadily accumulating among various patient populations without overt infections[19–21] and even among apparently healthy individuals.[22–25] Furthermore, both quantitative and qualitative changes in the circulating microbial signatures have recently been implicated in the pathogenesis of conditions linked to chronic inflammation, such as cardiovascular disease, potentially through their immunostimulatory, atherogenic, and cardiotoxic properties.[26–28] These results suggest that a circulating microbiome exists in patients with ESRD and may contribute to the high rates of chronic inflammation and premature cardiovascular mortality in these patients. Nevertheless, to the best of our knowledge, no previous work has described the characteristics and roles of the circulating microbiome in premature cardiovascular mortality in ESRD.

We hypothesized that the microorganisms, including not only Bacteria but also Archaea and Fungi, are chronically present in the cell-free blood fraction of patients with ESRD, and that individuals who died of a cardiovascular event would have different quantitative and qualitative circulating microbial profiles compared with those without such an event. In this pilot case-control study, we therefore aimed to measure the circulating cell-free microbial profile in the serum of patients with ESRD receiving maintenance hemodialysis and to examine its association with cardiovascular death in these patients.

MATERIALS AND METHODS

Study Design

This was a prospective study of anonymized samples and statistically deidentified clinical data obtained from a biorepository assembled by DaVita Clinical Research (Minneapolis, MN). Anonymized samples and statistically deidentified data were made available to the researcher for academic research via a grant program called BioReG.

Study Population

The DaVita Clinical Research biorepository comprises blood samples and clinical data from 4028 individuals with prevalent end-stage renal disease who received hemodialysis at a large dialysis organization between May 2011 and October 2013.[29] The biorepository sampling protocol was reviewed and approved by an Institutional Review Board (IRB) (Quorum IRB, Seattle, WA), and patients provided written informed consent prior to the initiation of sample collection. Patients with hemoglobin $<$8.0 g/dl, who were $<$18 years of age, who were pregnant, or who had any physical, mental, or medical condition that prevented the ability to provide informed consent were excluded from participation.

For the present pilot case-control study, we used biospecimens and data at baseline (i.e., first blood sampling date) from a total of 34 hemodialysis patients within the repository housed at the University of Tennessee Health Science Center (UTHSC) (UT-DaVita hemodialysis cohort; n = 978).[30] Cases (n = 17) were hemodialysis patients who died of a CV event, whereas controls (n = 17) were those who remained alive over the entire follow-up, matched 1:1 by age, sex, race, and dialysis vintage to account for major nonmodifiable cardiovascular risk factors. The study was approved by the IRB at UTHSC (IRB protocol numbers 16-04357-XP and 17-05299-XP).

Biorepository Biospecimen and Clinical Data Collection

Under the biorepository study protocol, blood samples were collected from each subject at baseline and thereafter every 3 months for up to 1 year. Pre-dialysis blood samples were collected and processed according to a standardized protocol: specimens were shipped on refrigerated packs on the day of collection to a centralized laboratory, where they were aliquoted and stored at −80°C. Specimens with cause for rejection (e.g., unspun tubes, insufficient volume, or thawed specimens) or that were received $>$48 hours from the time of collection were rejected. Anonymized plasma samples were shipped from the centralized laboratory to the researchers on dry ice at −80°C. In the present

case-control study, we used blood samples collected only at baseline.

Clinical and hemodialysis treatment data for each biorepository subject were collected by the large dialysis organization during the course of routine care and were maintained in the organization's electronic health record. Clinical and hemodialysis treatment data were provided to the researchers by DaVita Clinical Research in statistically deidentified form. Cardiovascular death was defined as death caused by acute myocardial infarction, atherosclerotic heart disease, cardiomyopathy, cardiac arrhythmia, cardiac arrest, or congestive heart failure.

DNA Extraction, Quantification, and Metagenomic Sequencing

Circulating microbial DNA was extracted from serum samples (200−500 μl) using lyticase and proteinase K according to our previously published methods.[31] 16S and Internal Transcribed Spacer (ITS) ribosomal RNA (rRNA) copy numbers (per microliter [μl] of serum) were assessed using quantitative polymerase chain reaction (qPCR) in samples of 32 (of 34) patients who had sufficient samples for DNA quantification using qPCR primers for 16S (F: 5′-TCC TAC GGG AGG CAG CAG T-3′, and R: 5′-GGA CTA CCA GGG TAT CTA ATC CTG TT-3′) and ITS (F: 5′-GTG AAT CAT CGA ATC TT GAA-3′, and R: 5′-TCC TCC GCT TAT TGA TAT GC-3′). Purified DNA samples of all 34 patients underwent amplicon sequencing at the Argonne National Laboratory (Lemont, IL), using the NextGen Illumina MiSeq platform for 16S and ITS (Supplementary Appendix). Isolated DNA was amplified using universal Bacterial and Archaeal primers for the 16S rRNA-encoding gene (F: 5′-AGA GTT TGA TCC TGG CTC AG-3′, and R: 5′-TGC TGC CTC CCG TAG GAG T-3′ for Bacteria and Archaea) and modified versions of the fungal primer set for the ITS region of ribosomal DNA (ITS1F and ITS2).[32] To minimize potential contamination, blank sample controls were run through all steps of the workflow.

Microbiome Data Processing

Sequencing data were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) 1.9.1. Sequences were first demultiplexed, then denoised and clustered into sequence variants. Representative bacterial sequences were aligned via Python Nearest Alignment Space Termination (PyNAST), and taxonomy was then assigned using the Ribosomal Database Project (RDP) Classifier to determine community profiles. For Fungi, sequences were aligned, and taxonomy was assigned using the UNITE (dynamic setting) database.[33] Processed data were imported into Calypso 8.84 for further analysis and data visualization.[34] Bacterial α diversity was assessed by Shannon, Simpson, Chao1, Richness, Fisher's alpha, and Evenness indices.[35–37] β Diversity of Bacteria was assessed using principal coordinate analysis (PCoA) by Bray−Curtis, and significance was determined using permutational multivariate analysis of variance (adonis), which measured the interindividual differences in taxa distribution within each sample (intersample).[38] Fungal operational taxonomic units were assessed for α diversity using Chao1 indices.[39] Community differences were analyzed by Spearman's rank correlation coefficient, which is presented as heatmaps. Network analysis was performed to explore possible correlations between key microbial taxa using Spearman correlations, where positive correlations with a false discovery rate−adjusted $P < 0.05$ were presented as edges.[40]

Biomarker Measurements

In addition to the variables available from the laboratory measurements obtained during routine care, plasma LPS and specific inflammatory markers including C-reactive protein (CRP), tumor necrosis factor−α (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein−1 (MCP-1), and nuclear factor erythroid 2−related factor 2 (Nrf2) were additionally measured in our pilot study. Plasma LPS levels (EU/ml) were quantified in duplicate using a Pierce LAL Chromogenic Endotoxin Quantitation Kit (ThermoFisher Scientific, Waltham, MA) as per the manufacturer's protocol. Plasma levels of CRP, TNF-α, IL-6, and MCP-1 were measured and verified using the Magnetic Luminex (Magpix) platform from R&D systems (R&D Systems, Minneapolis, MN) following the manufacturer's recommendations.[41] Nrf2 levels in whole blood were quantified by its relative gene expression against GAPDH using TaqMan Gene Expression Assays specific to Nrf2 (Hs00975961_g1; Applied Biosystems, Carlsbad, CA) and GAPDH (Hs02758991_g1; Applied Biosystems, Carlsbad, CA), as previously described.[30]

Statistical Analysis

Baseline patient characteristics by cardiovascular case status were presented as number (percentage) for categorical variables and as mean (SD) for continuous variables with a normal distribution or as median (interquartile interval [IQI]) for those with a skewed distribution. Variables with a skewed distribution were treated as log-transformed continuous variables, as appropriate. Differences between groups were assessed using the Fisher exact test, *t* test, or Wilcoxon rank-sum test, as appropriate. Because Actinobacteria and Proteobacteria were the only phyla significantly different in proportion between groups, only these 2 bacterial phyla were considered in further analyses. Correlations of serum 16S and ITS rRNA levels, α

diversity (Shannon and Chao1 Indices for bacterial and fungal communities, respectively), proportions of Actinobacteria and Proteobacteria, and selected continuous parameters (i.e., age, dialysis vintage, plasma LPS, inflammatory markers [i.e., CRP, TNF-α, IL-6, MCP-1, and Nrf2]) were evaluated using the Spearman rank correlation (rho).

We fit multivariable conditional logistic regression models to examine the risk of cardiovascular death associated with circulating microbial signatures, using serum 16S and ITS rRNA levels, Shannon and Chao1 Indices (for bacterial and fungal communities, respectively), and proportions of Actinobacteria and Proteobacteria as exposures of interest, respectively. Given the limited sample size of this pilot study, these predictors were treated as continuous variables, and the following incremental models were used to account for potential confounders on the basis of theoretical consideration and data availability: model 1 was unadjusted; model 2 included age and dialysis vintage to account for residual imbalance of these continuous matching factors; and model 3 was additionally adjusted for vascular access type. Because no between-group dissimilarity was observed for diabetes, ischemic heart disease, liver disease, HIV/AIDS, malignancies, infectious hospitalization, culture-positive bacteremia, and antibiotic use, as well as categorical matching factors (i.e., sex and race), these variables were not accounted for in this analysis. All analyses were performed in patients with complete data available.

A threshold of statistical significance was set at the level of $P < 0.05$ for all analyses unless otherwise specified. Statistical analyses were conducted in STATA/MP Version 15 (StataCorp, College Station, TX).

RESULTS

Baseline Characteristics

Patients' baseline characteristics by cardiovascular case status are presented in Table 1. Cases and controls were of similar age at baseline (means of 63.4 ± 9.9 and 61.9 ± 10.0 years, respectively) and by design did not differ for other matching factors, including sex (52.9% male in both), race (70.6% African American in both), and dialysis vintage (5.2 ± 3.0 and 5.4 ± 2.8 years, respectively). Compared with controls, cases were less likely to use a dialysis catheter and tended to have higher levels of blood hemoglobin and plasma CRP, TNF-α, and IL-6, although none of the differences reached statistical significance. In both groups, no patients had evidence of active bacterial infection, including infectious hospitalization, culture-positive bacteremia, and antibiotic use (Table 1).

Levels of Circulating Microbiome

16S rRNA was readily detectable in all serum samples examined (n = 32), whereas ITS rRNA was detected in all but 3 serum samples examined (i.e., 2 in cases and 1 in controls). There was no significant difference in serum 16S rRNA copy numbers between cases and controls (58,672 ± 19,546 and 64,424 ± 26,259 copy numbers/μl, respectively, $P = 0.48$) (Figure 1). Compared with cases, controls tended to have higher serum ITS rRNA copy numbers, but no significant between-group difference was observed (8,728 [6,123, 19,426] and 19,357 [8,320, 22,144] copy numbers/μl in cases and controls, respectively) (Supplementary Figure S1). Although our analysis targeted inclusion of Archaeal taxa, almost no evidence (minimal reads in a single control patient) was found for the existence of intact Archaeal DNA in circulation.

No significant correlations were observed for 16S and ITS copy numbers with age, dialysis vintage, LPS, and inflammatory markers, respectively (Table 2 and Supplementary Table S1).

Composition of Circulating Microbiome

α and β Diversity

There were no significant differences in bacterial α diversity, as assessed by the Shannon Index, between cases and controls (2.3±0.4 and 2.3±0.5, respectively, $P = 0.60$) (Figure 2a) as well as by the Simpson, Chao1, Richness, Fisher α, or Evenness Index (Supplementary Figure 2A−E).

We did not observe major separation in bacterial β diversity between groups (adonis $R^2 = 0.026$; $P = 0.63$) (Figure 2b). Fungal community α diversity assessed using the Chao1 Index demonstrated significantly increased diversity in cases versus controls (36.5 ± 9.0 vs. 31.2 ± 5.1, $P = 0.044$) (Supplementary Figure S3).

In the correlation analysis, except for a significant positive correlation between bacterial α diversity (Shannon Index) and CRP levels (rho=0.40, P=0.025) (Table 2), none of the correlations with bacterial and fungal α diversity were statistically significant (Table 2, Supplementary Table S1).

Taxonomic Analysis

Taxonomic analysis demonstrated a striking difference in the bacterial community membership between groups. Globally, this is displayed by the heatmap in Figure 3a. Although the circulating microbial taxa were dominated by Firmicutes (~50%) followed by Actinobacteria and Proteobacteria at the phylum level in each group, cases (vs. controls) displayed significantly greater Actinobacteria (29.0% vs. 17.7%, $P < 0.01$) and less Proteobacteria (8.9% vs. 25.2%, $P = 0.013$) (Figure 3b, c). At the genus level, *Staphylococcus* was markedly elevated in cases compared with controls,

Table 1. Baseline patient characteristics by cardiovascular case status

Characteristic	Cases (n = 17)	Controls[a] (n = 17)	*P*
Age, yrs	63.4 ± 9.9	61.9 ± 10.0	0.95
Male sex	9 (52.9)	9 (52.9)	1.00
Race			1.00
White	4 (23.5)	4 (23.5)	
African American	12 (70.6)	12 (70.6)	
Others	1 (5.9)	1 (5.9)	
Dialysis vintage, yrs	5.2 ± 3.0	5.4 ± 2.8	0.85
Dialysis membrane materials			1.00
PAES/PVP	14 (82.3)	13 (76.4)	
Polysulfone	2 (11.8)	2 (11.8)	
Cellulose triacetate	1 (5.9)	2 (11.8)	
Vascular access type			0.34
Arteriovenous fistula	14 (82.3)	10 (58.8)	
Arteriovenous graft	2 (11.8)	3 (17.7)	
Catheter	1 (5.9)	4 (23.5)	
Body mass index, kg/m^2	31.4 ± 8.2	31.3 ± 6.2	0.97
Systolic BP, mm Hg	147.1 ± 23.3	154.5 ± 22.5	0.35
Diastolic BP, mm Hg	77.5 ± 16.8	78.3 ± 20.7	0.90
Body temperature, °F	97.0 ± 1.0	97.1 ± 0.8	0.85
Charlson Comorbidity Index	5.7 ± 1.7	6.0 ± 1.7	0.62
Comorbidities			
Diabetes mellitus	12 (70.6)	12 (70.6)	1.00
Ischemic heart disease	3 (17.7)	3 (17.7)	1.00
Congestive heart failure	3 (17.7)	1 (5.9)	0.60
Liver disease	1 (5.9)	1 (5.9)	1.00
HIV/AIDS	0 (0)	0 (0)	1.00
Malignancies	0 (0)	0 (0)	1.00
Infectious hospitalization	0 (0)	0 (0)	1.00
Culture-positive bacteremia	0 (0)	0 (0)	1.00
Laboratory parameters			
Blood hemoglobin, g/dl	11.3 ± 1.4	10.5 ± 0.9	0.054
Serum albumin, g/dl	4.0 ± 0.3	3.9 ± 0.3	0.38
Serum calcium, mg/dl	9.3 ± 0.6	9.0 ± 0.7	0.13
Serum phosphorus, mg/dl	5.2 ± 1.8	5.2 ± 1.6	0.90
Serum ALP, U/l	106.2 ± 35.8	109.9 ± 58.2	0.82
Serum intact PTH, pg/ml	409 [288, 692]	351 [253, 492]	0.33
Plasma LPS, EU/ml	0.10 [0.07, 0.12]	0.12 [0.11, 0.18]	0.097
Plasma CRP, mg/l	2.4 [0.7, 3.8]	1.8 [0.9, 3.0]	0.98
Plasma TNF-α, pg/ml	9.9 ± 2.6	8.9 ± 3.3	0.36
Plasma IL-6, pg/ml	5.3 [2.4,10.6]	2.7 [2.2, 3.6]	0.11
Plasma MCP-1, ng/ml	156.8 ± 61.5	173.8 ± 45.2	0.37
Nrf2 expression, $\times 10^{-2}$, RQ)	6.9 ± 0.9	7.4 ± 1.2	0.14
Medications			
Antibiotics	0 (0)	0 (0)	1.00
Oral irons	0 (0)	0 (0)	1.00
Immunosuppressants	0 (0)	0 (0)	1.00
ESAs	13 (76.5)	13 (76.5)	1.00
Phosphate binders	15 (88.2)	15 (88.2)	1.00
Vitamin D analogs	5 (29.4)	3 (17.6)	0.69
NSAIDs	0 (0)	0 (0)	1.00

Data are presented as number (percentage), mean ± SD, or median [interquartile interval].
ALP, alkaline phosphatase; BP, blood pressure; CRP, C-reactive protein; ESAs, erythropoiesis-stimulating agents; IL-6, interleukin-6; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein–1; NSAIDs, nonsteroidal anti-inflammatory drugs; Nrf2, nuclear factor erythroid 2–related factor 2; PAES/PVP, polyarylethersulfone/polyvinylpyrrolidone; RQ, relative quantity; TNF-α, tumor necrosis factor–α.
[a]Matched by age, sex, race, and dialysis vintage.

whereas *Sphingomonas*, *Pseudomonas, Dermacoccus,* and *Thermoanaerobacterium* were greater in controls, albeit not reaching statistical significance (Figure 3d). Network analysis independently confirmed the association of co-occurring bacterial genera between the respective groups (Figure 3e). Although no significant

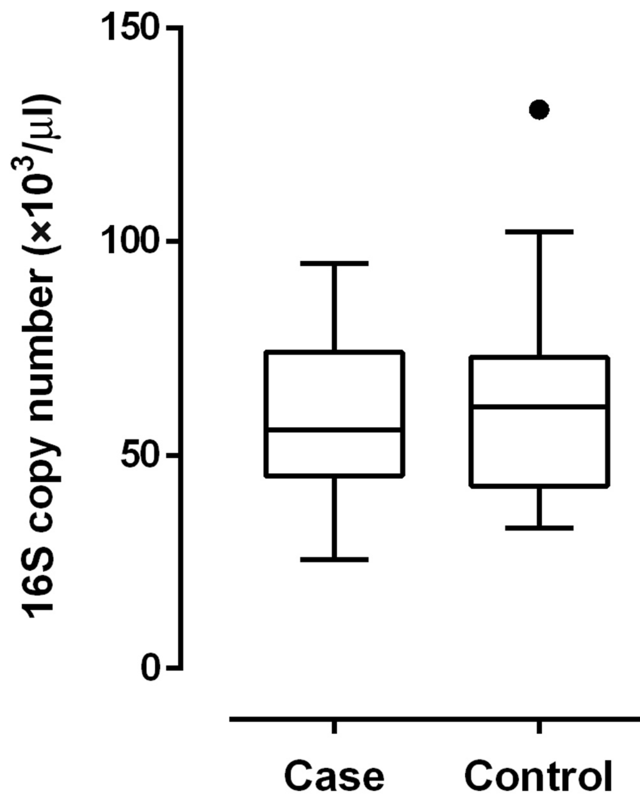

Figure 1. 16S rRNA copy numbers (per microliter [μl] of serum) in cardiovascular cases and controls. rRNA, ribosomal RNA.

taxonomic differences were observed in the fungal community between groups, cases (vs. controls) exhibited differential fungal community signatures at the genus level (Supplementary Figure S4).

In the correlation analysis, the Actinobacteria phylum had a significant negative correlation with Nrf2 levels (rho = −0.41, P = 0.015) (Table 2), with lower Nrf2 levels detected in patients with a higher proportion of Actinobacteria. In contrast, a significant positive correlation with Nrf2 levels was observed for the Proteobacteria phylum (rho = 0.42, P = 0.013) (Table 2).

Associations of Circulating Microbial Signatures With Cardiovascular Death

Table 3 shows the associations of 16S rRNA levels, bacterial α diversity (Shannon Index), and proportions of Actinobacteria and Proteobacteria phyla with cardiovascular death, using univariable and multivariable conditional logistic regression analyses. In the univariable model, Actinobacteria and Proteobacteria phyla were marginally associated with cardiovascular death, with its higher risk seen in patients with higher and lower proportions of Actinobacteria and Proteobacteria, respectively (odds ratios [ORs] [95% confidence interval {CI}] for 1% increase = 1.11 [0.99−1.22] and 0.92 [0.84−1.01], respectively, in model 1) (Table 3). These marginal associations of Actinobacteria and Proteobacteria phyla with cardiovascular death remained largely similar even after adjustment for age, dialysis vintage, and vascular access (adjusted ORs [95% CI] = 1.12 [0.98−1.29] and 0.88 [0.76−1.02], respectively, in model 3) (Table 3). No significant associations were observed for fungal ITS rRNA levels and α diversity with cardiovascular death (Supplementary Table S2).

DISCUSSION

In this pilot case-control study of ESRD patients receiving maintenance hemodialysis, we found that patients who died of a cardiovascular event had significantly greater Actinobacteria and less Proteobacteria phyla in their serum than those without a fatal cardiovascular event, although 16S rRNA levels and bacterial α diversity were similar between the 2 groups. Furthermore, we demonstrated that a higher proportion of Actinobacteria and a lower proportion of Proteobacteria were marginally associated with higher risk of cardiovascular death, independent of age, sex, race, dialysis vintage, and vascular access type.

With recent scientific interest in the microbiome, mounting evidence points to the role of the circulating microbiome in the pathogenesis of cardiovascular disease, potentially through the processes mediated by its quantitative and/or qualitative changes.[42] In terms of quantitative changes, bacterial DNA contains unmethylated cytosine–guanine dinucleotide, with 2 purine 5′ and 2 pyrimidine 3′ (CpG) terminations, which are the structures required to interact with toll-like receptors (TLRs) expressed on both immune and

Table 2. Correlations of levels and composition of the circulating microbiome with selected clinical parameters in hemodialysis patients

Characteristic	Age	Vintage	LPS	CRP[a]	TNF-α	IL-6[a]	MCP-1	Nrf2
16S rRNA	−0.18	0.014	0.11	0.27	0.30	−0.20	−0.33	−0.078
16S Shannon Index	0.093	0.081	−0.017	0.40[b]	0.016	0.094	0.12	0.22
Actinobacteria	0.26	0.13	−0.039	−0.13	0.20	0.095	−0.086	−0.41[b]
Proteobacteria	−0.30	0.17	0.26	0.28	0.0011	0.13	0.10	0.42[b]

Data are presented as Spearman rank correlation (rho).
CRP, C-reactive protein; IL-6, interleukin-6; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein−1; Nrf2, nuclear factor erythroid 2−related factor 2; rRNA, ribosomal RNA; TNF-α, tumor necrosis factor−α.
[a]Values were log transformed.
[b]P <0.05.

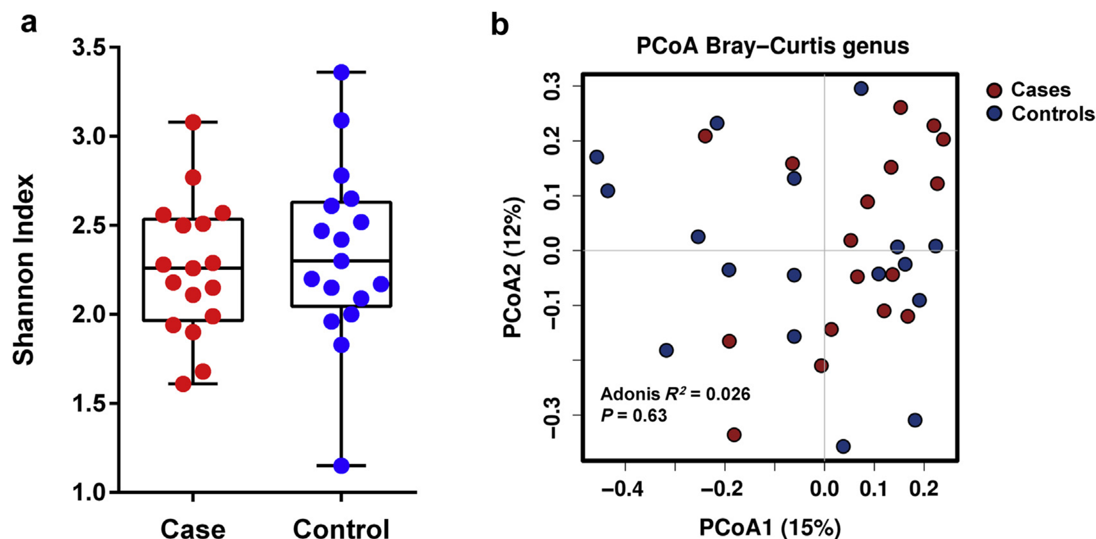

Figure 2. Bacterial (a) α diversity assessed using Shannon Index and (b) β diversity (Bray–Curtis) in serum of cardiovascular cases and controls.

nonimmune (fibroblast, endothelial, epithelial, and cardiomyocyte) cells.[43–45] Increased bacterial DNA can therefore trigger various cell signaling pathways including key regulators of inflammation (e.g., the Nrf2-antioxidant response element pathway)[45] and have been involved in endothelial injury through induction of endothelial cell apoptosis.[46] Furthermore, bacterial DNA has also been shown to cause dose-dependent suppression of cardiac myocyte contraction *in vitro*.[47] Although our pilot results showed no significant association of 16S rRNA copy numbers with cardiovascular death, a few larger clinical studies have demonstrated the independent association of higher plasma bacterial DNA levels with higher risk of cardiovascular events in ESRD patients.[48,49]

In contrast to these observations, less is known about the roles of qualitative and compositional changes in the circulating microbiome in cardiovascular disease. In a pioneering cohort study investigating the longitudinal association between circulating microbial signatures and cardiovascular events in the general population, a higher (vs. lower) relative abundance of Proteobacteria phylum in peripheral blood leukocytes was significantly associated with a higher risk of incident cardiovascular events, independent of traditional cardiovascular risk factors.[26] Similar to this finding, a subsequent cross-sectional study demonstrated a significantly higher relative abundance of Proteobacteria phylum in whole blood of patients with cardiovascular disease compared with that of apparently healthy individuals.[27] These studies, however, did not include patients with prevalent kidney disease, including those with ESRD on hemodialysis, who display unique phenotypic features (e.g., premature cardiovascular morbidity and mortality) that are distinct from those of other patient populations.[11,50] In addition, the circulating microbial signatures in these previous studies was assessed using leukocyte-containing blood fractions, the nature of which may be different from that of the circulating "cell-free" microbial signatures. More specifically, compared with microbial DNA that is located predominantly in the buffy coat (which contains the platelets and leukocytes) presumably due to the bacterial entrapment by leukocytes,[24] the circulating cell-free microbiome located in the serum or plasma may exert its potential biological effects on cardiac myocytes and on immune system cells in a more direct manner (through their surface receptors [e.g., TLR-9][43]), which could therefore serve as a novel diagnostic/prognostic biomarker with a potential for immediate clinical applicability. For these reasons, we used serum samples to assess the circulating microbial signatures in the present study. Despite the perceived concern about the detectability of microbial DNA in the cell-free blood fraction, we have successfully shown the detectability of circulating microbial signatures, including not only Bacteria but also Fungi, in a serum fraction of ESRD patients without clinical evidence of systemic infection. Most importantly, we preliminarily demonstrated, for the first time, that the qualitative changes in microbial community membership in circulation may be associated with risk of cardiovascular death. Although our results showing a lower proportion of Proteobacteria in cardiovascular cases (vs. controls) seems contradictory to the findings of the aforementioned previous studies, this may be explained by the differences in study population and/or blood fractions used to assess the

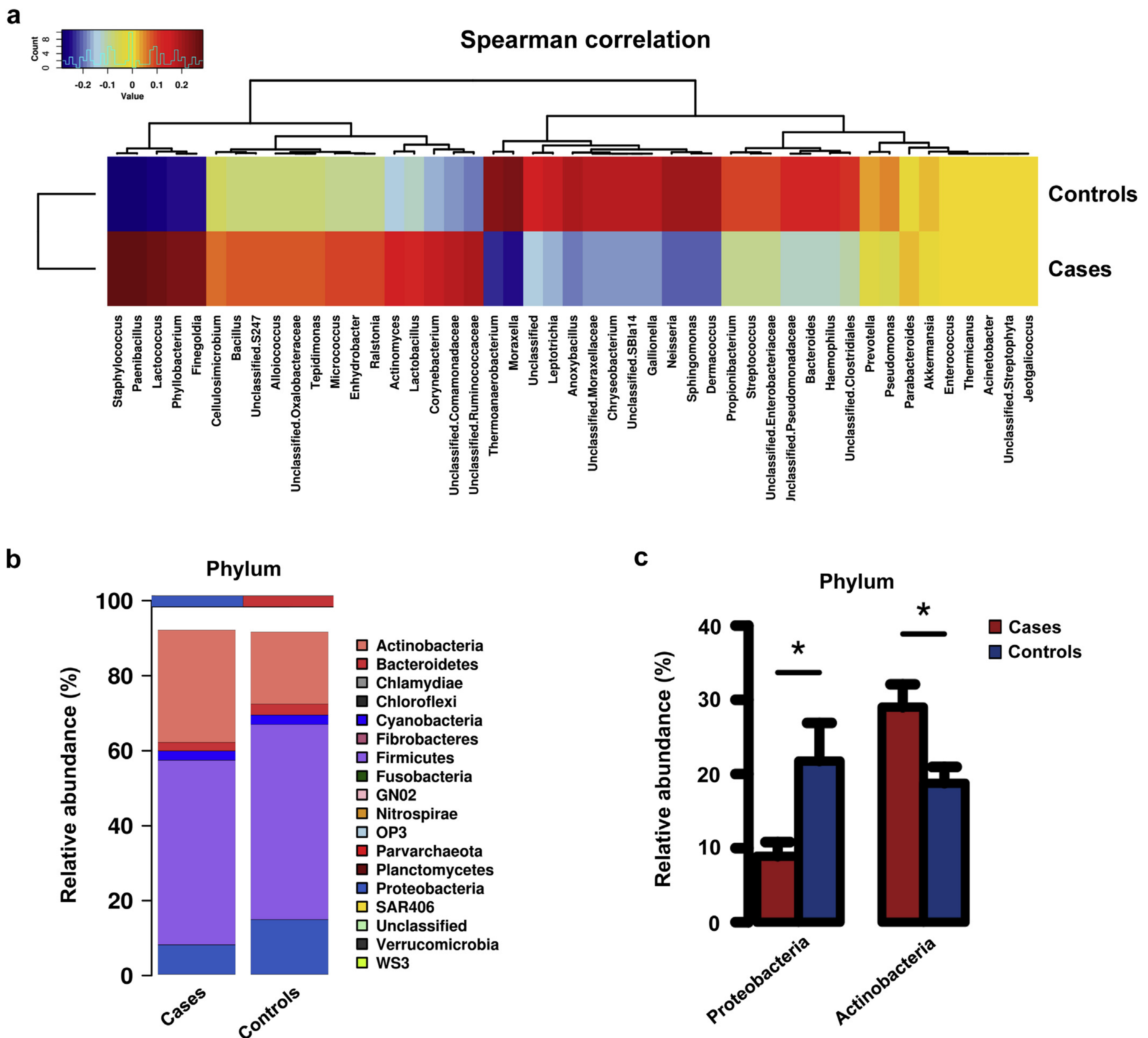

Figure 3. Compositional changes in the circulating microbiome in cardiovascular cases and controls. (a) Heatmap of bacterial community at the genus level. (b) Relative abundance of bacterial taxa* at the phylum level. (c) Relative abundance of Proteobacteria and Actinobacteria phyla. (Continued)

circulating microbial signatures between studies. Nonetheless, it is important to note that similar taxonomic changes (i.e., greater Actinobacteria and less Proteobacteria phyla) have also been reported in a plasma fraction of patients with cardiovascular disease (vs. healthy individuals).[28]

Although the precise mechanisms underlying the association between qualitative changes in the circulating microbial signatures and cardiovascular disease remain unclear, the significant correlations of Actinobacteria and Proteobacteria phyla with Nrf2 levels observed in this study may be of a particular value, with potential clinical and research implications. Nrf2, a master regulator of antioxidative responses, is known to play a critical role in the regulation of innate immunity against bacterial infection.[45] The dysregulation of Nrf2 activation has in turn been implicated in the pathogenesis not only of immune dysfunctions but also of various pathological conditions such as neurodegeneration, cancer, and cardiovascular disease,[51–54] all of which are commonly seen in patients with ESRD. Albeit speculative, the lower Nrf2 levels associated with both greater Actinobacteria and lesser Proteobacteria phyla may therefore serve as a possible explanation for the observed association between these taxonomic changes and risk of cardiovascular death in this population. This may also be supported by a recent study demonstrating the inverse correlation

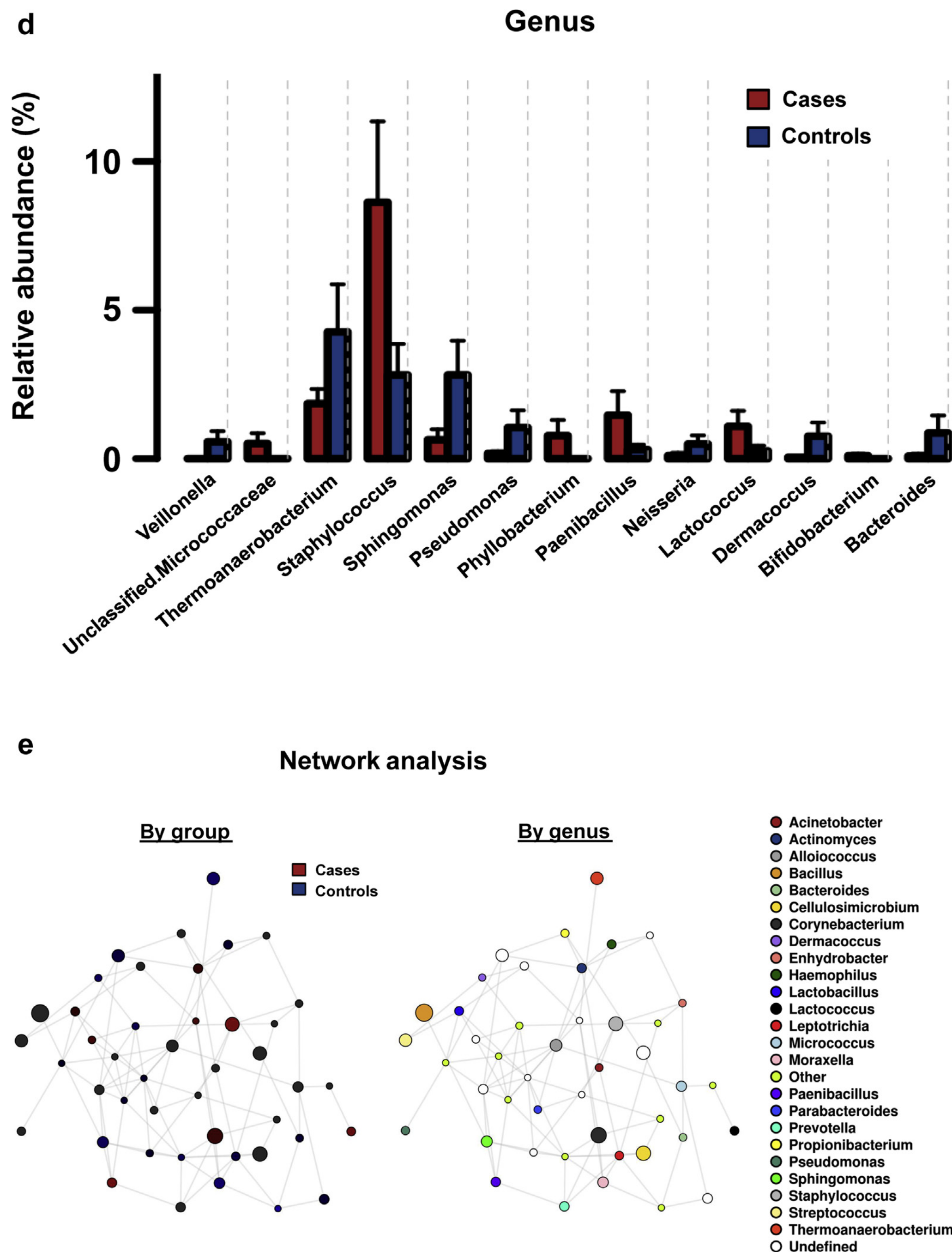

Figure 3. (Continued) (d) Relative abundance of selected bacterial taxa at the genus level. (e) Network analyses of bacterial community by group and by genus. *Bacteria phyla with a mean relative abundance >1% are presented.

between Proteobacteria phylum in subgingival plaque and systemic inflammation,[55] suggesting a possible influence of oral microbiota on the circulating microbial signatures. Given the *Staphylococcus* genus being the dominant microbial component in dysbiotic oral microbiota,[56,57] our results showing a higher proportion of *Staphylococcus* genus in the blood of cases (vs. controls) might further support this speculation. These plausible mechanisms may deserve future in-depth investigation.

The study results must be interpreted in light of several limitations. Our study sample was not representative of patients with ESRD who are heterogeneous with various etiologies and comorbidities. Because of the small sample size of this pilot study, we were unable to fully account for potential confounders despite matching. Also, we were underpowered to detect a clear association between circulating microbial signatures and cardiovascular death, and a type II error (false negative) cannot be excluded. It is

Table 3. Odds ratios and 95% confidence interval for cardiovascular death associated with levels and composition of the circulating microbiome in hemodialysis patients

Characteristics	Model 1		Model 2		Model 3	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
16S rRNA[a], per log10 ng/μl	0.28 (0.005–15.1)	0.53	0.13 (0.001–16.1)	0.40	0.38 (0.002–85.7)	0.73
Shannon Index, per unit	0.75 (0.21–2.71)	0.66	1.09 (0.24–4.87)	0.91	0.92 (0.17–4.92)	0.71
Actinobacteria, per percent	1.11 (0.99–1.22)	0.053	1.14 (0.99–1.30)	0.067	1.12 (0.98–1.29)	0.086
Proteobacteria, per percent	0.92 (0.84–1.01)	0.095	0.90 (0.81–1.01)	0.080	0.88 (0.76–1.02)	0.099

All models matched for age, sex, race, and dialysis vintage. Model 1 is unadjusted: model 2 is adjusted for age and dialysis vintage to account for residual imbalance; and model 3 is adjusted for the variables in model 2 plus vascular access type.
CI, confidence interval; OR, odds ratio; rRNA, ribosomal RNA.
[a]Values were log transformed.

impossible to determine whether circulating microbial DNA were derived from living or dead microorganisms. Our study lacked associated measurement of microbiomes in other body sites, such as stool, oral, and skin microbiomes, which may have influenced the circulating microbial signatures. Concurrent measurement of microbiomes at different body sites would allow comparison of their composition and would help to elucidate the biological link between them. Nevertheless, it is possible that the circulating microbiome could exert its biological effects as a downstream site of action in the entire body, reflecting the dysbiotic changes of highly complex microbial communities in different body sites, potentially making the circulating microbiome (vs. microbiome from other body sites) a more promising biomarker and therapeutic target in ESRD patients. Similarly, although contamination of blood cannot be completely excluded, associational analyses should not be meaningfully influenced, as the contamination is likely nondifferetial between cases and controls. Finally, as with all observational studies, we cannot infer any causal relationships and eliminate the possibility of unmeasured confounders that might have affected the association between the circulating microbial signatures and cardiovascular death.

In conclusion, in this pilot case-control study of prevalent hemodialysis patients, we found a taxonomic difference in circulating cell-free microbiome between patients who died of a cardiovascular event and those who did not, with significant correlation with Nrf2 levels and marginal association with subsequent risk of cardiovascular death. Our findings suggest a potential pathogenic contribution of changes in the circulating cell-free microbial signatures to premature cardiovascular mortality in patients with ESRD. Further in-depth and larger studies are needed to clarify the characteristics and roles of circulating microbiome toward the development of a novel diagnostic and prognostic biomarker and personalized therapeutic strategies for premature mortality in these patients.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATRIAL

Supplementary File (PDF)

Supplementary Appendix. Protocol for 16S ribosomal RNA (rRNA) amplicon library preparation and sequencing

Table S1. Correlations of fungal ITS copy numbers and α diversity in serum of hemodialysis patients with selected clinical parameters

Table S2. Odds ratios and 95% confidence interval for cardiovascular death associated with fungal ITS copy numbers and α diversity in serum of hemodialysis patients

Figure S1. ITS rRNA copy numbers (per uL of serum) in cardiovascular cases and controls.

Figure S2. Bacterial α diversity assessed using (A) Simpson, (B) Chao1, (C) Richness, (D) Fisher alpha, and (E) Evenness indices in serum of cardiovascular cases and controls.

Figure S3. Fungal community α diversity assessed using Chao1 index in serum of cardiovascular cases and controls.

Figure S4. Heatmap of fungal community at the genus level in serum of cardiovascular cases and controls.

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