GUT MICROBIOME AND CHRONIC KIDNEY DISEASE

ANA MERINO RIBAS

Universitat Autònoma de Barcelona

DOCTORAL THESIS

GUT MICROBIOME AND CHRONIC KIDNEY DISEASE

AUTHOR

ANA MERINO RIBAS

SUPERVISORS

Dra. Maria Benedita Almeida-Garret Sampaio-Maia

Dra. Ioana Elena Bancu Dumitrescu

TUTOR

Dra. María Luisa Pedro-Botet Montoya

Doctoral Program in Medicine

Department of Medicine

Universitat Autònoma de Barcelona

Barcelona, 2023

ACKNOWLEDGEMENTS

A mis padres, Luis y Carmen, por ser mis mayores referentes a nivel personal y profesional, por dármelo todo y por ayudarme siempre en todo lo que ha estado en sus manos. A Iago por acompañarme en todo el proceso y, a pesar de las adversidades, conseguir hacerme feliz cada día. A mi hermano, Luis, por estar a mi lado y darme energía positiva con sus excelsas obras culinarias.

A mis directoras de tesis, Ioana y Benedita, no sólo por darme todo su apoyo para la realización de esta tesis, sino también por ser, desde el inicio de mi residencia en Nefrología, mis mayores mentoras profesionales, con las que trabajar siempre ha sido mucho más fácil y productivo.

Al grupo Nephrology & Infectious Diseases del i3S por su profesionalidad y por permitirme participar en muchos de sus proyectos.

A en Jordi, per ser el millor cap de servei que mai hagués pogut imaginar i per ajudar-me sempre en tot el que he necessitat. Als meus companys de Nefrologia, la Nàdia, la Marcela, la Cris, la Lisbet, en Pere, la Fredzzia, la Yanel, en Gabri, la Jehimy, la Laura i la Leyre, per recolzar-me en tot el procés i per aconseguir entre tots un ambient laboral idoni.

A tota la resta de la meva família i a les meves amigues pel recolzament i per ser-hi sempre.

I als pacients, tant catalans com portuguesos, que han participat en aquesta tesi doctoral.

LIST OF ABBREVIATIONS

BNP	B-type natriuretic peptide
CA	Calcium acetate
CKD	Chronic kidney disease
CRP	C-reactive protein
CVD	Cardiovascular disease
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
GBD	The Global Burden of Disease
HD	Hemodialysis
HDL	High-density lipoprotein
HPLC	High-performance liquid chromatography
IL	Interleukin
KDOQUI	Kidney Disease Outcomes Quality Initiative
LDL	Low-density lipoprotein
LPS-BP	Lipopolysaccharide-binding protein
OTUs	Operational taxonomic units
PCS	P-cresol sulphate
PD	Peritoneal dialysis
PTH	Parathyroid hormone
sCD14	Soluble CD14
SCFAs	Short-chain fatty acids
SFO	Sucroferric oxyhydroxide
SD	Standard deviation
SV	Sedimentation velocity
TLR	Toll-like receptor
TMAO	Trimethylamine N-oxide
TNF-α	Tumor necrosis factor-α
VC	Vascular calcification
VDR	Vitamin D receptor
VSMC	Vascular smooth muscle cells
3-IAA	3-indole-acetic acid
3-INDS	3-indoxyl sulphate

Index of Figures

Index of Tables

ÍNDEX

RESUM

Introducció

Els microbiomes intestinal, en sang i urinari poden patir alteracions (degut a l'acumulació de toxines urèmiques, les restriccions dietètiques, o els tractaments farmacològics, entre d'altres) a pacients amb malaltia renal crònica (MRC). Aquest fet pot contribuir a la inflamació crònica d'aquests pacients, incrementant el seu risc cardiovascular i la mortalitat, especialment en aquells pacients en hemodiàlisi (HD) i en diàlisi peritoneal (DP). La calcificació vascular (CV) és una condició freqüent a la MRC i un factor de risc ben establert pel desenvolupament de malaltia cardiovascular, pel que la seva òptima estimació a pacients amb MRC podria ser molt valuosa.

Objectius

L'objectiu principal fou avaluar el microbioma humà a pacients amb MRC en teràpia renal substitutiva (HD o DP). Concretament caracteritzar el microbioma intestinal a HD, els microbiomes intestinal, en sang i urinari a DP. També avaluar la relació entre el microbioma intestinal i el tractament amb quelants del fòsfor a pacients en HD, així com avaluar la relació entre els microbiomes intestinal i en sang amb CV i risc de mortalitat a pacients en DP.

Pacients i Mètodes

Per a l'elaboració de la present tesi doctoral, vam realitzar estudis a dues poblacions diferents de pacients amb MRC:

- Pacients amb MRC en HD: El microbioma intestinal de 12 pacients en HD fou analitzat i es van descriure els canvis comparant 2 grups de pacients segons la pressa d'acetat càlcic (AC) o sucroferric oxihidroxid (SFO), durant 20 setmanes de seguiment.

- Pacients amb MRC en DP: Els microbiomes intestinal, en sang i urinari foren analitzats a 46 pacients en DP. A 44 dels 46 pacients en DP es van valorar les diferències entre els microbiomes intestinal i en sang en associació amb la severitat de la CV (mesurada per l'Índex d'Adragao), i el risc de mortalitat (mesurat per l'Índex de comorbiditat de Charlson).

Les comunitats microbiològiques foren analitzades mitjançant amplificació i seqüenciació de les regions V3-V4 del 16S rRNA.

Resultats

- Pacients amb MRC en HD: Pel que fa a les variables clíniques i analítiques, no es trobaren diferències estadísticament significatives quan comparem grups AC i SFO. Quan es va analitzar el microbioma intestinal, vam observar que tots els pacients eren molt diferents entre ells ($p = 0.001$), i aquestes diferències es mantenien durant les 20 setmanes de tractament. Les mostres del mateix pacient clusteritzaven amb ell mateix, independentment del tractament administrat i de la setmana de tractament.
- Pacients amb MRC en DP: Els pacients en DP presentaven diferents perfils taxonòmics quan es comparaven microbiomes intestinal, en sang i urinari. El microbioma intestinal es trobava dominat per Bacillota i Bacteroidota, el microbioma en sang per Pseudomonadota i Actinomycetota, i el microbioma urinari es trobava dominat per Bacillota, Actinomycetota i Pseudomonadota. A més a mes, el microbioma urinari es caracteritzava per una diversitat menor als microbiomes intestinal i en sang. Es van observar canvis relatius a taxes específiques comparant pacients en DP amb i sense CV, concretament a *Coprobacter*, *Coprococcus 3*, *Lactobacillus* i *Eubacterium eligens* a la microbiota intestinal, i a *Cutibacterium*, *Pajaroellobacter*,

Devosia, *Hyphomicrobium* i *Pelomonas* en sang. També es va observar una associació entre CV i índex de mortalitat a pacients en DP, i els pacients amb índex de mortalitat més elevat corroboraven els canvis a *E. eligens* intestinal i *Devosia* en sang. El factor soluble CD14 es trobava positivament correlacionat amb la severitat de la CV, i uns valors superiors a 3.5 µg/mL foren relacionats amb un increment de *Lactobacillus*, *Dermabacter* i *Gardnerella* al microbioma urinari.

Conclusions

El present treball descriu el microbioma intestinal d'un grup de pacients en HD i els microbiomes intestinal, en sang i urinari d'un altre grup de pacients en DP. Vam concloure que el tractament amb AC i SFO no modificava el microbioma intestinal a pacients en HD. Vam observar una correlació positiva entre CV i risc de mortalitat, i aquells pacients amb CV i amb risc de mortalitat més elevat presentaven canvis a *E. eligens* intestinal i a *Devosia* en sang. El factor soluble CD14 es va correlacionar positivament amb la severitat de la CV als nostres pacients en DP, i nivells més elevats de factor soluble CD14 es van associar a un increment de *Lactobacilllus*, *Dermabacter* i *Gardnerella* al urobioma.

ABSTRACT

Introduction

Gut, blood, and urinary microbiomes can be altered (due to uremic toxins accumulation, dietary restrictions, or drugs, among others) in patients with chronic kidney disease (CKD). This may contribute to chronic inflammation and increases cardiovascular risk and mortality, especially in those undergoing hemodialysis (HD) and peritoneal dialysis (PD). Vascular calcification (VC) is a frequent condition in CKD and a well-established risk factor for the development of cardiovascular disease and its assessment in CKD patients could be very valuable.

Objectives

The main objective was to evaluate the human microbiome in CKD patients on renal replacement therapy (HD and PD). Specifically, to characterize the gut microbiome in HD patients, and the gut, blood, and urinary microbiomes in PD patients. Also to evaluate the relation between the gut microbiome and phosphate binders treatment in HD patients, and between the gut and blood microbiomes and VC and mortality risk in PD patients.

Patients and Methods

For this thesis, studies were performed in two different CKD populations:

- CKD patients on HD: The gut microbiome of 12 HD patients was analyzed and described the changes between taking calcium acetate (CA) and sucroferric oxyhydroxide (SFO), in a 20-weeks follow-up.
- CKD patients on PD: The gut, urinary, and blood microbiomes were analyzed in 46 PD patients. In 44 of the 46 PD patients, we evaluated differences in the gut and blood microbiomes in association with the severity of VC (by Adragao score), and mortality risk (by Charlson Comorbidity Index).

The microbiome communities were analyzed by amplification and sequencing of the V3-V4 region of the bacterial 16S rRNA gene.

Results

- CKD patients on HD: Regarding clinical variables and laboratory parameters, no statistically significant differences were observed between CA or SFO. When analyzing the gut microbiome, we found that all HD patients were different among themselves ($p = 0.001$), and these differences were kept along the 20 weeks of treatment. The clustering analysis in microbial profiles grouped the samples of the same patient independently of the treatment followed and the week of treatment.
- CKD patients on PD: PD patients presented distinct taxonomic profiles among gut, blood, and urine. Gut microbiome was dominated by Bacillota and Bacteroidota, blood microbiome was dominated by Pseudomonadota and Actinomycetota, and urobiome was dominated by Bacillota, Actinomycetota, and Pseudomonadota. Also, urobiome was characterized by a lower Shannon diversity than gut and blood microbiomes. Relative changes were observed in specific taxa between PD patients with and without VC, namely *Coprobacter*, *Coprococcus 3*, *Lactobacillus*, and *Eubacterium eligens* in the gut microbiome, and *Cutibacterium*, *Pajaroellobacter*, *Devosia*, *Hyphomicrobium*, and *Pelomonas* in the blood. An association between VC and all-cause mortality risk in PD patients was also observed, and patients with higher mortality risk corroborate the changes of *E. eligens* in the gut and *Devosia* in the blood. Soluble CD14 (sCD14) was positively correlated with VC severity, and more than 3.5 µg/mL sCD14 levels were related to an increase in *Lactobacillus*, *Dermabacter*, and *Gardnerella* in the urobiome.

Conclusions

The present work describes the gut microbiome of a group of HD patients and the gut, blood, and urinary microbiomes of another group of PD patients. We could conclude that the treatment with CA and SFO does not modify the gut microbiome in HD patients. We found a positive correlation between VC and all-cause mortality risk and those patients with VC and higher mortality risk presented changes in *E. eligens* in the gut and *Devosia* genus in the blood. SCD14 was positively correlated with VC severity in our PD patients and higher sCD14 levels were related to an increase in *Lactobacilllus*, *Dermabacter*, and *Gardnerella* in the urobiome.

Introduction

1. INTRODUCTION

1.1. Chronic kidney disease - a worldwide public health problem

Chronic kidney disease (CKD) is defined by a progressive loss of kidney function, entailing a decrease of the ability of the kidneys to excrete liquids and toxic products in the urine (1). The Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines (from the National Kidney Foundation) define CKD as a decrease in kidney function that occurs in 3 months or more, irrespective of the cause, accompanied by an estimated glomerular filtration rate (eGFR) lower than 60 mL/min per 1.73 m^2, and/or by markers of kidney damage (e.g., albuminuria, altered morphology) (1,2). CKD may progress to different stages and eGFR is useful for staging CKD, in mL/min per 1.73m^2: more than 90 (stage 1); 60–89 (stage 2); 45–59 (stage 3); 30–44 (stage 4); 15–29 (stage 5); less than 15 (stage 5) (1,2). CKD stages 4 and 5, also known as end-stage renal disease (ESRD), may promote the accumulation of dangerous levels of fluid, electrolytes (e.g., potassium, phosphate), and wastes (such as urea) in the human body, leading to acute serious complications (1). When patients progress to ESRD, they may require renal replacement therapy in the modality of hemodialysis (HD), peritoneal dialysis (PD), and/or kidney transplantation (1,2).

Nowadays, CKD is a worldwide public health problem, with an increasing prevalence, a high economic burden, and elevated morbidity and mortality (1,3,4). Globally, the number of individuals with all-stage CKD reached almost 700 million in 2017, which was a higher prevalence than other chronic diseases such as diabetes, osteoarthritis, chronic obstructive pulmonary disease, asthma, or

depressive disorders (4). From 2017 to March 2020, in the United States, the prevalence of CKD was 14.0%, based on *United States Renal Data System* (5). In Europe, CKD presents an estimated prevalence among 3.3-17.3% (6). In Spain, the estimated prevalence was 15.1% (between 2008 and 2010) (7). In Catalunya, analyzing the year 2020 register, the prevalence of patients on renal replacement (including HD, PD, and kidney transplant) was of 11593 patients (1490 per million of population) (8).

As mentioned above, CKD presents high morbidity and mortality (3–5). In 2020, CKD reached in the US a mortality rate of 127.4 deaths per 1000 person-year, being noticeably higher in CKD stages 4 and 5 (230.5 deaths per 1000 person-year) (5). The Global Burden of Disease (GBD) ranks CKD as the 12th leading cause of death out of 133 conditions (4). CKD resulted in 1.2 million deaths in 2017, a number that has been projected to rise by 2040 to 2.2 million in a best-case scenario and up to 4 million in a worst-case scenario (3). In CKD patients, the main causes of death are cardiovascular disease (CVD) and infectious complications (3–5).

1.2. End-stage renal disease and infectious complications

As mentioned above, there are 3 modalities of renal replacement therapy: HD, PD, and kidney transplantation. HD consists of purifying the blood of ESRD patients extracorporeally (9). HD implies the existence of a vascular access (an arteriovenous fistula/grafts or a central venous catheter), a blood circuit and a dialysis solution circuit, with both circuits meeting at an external filter, the dialyzer (9). HD is usually done in the hospital or at an outside center, thrice a week, and by specialized staff (10). During the procedure, excess fluid, and metabolic wastes are removed from the blood through the dialyzer (10). In PD the peritoneum acts as a

high vascularized dialysis membrane allowing the removal of water and solutes from the internal milieu (11). In PD modality, the exchanges must be done daily by the patient at home through a long-term PD catheter (11). Both modalities, HD and PD are similar in terms of surveillance and efficacy (9–11). In comparison to HD, PD might promote a better quality of life (because it is a home dialysis modality) and may preserve the residual renal function for longer periods (10,11). Nonetheless, PD implies a training and a responsibility of the patient on the technique and on its hygienic and aseptic measures (9–11).

A major problem related to the procedure of PD and HD are infections (10). The possibility of infection in HD patients using a central venous catheter as a vascular access is quite high (10,12). Catheter-related bacteremia in HD patients is usually caused by migration through the catheter of bacteria from the skin to the bloodstream or directly by inoculation from the biofilm in the inner surface of the catheter to the bloodstream. The most common etiological agents of HD catheter-related bacteriemia are *Staphylococcus aureus*, other coagulase-negative staphylococci, and gram-negative bacilli (13). Infectious episodes are also one of the main weakness of PD programs, with peritonitis and exit-site/tunnel infections remaining as the most common and relevant concerns for these patients (14,15). While gram-positive agents (mainly *Staphylococcus* spp. and *Streptococcus* spp.) are responsible for most PD-related infections, gram-negative bacteria (mainly *Pseudomonas* spp. and members of the family Enterobacteriaceae) are more likely to promote more severe infections with poorer outcomes (12,15)

1.3. End-stage renal disease, cardiovascular disease, and vascular calcification

CVD is the leading cause of death among CKD patients, manifested as coronary artery disease, heart failure, arrhythmias, and sudden cardiac death (16). With a bidirectional relationship, CKD has also been recognized as a risk factor for CVD independently of other conventional risk factors for CVD, such as high blood pressure, diabetes, dyslipidemia, and smoking (17). Indeed, CKD is a cardiovascular risk multiplier in patients with hypertension and diabetes (18). CKD causes a systemic and chronic proinflammatory state, contributing to vascular and myocardial remodeling processes resulting in atherosclerotic lesions, vascular calcification, and vascular senescence as well as myocardial fibrosis and calcification of cardiac valves (19). Consequently, CKD mimics an accelerated aging of the cardiovascular system.

Despite being CVD the leading cause of death in CKD patients, it is very difficult to estimate cardiovascular risk in CKD patients, especially in ESRD. Whereas some adjustment may improve the performance of standard cardiovascular risk assessment methods in early-stage CKD, standard risk prediction methods work poorly in ESRD patients (17). ESRD appears to modify the effects of standard risk factors (hypercholesterolemia, blood pressure, and high glucose), and the increased rates of sudden death and heart failure are not captured by standard risk methods. Entirely new cardiovascular risk models could be needed in ESRD (17).

Vascular calcification (VC) and its severity has long been recognized as an important CVD risk factor in CKD patients, especially in ESRD patients (20). VC is an active and highly regulated cellular process defined by the deposition of calcium-

phosphate crystals within the intima and media layers of the vasculature and/or heart valves. VC increases as eGFR declines and it is associated with CVD mortality in ESRD (17). Resulting cardiovascular calcifications are markedly accelerated in patients with CKD, and even children with advanced CKD frequently exhibit VC (19). The histological prevalence of VC in radial arteries was 45-fold greater in patients with CKD compared with those without CKD (19). Besides well-known traditional risk factors for VC such as age, male gender, diabetes, dyslipidemia, hypertension, smoking and inflammation, VC in CKD patients (since early stages until ESRD) is additionally driven by deregulations in mineral metabolism and phenotypic changes in vascular smooth muscle cells (VSMC) (21). Multifaceted intricate mechanisms in CKD-induced VC also comprise the instability and liberation of matrix extracellular vesicles containing calcium and phosphate from bone and VSMC, and elastin (most abundant protein in media wall) degradation, promoting calcium deposition (22). Several factors have been related with VC in CKD, such as biomarkers of inflammation (for example high-sensitivity C-reactive protein (CRP), interleukin (IL)-6, tumor necrosis factor-α (TNF-α)), and of monocyte activation (for example soluble CD14 and CD163) (23). In fact, the mineral bone disorder associated with CKD is characterized by one or more abnormalities in circulating minerals and their regulating hormones, bone abnormalities, and VC (20).

It is believed that if we can estimate VC in ESRD patients, we may assess cardiovascular risk in these patients. Some evidence suggest that VC can be estimated in ESRD patients by simple radiographies (and scoring the Kauppila or the Adragao score through hands and pelvic radiographies), carotid ultrasound, or by computed tomography (specially in coronary arteries), among others (24). Mounting evidence indicates that the gut dysbiosis associated with CKD may be

involved in the pathogenesis of bone–vascular axis (20,21). Recent data suggest that an increased protein fermentation, and consequent uremic toxins production, decreased carbohydrate fermentation, vitamin K deficiency, and gut-derived inflammation may, alone or together, drive to a vascular and skeletal pathobiology in CKD patients (20,21).

1.4. The human gut microbiome

The human microbiome was defined by the Human Microbiome Project (25) as all microorganisms that inhabit the human body and regulate the human metabolism, physiology, and immunity interfering in human health (26). The human microbiome includes microorganisms belonging to taxonomic groups of bacteria, archaea, fungi, protozoa, and virus; despite that, it is believed that bacteria are the most relevant and numerous group and the focus of most studies (26).

The healthy human gut microbiome is extremely diverse, encompassing over 1000 species, and relative abundances and microbial identities may vary significantly between individuals (27). However, the healthy gut microbiome is usually dominated by the phyla Bacillota (previously Firmicutes), and Bacteroidota (previously Bacteroidetes), representing the 90% of the gut microbiome. Actinomycetota (previously Actinobacteria), Pseudomonadota (previously Proteobacteria), Fusobacteriota (previously Fusobacteria), and Verrucomicrobiota (previously Verrucomicrobia) also shape the healthy gut microbiome but in lower proportions (26–28). *Clostridium* represents 95% of Bacillota members in the gut human microbiome, while *Bacteroides* and *Prevotella* are predominant among the Bacteriodota members (28).

Gut microbiome has several functions including vitamin synthesis, improve food digestion, and protect the host against the colonization by harmful bacteria through several mechanisms, such as competition for available nutrients, modulation of the mucus barrier, production of bacteriocins and other antimicrobial peptides, and enhancement of the host's innate immunity (e.g., secretion of IgA, activation of Toll-like cell receptors) (28,29). Factors such as diet, drugs intake, disease, psychological and physical stress, immunity, among others, may modify the structure and diversity of the gut ecosystem (30,31). A disturbed gut microbiome may facilitate infection by pathogens and the overgrowth of pathobionts (29,31). Pathobionts are microorganisms which, when present in low proportion in the healthy gut microbiome, are symbiotic and do not negatively affect the host health. However, when there is a disruption of the gut milieu, they may selectively expand and shift to a pathological role (29,32).

A disturbed or unbalanced gut microbiome, described as gut dysbiosis, is currently recognized as a key factor in the pathogenesis of several chronic and inflammatory diseases, such as obesity, insulin resistance, diabetes, inflammatory bowel disease, cancer, among others, including CKD (33–36).

1.5. Gut dysbiosis in chronic kidney disease

Increasing evidence indicates that several factors contribute to gut dysbiosis in CKD patients, such as uremic toxins accumulation, chronic inflammatory status, pharmacologic therapies (e.g., corticosteroids, immunosuppressive agents, antibiotics, phosphate binders), dietary restrictions, and other associated comorbidities (such as hypertension, diabetes, and obesity, among others) (33,36). Moreover, HD, PD, and kidney transplant may also impact on the gut microbiome

(37). Indeed, a recent systematic review showed that patients in any stage of CKD, ranging from early CKD to ESRD, exhibited substantial differences in gut microbiome composition compared to healthy individuals (38). Several data suggest that the microbiota in early CKD may be closer to that of healthy individuals, whereas in advanced CKD, a severe dysbiotic ecosystem is more common.

The progression of CKD to ESRD is associated with the accumulation of toxic metabolites in the blood. This accumulation may be related to enhanced generation of toxins from the dysbiotic microbiome accompanied by their reduced elimination by impaired kidneys (39). These accumulated organic waste products are known as uremic toxins (40,41). The accumulation of uremic toxins may promote some detrimental consequences, such as vascular and organ dysfunction, renal injury, cardiovascular damage, mineral and bone disorder, intestinal barrier dysfunction, and muscle wasting, among others, being associated to an increase in morbidity and mortality (41–43). As eGFR decreases with CKD progression the levels of uremic toxins increase, reaching levels 10-fold higher than in healthy individuals. This is particularly important for P-cresol sulphate (PCS) and 3-indoxyl sulfate (3-INDS), whose clearance do not fall markedly during each HD treatment, inversely to trimethylamine N-oxide (TMAO) (44). Moreover, in the kidneys, some uremic toxins can favor interstitial fibrosis, cellular senescence, kidney fibrosis, and matrix expansion and oxidative stress (42). Most of the uremic toxins are secreted into the gut altering intestinal milieu, inducing changes in the structure, composition, and function of the gut microbiome. Uremic toxins also serve as alternative substrates for gut microbiome, and when metabolized, harmful molecules are produced (45). Aranov et al. (46) compared HD patients with and without colon and observed that colonic microbes may produce a significant proportion of uremic solutes, most of

which remain unidentified. PCS, 3-INDS, indole-3-acetic acid (3-IAA), and trimethylamine N-oxide (TMAO), among others, are some examples of these uremic toxins of microbial origin.

A growing body of evidence shows that there is a CKD-associated gut dysbiosis that accompanies the progression of CKD through its different stages. A major example is related to the accumulation of urea in the body fluids of CKD patients in the context of a reduced eGFR, and its diffusion to the gastrointestinal tract that leads to the expansion of urease-producing bacteria, accounting for greater ammonia generation and a consequent increase of intestinal pH (47). In addition, these urea by-products degrade the epithelial tight junction, allowing the translocation into systemic circulation of whole or parts of bacteria (such as endotoxins or other antigens), and their metabolites (as toxins and gut-derived uremic toxins) (21,48). Moreover, in ESRD there is also an increased concentration of uric acid and oxalate secretion in the gastrointestinal tract because the colon becomes the main route of excretion, promoting the overgrowth of uricase-producing microbes. The proliferation of these proteolytic bacterial species (i.e., uricase- and urease-producing species) potentiates the establishment of a dysbiotic state in the gut of CKD patients. Furthermore, the disruption of epithelial tight junctions and the consequent translocation from the gut into systemic circulation of gut-derived bacterial products strongly activates innate immunity and systemic inflammation (49).

Moreover, dietary recommendations in CKD including restricted intake of potassium, phosphate, and sodium result in a low intake of fermentable carbohydrates and this may lead to a further expansion of proteolytic species and

an increased generation of bacterial toxins (50,51). Short-chain fatty acids (SCFAs) (propionate, acetate, and butyrate) are a by-product of the fermentation of nonabsorbable complex carbohydrates. Adequate levels of SCFAs have been proven to be critical for sustaining the health of the gut microbiome (52). Some studies demonstrated that with CKD progression there is a gradual reduction in butyrate-producing bacteria and in SCFA levels, specifically butyrate, proposing an association with concomitant inflammation (53,54). The imbalanced intestinal microbiome in CKD patients comprises a decreased prevalence of carbohydrate fermentation-inducing microorganisms and increased prevalence of those that induce protein fermentation (38). Moreover, ESRD and specially HD are associated with a perturbation of the intestinal barrier, causing ischemia, and consequently facilitating the translocation of endotoxins or other bacterial-derived products through the gut that may potentiate systemic inflammation (50,55).

Another very important factor that favors dysbiosis are pharmacologic therapies. It is well known that patients with ESRD are usually poly-medicated. Iron supplementation, immunosuppressants, or antibiotics, frequently used in our patients, have been demonstrated to alter the gut microbiome (56–58). Moreover, while other factors that alter gut microbiome such as comorbidities and diet are difficult or impossible to control by the nephrologist, we can choose drugs that are less harmful to our patients. However, the effects on the gut microbiome of some drugs widely used in CKD patients remain unknown. Most patients with ESRD, especially those on dialysis, tend to hyperphosphatemia and need high doses of different types of phosphate binders to correct this condition. Phosphate binders can be classified as calcium and non-calcium-based phosphate binders. It has been described that both groups of phosphate-binding agents can potentially produce

changes in the composition of the gut microbiome (59–63). Recently, new non-calcium-based phosphate-binding agents have been approved for the treatment of hyperphosphatemia in ESRD patients. Some of these new agents, such as sucroferric oxyhydroxide (SFO) and ferric citrate, hold iron in their compositions. It is believed that, given the critical role of iron in microbial growth and virulence, the large iron load administrated with these drugs, may alter gut microbiome composition (64,65). Nevertheless, there is still little evidence about the effects of these new phosphate binders on the gut microbiome.

Dialysis therapies, including HD and PD, seem to have a noticeable impact on the human microbiome, which can be explained by several factors, described below (37).

1.5.1. The gut microbiome in hemodialysis

Few studies have described gut microbiome alterations in HD patients. Most of these studies compared HD patients with healthy controls, finding significant differences regarding the composition of the gut microbiome between these two groups. However, as most of these studies compare the microbiome of HD patients with healthy controls, and not with CKD patients not on HD, it is not clear if the alterations reported are a consequence of HD, of CKD itself, or even associated with the additional treatment or with other comorbidities.

Few evidence reported an increase in Actinomycetota, Pseudomonadota (primarily Gammaproteobacteria), and Bacillota (especially the subphylum Clostridia) in HD patients (66). A study by Stadlbauer et al. (67) found a significantly lower α-diversity in HD patients, accompanied by an increase in

potentially pathogenic species and a decrease in beneficial species of gut bacteria. In pediatric patients, Crespo-Salgado et al. (68) compared the microbiome of healthy children, kidney transplant recipients, PD, and HD patients and reported that the relative abundance of Bacteroidota was significantly increased in HD patients compared to healthy controls, while the relative abundance of Pseudomonadota was significantly decreased in HD patients compared to PD patients. Shivani et al. (69) observed that the most abundant genus identified in CKD patients on HD and on PD was *Bacteroides*, and that at species level HD patients showed significantly higher abundance in *B. ovatus*, *B. caccae*, *B. uniformis* than healthy controls and PD patients. Shi et al. (70) found in the gut microbiome of HD patients compared with healthy controls an increase in the proteolytic bacteria *B.fragilis*, in several species of *Clostridium* genus, and in *Ruminococcus*. Another recent study (71) compared the gut microbiome of HD patients with healthy controls and observed differences regarding beta diversity, due to decreased Bacteriodetes and increased Bacillota and Pseudomonadota in HD patients.

Lun et al. (72) also included the comparison of pre-dialysis CKD patients, besides evaluating the gut microbiome of HD patients and healthy controls. The authors found that *Holdemanella*, *Megamonas*, and *Prevotella* were detected in healthy controls, also detected at decreased levels in CKD patients and not detected in HD samples, and that *Dielma* and *Scardovia* were absent in controls but detected in CKD and HD patients. Li et al. (73) compared the gut microbiome of CKD patients, HD patients, and healthy controls and reported that CKD patients, with or without HD, exhibited a

significantly higher relative abundance of *Neisseria* and *Lachnoclostridium* and lower abundance of *Faecalibacterium*.

In conclusion, HD appears to impact the microbiome in a specific way. The microbiome of HD patients is associated with less diversity and with an increase in potentially pathogenic species, particularly of the phyla Pseudomonadota, Actinomycetota, and Bacillota. The HD-associated perturbation of the intestinal barrier (commonly leading to mesenteric ischemia), the strict dietary restrictions, and the long-term medications intake in these patients seem to favor this dysbiotic state in the gut of HD patients. Despite that, more studies specifically comparing HD patients with pre-dialysis CKD patients are required.

1.5.2. The gut microbiome in peritoneal dialysis

Few studies have documented the effects of PD on the gut microbiome. Some studies have found differences in the gut microbiome composition and function between dialysis, ESRD patients, and healthy controls (67,68,74). However, these results were not consistent and, more importantly, these studies did not differentiate dialysis ESRD patients from pre-dialysis ESRD patients, so the observed changes in the gut microbiota of dialysis patients can be a consequence of dialysis or ESRD itself (or both) (37). More recent studies have included pre-dialysis ESRD patients.

Some studies report that PD patients present a significantly decreased bacterial diversity in comparison to healthy controls and non-dialysis CKD patients (68,75) and in one study this has been associated with a worse nutritional status measured by albumin levels (76). Mounting evidence

suggest that the gut microbiome of PD patients is dominated by urease containing-, indole- and PCS-forming bacteria (belonging to *Escherichia* genus and Enterobacteriaceae and Enterococcaceae families), whereas families linked with production of probiotic butyrate and SCFAs and carbohydrate fermentation appear to be markedly reduced (76,77). Some PD parameters that have been related with a reduction in SCFAs production are long dialysis duration, high peritoneal glucose exposure, and loss of residual renal function (76). A recent study also correlated PD-protein-energy wasting with a decrease in butyric acid-producing bacteria, namely *Roseburia* and *Phascolarctobacterium*, and with an increase in *Escherichia* which may be associated with higher intestinal permeability, inflammation, and nutritional imbalance, with the higher morbidity and mortality that this encompasses (78). Some studies have identified the genus *Blautia* and *Dorea* increased in the gut microbiome of PD patients when compared with non-dialysis CKD patients (69,79). Luo et al. (80) described a decrease in the relative abundance of *Dorea*, *Clostridium*, and SMB53 in PD patients with peritonitis, suggesting that they may have anti-inflammatory roles. Also, this study suggested that *Bacteroides* and *Phascolarctobacterium* may be associated with cardiovascular mortality, proposing that gut bacteria may exert an impact on patient prognosis. A recent study also correlated a decrease in gut microbiome producers of SCFAs with peritonitis, proposing a decrease in the Bacillota/Bacteroidota ratio as potential biomarker of *Escherichia coli* peritonitis (80). Another study observed an increase in Bacteroidota and Synergistetes in PD patients with PD-related peritonitis (80). A recent study (75) observed decreases in acetate producer *Bifidobacterium* and in butyrate

producers *Faecalibacterium* and *Subdoligranulum* that suggest a diminished ability to generate SCFAs causing a potential gut barrier dysfunction and systemic inflammation in PD patients.

In conclusion, PD per se appears to impact the gut microbiome. PD patients were associated with a less diverse microbiome, dominated by urease containing-, indole-, and p-cresyl-forming bacteria and with a lack of SCFAs producers. Dialysis duration and glucose exposure are two factors that may condition the severity of the dysbiotic state in the gut of PD patients.

1.6. Beyond the gut microbiome in chronic kidney disease

Beyond the gut, recent reports suggest the existence of alterations in other microbiomes in CKD patients, such as blood, salivary, and urine microbiomes (81–85). Increasing body of evidence supports the existence of a human blood microbiome with relevance in health and disease, although its origin, structure, and function remain unrevealed (86,87). Different reports suggested that shifts in the blood microbiome might be associated with some chronic inflammatory diseases, such as chronic liver disease, autoimmune diseases, obesity, among others, including CKD (81). The existence of a highly diversified blood microbiome, including metagenomic profiles even in healthy human donors, has been found using 16S ribosomal DNA measurement (88). Given the fact that high urea levels in CKD are converted to ammonia, resulting in a disruption of intestinal tight junctions and, as a consequence, a translocation of gut toxins into blood (89), it is thought that CKD patients may present a different quantitative and qualitative microbial profile in the blood compared with controls. Shah et al. (82), observed in the blood microbiome of a small number of CKD patients when compared with healthy

controls, lower α diversity and significant taxonomic variations, with a significant rise in the Pseudomonadota phylum in CKD patients. Nonetheless, studies to clarify the role of the blood microbiome in CKD patients, specifically in dialysis patients are lacking.

The accumulation of substances in the body as a result of a decrease in the eGFR, also can cause alterations in the oral cavity, with higher levels of urea, ammonia and pH being commonly detected in the saliva of CKD patients, including ESRD patients (83). Changes in the oral microbiome have been related to CKD progression (83,90). A study of our group (91) observed in PD patients a dysbiosis of the commensal oral microbiome, namely a proliferation of clinically relevant *Enterobacteriaceae*, potentially harboring acquired antibiotic resistance genes and highlights the importance of the oral cavity as a reservoir for pathobionts and antibiotic resistances in PD patients.

Urine has been considered throughout the history a sterile fluid. However, there is evidence showing that the urinary tract of healthy individuals without urinary infection is dominated by different kinds of viable microbes, and that the distribution pattern of these microorganisms may affect the urinary tract health (92). Urinary dysbiosis has been associated with urinary tract infections and overactive bladder syndrome (93,94), nephrolithiasis (95), urinary tract neoplasms (96), and other systemic comorbidities such as diabetes, dyslipidemia, older age, gender, and recent antibiotic intake (97,98). Some studies have observed a different urine microbiome composition in CKD patients when compared to individuals with normal kidney function (84,85). Interestingly, it has been also observed that the urobiome diversity decreases as kidney function gets worse. Besides, some studies analyzed

the urinary microbiome of kidney transplant recipients and revealed an overall decrease in diversity, also changes in the urobiome related to chronic allograft dysfunction, and an emergence of opportunistic pathogens promoting antibiotic resistances and increasing the susceptibility to infection in these patients (93,99). There are still no studies describing the urinary microbiome in PD patients.

Hypothesis and Objectives

2. HYPOTHESIS

1. Chronic inflammation and mortality and cardiovascular risks related to CKD are associated to changes in the human microbiome, especially in ESRD patients on renal replacement therapy.

2. Phosphate binders alter the gut microbiome in CKD patients on dialysis.

3. Changes in gut and blood microbiomes are associated to vascular calcification, all-cause mortality risk, and cardiovascular disease in CKD patients on dialysis.

4. Changes in the urinary microbiome are associated to specific clinical conditions in CKD patients on dialysis.

3. OBJECTIVES

3.1. Main objective

To evaluate the human microbiome in ESRD patients on renal replacement therapy (HD and PD): explore modulatory factors and links to cardiovascular and mortality risks.

3.2. Secondary objectives

a) To characterize the gut microbiome in HD patients, and the gut, the blood, and the urinary microbiomes in PD patients.

b) To evaluate the relation between the gut microbiome and the type of phosphate binder taken in CKD HD patients: calcium acetate or sucroferric oxyhydroxide.

c) To evaluate the relation between the changes in the gut and blood microbiomes, vascular calcification, and all-cause mortality risk in CKD PD patients.

d) To evaluate the relation between the changes in the urinary microbiome and some clinical parameters in CKD PD patients.

Methods

4. PATIENTS AND METHODS

For this thesis, studies were performed in two different CKD populations:

- CKD patients on HD. The first population included 12 CKD patients on HD in Hospital Universitari Germans Trias i Pujol. In this population we analyzed the gut microbiome and described the changes between taking different types of phosphate binders, comparing calcium acetate (CA) and sucroferric oxyhydroxide (SFO), in a 5-month follow-up.

- CKD patients on PD. The second population included 46 CKD patients on PD in Centro Hospitalar Universitário de São João. In this population we analyzed the gut, urinary, and blood microbiomes. In 44 of the 46 PD patients, we also evaluated the differences in the gut and blood microbiomes in association with the severity of vascular calcification (VC), and the risk of mortality, as well as the link between the urinary microbiome and clinical parameters.

The methodology of these 2 studies is described below.

4.1. Study design, subjects, and sample collection

4.1.1. Study design, subjects, and sample collection: CKD patients on HD

To evaluate the changes in the gut microbiome of HD patients taking different types of phosphate binders, comparing calcium acetate (CA) and sucroferric oxyhydroxide (SFO), we performed a first study. This cross-sectional observational study included 12 HD patients in Hospital Universitari Germans Trias i Pujol with a 5-month follow-up. This study was approved by the Clinical

Research Ethics Committee of the Hospital Universitari Germans Trias i Pujol (PI-16-169, NCT5551048) and conformed to the principles outlined in the Declaration of Helsinki. All the HD subjects were recruited from the Hemodialysis Department of the Hospital Universitari Germans Trias i Pujol, in Badalona, Spain.

All patients were aged above 18 years old and had been on HD for at least 1 year (4 h sessions, 3 sessions per week). All participants were recruited voluntarily after receiving detailed information on the study protocol. Written informed consent was obtained from all patients.

Exclusion criteria included age under 18 years old, inability to give informed consent, history of gastrointestinal disease, hospitalization, and history of infection and antibiotics intake in the last 3 months.

Relevant clinical and demographic information was gathered for each participant at baseline. Clinical characteristics collected were gender, age, CKD etiology, history of high blood pressure, diabetes mellitus, dyslipidemia, and history of CVD (peripheral vascular disease, ischemic cardiomyopathy, or cerebrovascular disease), and neoplasm.

Information regarding their vascular access and their previous phosphate-binder treatment at the beginning of the study was also gathered (nine received CA, one received calcium carbonate, and two were not previously treated for hyperphosphatemia).

The patients were divided into two groups and their treatment for hyperphosphatemia changed: 5 patients were placed in CA group (4 continuing CA therapy and 1 patient changing from calcium carbonate

therapy) and 7 were switched to SFO (5 changing from CA therapy and 2 starting phosphate-binding treatment).

Stool samples were self-collected from all HD patients receiving phosphate binders, 5 in the CA group and 7 in the SFO group. Blood samples from routine controls were also collected. Stool samples were collected in DNA-free sterile containers and were immediately frozen and stored at -80 ºC for microbiome analysis.

The samples (blood and fecal samples) were collected in a 5-month follow-up: at baseline, 4, 12, and 20 weeks after treatment initiation.

In blood samples the following parameters were analyzed: hemoglobin, ferritin, transferrin saturation index, calcium, phosphate, parathyroid hormone (PTH), CRP, sedimentation velocity (SV), and albumin.

4.1.2. Study design, subjects, and sample collection: CKD patients on PD

We performed a second study in PD patients, to characterize the gut, urinary, and blood microbiomes of PD patients, and to associate the gut and blood microbiomes to the severity of vascular calcification (VC) and the risk of mortality, as well as to describe the link between the urinary microbiome and clinical parameters. This cross-sectional observational study included 46 PD patients in Centro Hospitalar Universitário de São João in Porto, Portugal, between 2018 and 2019. This study was approved by the local Ethics Committee (approval references 200/18), in accordance with the 1964 Helsinki declaration and its later amendments.

All participants were recruited voluntarily after receiving detailed information on the study protocol. Written informed consent was obtained from all patients.

Exclusion criteria included age under 18 years old, inability to give informed consent, history of gastrointestinal disease, hospitalization, and history of infection and antibiotics intake in the last 3 months.

Relevant clinical and demographic information was gathered for each participant. Clinical characteristics collected were gender, age, CKD etiology, history of high blood pressure, diabetes mellitus, dyslipidemia, obesity (defined as body mass index of 30 kg/m^2 or higher), history of CVD (peripheral vascular disease, ischemic cardiomyopathy, or cerebrovascular disease), and neoplasm. Their pharmacological treatment (at the time of sample collection) and infection history (more than 3 months before sample collection) were also gathered. Parameters related to PD technique were also collected such as PD duration, PD modality, creatinine clearance, residual renal function, and Kt/V (urea). Kt/V (urea) defines the adequacy to PD measuring urea weekly clearance and normalizing it by urea estimated distribution volume (100).

VC was estimated in all patients using Adragao score (24), which estimates VC in ESRD patients through hands and pelvic radiographies. The Charlson Comorbidity Index was also calculated predicting 10-years survival in patients with multiple comorbidities (101). VC by Adragao score was evaluated in 44 of the 46 PD patients.

Blood samples were collected in the PD unit, and the self-collected stool and mid-stream urine specimens were brought refrigerated by the patient within 48 h and 4 h after collection, respectively. Whole blood, urine, and stool samples, after aliquoted, were collected in DNA-free sterile containers and were immediately frozen and stored at -80 ºC for microbiome analysis. Plasma was obtained after blood centrifugation (1500x g, 15 min, 4 ºC) and stored at -80 ºC for biochemical analysis.

In blood samples, apart from the blood microbiome, the following parameters were also analyzed: hemoglobin, ferritin, calcium, phosphate, calcium-phosphate product, urea, albumin, cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, PTH, CRP, SV, B-type natriuretic peptide (BNP), and proteinuria.

In plasma samples markers of intestinal translocation were measured, such as endotoxins, Lipopolysaccharide-binding protein (LPS-BP), Toll-like receptor 4 (TLR4), and soluble CD14 (sCD14); other inflammatory parameters a part from CRP, ferritin, and SV, such as IL-1, IL-6, TNF-α, and the anti-inflammatory IL-10; and uremic toxins such as PCS, 3-INDS, 3-IAA, and TMAO.

4.2. Sample processing and microbiome analysis

Fecal DNA from HD patients was extracted by Powersoil DNA Isolation Kit MoBio, and a 16S rRNA sequencing library was constructed targeting the V3 and V4 hypervariable regions. Sequencing was performed on a MiSeq platform (2 x 300). Operational taxonomic units (OTUs) table construction, taxonomic

assignment, and descriptive and statistical analyses were performed using R version 3.4.2. and different packages (DADA 2, vega, ggplot, phyloseq) and the Greengenes rRNA database.

Regarding PD patients, genomic DNA was isolated in a strictly controlled environment at Vaiomer SAS (Labège, France) as previously described (82). Total DNA was extracted from feces, whole blood, and urine (100 μL) using a specific Vaiomer protocol carefully designed to minimize any risk of contamination between samples from the experimenters or the environment. Negative controls (molecular grade water added in an empty tube, the same used for sample storage and PD solution) were extracted, amplified, and sequenced at the same time as the samples. PCR amplification was performed using universal primers targeting the V3-V4 region of the bacterial 16S rRNA gene (340F-781R). Illumina sequencing length, by use of the 2 x 300 paired-end MiSeq kit V3, was designed to encompass the 476-base pair amplicons. Sample multiplexing and sequencing library generation were conducted, as previously described (45). qPCR was used to quantify the DNA concentration in the pool employing a 7900HT Fast Real-Time PCR System (Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA) and KAPA Library Quantification Kits for Illumina Platform (Kapa Biosystems, Inc., Wilmington, NC, USA). The final pool, at a concentration after dilution between 5 and 20 nM, was used for sequencing as suggested previously (45). The sequencing steps were performed using a paired-end sequencing run in a MiSeq Illumina device.

The targeted gene regions were analyzed using the FROGS bioinformatics pipeline established by Vaiomer SAS (Labège, France) (102). The following filters were applied as previously suggested (68): (1) amplicons with a length <

350 nt or a length > 480 nt were removed; (2) amplicons without the two PCR primers were removed (10% of mismatches were authorized); (3) amplicons with at least one ambiguous nucleotides ('N') were removed; (4) OTUs identified as chimera (with search v1.9.5) in all samples in which they were presented were removed; (5) OTUs with an abundance lower than 0.005% of the whole dataset abundance were removed, and (6) OTUs with a strong similarity (coverage and identity ≥80%) with the phiX (library used as a control for Illumina sequencing runs) were removed. OTUs were produced via single-linkage clustering, and taxonomic assignment was performed by Blast+ v2.2.30+ with the databank RDP v11.4.

4.3. Biochemical analysis

Routine clinical analyses were collected from our patients' clinical records, namely, urea, proteinuria, albumin, hemoglobin, cholesterol, LDL, HDL, triglycerides, phosphorus, calcium, calcium phosphate product, ferritin, transferrin saturation index, BNP, PTH, SV, CRP, creatinine clearance, residual renal function, and Kt/V (urea). TNF-α, IL-1, IL-6, IL-10 were determined in plasma by Luminex Multiplex Assay (Millipore Corporation, Billerica, MA, USA). ELISA kits were used to evaluate LPS-BP (LPS-BP, Cloud-clone Corp.®, Katy, TX, USA), TLR4 (TLR4, Cloudclone Corp.®, Katy, TX, USA), and sCD14 (sCD14, Quantikine® ELISA, R&D Systems, Inc., Minneapolis, MN, USA), and TMAO (MyBiosource®, San Diego, CA, USA) whereas endotoxins were evaluated by Traditional Kinetic Limulus Amebocyte Lysate (LAL) Assay (LonzaWalkersville, Inc., Walkersville, MA, USA).

Uremic toxins were quantified following the method described by Calaf et al. (103) with modifications. PCS, 3-INDS, 3-IAA were detected by high-performance liquid chromatography (HPLC) with fluorescence detection (275 and 330 nm). Elution was performed in gradient mode using as mobile phase a mixture of (A) aqueous NaH_2PO_4 buffer (20 mM, pH 4.6), containing tetrabutyl ammonium iodide (TBAI, 5 mM), and (B) acetonitrile, at a flow rate of 1.5 mL/min, and injection volume of 20 μL. Prior to HPLC analysis, 100 μL of each plasma standard or sample was added to 300 μL of ethanol containing 0.22 mg/L of internal standard 4-ethylphenol. After vortexing during 30 s, 100 mg of NaCl were added and mixed vigorously. After 10 min, 700 μL of component (A) of mobile phase was further added following centrifugation at 18,000x g for 10 min at 4 ºC and supernatant analysis by HPLC.

4.4. Statistics

All the results are represented as mean ± standard deviation (SD) or in percentage (%). Statistical analysis was performed using SPSS Statistics version 27 (IBM). The categorical variables were described through absolute or relative frequencies (%) and analyzed using the Pearson's chi-square test or Fisher's exact test when more than 1 cell displayed expected counts less than 5. Continuous variables were described using mean ± SD and analyzed by Student's t test for independent samples when following a normal distribution, or by Mann-Whitney U test when there was no normality of the data. Normality was assessed by the Shapiro-Wilk test. A partial correlation between VC and all-cause mortality risk, while controlling of the effect of age and sex, was performed

using JASP-stats software. For all analysis, statistical significance was assumed when p values were less than 0.05.

Primer v7 (PRIMER-e, Auckland, New Zealand) was used for the calculation of diversity indices, non-metric multidimensional scaling and principal coordinate analyses, and other multivariate analyses, mainly ANOSIM and PERMANOVA, were used to test the significance of beta-diversity. The percentage of OTUs data per sample was used for these analyses, followed by squared root transformed data, resemblance matrices of similarity data types using Bray-Curtis similarities, adding dummy value and testing 4999 permutations. The reads in each sample were converted into percentage values according to the total number of sequences in the sample to eliminate the effect of the final number of reads (104). Post-hoc analyses were done in STAMP 2.1.3 (105) for multiple groups using one-way analysis of variance (ANOVA), Tukey-Kramer (0.95) and Eta-squared for effect size, while, with two groups, analysis using Welch’s t-test was conducted (two-sided, Welch’s inverted for confidence interval method).

Results

5. RESULTS

5.1. CKD patients on HD

5.1.1. Clinical characterization: CKD patients on HD

The clinical characterization of CKD patients on HD classified according to group of phosphate-binding agent taken (CA or SFO) is described in Table 1. The main clinical parameters were not different between HD patients assigned to CA or SFO groups at baseline. Although not statistically significant, CA group presented an increased prevalence in history of arterial hypertension, dyslipidemia, peripheral vascular disease, stroke, ischemic cardiomyopathy than the SFO group. HD patients assigned to SFO group presented a greater incidence of catheter as vascular access, but also not statistically significant.

Table 1: Clinical characterization of HD patients undergoing calcium acetate (CA) or sucroferric oxyhydroxide (SFO) as phosphate-binding agent.

Clinical parameter	CA	SFO
Age, years	66.8±13.9	61.1±16.7
Women, %	40.0%	42.9%
Arterial hypertension, %	100.0%	85.7%
Dyslipidemia, %	60.0%	42.9%
Diabetes mellitus, %	40.0%	42.9%
Peripheral vascular disease, %	40.0%	14.3%
Stroke, %	40.0%	14.3%
Ischemic cardiomyopathy, %	40.0%	14.3%
Neoplasm, %	20.0%	28.6%
Catheter as a vascular access, %	60.0%	71.4%

Values are means ± standard deviation or relative frequencies (%).

5.1.2. Laboratory parameters: CKD patients on HD

At baseline, no patient was treated with SFO, some were treated with CA in both groups of treatment, and 2 patients in the SFO group have no

phosphate-binding treatment. At this time point, no statistically significant differences were found regarding laboratory parameters, such as hemoglobin, ferritin, transferrin saturation index, calcium, phosphate, PTH, CRP, SV, and albumin (Table 2). Collectively, SFO group presented at baseline higher transferrin saturation index, and lower values of CRP than CA group, but those differences were not statistically significant. PD patients assigned to CA group presented an increased trend to hyperphosphatemia at baseline, but this was also not statistically significant.

Table 2 shows the evolution of the laboratory parameters over the different time points. In CA group, 20-week calcium was higher than in the SFO group with statistical significance ($p = 0.02$). SV was increased in CA group at week 12 of treatment when compared with SFO group, with statistical significance ($p = 0.04$). Also, a statistically significant lower albumin was observed in SFO group at 20-week treatment when comparing with CA group ($p < 0.01$). The ferritin levels at baseline and after 20 weeks of treatment were high in the two groups, and both groups get normal levels of phosphate at week 20 of treatment, with no statistically significant differences. The levels of transferrin saturation index, PTH, CRP, SV, and albumin, at 20 weeks of treatment were similar in both groups.

Table 2: Laboratory clinical data of HD patients undergoing calcium acetate (CA) or sucroferric oxyhydroxide (SFO) as phosphate-binding agent.

Laboratory parameter	CA	SFO
Ferritin, ng/ml		
Basal	1451.8±1299.3	1185.1±268.2
4 weeks	1670.4±1326.9	1166.0±187.2
12 weeks	1722.2±1622.0	1056.8±327.9
20 weeks	1691±1557.1	1149.8±360.0
Transferrin saturation, %		

Basal	29±8.9	53.1±29.4
4 weeks	43.4±19.7	46.7±28.9
12 weeks	38.8±19.3	41.4±18.6
20 weeks	32.4±14.0	42.0±15.6
Calcium, mg/dl		
Basal	9.34±0.3	9.1±0.3
4 weeks	9.24±0.3	8.9±0.4
12 weeks	9.02±0.5	8.7±0.7
20 weeks	10.06±0.7	8.9±0.5*
Phosphate, mg/dl		
Basal	5.16±2.1	4.4±2.2
4 weeks	4.88±1.4	4.4±1.8
12 weeks	4.42±1.7	5.5±2.7
20 weeks	3.26±0.8	4.7±2.9
Parathormone, pg/ml		
Basal	242.1±182.7	216.9±259.2
4 weeks	309.4±211.2	244.1±267.7
12 weeks	327.1±196.9	134.4±108.8
20 weeks	181.5±136.8	
C-reactive protein, mg/ml		
Basal	16.9±20.6	4.2±2.6
4 weeks	12.02±6.0	5.6±7.0
12 weeks	12.54±7.3	4.4±3.9
20 weeks	7.78±6.3	4.2±1.7
Sedimentation velocity, mm		
Basal	51.4±24.2	45.3±19.1
4 weeks	46.8±24.2	39.2±18.3
12 weeks	61.6±26.9	26.4±17.6*
20 weeks	47.6±7.2	38.4±19.4
Albumin, g/l		
Basal	39.04±2.2	35.7±3.0
4 weeks	37.54±1.6	35.4±2.4
12 weeks	37±3.7	33.9±4.0
20 weeks	39.5±1.8	32.8±2.3*
Hemoglobin, g/dl		
Basal	11.26±0.9	10.9±1.4
4 weeks	11.42±0.8	10.1±2.9
12 weeks	10.14±1.3	11.8±1.3
20 weeks	10.55±0.8	10.7±1.2

Values are means ± standard deviation. *Values in SFO are significantly different from CA.

5.1.3. Gut microbiome analysis: CKD patients on HD

The samples of all time points (baseline, week 4, week 12, and week 20) were collected in eight out of the total twelve individuals, in a total of 38 stool

and blood samples. From the initial set of 12 patients, patient 7 (SFO group) dropped out because he was derived to another hospital due to clinical reasons, patient 3 (SFO group) received a kidney transplant before the collection of 20-week samples, patient 9 (SFO group) died before the collection of 12-week samples, and good-quality samples for gut microbiome analysis were only obtained from week 12 and week 20 in patient 8 (SFO group).

The set of 38 fecal samples showed over 2 million reads, then classified using the Greengenes database. A high number of ASVs (33,734) were found among the tested samples and classified as belonging to the kingdom Bacteria. Shannon diversity was measured in each sample and the group of 38 samples showed values for Shannon diversity ranging from 6.2 to 7.7.

Interestingly, all patients were very different among themselves ($p = 0.001$) when comparing one patient with another patient at baseline (Figure 1). These differences among the patients were kept along the 20 weeks of treatment; and there were no significant differences ($p > 0.05$) when the samples were grouped by week of treatment (baseline, 4, 12, or 20 weeks). It is important to note that the gut microbiome was found stable throughout the 20 weeks of this study in patients that were on CA before the study and maintained that drug within the study protocol, and in patients who changed phosphate-binding agents (from no treatment, CA, or calcium carbonate to CA or SFO).

When the microbial profiles of the patients treated with CA versus SFO considering all time points were compared, statistical differences were found (Figure 1); and these differences were confirmed by ANOSIM ($p = 0.002$) and

PERMANOVA (p = 0.001). This statistical analysis was done independently of the differences observed at baseline.

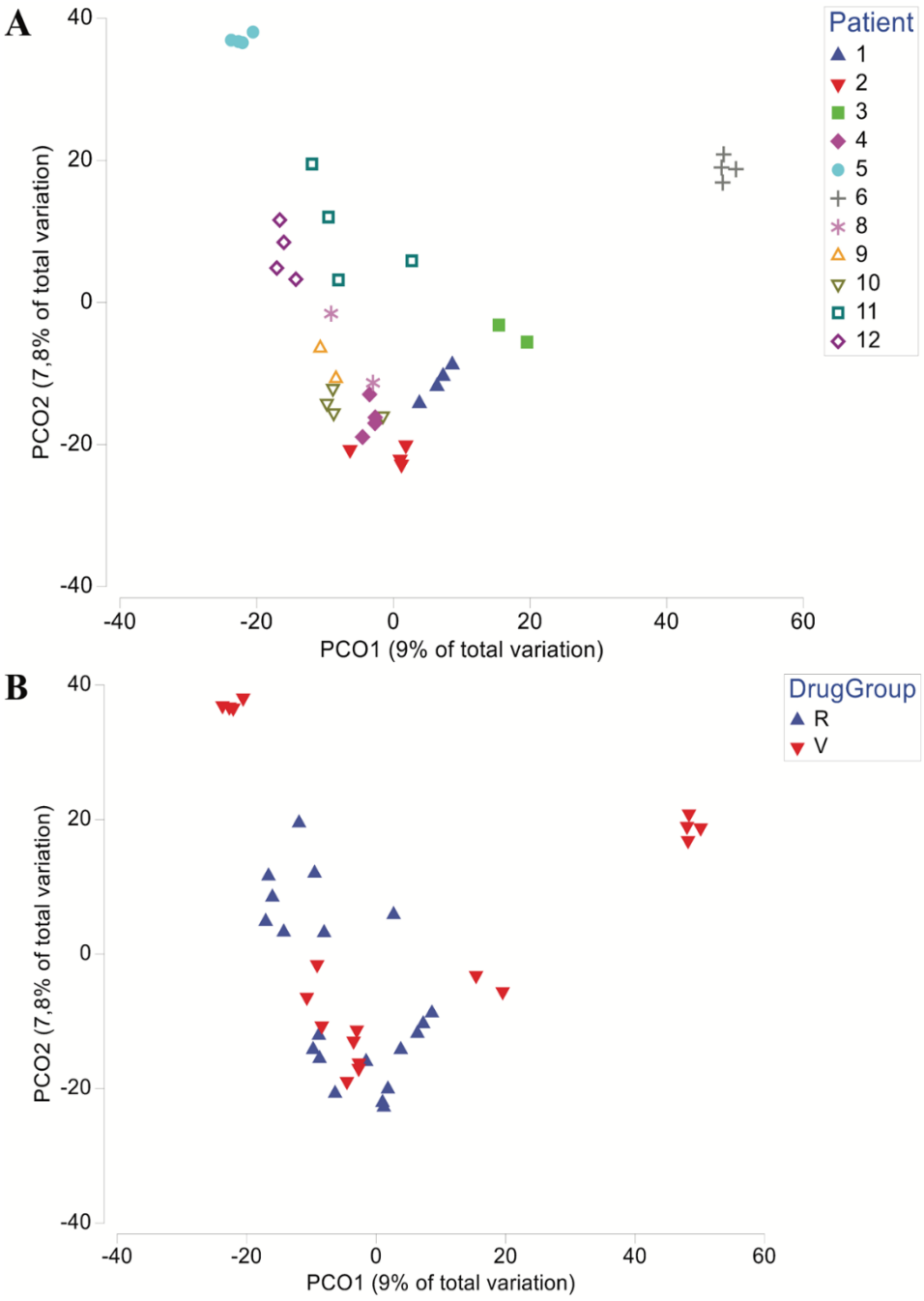


Figure 1: A Principal Co-ordinate Analysis (PCO) of the microbiome profiles for multiple patients. B PCO of the microbiome profiles for drug treatments (calcium acetate versus sucroferric oxyhydroxide).

The bacterial communities were studied, and Bacteroidota and Bacillota were the most common phyla found in the fecal samples, followed by

Pseudomonadota, Actinomycetota, and Verrucomicrobiota. Looking for more specific compositional differences, multiple taxonomical levels among these samples were compared. When analyzing the bacterial composition at the genus level, *Bacteroides* was the most prevalent in both groups of patients, independently if they were treated with either CA or SFO (Figure 2). The microbial profiles were very distinct among patients and, once again, the clustering analysis grouped the samples of the same patient independently of the treatment followed and the stage of the treatment (baseline, 4, 12, or 20 weeks).

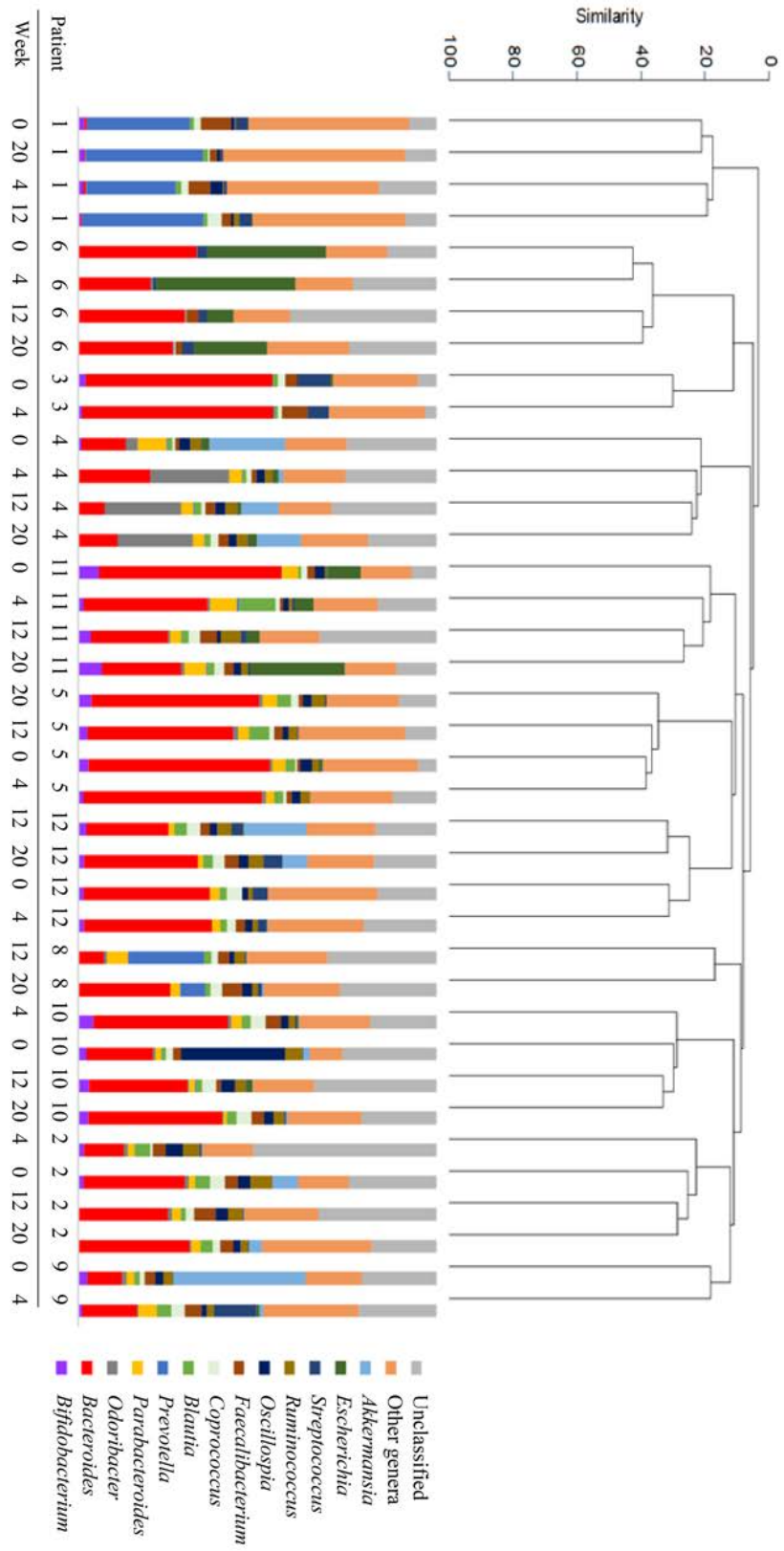


Figure 2: Clustering analysis and microbiome profiles (at genus level) for the samples considered in this study.

When considering all the time points (all patients and all weeks), it was possible to find statistical differences ($p < 0.05$) for the microbial communities when comparing the samples for multiple variables, including gender, ischemic cardiomyopathy, the use of a catheter as vascular access, or age (the patients were organized in three groups: under 45, range 61–69, above 71). These statistical differences could not be observed when each treatment stage (baseline, 4, 12, or 20 weeks) was considered separately; therefore, no differences were observed for the variables gender, age, ischemic cardiomyopathy, catheter use, and phosphate binder treatment (CA versus SFO).

5.2. CKD patients on PD

5.2.1. Clinical and laboratory characterization: CKD patients on PD

The detailed clinical characterization of patients on PD included in this study is shown in Table 3. This population include 46 participants, mostly males and with a median age of 56.1 ± 10.66 years old. Almost half patients were on automated PD. Most patients were on iron supplementation and on phosphate binders. The laboratory parameters are shown in Table 3, including markers of intestinal translocation, inflammatory parameters, and uremic toxins.

Table 3: Clinical characterization of chronic kidney disease patients on peritoneal dialysis (PD).

Clinical characterization	**PD patients (n=46)**
Gender, female	15 (33%)
Age, years	56.10±10.66
Time in PD, years	2.68±2.45
Type of PD, automated	22 (48%)
With history of peritonitis	11 (24%)

Iron supplementation	35 (76%)
Hipouricemiants	18 (39%)
Phosphate binders	39 (85%)
Diabetes	14 (30%)
Obesity, Body Mass Index>30	5 (11%)
Cardiovascular disease	10 (22%)
Neoplasm	9 (20%)
Creatinine clearance, L/week	114.09±56.21
Urea, mg/dl	126.37±37.02
Urea depuration, Kt/V (urea)	2.22±0.52
Renal clearance total, ml/min	5.62±3.94
Residual diuresis, cc/24h	1301.04±830.69
Proteinuria, g/24h	1.01±1.25
Albumin, g/l	37.03±3.33
Cholesterol, mg/dl	171.48±55.86
CRP, mg/l	5.25±8.33
Ferritin, ng/ml	361.75±218.09
Hemoglobin, g/dl	11.5±61.42
SV, mm	63.28±25.41
sCD14, µg/ml	5.13±2.10
lL-10, pg/ml	17.98±14.44
lL-6, pg/ml	2.82±6.18
IL-1β, pg/ml	1.32±0.92
TNF-α, pg/ml	11.47±4.33
TLR4, pg/ml	632.85±442.02
LBP, pg/ml	39.94±17.17

Results expressed in mean ± standard deviation (mean ± SD) for continuous variables and number of patients (n) and prevalences (%) for categoric variables.

5.2.2. Vascular calcification in CKD patients on PD

In a subgroup of 44 PD patients, VC was evaluated by Adragao score. The mean Adragao score was 2.98 ± 2.74 and included 26.1% patients without VC (Adragao score = 0); 30.4% with moderate VC (Adragao score of 1 or 2), and 39.1% with severe VC (Adragao score higher than 2). In this study, PD patients with moderate or severe VC were compared with patients without VC. Demographic and clinical characteristics of the studied PD population with and without VC are shown in Table 4.

PD patients with moderate or severe VC were older and included more males than PD patients without VC. Concerning the comorbidities, no differences were found in history of arterial hypertension (present in 95.5% of the studied population), obesity (11.4% of the studied population), or CVD (25.0% of the studied population). A significantly higher prevalence of patients with diabetes mellitus was observed in the group with VC in comparison to the group without VC (43.8% vs. 8.3%, p = 0.035).

Table 4: Demographic and clinical characterization of chronic kidney disease patients on peritoneal dialysis (PD) with and without vascular calcification (VC).

Demographic data	**PD (n = 44)**	**PD Without VC (n = 12)**	**PD with VC (n = 32)**	**p-Value**
Age, years	56.1±10.9	47.7±11.5	59.4±8.8	**<0.001[a]**
Sex, % male	65.9%	33.3%	78.1%	**0.011[d]**
PD parameters				
PD duration, months	33.4±30.0	36.3±43.4	30.9±23.8	0.668[b]
PD type, %				>0.999[d]
APD	52.3%	50.0%	53.1%	
CAPD	47.7%	50.0%	46.9%	
Creatinine clearance, L/week	114.8±56.8	105.7±45.1	118.2±60.8	0.668[b]
Residual renal function, mL/min	5.6±4.0	5.8±3.8	5.6±4.1	0.706[b]
Kt/V (urea)	2.2±0.5	2.6±0.6	2.1±0.4	**0.004[b]**
Charlson Index, %				**0.003[c]**
Low (≤2)	18.2%	50.0%	6.3%	
Moderate (3-4)	31.8%	25.0%	34.4%	
Severe (≥5)	50.0%	25.0%	59.4%	
Biochemical parameters				
Urea, mg/dL	125.0±37.0	127.6±20.1	124.0±41.8	0.780[a]
Proteinuria, g/24 h	1.0±1.2	0.9±1.0	1.0±1.2	0.342[b]
Albumin, g/L	37.1±3.3	37.0±2.6	37.1±3.6	0.944[a]
Hemoglobin, g/dL	11.5±1.4	11.0±0.9	11.7±1.6	0.133[a]
Cholesterol, mg/ dL	171.0±56.8	169.9±42.8	171.4±61.8	0.825[b]
LDL, mg/dL	95.7±42.6	99.9±33.7	94.0±46.1	0.547[b]
HDL, mg/dL	45.6±10.7	47.4±9.3	45.0±11.3	0.267[b]
Triglycerides, mg/dL	158.6±68.4	129.8±42.9	169.4±73.5	0.169[b]
P, mg/dL	5.0±1.1	5.72±1.05	4.73±1.02	**0.011[a]**
Ca, mg/dL	9.02±0.89	9.39±0.85	8.84±0.89	0.073[a]
Ca • P product	43.83±10.63	52.08±9.32	40.67±9.70	**0.002[b]**

Ferritin, ng/mL	361.3±222.9	316.1±221.3	378.3±224.6	0.419[a]
BNP, pg/ml	143.1±119.2	87.0±36.6	163.1±131.9	0.124[b]
PTH,pg/mL	4625±280.0	485.5±366.4	453.9±246.7	0.866[b]
SV,mm	64.2±25.6	67.2±18.7	63.1±27.9	0.644[a]
CRP, mg/L	5.3±8.5	4.8±7.7	5.5±8.9	0.907[b]
TNF-α, pg/mL	11.4±4.3	10.4±2.8	11.7±4.7	0.524[b]
IL-1β,pg/ml	1.3±0.93	1.3±1.0	1.3±0.9	0.969[b]
IL-10, pg/mL	17.7±14.7	17.5±16.7	17.8±14.2	0.825[b]
IL-6, pg/mL	2.9±6.3	5.4±10.3	2.0±3.8	0.687[b]
Endotoxins, EU/ml	3.8±0.8	3.8±0.4	3.7±0.8	0.978[a]
LPS-BP, µg/mL	39.9±17.1	32.2±13.4	41.2±18.3	0.442[b]
TLR-4, pg/mL	624.4±439.2	699.1±464.5	596.4±433.7	0.630[b]
sCD14, µg/ mL	5.0±2.1	4.4±2.0	5.3±2.1	0.224b
T-MAO	0.52±0.62	0.47±0.40	0.57±0.70	0.854[b]
PCS, mg/L	33.5±19.1	36.4±18.0	32.3±19.7	0.341[b]
3-INDS, mg/L	23.7±14.6	24.1±9.6	23.5±16.22	0.442[b]
3-IAA, mg/L	1.1±1.2	1.0±0.5	1.1±1.4	0.169[b]

Results are shown in absolute or relative frequencies (%) or mean ± standard deviation (SD). CKD, chronic kidney disease; PD, peritoneal dialysis; APD, Automated Peritoneal Dialysis; CAPD, continuous ambulatory peritoneal dialysis; Ccreat, creatinine clearance; residual renal function; Kt/V (urea); LDL, low-density lipoprotein; HDL, High-density lipoprotein; P, phosphorous; Ca, calcium; Ca·P product, calcium phosphate product; BNP, B-type natriuretic peptide; PTH, Parathyroid hormone; SV, sedimentation velocity, CRP,C-reactive protein; TNF-α, Tumor necrosis factor-α; IL, Interleukin; LPS-BP, Lipopolysaccharide-binding protein; TLR-4, Toll-like receptor 4; sCD14, soluble CD14; TMAO, trimethylamine N-oxide; PCS, p-cresol sulphate; 3-INDS, 3-indoxyl sulfate; 3-IAA, indole-3-acetic acid. p values were calculated using the following statistical analysis: [a]Student's t-test, [b]Mann-Whitney U test, [c]Pearson Chi-square test, and [d]Fisher test.

Most PD technical parameters did not differ significantly between patients with and without VC, except total Kt/V (urea), which was lower in PD patients with VC (Table 4). In addition, this parameter was inversely correlated with VC severity (Spearman correlation, correlation coefficient = -0.437, $p < 0.01$). The time on PD did not significantly change between both groups.

The analysis of the mean values of Charlson Comorbidity Index showed that PD patients with VC presented a significant increase in all-cause mortality risk compared with PD patients without VC (5.6 ± 2.2 vs. 3.92 ± 3.0, $p < 0.05$).

Accordingly, PD patients with VC included twice as many patients with severe Charlson Comorbidity Index scores than patients without VC (Table 4). VC severity was significantly positively correlated with all-cause mortality risk (Spearman correlation, correlation coefficient (r) = 0.538, $p < 0.001$), meaning that patients with more severe VC presented higher mortality risk. Moreover, by multivariable analysis VC was correlated with the all-cause mortality risk, independently of sex and age.

Pharmacological therapies did not differ significantly between patients with or without VC regarding iron supplementation, erythropoietin, laxatives, hypouricemic agents, statins, calcimimetics, calcium-based phosphate binders, non-calcium-based phosphate binders, and vitamin D. However, the percentage of PD patients on vitamin D analogues and activators of vitamin D receptor (VDR) (including alpha D, calcitriol, paricalcitol, and VDR selective activators) was 100% in patients without VC whereas it was ~72% in patients with VC, representing a statistically significant difference ($p < 0.05$). Further, two patients were on corticosteroids, both with severe VC (Adragao score of 8), and only three patients were not on anti-hypertensive drugs, all with VC.

Regarding biochemical parameters, only phosphorous plasma levels were significantly lower in PD patients with VC than patients without VC. Moreover, markers of inflammation (IL-1, IL-6, TNF-α, and the anti-inflammatory IL-10), markers of intestinal translocation (endotoxins, LPS-BP, TLR4, and sCD14), and uremic toxins of microbial origin (T-MAO, PCS, 3-INDS, and 3-IAA) did not differ significantly between patients with or without VC. Regarding sCD14, although no statistically significant differences were found between PD patients with and without VC, a positive correlation was observed between

sCD14 levels and VC severity ($r = 0.338$, $p < 0.05$). So, PD patients with more severe VC presented higher plasma values of sCD14.

5.2.3. Gut, blood, and urinary microbiome analysis of CKD patients on PD

In the total population of 46 PD patients, the bacterial microbiomes evaluated resulted in a total of 1,127 OTUs obtained from the analysis of gut, blood, and urine samples. A set of 583 OTUs were found in urine samples, while there were 542 OTUs in gut samples, and 514 OTUs in blood samples. Regarding Shannon diversity, urine showed lower diversity in comparison to gut and blood diversities: the median values were 2.29 for urine (ranging from 1.19 to 4.14), 2.66 for blood (1.79-3.09), and 3.41 for gut (2.39-4.09), with significance difference among all groups of samples ($p<0.001$). The taxonomic profiles among the three habitats were distinct (Figure 3). Gut microbiome was dominated by Bacillota and Bacteroidota at phylum level, and by Ruminococcaceae, Bacteroidaceae, Lachnospiraceae, and Prevotellaceae at family level. Blood microbiome was dominated by Pseudomonadota and Actinomycetota at phylum level, and by Pseudomonadaceae, Burkholderiaceae, and Legionellaceae at family level. Urine samples were dominated by Bacillota, Actinomycetota, and Pseudomonadota at family level, and Streptococcaceae, Enterobacteriaceae, Lactobacillaceae, Family XI, and Bifidobacteriaceae at family level. The microbiome of the tested samples was significantly different when comparing urine, gut, and blood ($p=0.001$, Figure 4).

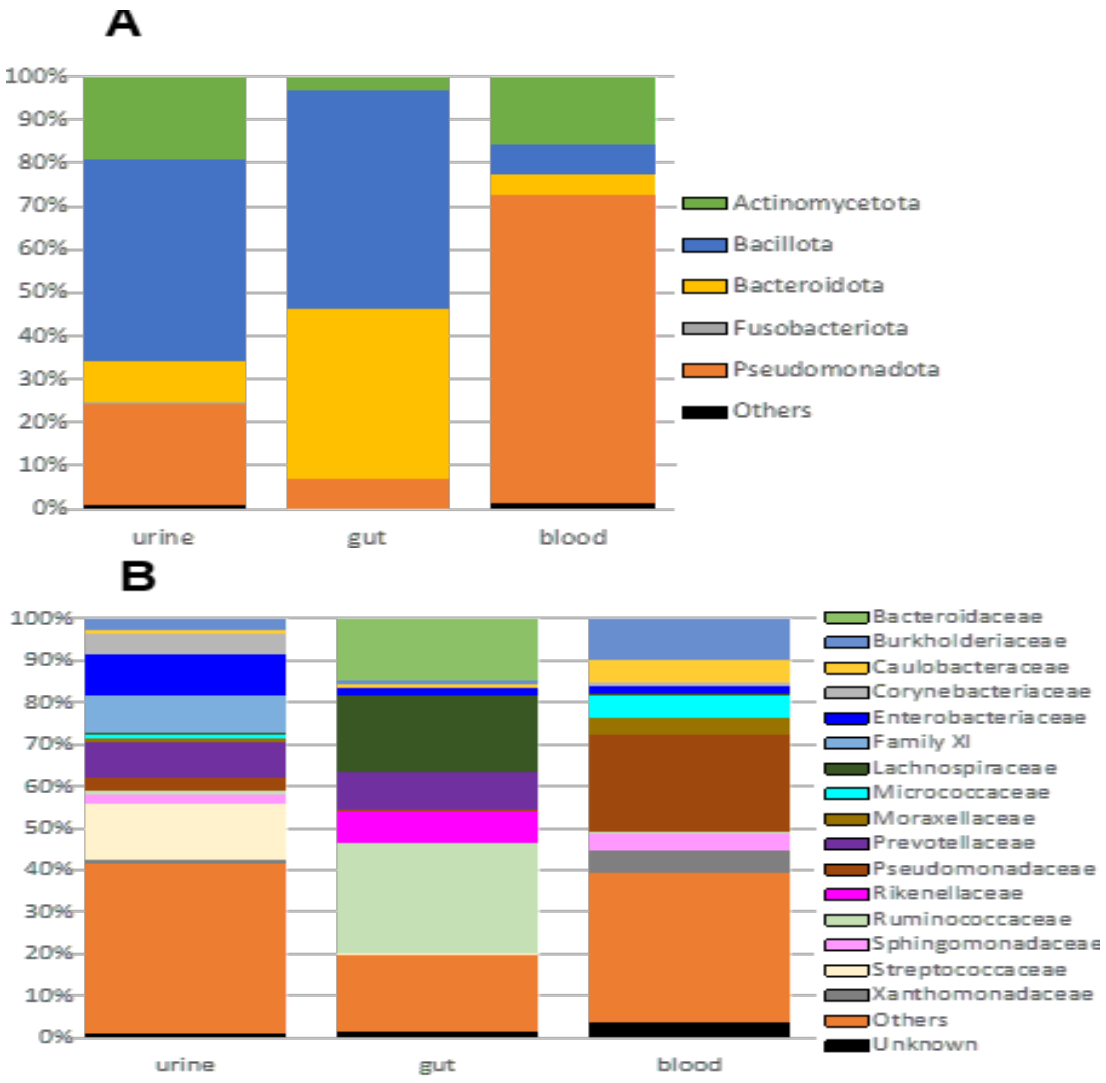


Figure 3: The taxonomic profiles of gut, blood, and urine samples obtained from patients on peritoneal dialysis: (A) phylum, and (B) family levels.

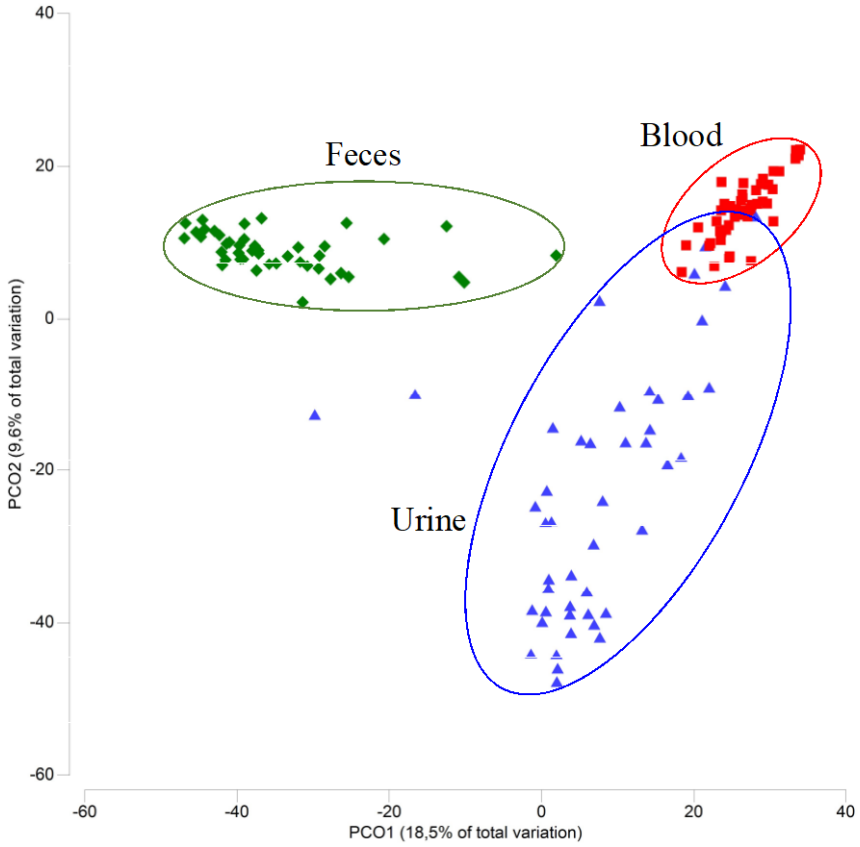


Figure 4: Principal coordinate analysis of fecal, blood and urinary samples collected from patients on PD.

5.2.4. Gut and blood microbiome analysis and the relation with vascular calcification and all-cause mortality risk: CKD patients on PD

Similar values of diversity were observed in both groups of patients (with or without VC) separately regarding gut and blood samples. Beta-diversity

assessment did not show differences in the gut and blood microbial communities when comparing PD patients with and without VC (Figure 5).

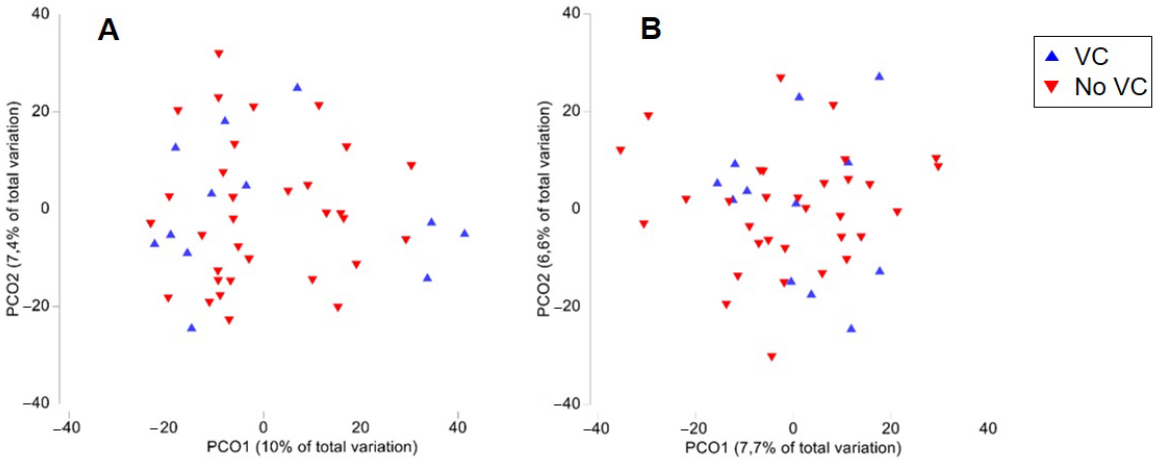


Figure 5: Principal coordinates analysis (PCO) of gut (A) and blood (B) microbiome in PD with vascular calcification (VC) or without vascular calcification (no VC).

ANOSIM and PERMANOVA confirmed the PCO observations, as the groups for both analyses were not significantly different ($p > 0.1$). Therefore, the taxonomic profiles of the gut and blood microbiomes were similar at phylum and family taxonomic levels within each group of PD patients with or without VC. Nonetheless, relative changes of specific rare and/or less abundant taxa were observed between PD patients with and without VC, namely *Coprobacter*, *Coprococcus 3*, *Lactobacillus*, and *Eubacterium eligens* group in the gut microbiome, and *Cutibacterium*, *Pajaroellobacter*, *Devosia*, *Hyphomicrobium*, and *Pelomonas* in the blood microbiome (Figure 6).

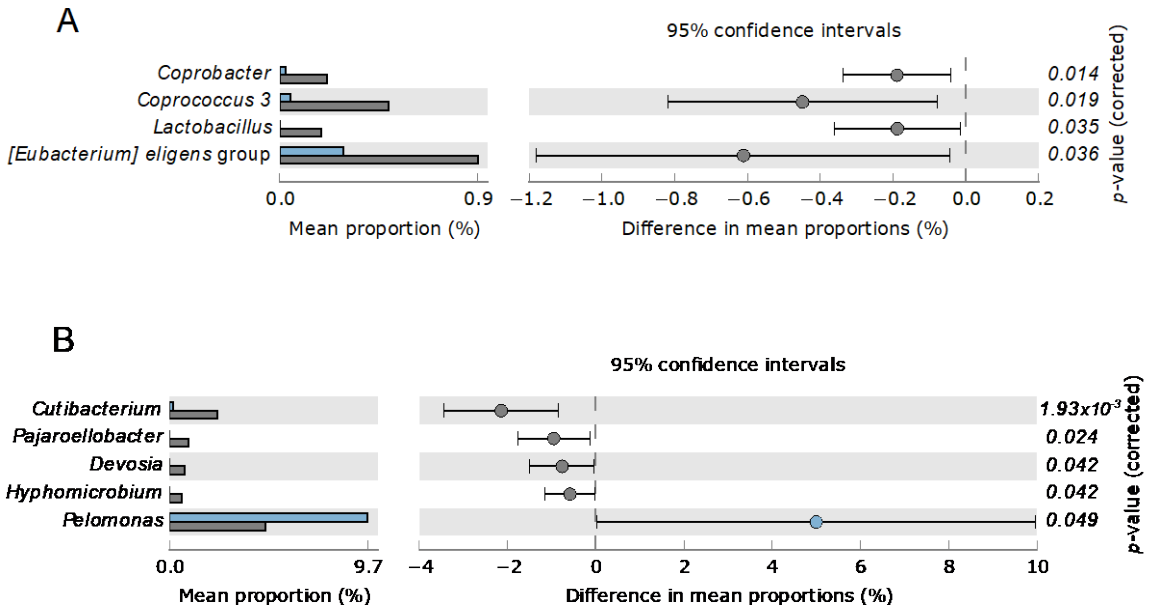


Figure 6: Relative changes of gut (A) and blood (B) bacterial taxa at the genus/family level comparing PD patients with vascular calcification (grey bars) and PD patients without vascular calcification (blue bars).

Given the correlation between VC and all-cause mortality risk, the gut and blood microbiome were compared between PD patients with low all-cause mortality risk (Charlson Comorbidity Index scores of 2 or less) and moderate or severe all-cause mortality risk (Charlson Comorbidity Index scores of 3 or more) (Figure 7). Among the taxonomic differences observed in PD patients with and without VC, patients with moderate or severe all-cause mortality risk presented higher relative abundance in *E. eligens* group in the gut microbiome and *Devosia* in the blood microbiome when compared to patients with low all-cause mortality risk.

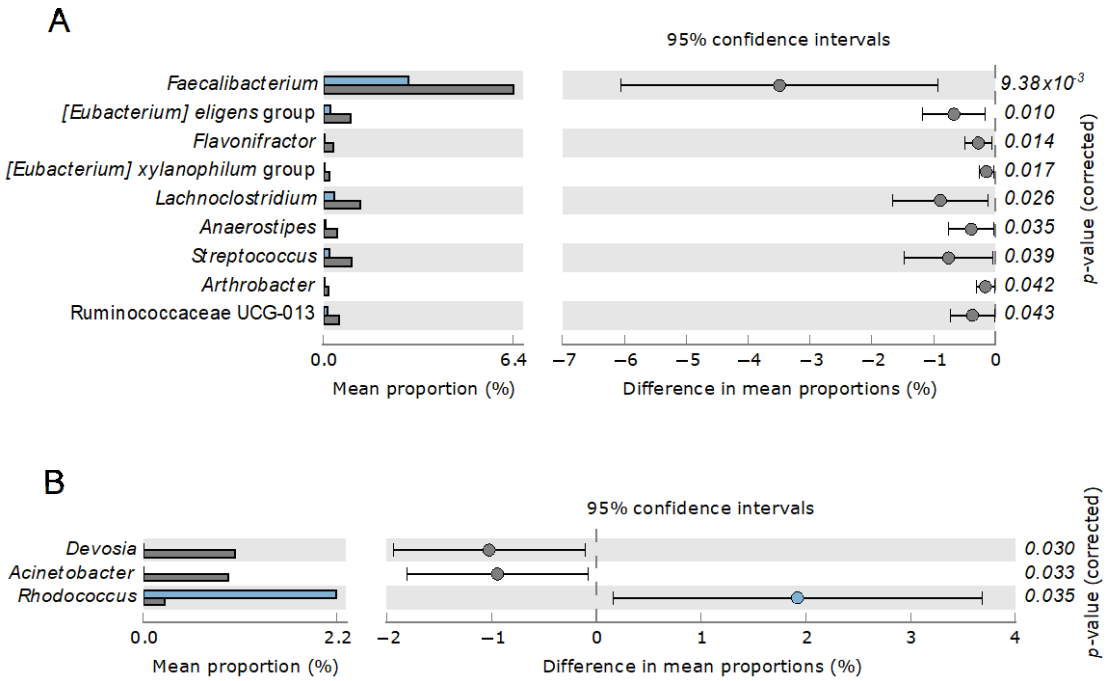


Figure 7: Relative changes of gut (A) or blood (B) bacterial taxa at the genus/family level comparing PD patients with low all-cause mortality risk (Charlson Index ≤2, blue bars) and PD patients with moderate or severe all-cause mortality risk (Charlson Index ≥3, grey bars).

Given that PD patients with VC included more males and older participants, we further investigate if sex and age would play a role in the relative changes of gut and blood microbiomes (Figure 8, Figure 9). Male participants also showed higher levels of *E. eligens* group in the gut in comparison to females. Although *Hyphomicrobium* was elevated in patients with VC compared to patients without VC, *Hyphomicrobium* was present in adult participants but not in senior participants. Therefore, except for *E. eligens* group, the results suggest that the variation of the specific taxa in Figures 6 and 7 are mostly explained by VC in PD patients.

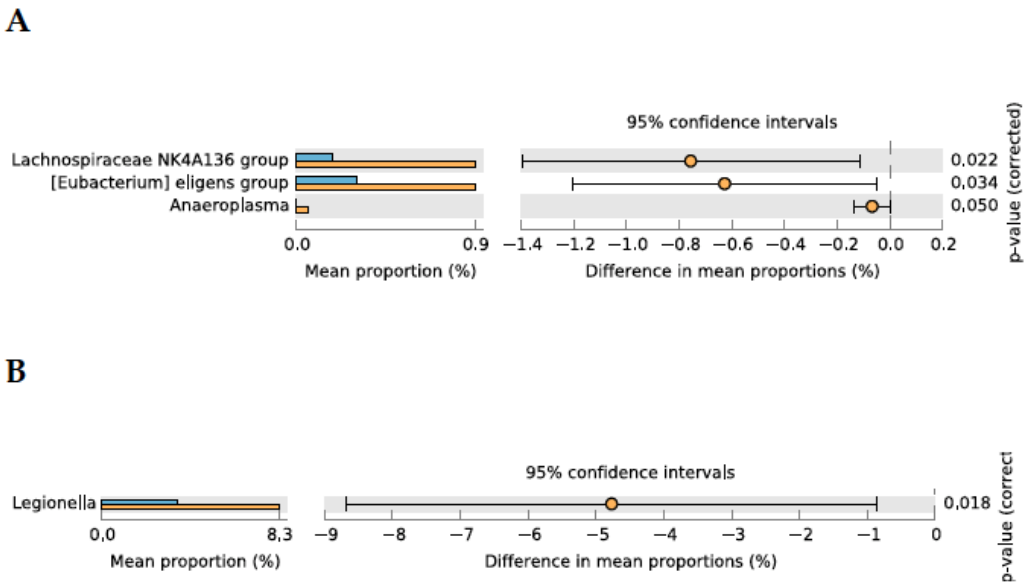


Figure 8: Relative changes in gut (A) and blood (B) bacterial taxa at the genus/family level in peritoneal dialysis patients comparing males (yellow bars) with females (blue bars).

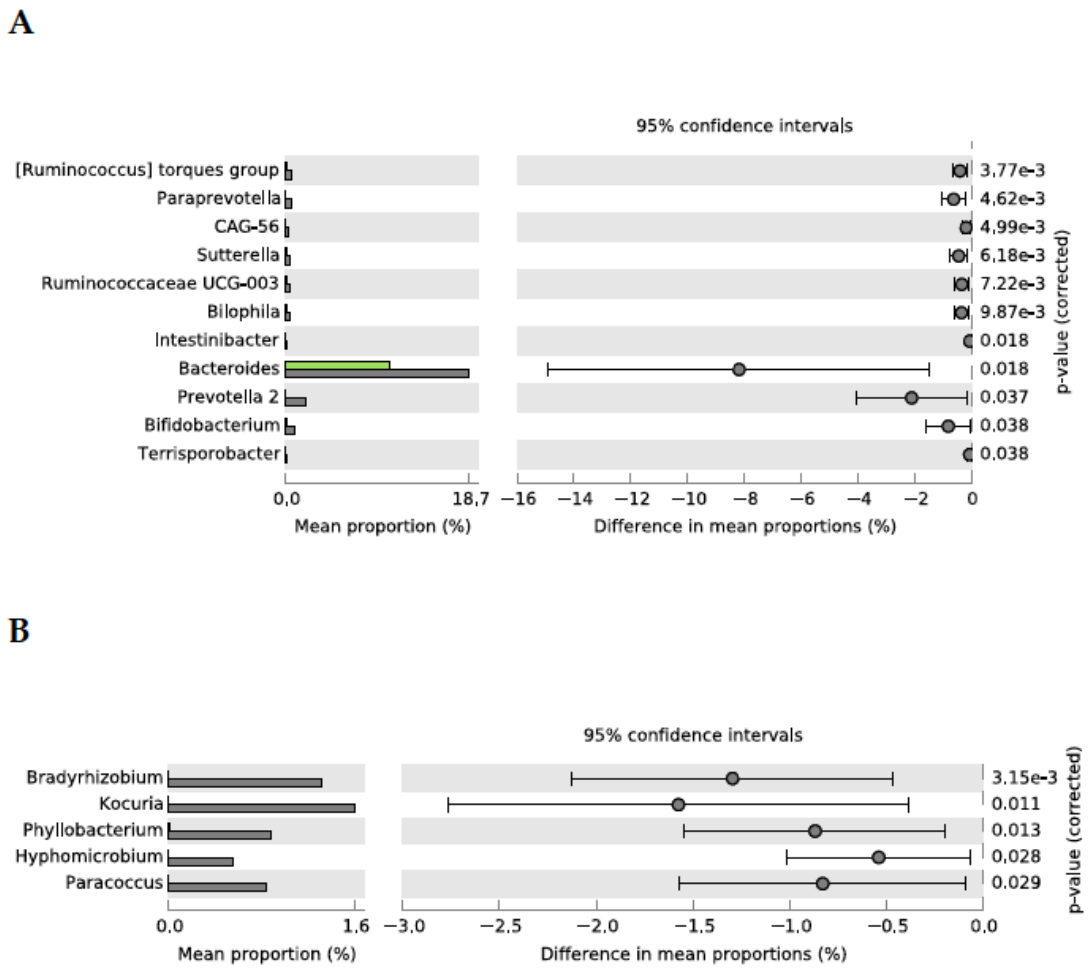


Figure 9: Relative changes of gut (A) or blood (B) bacterial taxa at the genus/family level in peritoneal dialysis patients comparing adulthood (until 65 years old, grey bars) with senior (> 65 years old, green bars).

5.2.5. Urobiome in CKD patients on PD

The taxonomic profiles allowed to organize the urinary samples in multiple subtypes, comprising samples dominated by *Lactobacillus*, *Staphylococcus*, *Streptococcus*, *Gardnerella*, *Prevotella*, *Escherichia-Shigella*, or others (Figure 10). The meaning of finding these subtypes is complex, but it is not related to active urinary infections (because patients with active infections were excluded from this study). All urinary samples of *Lactobacillus* subtype

were linked to female patients, while *Staphylococcus* and *Anaerococcus* dominated samples were exclusively found in male patients.

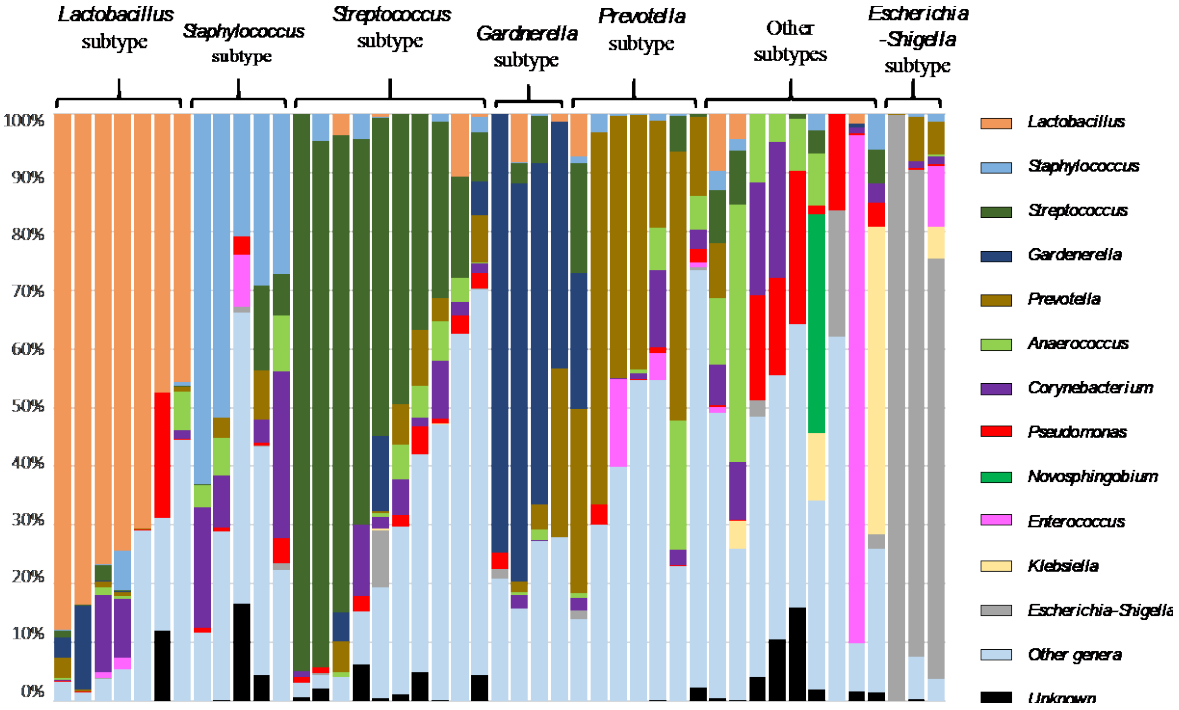


Figure 10: Sample subtypes observed among the urobiome of patients on peritoneal dialysis. Each subtype is dominated by a specific taxonomic group (genus), namely *Lactobacillus*, *Staphylococcus*, *Streptococcus*, *Gardnerella*, *Prevotella*, *Escherichia-Shigella*, or other subtypes (no specific genus dominates this subset of samples).

When comparing the urinary, the gut, and the blood microbiomes in PD patients, *Gardnerella* OTUs were exclusively found in urinary samples (in 20 out of 46 samples). *Dermabacter* and *Atopobium* OTUs were rarely observed in gut and blood samples (in 1 and 2 samples, respectively), but frequently found in urine (6 samples were positive for *Dermabacter* and 13 samples for *Atopobium*). There were other taxonomic groups only found in urinary samples (Table 5 and Table 6). When urine OTUs were compared with OTUs in gut and blood samples, some similarities were observed. There were three OTUs (identified as *Pseudomonas* and *Stenotrophomonas*) common for all PD patients included in this study, and two OTUs (identified as *Pelomonas*

and *Escherichia-Shigella*) presented in more than 90% of the patients. Interestingly, there were some OTUs found in multiple samples from a single patient (e.g., urine and blood). A set of 54 OTUs were common to multiple samples from a single patient, being 39 of these OTUs (72%) found in urinary and stool samples. Only 9 OTUs (17%) were simultaneously observed in urinary and blood samples (17%), and 6 OTUs (11%) were observed in stool and blood samples. Similarities were also high when the analysis was extended to OTUs present in more than one patient, suggesting a high interplay between taxa in urinary and gut microbiomes.

Table 5: Taxonomic groups found exclusively in urine samples.

Phylum	Class	Order	Family	Genus	N reads
Actinomycetota	Actinomycetota	Bifidobacteriales	Bifidobacteriaceae	*Gardnerella*	32
Bacillota	Clostridia	Clostridiales	Family XI	*Finegoldia*	31
Bacillota	Clostridia	Clostridiales	Family XI	*Ezakiella*	24
Actinomycetota	Actinomycetota	Actinomycetales	Actinomycetaceae	*Varibaculum*	20
Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	*Prevotella 6*	18
Bacillota	Clostridia	Clostridiales	Lachnospiraceae	*Howardella*	13
Actinomycetota	Actinomycetota	Actinomycetales	Actinomycetaceae	*Mobiluncus*	12
Bacillota	Negativicutes	Selenomonadales	Veillonellaceae	*Negativicoccus*	10
Bacillota	Clostridia	Clostridiales	Family XI	*Murdochiella*	9
Pseudomonadota	GammaPseudomonadota	Enterobacteriales	Enterobacteriaceae	*Proteus*	9
Bacillota	Bacilli	Lactobacillales	Aerococcaceae	Multi-affiliation	8
Actinomycetota	Actinomycetota	Micrococcales	Dermacoccaceae	*Dermacoccus*	5
Bacteroidota	Bacteroidia	Flavobacteriales	Weeksellaceae	*Bergeyella*	5
Actinomycetota	Actinomycetota	Actinomycetales	Actinomycetaceae	*Actinobaculum*	5
Bacillota	Clostridia	Clostridiales	Family XI	*Parvimonas*	4
Patescibacteria	Gracilibacteria	Absconditabacteriales (SR1)	Unknown	Unknown	3
Pseudomonadota	AlphaPseudomonadota	Sphingomonadales	Sphingomonadaceae	*Altererythrobacter*	3
Pseudomonadota	AlphaPseudomonadota	Sphingomonadales	Sphingomonadaceae	*Novosphingobium*	4
Bacillota	Clostridia	Clostridiales	Lachnospiraceae	*Catonella*	3
Bacillota	Clostridia	Clostridiales	Lachnospiraceae	*Stomatobaculum*	3
Bacillota	Clostridia	Clostridiales	Lachnospiraceae	*Lachnoanaerobaculum*	2
Pseudomonadota	AlphaPseudomonadota	Rhizobiales	Methyloligellaceae	Unknown	1
Pseudomonadota	GammaPseudomonadota	Enterobacteriales	Enterobacteriaceae	*Pantoea*	1
Actinomycetota	Actinomycetota	Micrococcales	Micrococcaceae	*Pseudoglutamincibacter*	2
Actinomycetota	Actinomycetota	Micrococcales	Multi-affiliation	Multi-affiliation	1
Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotellaceae UCG-001	1
Bacillota	Clostridia	Clostridiales	Peptostreptococcaceae	*Filifactor*	1
Bacillota	Negativicutes	Selenomonadales	Veillonellaceae	*Megasphaera*	2
Fusobacteriota	Fusobacteriia	Fusobacteriotales	Leptotrichiaceae	*Sneathia*	2
Pseudomonadota	AlphaPseudomonadota	Rhizobiales	Rhizobiaceae	*Mesorhizobium*	2
Pseudomonadota	GammaPseudomonadota	Pasteurellales	Pasteurellaceae	*Actinobacillus*	1
Pseudomonadota	GammaPseudomonadota	Vibrionales	Vibrionaceae	*Vibrio*	1
Actinomycetota	Actinomycetota	Micrococcales	Demequinaceae	Multi-affiliation	1
Actinomycetota	Actinomycetota	Micrococcales	Micrococcaceae	*Nesterenkonia*	1
Actinomycetota	Actinomycetota	Micrococcales	Promicromonosporaceae	*Cellulosimicrobium*	1

Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	*Prevotella 1*	1
Elusimicrobia	Lineage Iic	Unknown	Unknown	Unknown	1
Bacillota	Clostridia	Clostridiales	Lachnospiraceae	*Shuttleworthia*	1
Bacillota	Clostridia	Clostridiales	Ruminococcaceae	*Fastidiosipila*	1

Table 6: Taxonomic groups found exclusively in urine samples with one exception (one read either in feces or blood samples).

Phylum	**Class**	**Order**	**Family**	**Genus**	**urine**	**gut**	**blood**
Bacillota	Clostridia	Clostridiales	Family XI	*Anaerococcus*	125	1	0
Bacteroidota	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Porphyromonas*	52	1	0
Fusobacteriota	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	*Fusobacterium*	31	1	0
Epsilonbacteraeota	Campylobacteria	Campylobacterales	Campylobacteraceae	*Campylobacter*	22	1	0
Fusobacteriota	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	*Leptotrichia*	20	0	1
Actinomycetota	Coriobacteriia	Coriobacteriales	Atopobiaceae	*Atopobium*	18	0	1
Bacillota	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	18	1	0
Bacillota	Clostridia	Clostridiales	Peptostreptococcaceae	*Peptostreptococcus*	15	1	0
Actinomycetota	Actinomycetota	Bifidobacteriales	Bifidobacteriaceae	*Alloscardovia*	13	1	0
Bacteroidota	Bacteroidia	Flavobacteriales	Flavobacteriaceae	*Capnocytophaga*	10	0	1

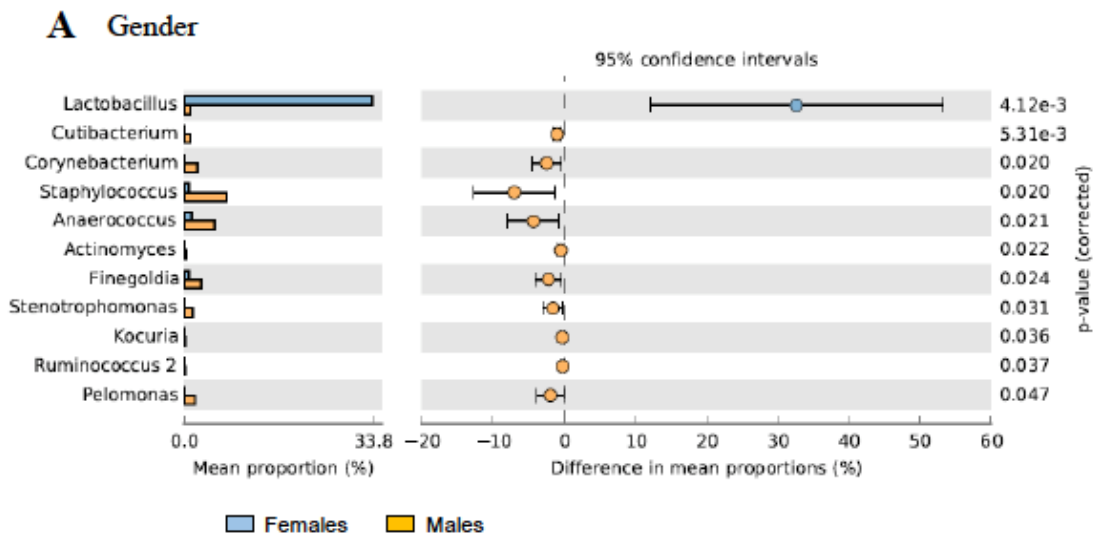
Demographic and clinical variables of PD patients were studied to find correlations with the urinary microbiome, being the largest significance found for gender (p = 0.001). *Lactobacillus* was linked to females and other families, such as *Staphylococcus* and *Anaerococcus*, to males (Table 7 and Figure 11). Figure 11 shows the taxonomic groups significantly affected by the separation of urinary samples in two groups according to different clinical variables.

PD patients with more than 3.5 µg/mL sCD14 levels showed increased levels of *Lactobacilllus*, *Dermabacter*, and *Gardnerella* in urine microbiome (p = 0.03) when compared with patients with equal or lower levels of sCD14. Residual diuresis lower than or equal to 1500 ml/24h, proteinuria, and creatinine clearance lower than 50 L/week were also associated to differences in the PD urobiome. Also of note, lower relative abundance of *Atopobium*, *Dermabacter*, and *Gardnerella* were found in diabetic PD patients (p = 0.055; p = 0.04 by removing two outgroup samples).

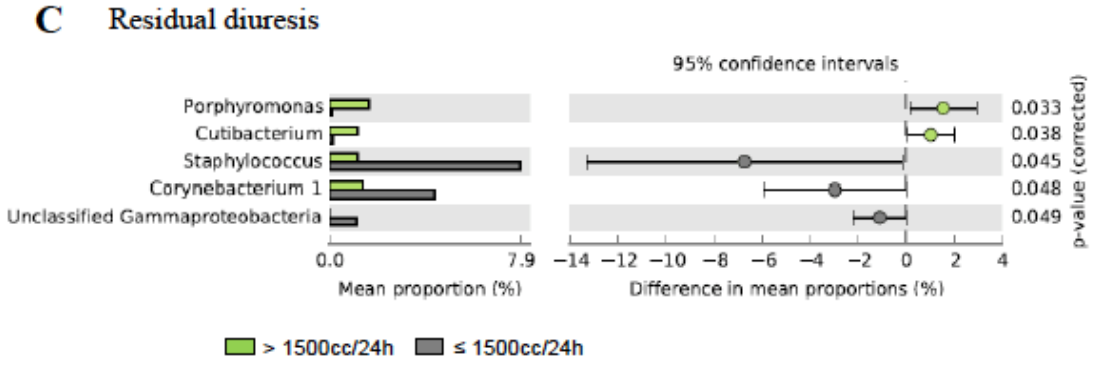
Table 7: Demographic and clinical characterization of PD patients and the association of these variables with urinary microbiome.

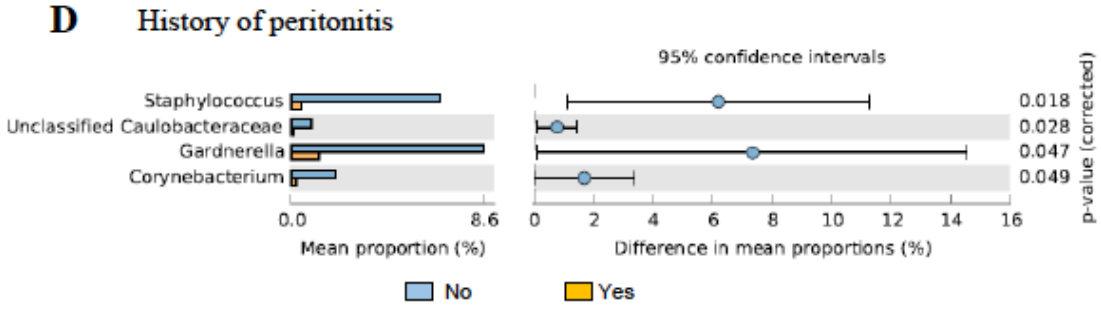
Variable	Mean ± SD	Specificity	n (%)
Gender, female		Female	15 (33%)
Age, years	56.10±10.66	More than 65 years	9 (20%)
		Less than 51 years	14 (30%)
Time in PD, years	2.68±2.45	More than 5 years	5 (11%)
		Less than 1 year	12 (26%)
Type of peritoneal dialysis		Automated	22 (48%)
History of peritonitis		Presence	11(24%)
Iron supplementation		On	35 (76%)
Hypouricemic		On	18 (39%)
Phosphate binders		On	39 (85%)
Diabetes		Presence	14 (30%)
Obesity		BMI higher than 30	5 (11%)
Cardiovascular disease		Presence	10 (22%)
Neoplasm		Present or previous	9 (20%)
Adragao Index		No calcification	12 (26%)
Charlson Index		Less than 2	12 (26%)
Creatinine clearance, L/week	114.09±56.21	Less than 50L/week	3 (7%)
Urea, mg/dl	126.37±37.02	Higher than 150mg/dl	10 (22%)
Urea depuration, Kt/V (urea)	2.22±0.52	Less than 1.7	6 (13%)
Renal clearance total, ml/min	5.62±3.94	Less than 10ml/min	18 (39%)
Residual diuresis, cc/24h	1301.04±830.69	Higher than 1500cc/24h	19 (41%)
Proteinuria, g/24h	1.01±1.25	Presence	39 (85%)
Albumin, g/l	37.03±3.33	Less than 35g/l	11(24%)
Cholesterol, mg/dl	171.48±55.86	Higher than 200mg/dl	9 (20%)
C- reactive protein, mg/l	5.25±8.33	Higher than 10mg/L	6 (13%)
Ferritin, ng/ml	361.75±218.09	Higher than 600ng/ml	6 (13%)
Hemoglobin, g/dl	11.5±0.6	Higher than 12g/dl	15 (33%)
Sedimentation velocity, mm	63.28±25.41	Higher than 81mm	14 (30%)
Soluble CD14, ug/ml	5.13±2.10	Less than 3.5µg/ml	9 (20%)
IL-10, pg/ml	17.98±14.44	Less than 11.1pg/ml	17 (37%)
IL-6, pg/ml	2.82±6.18	Equal or higher than 1.3pg/ml	18 (39%)
IL-1β, pg/ml	1.32±0.92	Less than 1.0pg/ml	22 (48%)
TNF-α, pg/ml	11.47±4.33	Less than 6.0pg/ml	3 (7%)
TLR4, pg/ml	632.85±442.02	Less than 451pg/ml	19 (41%)
LBP, pg/ml	39.94±17.17	Equal or higher than 41pg/ml	18 (39%)

Results expressed in mean and standard deviation (Mean ± SD) for continuous variables and number of patients (n) and prevalence (%) for categoric variables. For statistical analysis, continuous variables were transformed in categoric variables.


A Gender
95% confidence intervals
Lactobacillus
Cutibacterium
Corynebacterium
Staphylococcus
Anaerococcus
Actinomyces
Finegoldia
Stenotrophomonas
Kocuria
Ruminococcus 2
Pelomonas
4.12e-3
5.31e-3
0.020
0.020
0.021
0.022
0.024
0.031
0.036
0.037
0.047
p-value (corrected)
0.0
33.8
Mean proportion (%)
−20 −10 0 10 20 30 40 50 60
Difference in mean proportions (%)
Females
Males


B CD14
95% confidence intervals
Lactobacillus
Dermabacter
Gardnerella
3.35e-3
0.026
0.048
p-value (corrected)
0.0
15.0
Mean proportion (%)
−25 −20 −15 −10 −5 0
Difference in mean proportions (%)
≤ 3.5µ/mL
> 3.5µ/mL


C Residual diuresis
95% confidence intervals
Porphyromonas
Cutibacterium
Staphylococcus
Corynebacterium 1
Unclassified Gammaproteobacteria
0.033
0.038
0.045
0.048
0.049
p-value (corrected)
0.0
7.9
Mean proportion (%)
−14 −12 −10 −8 −6 −4 −2 0 2 4
Difference in mean proportions (%)
> 1500cc/24h
≤ 1500cc/24h


D History of peritonitis
95% confidence intervals
Staphylococcus
Unclassified Caulobacteraceae
Gardnerella
Corynebacterium
0.018
0.028
0.047
0.049
p-value (corrected)
0.0
8.6
Mean proportion (%)
0 2 4 6 8 10 12 14 16
Difference in mean proportions (%)
No
Yes

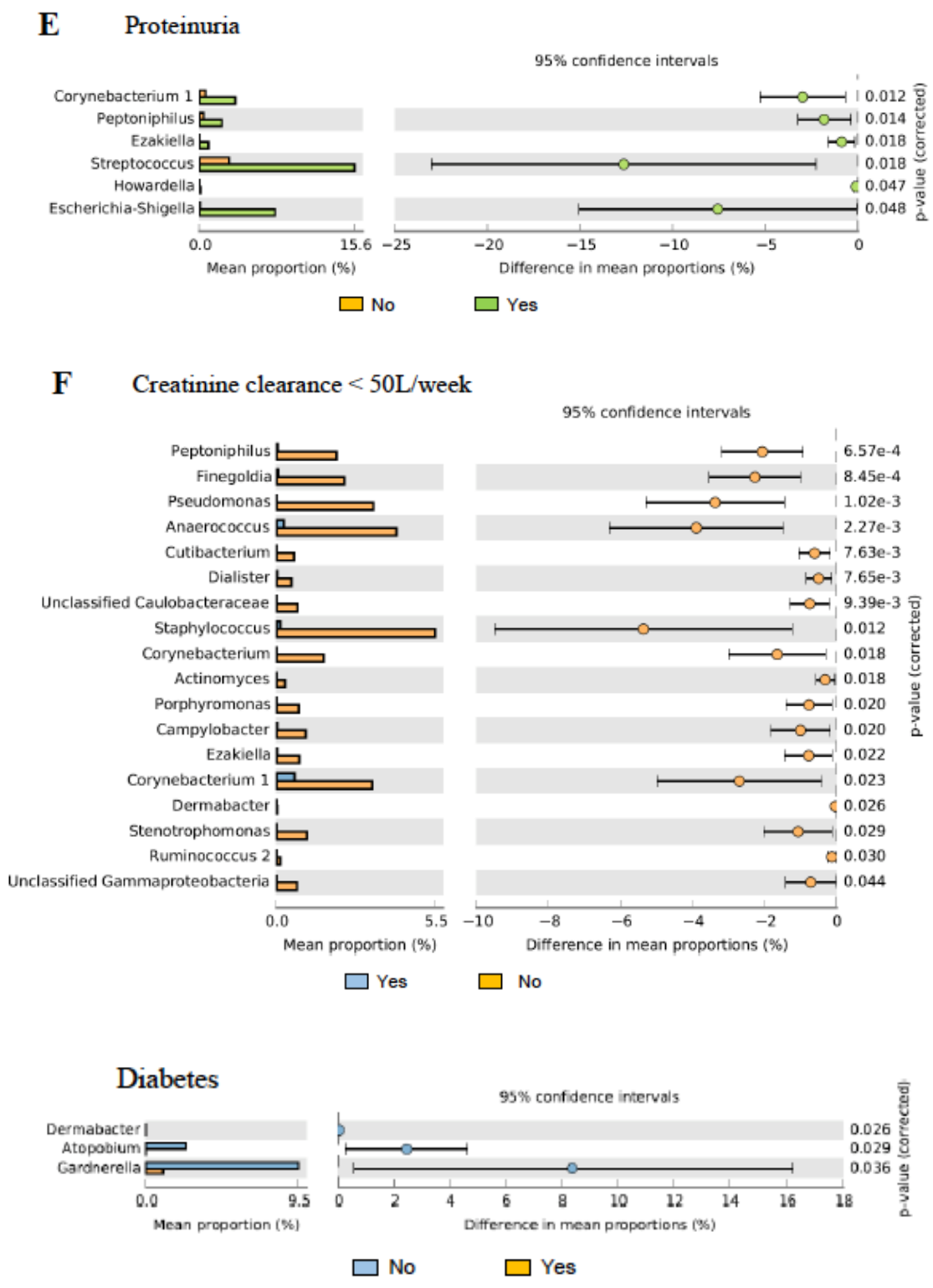


Figure 11: post-hoc analyses describing prevalence differences of multiple taxonomic groups (and p values associated to each taxa) considering the clinical and demographic factors associated to urobiome; all the remaining taxa not displayed in the figures are similar in both groups of patients – the figure displays only the differences. Gender, sCD14, residual diuresis, history of peritonitis, proteinuria and creatinine clearance were significantly associated to urobiome profiles ($p<0.05$; see Table 7 with values for the analysis of

similarities (ANOSIM) and confirmed by permutational multivariate analysis of variance (PERMANOVA). Post-hoc analyses were done in STAMP 2.1.3.

History of peritonitis (occurred more than 3 months before samples collection) was also associated with differences in the urinary microbiome, being *Gardnerella*, *Staphylococcus*, and *Corynebacterium* decreased in such patients (Figure 11). Previous peritonitis was reported in 11 patients, being caused by *Staphylococcus epidermidis* (n=2), *Streptococcus salivarius* (n=2), *Rhizobium radiobacter*, *Klebsiella oxytoca*, *Streptococcus mitis*, *Pantoea spp.*, *Serratia marcescens*, while no agents were identified in 4 cases. Also, there were 4 cases of reported urinary infections (occurring more than 3 months before sample collection) among these patients, being caused by *E. coli* (n=2), *Streptococcus haemolyticus* (n=1), and one unidentified agent. Neither the history of peritonitis, nor previous urinary infection were associated to the specific subtypes described above.

In this work, we also measured markers of intestinal translocation (endotoxins, LPS-binding protein, TLR4, and sCD14), inflammatory parameters (CRP, ferritin, SV, IL-1, IL-6, TNF-α, and the anti-inflammatory IL-10), and other routine laboratory parameters (such as urea, proteinuria, albumin, hemoglobin, cholesterol and its different fractions, triglycerides, calcium, PTH, BNP), but no statistically significant differences were found (Table 7). No difference was observed on the urinary microbiome of patients depending on their PD modality (automated or manual).

Discussion

6. DISCUSSION

6.1. Chronic kidney disease is associated with changes in gut, blood, and urinary microbiomes

6.1.1. Hemodialysis is associated with gut dysbiosis

In CKD patients on HD, we analyzed the gut microbiome of 12 HD patients in Hospital Universitari Germans Trias i Pujol. The gut microbiome in our HD patients is dominated by Bacteroidota and Bacillota phyla, being Actinomycetota, Pseudomonadota, and Verrucomicrobiota in the second line of colonization, and this is in accordance with previous reports (45,71). These described in HD patients' dominant phyla are the same as described in healthy individuals (26–28). Although the gut microbiome of HD patients seems similar to healthy individuals at phyla level, some studies that compare HD patients to healthy controls report that HD patients present higher relative abundances of Bacteroidota and Bacillota (37). Moreover, studies in HD patients report an increase in potentially pathogenic species, particularly of the phyla Pseudomonadota, Actinomycetota, and Bacillota (106). The content of Pseudomonadota in the gut microbiome of healthy people has been reported to be less than 1% (107). Some studies propose that an increase in the relative abundance of Pseudomonadota would be a potential diagnostic signature of dysbiosis and risk of disease, and this increase has been observed in studies comparing HD patients to healthy controls (108,109). In our HD study, we do not have healthy controls to compare, so it is no possible to calculate the comparative proportions between different phyla. Nowadays, most microbiome analysis are only used in an

experimental field, so there are not reference values to describe what is “normal”. To discover the changes in the microbiome of a diseased population, we need healthy controls or a different group of patients for comparison.

In the present study, differences in the gut microbiome of HD patients were observed compared by age or gender. In accordance, some alterations have been demonstrated in the gut microbiome by aging (110). Elderly patients, especially those with high frailty scores, present relative proportions of Bacteroidota predominating, less microbial diversity, and decreases in *Bifidobacteria*, *Bacteroides/Prevotella*, *Lactobacillus*, and *Clostridium* cluster IV, when compared with young individuals, who present more microbial diversity and higher proportions of Bacillota, among others (111,112). There is also mounting evidence supporting that there are alterations in the gut microbiome when comparing women and men (113,114). In this study in HD patients, some differences were observed in the gut microbiome according to gender and age, but the differences found among each patient were much more pronounced. So, microbial profiles were very distinct among patients and, when the samples of all the different time-points were considered, the clustering analysis grouped the samples of the same patient, independently of the clinical condition analyzed (gender, age, ischemic cardiomyopathy, or catheter as vascular access).

It is essential to consider that the present study presents some limitations. On the one hand, the size of the patient sample is small, so it is difficult to draw solid conclusions, especially on the effects of the clinical and biochemical variables analyzed. To validate our results, a larger study, with

an increased number of patients is needed. On the other hand, our patients display different backgrounds, with distinctive comorbidities and different pharmacological therapies which can influence on the gut microbiome. So, our study alerts about the high variability of profiles found on the gut microbiome of HD patients.

6.1.2. Peritoneal dialysis is associated with gut dysbiosis

In CKD patients on PD, we studied in one time-point the gut, blood, and urinary microbiomes of 46 PD patients in Centro Hospitalar Universitário de São João. The gut microbiome of this PD population was dominated by Bacillota and Bacteroidota at the phylum level (115), as described in the previous subchapter in healthy individuals and HD patients. At the family level, the gut microbiome of PD patients was dominated by Ruminococcaceae, Bacteroidaceae, Lachnospiraceae, and Prevotellaceae, according to other studies describing the gut microbiome of PD patients (72,75,77,79). Jiang et al. (77) related Ruminococcaceae with a declined residual renal function in PD patients. In line with this, some studies suggest that uremic condition resulted from impaired renal function may favor the growth of some genera belonging to Ruminococcaceae family (116). We have observed a similar gut microbiome in our PD patients to that previously described in other ESRD populations. Although this seems to be not very innovative, given the fact that there are very few studies in PD patients, with our results we might make our contribution to PD microbiome knowledge.

6.1.3. Peritoneal dialysis and changes in the blood microbiome

Since human blood has traditionally been considered an entirely sterile environment, comprising only blood-cells, platelets and plasma, the detection

of microbes in blood was consistently interpreted as an indication of infection (86,88). Nonetheless, although still controversial, there is evidence supporting the existence of a healthy and a diseased non-infectious human blood-microbiome (81,86,88,117,118). In our PD patients, the results of this thesis show that the blood microbiome is dominated by Pseudomonadota and Actinomycetota at the phylum level and by Pseudomonadaceae, Burkholderiaceae, and Legionellaceae at the family level (115). Pseudomonadota is a major phylum of Gram-negative bacteria, which includes a wide variety of pathogens such as *Escherichia*, *Salmonella*, *Vibrio*, *Yersinia*, *Pseudomonas*, *Burkholderia*, *Legionella*, and many other genera. Pseudomonadota is higher both in the gut and blood in many chronic inflammatory diseases, including inflammatory bowel disease, metabolic syndrome, CVD, and chronic lung diseases (81,117,119).

Regarding CKD patients, Shah et al. (82), carefully profiled blood microbial DNA from non-dialysis CKD patients and compared that microbiome with gut and blood microbiome from healthy controls. They observed that Pseudomonadaceae and Enterobacteriaceae families were significantly higher in the blood microbiome of CKD patients. They also demonstrated higher Pseudomonadota and Actinomycetota predominance in the blood in contrast to Bacteroidota and Bacillota predominance in the gut, in line with our findings in PD patients. An important question raised by Shah et al. (82) results and by our findings is whether microbial DNA in the blood is derived from microorganisms of the colon or from other body habitats (120). However, taking in consideration that both studies found that more than half of the 16S rDNA sequences in the blood of the participants were derived from

Pseudomonadota phylum, which represents a small minority of the normal colon microbial population, this suggests that the blood microbiome is not derived from the colon microbiome in CKD patients, and other body sites must be explored (120).

It is also relevant to note that families found in the blood microbiome of our PD patients include serious clinical pathogens, such as Pseudomonadaceae, Burkholderiaceae, and Legionellaceae. Although PD patients with history of infections in the last 3 months were excluded from this study, when evaluating the history of previous infections, five presented previous *Pseudomonas aeruginosa* infections (between 4 months to 2 years before sample collection), with this pathogen being isolated in the catheter exit-site in four of these five patients, and in the respiratory tract in the remaining patient (115). However, it is important to highlight that our PD patients were stable and not infected at the time of blood samples collection. The detection of such families in the blood microbiome of stable PD patients, may be explained by the translocation of phagocyted microbial cells of microorganisms from other body sites, such as the gut, the oral cavity, the PD catheter biofilm, or even from PD solutions (81,120). These findings also support the existence of a healthy and a diseased non-infectious human blood microbiome (81,86,88,117,118), with relevance in acute diseases, but also in a moment of stability and chronicity of these diseases.

6.1.4. Peritoneal dialysis and changes in the urinary microbiome

The existence of an urobiome has been recognized since 2010 (121) and multiple studies described its diversity and complexity (85,92,97). The urinary microbiome has not been previously described in PD patients. Most PD

patients preserve residual renal function and we thought that, given the easy and not harmful urine collection procedure, it was useful to analyze the urobiome in PD patients and understand its relation to clinical conditions. In our study in PD patients, the urobiome was dominated by Bacillota, Actinomycetota, and Pseudomonadota, specifically by the families Streptococcaceae, Enterobacteriaceae, Lactobacillaceae, and Bifidobacteriaceae and it is similar to the urobiome previously described for CKD patients stages 3 to 5 that were not on dialysis (85). The urine subtypes we described in this set of samples are dominated by the genera *Lactobacillus*, *Staphylococcus*, *Streptococcus*, *Gardnerella*, *Prevotella*, and *Escherichia-Shigella*. And this is in concordance with previously described subtypes in CKD humans and CKD cats (84,85,122) suggesting that those subtypes may be transversal to distinct groups of CKD patients (85) and may have a role in CKD pathophysiology.

It has been recognized the relation of the urinary microbiome on recurrent urinary infections among women (123). In the present work, PD patients with history of infections and antibiotic intake in the last 3 months were excluded. History of catheter exit-site infections and history of urinary infections were not associated to urinary microbiome differences in PD patients. Nevertheless, history of peritonitis (more than 3 months before sample collection) was described as a factor associated to changes in the urobiome in this study, being *Gardnerella*, *Staphylococcus*, and *Corynebacterium* decreased in these patients. This fact suggests that peritonitis occurrence may promote a microbial translocation to the bladder, and this may alter the urobiome persistently even after successful treatment. It would be interesting

to have a long-term follow-up of infectious events to better evaluate if the urobiome may have an impact on infectious episodes.

This is the first study that analyze the urinary microbiome in PD patients. In our PD patients, we found that gender and diabetes were associated to differences in the urobiome, while age did not alter this microbiome. In previous reports, some clinical conditions, such as diabetes, dyslipidemia, older age, or gender (85,97) have been associated with fluctuations in the urinary microbiome of non-CKD patients. Regarding the gender, in the present study, all urinary samples of *Lactobacillus* were linked to female PD patients, while *Staphylococcus* and *Anaerococcus* dominated samples were exclusively found in male PD patients, as described in a previous study analyzing non-dialysis CKD women and men (85). *Lactobacillus* has been also previously described in healthy women (124). Lower relative abundances of *Atopobium*, *Dermabacter*, and *Gardnerella* were found in diabetic PD patients. *Gardnerella* has also been shown to be depleted in the urobiome of diabetic patients compared to healthy controls and was not found in a diabetes plus dyslipidemia cohort comparing with other cohorts (diabetes only, diabetes plus hypertension, and diabetes plus hypertension and dyslipidemia) (125,126).

Residual diuresis, proteinuria, and creatinine clearance were associated to changes in the urobiome of our PD patients. We observed higher levels of *Corynebacterium* and *Staphylococcus* in patients with residual diuresis ≤1500ml/24h. The genera *Staphylococcus* and *Corynebacterium*, among others, have been shown to be increased in patients with urinary tract infection (127). The reduction in the residual diuresis is an indicator of loss of

residual renal function and a risk factor for poor outcomes and prognosis in PD patients (100). Some species of *Corynebacterium* have a potent ability to metabolize urea and some studies relate an increase of this bacterium to urinary calculi (128). We also observed that proteinuria and creatinine clearance < 50 L/week were associated to multiple different taxonomic groups in the PD urobiome. Still, such findings result from a low number of samples analyzed and should be further explored.

The levels of inflammatory parameters and intermediate size molecules clearance may be related to specific taxonomic groups frequently present in urine (123), but confirmatory studies supporting these associations are still lacking. Interestingly, in our study PD patients with higher sCD14 levels presented an increase in *Lactobacillus*, *Dermabacter*, and *Gardnerella* in the urobiome. CD14 is a human monocyte differentiation antigen that acts as a pattern recognition receptor by binding to pathogen-associated molecular patterns such as LPS, working as TLR co-receptor for the detection of infections (129). Particularly in CKD, we have also associated in our PD study, as described in subchapter 5.6, higher levels of sCD14 to VC, CVD, and all-cause mortality.

6.1.5. Differences between gut, blood, and urinary microbiome in peritoneal dialysis patients

In the present study, the urinary microbiome of PD patients showed a lower biodiversity (Shannon index) compared to gut and blood microbiomes, as described for other non-dialysis CKD patients (85). In this study, the urinary microbiome of PD patients was distinct from the gut and blood microbiome and presented some exclusive taxa such as *Gardnerella*. *Gardnerella* has

been increasingly recognized as a common and often abundant member of the female urinary microbiome. Some studies even suggest that the presence of *Gardnerella* is associated with urological or gynecological disorders (130,131). *Gardnerella* has been also identified in the urobiome of patients with history of high blood pressure (95). The inoculation of *Gardnerella* into the bladders of mice results in urothelial exfoliation, urothelial turnover, and increased susceptibility to subsequent urinary tract infections caused by pathogenic *Escherichia coli* (132). In our PD population, we observed an increase in *Gardnerella* in PD patients with higher levels of sCD14, but we have observed a depletion of *Gardnerella* in PD patients with diabetes and in those with history of peritonitis more than 3 months before sample collection. Given these findings and considering that PD patients with history of infection in the last 3 months were excluded, we cannot clarify the role of *Gardnerella* in PD patients.

PD patients may present a different quantitative and qualitative microbial profile in gut, blood, and urine when compared to healthy individuals. Results showed shared OTUs between gut, blood, and urine microbiomes. These similarities can be influenced by the uremic environment that promotes the disruption of intestinal tight junctions, and the translocation of gut taxonomic groups and toxins into the blood and other body fluids (92). Nonetheless, this is a unicentric, cross-sectional study. For this reason, future studies should explore unrecognized mobility pathways of the human microbiome through different body habitats, and its role in infections and systemic inflammation in PD patients.

6.2. Phosphate binders and the gut microbiome in hemodialysis patients

This is the first study comparing the changes in the gut microbiome of HD patients taking CA versus SFO. The patients were divided into two groups and their treatment for hyperphosphatemia was changed: 5 patients were placed in CA group (4 continuing CA therapy and 1 patient changing from calcium carbonate therapy) and 7 were switched to SFO (5 changing from CA therapy and 2 starting phosphate-binding treatment). The gut microbiome was analyzed in stool samples collected at: baseline, 4, 12, and 20 weeks after treatment initiation. The main clinical parameters were not different between HD patients assigned to CA or SFO groups at baseline. In this study, there were no consistent differences in the bacterial composition of the gut microbiome between the two groups treated with these different phosphate-binding agents. Although different microbiome profiles were observed when both groups of treatment were compared, this different profile was already presented at baseline, and long-term treatment did not modify this diversity in any of the two groups. The treatment with CA or SFO during 4, 12, and 20 weeks in our HD population did not significantly modify baseline gut microbiome diversity nor composition in any of the two groups, as reported in our results (133).

Some other studies have previously reported the effects on the gut microbiome of phosphate binders. Regarding calcium-based phosphate-binding agents, an increase of fecal total SCFAs and a higher relative abundance of the genus *Clostridium* XVIII in healthy individuals taking calcium carbonate (63). Navarro-Gonzalez et al. (59) analyzed serum samples from HD patients taken either the non-calcium-based phosphate binder sevelamer or CA and concluded that treatment with sevelamer was associated with a significant decrease in high-sensitive CRP,

IL-6, serum endotoxin, and sCD14, independent predictors of mortality in HD patients. There are not previous studies that specifically analyze the effects of CA on the gut microbiome.

Recently, iron-based phosphate binders, such as ferric citrate and SFO, have been approved for the treatment of hyperphosphatemia in dialysis patients. Data suggested that the iron contained in these iron-based phosphate binders may switch gut microbiome because some gut bacteria use iron to increase their relative abundance (64,134,135). Moreover, it has been shown that an increase in the amount of iron reaching the colon may promote virulence of some pathogenic bacteria and a pro-inflammatory environment (57,136). Given these data, we expected that SFO treatment would promote changes in the gut microbiome, but our study shows that SFO treatment does not modify the gut microbiome in HD patients, nor CA treatment. Regarding previous studies analyzing the effects of iron-based phosphate binders on the gut microbiome, Lau et al. (65), compared fecal microbiome and uremic toxins in serum samples between CKD rats (who underwent 5/6 nephrectomy) and normal rats, randomly assigned to a regular diet or a diet containing 4% ferric citrate for 6 weeks. They observed that CKD rats had lower relative abundances of Bacillota, and *Lactobacillus* and a lower gut microbial diversity compared to normal rats, but they also described that ferric citrate treatment in CKD rats increased bacterial diversity almost to levels observed in control rats and that this treatment did not increase uremic toxins. In a recent study, Wu et al. (137), compared HD patients gut microbiome treated with either calcium carbonate or ferric citrate. They observed a significantly increased microbial diversity in the group treated with ferric citrate, with an increased abundance of Bacteroidota and a decreased abundance of Bacillota. Before the publication of our

results, Iguchi et al. (138), compared 3 months' changes in the gut microbiome and uremic toxins of HD patients treated with SFO versus no treatment for hyperphosphatemia. They also found no changes in the gut microbiome in patients treated with SFO throughout time. So, this study confirmed the long-term stability of the gut microbiome in HD patients treated with SFO for 5 months. A later publication confirmed this stability of the gut microbiome in HD patients and observed a stability of the oral microbiome four weeks after starting SFO treatment (139).

In this study on HD patients, no changes in the gut microbiome after 20 weeks of treatment were observed, independently of the phosphate binder taken. For the moment, when choosing a phosphate binder, we should rely on their power on the reduction of serum phosphate, the pill burden, the association to the progression of VC, the adverse events, or the gastrointestinal tolerance (140–143). Although the influence of these phosphate binders on the gut microbiome was expected, and remains possible, for now, there is no evidence that this aspect should influence our approach when treating hyperphosphatemia in ESRD patients.

6.3. Phosphate binders and clinical and biochemical parameters in hemodialysis patients

As reported, to search if a specific clinical variable could influence on this differentiated microbiome profile, the main clinical parameters at baseline of our HD patients were analyzed and not statistically significant differences were found between both groups of treatment. Patients 5 and 6 were a little bit out of order and it can be stated that patient 5 received vancomycin and tobramycin for 3 weeks (initiated before week 20 sample collection), while patient 6 presented chronic

diarrhea with repeatedly negative cultures and a possible wasting syndrome associated.

Regarding laboratory findings, as expected, HD patients treated with the calcium-based phosphate binder CA presented higher calcium levels than those treated with SFO. CA group compared with SFO group presented, although not statistically significant, increased levels of inflammatory parameters, such as SV, CRP, and ferritin. Such pleiotropic effect on diminishing inflammation was described for some phosphate binders other than calcium-based phosphate binders (143,144).

6.4. Vascular calcification, all-cause mortality risk, and the gut and blood microbiomes in peritoneal dialysis patients

To evaluate the differences in the gut and blood microbiomes in association with the severity of VC and the risk of mortality, a subgroup of 44 PD patients in Centro Hospitalar Universitário de São João was analyzed, VC was assessed by Adragao score, and all-cause mortality risk was estimated by Charlson comorbidity Index. Relative changes were observed in specific taxa when comparing PD patients with and without VC, namely an increase in *Coprobacter*, *Coprococcus 3*, *Lactobacillus*, and *Eubacterium eligens* group in the gut microbiome of PD patients with VC, and an increase in *Cutibacterium*, *Pajaroellobacter*, *Devosia*, and *Hyphomicrobium* and a decrease *Pelomonas* in the blood microbiome of PD patients with VC (115). An association between VC and all-cause mortality risk in PD patients was also observed, and patients with higher mortality risk corroborate the changes of *E. eligens* in the gut and *Devosia* genus in the blood.

Coprobacter, *Coprococcus 3*, *Lactobacillus*, and *E. eligens* were more abundant in the gut microbiome of PD patients with VC. Despite only a few taxa differed between the gut microbiome of PD patients with and without VC, these taxa represent relevant groups and some of these taxa are key players in the gut microbiome (145–148) Regarding this taxa and CVD, some studies relate *Lactobacillus* to cardiotoxicity and ST-segment elevation myocardial infarction (149), some studies associate an increase in *Eubacterium*, *Coprococcus* (some studies relate specifically *Coprococcus 3*), and *Lactobacillus* to CVD and stroke (150), and a study also relates *Lactobacillus* to increased homocysteine levels in patients with obstructive sleep apnea–hypopnea syndrome (151). Despite that, the role of *Lactobacillus* is controversial, and other studies observe a protective role of *Lactobacillus* in CVD, inflammatory response, and metabolic disorders (152).

Among the taxonomic differences observed in the gut microbiome of PD patients with or without VC, patients with higher mortality risk also demonstrated higher relative abundance in *E. eligens* group, highlighting a potential critical role of this taxon in PD patients. The increase in the relative abundance of *E. eligens* group is most frequently associated with a healthy status (153,154). For example, *E. eligens* was depleted in stool samples from atherosclerotic patients from Sweden and China cohorts and was appointed as promising probiotics and potential therapeutic target for atherosclerosis (153). However, a recent study identified that *E. eligens* group may have causal effects on increasing the risk of CKD (155). In our PD study, the microbiome differences associated to gender may have contributed to this result, given that participants with VC included more males, and male participants also presented higher *E. eligens* group prevalence in comparison to females. But, as the

results of the present study and other studies suggest, the increase in *E. eligens* may not always constitute a protective factor.

When comparing the blood microbiome of PD patients with and without VC, PD patients with VC presented an increase in *Cutibacterium*, *Pajaroellobacter*, *Devosia*, and *Hyphomicrobium*, and a decrease in the relative abundance of *Pelomonas*. Most of these groups appear sporadically in different areas of the human microbiome (skin, oral, gut) (156–159), but the real role of these genera remains unknown. We observed an increase in the relative abundance of *Devosia* genus both in PD patients with VC (when compared with PD patients without VC), as well as in PD patients with higher mortality risk. To our knowledge, *Devosia* has not been previously reported in the blood microbiome but has been found to be increased in the gut microbiota of colorectal cancer patients (159) and in rabbits with heat stress (160). Given these findings, the role of *Devosia* as a biomarker of CVD and mortality in CKD PD patients should be further explored.

6.5. Vascular calcification, all-cause mortality risk, and clinical and biochemical in peritoneal dialysis patients

When comparing PD patients with and without VC, patients with VC showed higher estimated mortality risk, corroborating previous reports (23). In this study, PD patients with VC included more males, older patients, and more diabetics in comparison with PD patients without VC. In fact, these three factors were previously recognized as major contributors to VC (161,162).

Moreover, PD patients with VC presented lower Kt/V (urea) values when compared with patients without VC. We must remark that, although we found lower Kt/V values in PD patients with VC, the Kt/V (urea) values observed in both groups are within

the recommended range by the International Society for PD (not less than 1.7) (100). To date, there has been a wide debate about the influence of dialysis dose on the prognosis of ESRD patients (163). Some studies suggest that a more efficient removal of urea leads to decrease morbidity and improves all-cause mortality(164), and even describe a negative correlation between Kt/V and pulse wave velocity as surrogate marker of VC (165). However, several reports do not support the potential benefit of high dose dialysis, and positively correlate Kt/V (urea) values with VC (163), contrary to our findings. In summary, the role of Kt/V (urea) in VC needs to be clarified.

When comparing phosphorous levels between PD patients with and without VC, patients without VC unexpectedly presented higher phosphorous levels. Higher levels of calcium-phosphate product were observed in patients without VC when compared with patients with VC but below the cut-off established for higher risk of VC and CVD (166). Previous reports (167,168) suggest that VC is marked by hyperphosphatemia and higher levels of calcium-phosphate product. Perhaps our results could be explained by some peculiarities in this study population. We performed a unique blood test, and we did not collect samples in different time-points, so it is possible that our PD patients with VC presented higher phosphorous levels in the past. Another argument is that PD patients on vitamin D analogues and activators of VDR (including alpha D, calcitriol, paricalcitol, and VDR activators) represented 100% of patients without VC, and only ~72% of patients with VC, denoting a significant difference ($p < 0.05$). The relationship between vitamin D and VC is complex. Moderate activation of VDR signaling protects against VC, but a deficient or excessive activation of VDR has been associated to VC (169). As some studies proved that the treatment with calcitriol and paricalcitol may protect against

VC (170), others found no differences in the presence of VC in PD patients treated with calcitriol (171). Vitamin D analogues and activators of VDR promote an increase in phosphate levels, so the higher intake of these drugs in the group without VC may collaborate on the higher levels of phosphorous in that group. Another argument to be looked at with caution is that, although not statistically significant, in this study the group with VC was treated in a higher proportion with calcium-based phosphate binders and in a lower proportion with non-calcium-based phosphate binders, resulting in better phosphorous control in that group. In clinical trials, calcium-based phosphate binders compared with non-calcium-based phosphate binders, have been related to promote hypercalcemia and consequently, to increase morbidity and mortality, CVD, and the progression of VC in ESRD patients (168,172,173).

6.6. Vascular calcification, all-cause mortality risk, and markers of intestinal translocation, inflammatory parameters, and uremic toxins in peritoneal dialysis patients

In the PD patients study, markers of intestinal translocation (endotoxins, LPS-BP, TLR4, and sCD14), inflammatory parameters (CRP, ferritin, SV, IL-1β, IL-6, TNF-α, and the anti-inflammatory IL-10), uremic toxins (PCS, 3-INDS, 3-IAA, and TMAO), and other routine laboratory parameters (such as urea, proteinuria, albumin, hemoglobin, cholesterol and its different fractions, triglycerides, calcium, PTH, BNP), were also measured but no statistically significant differences were found between PD patients with or without VC. Although some studies showed that markers of intestinal translocation, uremic toxins, or inflammatory parameters are

increased in CKD patients (174–176), we have found no differences in those parameters when comparing PD patients with and without VC. It should be noted that this PD study population did not include healthy controls for comparison, so we can only analyze the changes comparing PD patients with and without VC and we cannot define the changes that are promoted by CKD itself. The absence of differences between PD patients with or without VC could be associated with the relatively small number of patients included in this study.

Interestingly, sCD14 was positively correlated with VC severity. In accordance, plasma sCD14 levels have been independently associated with myocardial infarction, coronary heart disease, and all-cause mortality among men and women above 65 years old in the Cardiovascular Health Study (177). Longenecker et al. (178) observed that sCD14 was independently associated with coronary artery calcification measured by computed tomography and predicted the extent of subclinical disease in other vascular beds in HIV patients. Poesen et al. (179) demonstrated that sCD14 was elevated in patients with decreased kidney function and was associated with mortality and CVD in non-dialysis CKD patients during a median follow-up of 52–54 months. Other studies positively correlated higher levels of sCD14 level to markers of inflammation and negatively to nutritional status and concluded sCD14 to be an independent predictor of all-cause mortality in long-term HD patients (180,181). Together, these findings support a putative role of sCD14 in VC that should be explored in future studies in PD patients.

In summary, although no differences in uremic toxins, intestinal translocation markers, and inflammatory parameters were found among PD patients with and without VC, sCD14, a nonspecific marker of monocyte activation, was positively correlated with VC severity, suggesting its association with inflammation.

Collectively, these results open new avenues for biomarkers discovery in PD patients (115).

Conclusions

7. CONCLUSIONS

1. The gut microbiome of our HD patients is dominated by Bacteroidota and Bacillota phyla, being Actinomycetota, Pseudomonadota, and Verrucomicrobiota in the second line of colonization. The gut microbiome of our PD patients is dominated by Bacillota and Bacteroidota at the phylum level, and by Ruminococcaceae, Bacteroidaceae, Lachnospiraceae, and Prevotellaceae at family level. The blood microbiome of our PD patients is dominated by Pseudomonadota and Actinomycetota at the phylum level and by Pseudomonadaceae, Burkholderiaceae, and Legionellaceae at the family level. The urobiome showed lower diversity than the gut and blood microbiomes in our PD patients. The urobiome of our PD patients is dominated by Bacillota, Actinomycetota, and Pseudomonadota, specifically by the families Streptococcaceae, Enterobacteriaceae, Lactobacillaceae, and Bifidobacteriaceae

2. HD patients receiving CA presented a more diverse gut microbiome compared to those treated with SFO at baseline. The treatment for 5 months with either CA or SFO does not modify baseline diversity nor bacterial composition in the gut microbiome of HD patients. Although the influence of these phosphate binders on gut microbiome was expected, and remains possible, for now, there is no evidence that this aspect may influence our approach when treating hyperphosphatemia.

3. In PD patients, mortality risk estimated by Charlson Comorbidity Index is positively correlated to VC assessed by Adragao score. Relative changes when comparing PD patients with and without VC were observed in:

Coprobacter, *Coprococcus 3*, *Lactobacillus*, and *E. eligens* in the gut microbiome; and *Cutibacterium*, *Pajaroellobacter*, *Devosia*, *Hyphomicrobium*, and *Pelomonas* in the blood microbiome. PD patients with higher mortality risk corroborate the changes of *E. eligens* in the gut and *Devosia* genus in the blood.

4. Soluble CD14 is positively correlated with VC severity in our PD patients. PD patients with more than 3.5 µg/mL sCD14 levels show an increase of *Lactobacilllus*, *Dermabacter*, and *Gardnerella* in their urobiome when compared with patients with equal or lower levels of sCD14.

Future lines of investigation

8. FUTURE LINES OF INVESTIGATION

1. Our results suggest that *E. eligens* in the gut microbiome and *Devosia* in the blood microbiome are related to vascular calcification and all-cause mortality risk in PD patients. sCD14 is positively correlated with vascular calcification severity, also PD patients with higher sCD14 levels presented an increase in *Lactobacillus*, *Dermabacter*, and *Gardnerella* in the urobiome. Given that our sample size is small and that we do not have healthy controls to compare, future studies should further explore the role as VC biomarkers in CKD patients of *E. eligens* in the gut microbiome, *Devosia* in the blood microbiome and plasma sCD14, as well as the role of *Lactobacillus*, *Dermabacter*, and *Gardnerella* in the urobiome.

2. In our PD study we measured VC using Adragao score because the simplicity of that method is of great advantage in clinical studies. Despite that, scoring coronary artery calcification by computed tomography entails higher specificity and sensibility to estimate VC. In future studies, it could be an advantage to measure VC by computed tomography instead of Adragao score in ESRD patients.

3. In my thesis, both HD and PD populations had small sample sizes, and we did not have healthy controls to compare, so it is no possible to calculate the comparative proportions between different microbiome populations. Nowadays, most microbiome analyzes are only used in an experimental field, so there are not reference values to describe what is “normal”. To discover the changes in the microbiome of a diseased population, we need healthy controls or a different group of patients for comparison. Also, it would be

desirable if future microbiome studies in CKD patients can be done in larger populations, even multi-centric.

4. It has been recognized the relation of the urinary microbiome on recurrent urinary infections. In the present work, PD patients with history of infections and antibiotic intake in the last 3 months were excluded, but history of peritonitis (more than 3 months before sample collection) was described as a factor associated to changes in the urobiome in our PD patients, being *Gardnerella*, *Staphylococcus*, and *Corynebacterium* decreased in these patients. This fact suggests that peritonitis occurrence may promote a microbial translocation to the bladder, and this may alter the urobiome persistently even after successful treatment. It would be interesting to have a long-term follow-up of infectious events to better evaluate if the urobiome may have an impact on infectious episodes.

References and Annexes

9. BIBLIOGRAPHICAL REFERENCES

1. Kalantar-Zadeh K, Jafar TH, Nitsch D, Neuen BL, Perkovic V. Chronic kidney disease. The Lancet [Internet]. 2021;398(10302):786–802. Available from: http://dx.doi.org/10.1016/S0140-6736(21)00519-5
2. National Kidney Foundation. Clinical practice guidelines for chronic kidney disease : evaluation, classification and stratification. National Kidney Foundation; 2002.
3. Foreman KJ, Marquez N, Dolgert A, Fukutaki K, Fullman N, McGaughey M, et al. Forecasting life expectancy, years of life lost, and all-cause and cause-specific mortality for 250 causes of death: reference and alternative scenarios for 2016–40 for 195 countries and territories. The Lancet. 2018 Nov 10;392(10159):2052–90.
4. Bikbov B, Purcell CA, Levey AS, Smith M, Abdoli A, Abebe M, et al. Global, regional, and national burden of chronic kidney disease, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. The Lancet. 2020 Feb 29;395(10225):709–33.
5. Johansen KL, Chertow GM, Gilbertson DT, Ishani A, Israni A, Ku E, et al. US Renal Data System 2022 Annual Data Report: Epidemiology of Kidney Disease in the United States. American Journal of Kidney Diseases [Internet]. 2023 Mar;81(3):A8–11. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0272638622010617
6. Brück K, Stel VS, Gambaro G, Hallan S, Völzke H, Ärnlöv J, et al. CKD prevalence varies across the European general population. Journal of the American Society of Nephrology. 2016;27(7):2135–47.
7. Gorostidi M, Sánchez-Martínez M, Ruilope LM, Graciani A, de la Cruz JJ, Santamaría R, et al. Prevalencia de enfermedad renal crónica en España: impacto de la acumulación de factores de riesgo cardiovascular. Nefrología. 2018 Nov;38(6):606–15.
8. Informe estadístic del Registre de malalts renals de Catalunya Organització Catalana de Trasplantaments.
9. Kotanko P, Kuhlmann MK, Levin NW. Hemodialysis: Principles and Techniques. In: Comprehensive Clinical Nephrology: Fourth Edition. Elsevier; 2010. p. 1053–9.
10. Vadakedath S, Kandi V. Dialysis: A Review of the Mechanisms Underlying Complications in the Management of Chronic Renal Failure. Cureus. 2017 Aug 24;
11. Bajo Rubio A, Rivas B, del Peso Gilsanz G, José Fernández-Reyes M, Paz Madrid L. Modalidades de diálisis peritoneal. Prescripción y adecuación.
12. Li PKT, Chow KM. Infectious complications in dialysis-epidemiology and outcomes. Vol. 8, Nature Reviews Nephrology. 2012. p. 77–88.
13. Vandecasteele SJ, Boelaert JR, De Vriese AS. Staphylococcus aureus infections in hemodialysis: What a nephrologist should know. Vol. 4, Clinical Journal of the American Society of Nephrology. 2009. p. 1388–400.
14. Mujais S. Microbiology and outcomes of peritonitis in North America. Kidney Int. 2006;70(SUPPL. 103).
15. Liakopoulos V, Nikitidou O, Kalathas T, Roumeliotis S, Salmas M, Eleftheriadis T. Peritoneal dialysis-related infections recommendations: 2016 update. What is new? Vol. 49, International Urology and Nephrology. Springer Netherlands; 2017. p. 2177–84.

16. Sharma S, Sarnak MJ. Epidemiology: The global burden of reduced GFR: ESRD, CVD and mortality. Vol. 13, Nature Reviews Nephrology. Nature Publishing Group; 2017. p. 447–8.
17. Sarnak MJ, Amann K, Bangalore S, Cavalcante JL, Charytan DM, Craig JC, et al. Chronic Kidney Disease and Coronary Artery Disease: JACC State-of-the-Art Review. Vol. 74, Journal of the American College of Cardiology. Elsevier USA; 2019. p. 1823–38.
18. Matsushita K, Coresh J, Sang Y, Chalmers J, Fox C, Guallar E, et al. Estimated glomerular filtration rate and albuminuria for prediction of cardiovascular outcomes: A collaborative meta-analysis of individual participant data. Lancet Diabetes Endocrinol. 2015 Jul 1;3(7):514–25.
19. Jankowski J, Floege J, Fliser D, Böhm M, Marx N. Cardiovascular Disease in Chronic Kidney Disease Pathophysiological Insights and Therapeutic Options. Vol. 143, Circulation. Lippincott Williams and Wilkins; 2021. p. 1157–72.
20. Evenepoel P, Dejongh S, Verbeke K, Meijers B. The role of gut dysbiosis in the bone-vascular axis in chronic kidney disease. Toxins (Basel). 2020;12(5):1–18.
21. Rodrigues FG, Ormanji MS, Heilberg IP, Bakker SJL, de Borst MH. Interplay between gut microbiota, bone health and vascular calcification in chronic kidney disease. Eur J Clin Invest. 2021;51(9):1–14.
22. Yamada S, Giachelli CM. Vascular calcification in CKD-MBD: Roles for phosphate, FGF23, and Klotho. Vol. 100, Bone. Elsevier Inc.; 2017. p. 87–93.
23. Niu Q, Zhao H, Wu B, Tsai S, Wu J, Zhang M, et al. Study on the Prevalence of Vascular Calcification in Different Types of Arteries and Influencing Factors in Maintenance Peritoneal Dialysis Patients. Blood Purif. 2019 Mar 1;47(Suppl 1):8–16.
24. Adragao T, Pires A, Lucas C, Birne R, Magalhaes L, Gonçalves M, et al. A simple vascular calcification score predicts cardiovascular risk in haemodialysis patients. Nephrology Dialysis Transplantation. 2004 Jun;19(6):1480–8.
25. Proctor LM, Creasy HH, Fettweis JM, Lloyd-Price J, Mahurkar A, Zhou W, et al. The Integrative Human Microbiome Project. Nature. 2019 May 30;569(7758):641–8.
26. Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT, et al. Structure, function and diversity of the healthy human microbiome. Nature. 2012 Jun 14;486(7402):207–14.
27. Tuddenham S, Sears CL. The intestinal microbiome and health. Vol. 28, Current Opinion in Infectious Diseases. Lippincott Williams and Wilkins; 2015. p. 464–70.
28. Rinninella E, Raoul P, Cintoni M, Franceschi F, Miggiano GAD, Gasbarrini A, et al. What is the healthy gut microbiota composition? A changing ecosystem across age, environment, diet, and diseases. Microorganisms. 2019 Jan 1;7(1).
29. Kamada N, Chen GY, Inohara N, Núñez G. Control of pathogens and pathobionts by the gut microbiota. Vol. 14, Nature Immunology. 2013. p. 685–90.
30. Kho ZY, Lal SK. The human gut microbiome - A potential controller of wellness and disease. Vol. 9, Frontiers in Microbiology. Frontiers Media S.A.; 2018.
31. Winter SE, Bäumler AJ. Dysbiosis in the inflamed intestine: Chance favors the prepared microbe. Gut Microbes. 2014;5(1):71–3.
32. Gomes-Neto JC, Kittana H, Mantz S, Segura Munoz RR, Schmaltz RJ, Bindels LB, et al. A gut pathobiont synergizes with the microbiota to instigate inflammatory disease marked by immunoreactivity against other symbionts but not itself. Sci Rep. 2017 Dec 1;7(1).

33. Arnoriaga-Rodríguez M, Mayneris-Perxachs J, Burokas A, Contreras-Rodríguez O, Blasco G, Coll C, et al. Obesity Impairs Short-Term and Working Memory through Gut Microbial Metabolism of Aromatic Amino Acids. Cell Metab. 2020 Oct 6;32(4):548-560.e7.
34. Franzosa EA, Sirota-Madi A, Avila-Pacheco J, Fornelos N, Haiser HJ, Reinker S, et al. Gut microbiome structure and metabolic activity in inflammatory bowel disease. Nat Microbiol. 2019 Feb 1;4(2):293–305.
35. Gopalakrishnan V, Helmink BA, Spencer CN, Reuben A, Wargo JA. The Influence of the Gut Microbiome on Cancer, Immunity, and Cancer Immunotherapy. Vol. 33, Cancer Cell. Cell Press; 2018. p. 570–80.
36. Scheithauer TPM, Rampanelli E, Nieuwdorp M, Vallance BA, Verchere CB, van Raalte DH, et al. Gut Microbiota as a Trigger for Metabolic Inflammation in Obesity and Type 2 Diabetes. Vol. 11, Frontiers in Immunology. Frontiers Media S.A.; 2020.
37. Simões-Silva L, Araujo R, Pestana M, Soares-Silva I, Sampaio-Maia B. The microbiome in chronic kidney disease patients undergoing hemodialysis and peritoneal dialysis. Pharmacol Res [Internet]. 2018;130:143–51. Available from: http://dx.doi.org/10.1016/j.phrs.2018.02.011
38. Chung SY, Barnes JL, Astroth KS. Gastrointestinal Microbiota in Patients with Chronic Kidney Disease: A Systematic Review. Advances in Nutrition. 2019;10(5):888–901.
39. Rysz J, Franczyk B, Ławiński J, Olszewski R, Ciałkowska-Rysz A, Gluba-Brzózka A. The Impact of CKD on Uremic Toxins and Gut Microbiota. Toxins (Basel). 2021;13(4):1–23.
40. Glorieux G, Gryp T, Perna A. Gut-derived metabolites and their role in immune dysfunction in chronic kidney disease. Vol. 12, Toxins. MDPI AG; 2020.
41. Lim YJ, Sidor NA, Tonial NC, Che A, Urquhart BL. Uremic Toxins in the Progression of Chronic Kidney Disease and Cardiovascular Disease: Mechanisms and Therapeutic Targets. Vol. 13, Toxins. MDPI; 2021.
42. Sato E, Hosomi K, Sekimoto A, Mishima E, Oe Y, Saigusa D, et al. Effects of the oral adsorbent AST-120 on fecal p-cresol and indole levels and on the gut microbiota composition. Biochem Biophys Res Commun. 2020 May 7;525(3):773–9.
43. Garcia A, Macedo MH, Azevedo MJ, Pestana M, Sarmento B, Sampaio-Maia B. Effect of uremic state in intestine through a co-culture in vitro intestinal epithelial model. Int J Pharm. 2020 Jun 30;584.
44. Sirich TL, Funk BA, Plummer NS, Hostetter TH, Meyer TW. Prominent Accumulation in Hemodialysis Patients of Solutes Normally Cleared by Tubular Secretion. Journal of the American Society of Nephrology. 2014 Mar;25(3):615–22.
45. Sampaio-Maia B, Simões-Silva L, Pestana M, Araujo R, Soares-Silva IJ. The Role of the Gut Microbiome on Chronic Kidney Disease. Adv Appl Microbiol. 2016;96:65–94.
46. Aronov PA, Luo FJG, Plummer NS, Quan Z, Holmes S, Hostetter TH, et al. Colonic contribution to uremic solutes. Journal of the American Society of Nephrology. 2011 Sep;22(9):1769–76.
47. Wong J, Piceno YM, DeSantis TZ, Pahl M, Andersen GL, Vaziri ND. Expansion of urease- and uricase-containing, indole- and p-cresol-forming and contraction of short-chain fatty acid-producing intestinal microbiota in ESRD. Am J Nephrol. 2014;39(3):230–7.

48. Magnusson M, Magnusson KE, Sundqvist T, Denneberg T. Impaired intestinal barrier function measured by differently sized polyethylene glycols in patients with chronic renal failure. Gut. 1991;32(7):754–9.
49. Vaziri ND, Yuan J, Rahimi A, Ni Z, Said H, Subramanian VS. Disintegration of colonic epithelial tight junction in uremia: A likely cause of CKD-associated inflammation. Nephrology Dialysis Transplantation. 2012 Jul;27(7):2686–93.
50. Lau WL, Kalantar-Zadeh K, Vaziri ND. The Gut as a Source of Inflammation in Chronic Kidney Disease. Nephron. 2015;130(2):92–8.
51. Yang Q, Liang Q, Balakrishnan B, Belobrajdic DP, Feng QJ, Zhang W. Role of dietary nutrients in the modulation of gut microbiota: A narrative review. Vol. 12, Nutrients. MDPI AG; 2020.
52. Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Vol. 7, Gut Microbes. Taylor and Francis Inc.; 2016. p. 189–200.
53. Wang S, Lv D, Jiang S, Jiang J, Liang M, Hou F, et al. Quantitative reduction in short-chain fatty acids, especially butyrate, contributes to the progression of chronic kidney disease. Clin Sci. 2019;133(17):1857–70.
54. Jiang S, Xie S, Lv D, Zhang Y, Deng J, Zeng L, et al. A reduction in the butyrate producing species Roseburia spp. and Faecalibacterium prausnitzii is associated with chronic kidney disease progression. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology. 2016;109(10):1389–96.
55. McIntyre CW, Harrison LEA, Eldehni MT, Jefferies HJ, Szeto CC, John SG, et al. Circulating endotoxemia: A novel factor in systemic inflammation and cardiovascular disease in chronic kidney disease. Clinical Journal of the American Society of Nephrology. 2011 Jan 1;6(1):133–41.
56. Nazzal L, Roberts J, Singh P, Jhawar S, Matalon A, Gao Z, et al. Microbiome perturbation by oral vancomycin reduces plasma concentration of two gut-derived uremic solutes, indoxyl sulfate and p -cresyl sulfate, in end-stage renal disease. Nephrology Dialysis Transplantation. 2017;32(11):1809–17.
57. Kortman GAM, Reijnders D, Swinkels DW. Oral iron supplementation: Potential implications for the gut microbiome and metabolome in patients with CKD. Vol. 21, Hemodialysis International. 2017. p. S28–36.
58. Zaza G, Gassa AD, Felis G, Granata S, Torriani S, Lupo A. Impact of maintenance immunosuppressive therapy on the fecal microbiome of renal transplant recipients: Comparison between an everolimus-and a standard tacrolimusbased regimen. PLoS One. 2017 May 1;12(5).
59. Navarro-González JF, Mora-Fernández C, de Fuentes MM, Donate-Correa J, Cazaña-Pérez V, García-Pérez J. Effect of phosphate binders on serum inflammatory profile, soluble CD14, and endotoxin levels in hemodialysis patients. Clinical Journal of the American Society of Nephrology. 2011;6(9):2272–9.
60. Guida B, Cataldi M, Riccio E, Grumetto L, Pota A, Borrelli S, et al. Plasma p-Cresol Lowering Effect of Sevelamer in Peritoneal Dialysis Patients: Evidence from a Cross-Sectional Observational Study. PLoS One. 2013;8(8).
61. Lin C jui, Pan C feng, Chuang C kuang, Liu H liang, Huang SF, Chen HH, et al. Effects of Sevelamer Hydrochloride on Uremic Toxins Serum Indoxyl Sulfate and P-Cresyl Sulfate in Hemodialysis Patients. J Clin Med Res. 2017;9(9):765–70.
62. Biruete A, Hill Gallant KM, Lindemann SR, Wiese GN, Chen NX, Moe SM. Phosphate Binders and Nonphosphate Effects in the Gastrointestinal Tract

[Internet]. Vol. 30, Journal of Renal Nutrition. National Kidney Foundation, Inc.; 2020. p. 4–10. Available from: https://doi.org/10.1053/j.jrn.2019.01.004

63. Trautvetter U, Camarinha-Silva A, Jahreis G, Lorkowski S, Glei M. High phosphorus intake and gut-related parameters - Results of a randomized placebo-controlled human intervention study. Nutr J. 2018;17(1):1–11.
64. Wilson BR, Bogdan AR, Miyazawa M, Hashimoto K, Tsuji Y. Siderophores in Iron Metabolism : From Mechanism to Therapy Potential. Trends Mol Med [Internet]. 2016;xx:1–14. Available from: http://dx.doi.org/10.1016/j.molmed.2016.10.005
65. Lau WL, Vaziri ND, Nunes ACF, Comeau AM, Langille MGI, England W, et al. The phosphate binder ferric citrate alters the gut microbiome in rats with chronic kidney disease. Journal of Pharmacology and Experimental Therapeutics. 2018;367(3):452–60.
66. Vaziri ND, Wong J, Pahl M, Piceno YM, Yuan J, Desantis TZ, et al. Chronic kidney disease alters intestinal microbial flora. Kidney Int. 2013;83(2):308–15.
67. Stadlbauer V, Horvath A, Ribitsch W, Schmerböck B, Schilcher G, Lemesch S, et al. Structural and functional differences in gut microbiome composition in patients undergoing haemodialysis or peritoneal dialysis. Sci Rep. 2017 Dec 1;7(1).
68. Crespo-Salgado J, Vehaskari VM, Stewart T, Ferris M, Zhang Q, Wang G, et al. Intestinal microbiota in pediatric patients with end stage renal disease: A Midwest Pediatric Nephrology Consortium study. Microbiome. 2016;4.
69. Shivani S, Kao CY, Chattopadhyay A, Chen JW, Lai LC, Lin WH, et al. Uremic Toxin-Producing Bacteroides Species Prevail in the Gut Microbiota of Taiwanese CKD Patients: An Analysis Using the New Taiwan Microbiome Baseline. Front Cell Infect Microbiol. 2022 Apr 26;12.
70. Shi X, Gao B, Srivastava A, Izzi Z, Abdalla Y, Shen W, et al. Alterations of gut microbial pathways and virulence factors in hemodialysis patients. Front Cell Infect Microbiol. 2022 Aug 26;12.
71. Wu HY, Lin YT, Tsai WC, Chiu YL, Ko MJ, Yang JY, et al. Microbiota analysis in the hemodialysis population - Focusing on Enterobacteriaceae. Journal of Microbiology, Immunology and Infection. 2022 Apr 1;
72. Lun H, Yang W, Zhao S, Jiang M, Xu M, Liu F, et al. Altered gut microbiota and microbial biomarkers associated with chronic kidney disease. Microbiologyopen. 2019 Apr 1;8(4).
73. Li Y, Su X, Zhang L, Liu Y, Shi M, Lv C, et al. Dysbiosis of the gut microbiome is associated with CKD5 and correlated with clinical indices of the disease: A case-controlled study. J Transl Med [Internet]. 2019;17(1):1–13. Available from: https://doi.org/10.1186/s12967-019-1969-1
74. Wang IK, Lai HC, Yu CJ, Liang CC, Chang CT, Kuo HL, et al. Real-time PCR analysis of the intestinal microbiotas in peritoneal dialysis patients. Appl Environ Microbiol. 2012 Feb;78(4):1107–12.
75. Bao M, Zhang P, Guo S, Zou J, Ji J, Ding X, et al. Altered gut microbiota and gut-derived p-cresyl sulfate serum levels in peritoneal dialysis patients. Front Cell Infect Microbiol. 2022 Sep 27;12.
76. Hu J, Zhong X, Yan J, Zhou D, Qin D, Xiao X, et al. High-throughput sequencing analysis of intestinal flora changes in ESRD and CKD patients. BMC Nephrol. 2020 Jan 13;21(1).

77. Jiang N, Zhang C, Feng H, Yuan J, Ding L, Fang W, et al. Clinical characteristics associated with the properties of gut microbiota in peritoneal dialysis patients. Peritoneal Dialysis International. 2021 May 1;41(3):298–306.
78. Hu J, Zhong X, Liu Y, Yan J, Zhou D, Qin D, et al. Correlation between intestinal flora disruption and protein–energy wasting in patients with end-stage renal disease. BMC Nephrol. 2022 Dec 1;23(1).
79. Luo D, Zhao W, Lin Z, Wu J, Lin H, Li Y, et al. The Effects of Hemodialysis and Peritoneal Dialysis on the Gut Microbiota of End-Stage Renal Disease Patients, and the Relationship Between Gut Microbiota and Patient Prognoses. Front Cell Infect Microbiol. 2021 Mar 23;11.
80. Zhou J, Yang C, Lei W, Yang Z, Chen J, Lin H, et al. Exploration of the correlation between intestinal flora and Escherichia coli peritoneal dialysis-related peritonitis. BMC Nephrol. 2022 Dec 1;23(1).
81. Potgieter M, Bester J, Kell DB, Pretorius E. The dormant blood microbiome in chronic, inflammatory diseases. Vol. 39, FEMS Microbiology Reviews. Oxford University Press; 2015. p. 567–91.
82. Shah NB, Allegretti AS, Nigwekar SU, Kalim S, Zhao S, Lelouvier B, et al. Blood microbiome profile in CKD: A pilot study. Clinical Journal of the American Society of Nephrology. 2019 May 7;14(5):692–701.
83. Simões-Silva L, Silva S, Santos-Araujo C, Sousa J, Pestana M, Araujo R, et al. Oral yeast colonization and fungal infections in peritoneal dialysis patients: A pilot study. Canadian Journal of Infectious Diseases and Medical Microbiology. 2017;2017.
84. Hrbacek J, Tlaskal V, Cermak P, Hanacek V, Zachoval R. Bladder Microbiota Are Associated with Clinical Conditions That Extend beyond the Urinary Tract. Microorganisms. 2022 May 1;10(5).
85. Kramer H, Kuffel G, Thomas-White K, Wolfe AJ, Vellanki K, Leehey DJ, et al. Diversity of the midstream urine microbiome in adults with chronic kidney disease. Int Urol Nephrol. 2018 Jun 1;50(6):1123–30.
86. Castillo DJ, Rifkin RF, Cowan DA, Potgieter M. The healthy human blood microbiome: Fact or fiction? Front Cell Infect Microbiol. 2019;9(MAY).
87. D'Aquila P, Giacconi R, Malavolta M, Piacenza F, Bürkle A, Villanueva MM, et al. Microbiome in Blood Samples From the General Population Recruited in the MARK-AGE Project: A Pilot Study. Front Microbiol. 2021 Jul 26;12.
88. Païssé S, Valle C, Servant F, Courtney M, Burcelin R, Amar J, et al. Comprehensive description of blood microbiome from healthy donors assessed by 16S targeted metagenomic sequencing. Transfusion (Paris). 2016 May 1;56(5):1138–47.
89. Lau WL, Vaziri ND. The Leaky Gut and Altered Microbiome in Chronic Kidney Disease. Journal of Renal Nutrition. 2017 Nov 1;27(6):458–61.
90. Hu J, Iragavarapu S, Nadkarni GN, Huang R, Erazo M, Bao X, et al. Location-Specific Oral Microbiome Possesses Features Associated With CKD. Kidney Int Rep. 2018;3(1):193–204.
91. Costa CFFA, Merino-Ribas A, Ferreira C, Campos C, Silva N, Pereira L, et al. Characterization of Oral Enterobacteriaceae Prevalence and Resistance Profile in Chronic Kidney Disease Patients Undergoing Peritoneal Dialysis. Front Microbiol. 2021 Dec 14;12.
92. Brubaker L, Gourdine JPF, Siddiqui NY, Holland A, Halverson T, Limeria R, et al. Forming Consensus To Advance Urobiome Research [Internet]. 2021. Available from: https://doi.org/10

93. Wu JF, Muthusamy A, Al-Ghalith GA, Knights D, Guo B, Wu B, et al. Urinary microbiome associated with chronic allograft dysfunction in kidney transplant recipients. Clin Transplant. 2018 Dec 1;32(12).
94. Fok CS, Gao X, Lin H, Thomas-White KJ, Mueller ER, Wolfe AJ, et al. Urinary symptoms are associated with certain urinary microbes in urogynecologic surgical patients. Int Urogynecol J. 2018 Dec 1;29(12):1765–71.
95. Liu F, Zhang N, Jiang P, Zhai Q, Li C, Yu D, et al. Characteristics of the urinary microbiome in kidney stone patients with hypertension. J Transl Med. 2020 Mar 17;18(1).
96. Bučević Popović V, Šitum M, Chow CET, Chan LS, Roje B, Terzić J. The urinary microbiome associated with bladder cancer. Sci Rep. 2018 Dec 1;8(1).
97. Jones-Freeman B, Chonwerawong M, Marcelino VR, Deshpande A V., Forster SC, Starkey MR. The microbiome and host mucosal interactions in urinary tract diseases. Vol. 14, Mucosal Immunology. Springer Nature; 2021. p. 779–92.
98. Thomas-White K, Forster SC, Kumar N, Van Kuiken M, Putonti C, Stares MD, et al. Culturing of female bladder bacteria reveals an interconnected urogenital microbiota. Nat Commun. 2018 Dec 1;9(1).
99. Rani A, Ranjan R, McGee HS, Andropolis KE, Panchal D V., Hajjiri Z, et al. Urinary microbiome of kidney transplant patients reveals dysbiosis with potential for antibiotic resistance. Translational Research. 2017 Mar 1;181:59–70.
100. Brown EA, Blake PG, Boudville N, Davies S, de Arteaga J, Dong J, et al. International Society for Peritoneal Dialysis practice recommendations: Prescribing high-quality goal-directed peritoneal dialysis. Peritoneal Dialysis International. 2020 May 1;40(3):244–53.
101. Quan H, Li B, Couris CM, Fushimi K, Graham P, Hider P, et al. Updating and validating the charlson comorbidity index and score for risk adjustment in hospital discharge abstracts using data from 6 countries. Am J Epidemiol. 2011 Mar 15;173(6):676–82.
102. Escudié F, Auer L, Bernard M, Mariadassou M, Cauquil L, Vidal K, et al. FROGS: Find, Rapidly, OTUs with Galaxy Solution. Bioinformatics. 2018 Apr 15;34(8):1287–94.
103. Calaf R, Cerini C, Génovésio C, Verhaeghe P, Jourde-Chiche N, Bergé-Lefranc D, et al. Determination of uremic solutes in biological fluids of chronic kidney disease patients by HPLC assay. J Chromatogr B Analyt Technol Biomed Life Sci. 2011 Aug 1;879(23):2281–6.
104. Araujo R, Dunlap C, Franco CMM. Analogous wheat root rhizosphere microbial successions in field and greenhouse trials in the presence of biocontrol agents Paenibacillus peoriae SP9 and Streptomyces fulvissimus FU14. Mol Plant Pathol. 2020 May 1;21(5):622–35.
105. Parks DH, Beiko RG. Identifying biologically relevant differences between metagenomic communities. Bioinformatics. 2010;26(6):715–21.
106. Merino-Ribas A, Costa CFFA, Azevedo M, Alencastre I, Pestana M, Araujo R, et al. The gut microbiome in chronic kidney disease. In: Human-Gut Microbiome. Elsevier; 2022. p. 233–63.
107. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the Human Intestinal Microbial Flora [Internet]. Available from: www.sciencemag.org/cgi/content/full/1110591/DC1

108. Chao YT, Lin YK, Chen LK, Huang P, Hsu YC. Role of the gut microbiota and their metabolites in hemodialysis patients. Int J Med Sci. 2023;20(6):725–36.
109. Wu IW, Lin CY, Chang LC, Lee CC, Chiu CY, Hsu HJ, et al. Gut microbiota as diagnostic tools for mirroring disease progression and circulating nephrotoxin levels in chronic kidney disease: Discovery and validation study. Int J Biol Sci. 2020;16(3):420–34.
110. Buford TW. (Dis)Trust your gut: the gut microbiome in age-related inflammation, health, and disease. Microbiome. 2017;5(1):80.
111. Zwielehner J, Liszt K, Handschur M, Lassl C, Lapin A, Haslberger AG. Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of Bacteroides, bifidobacteria and Clostridium cluster IV in institutionalized elderly. Exp Gerontol [Internet]. 2009;44(6–7):440–6. Available from: http://dx.doi.org/10.1016/j.exger.2009.04.002
112. Claesson MJ, Jeffery IB, Conde S, Power SE, O'connor EM, Cusack S, et al. Gut microbiota composition correlates with diet and health in the elderly. Nature. 2012;488(7410):178–84.
113. Santos-Marcos JA, Rangel-Zuñiga OA, Jimenez-Lucena R, Quintana-Navarro GM, Garcia-Carpintero S, Malagon MM, et al. Influence of gender and menopausal status on gut microbiota. Maturitas. 2018;116(April):43–53.
114. Rizzetto L, Fava F, Tuohy KM, Selmi C. Connecting the immune system, systemic chronic inflammation and the gut microbiome: The role of sex. J Autoimmun. 2018;92(April):12–34.
115. Merino-Ribas A, Araujo R, Pereira L, Campos J, Barreiros L, Segundo MA, et al. Vascular Calcification and the Gut and Blood Microbiome in Chronic Kidney Disease Patients on Peritoneal Dialysis: A Pilot Study. Biomolecules. 2022 Jul 1;12(7).
116. Wang X, Yang S, Li S, Zhao L, Hao Y, Qin J, et al. Aberrant gut microbiota alters host metabolome and impacts renal failure in humans and rodents. Gut. 2020 Dec 1;69(12):2131–42.
117. Amar J, Lange C, Payros G, Garret C, Chabo C, Lantieri O, et al. Blood Microbiota Dysbiosis Is Associated with the Onset of Cardiovascular Events in a Large General Population: The D.E.S.I.R. Study. PLoS One. 2013 Jan 30;8(1).
118. Damgaard C, Magnussen K, Enevold C, Nilsson M, Tolker-Nielsen T, Holmstrup P, et al. Viable bacteria associated with red blood cells and plasma in freshly drawn blood donations. PLoS One. 2015 Mar 9;10(3).
119. Rizzatti G, Lopetuso LR, Gibiino G, Binda C, Gasbarrini A. Proteobacteria: A common factor in human diseases. Vol. 2017, BioMed Research International. Hindawi Limited; 2017.
120. Mair RD, Sirich TL. Blood microbiome in CKD should we care? Vol. 14, Clinical Journal of the American Society of Nephrology. American Society of Nephrology; 2019. p. 648–9.
121. Nelson DE, van der Pol B, Dong Q, Revanna K V., Fan B, Easwaran S, et al. Characteristic male urine microbiomes associate with asymptomatic sexually transmitted infection. PLoS One. 2010;5(11).
122. Kim Y, Carrai M, Y Leung MH, Chin J, Li J, H Lee PK, et al. Dysbiosis of the Urinary Bladder Microbiome in Cats with Chronic Kidney Disease. 2021; Available from: https://doi.org/10.1128/mSystems

123. Burnett LA, Hochstedler BR, Weldon K, Wolfe AJ, Brubaker L. Recurrent urinary tract infection: Association of clinical profiles with urobiome composition in women. Neurourol Urodyn. 2021 Aug 1;40(6):1479–89.
124. Pohl HG, Groah SL, Pérez-Losada M, Ljungberg I, Sprague BM, Chandal N, et al. The urine microbiome of healthy men and women differs by urine collection method. Int Neurourol J. 2020;24(1):41–51.
125. Liu F, Ling Z, Xiao Y, Yang Q, Wang B, Zheng L, et al. Alterations of urinary microbiota in type 2 diabetes mellitus with hypertension and/or hyperlipidemia. Front Physiol. 2017 Mar 3;8(MAR).
126. Penckofer S, Limeira R, Joyce C, Grzesiak M, Thomas-White K, Wolfe AJ. Characteristics of the microbiota in the urine of women with type 2 diabetes. J Diabetes Complications. 2020 Jun 1;34(6).
127. Joyce C, Halverson T, Gonzalez C, Brubaker L, Wolfe AJ. The Urobiomes of Adult Women With Various Lower Urinary Tract Symptoms Status Differ: A Re-Analysis. Front Cell Infect Microbiol. 2022 Jun 9;12.
128. Hong SY, Yang YY, Xu JZ, Xia QD, Wang SG, Xun Y. The renal pelvis urobiome in the unilateral kidney stone patients revealed by 2bRAD-M. J Transl Med. 2022 Dec 1;20(1).
129. Bas S, Gauthier BR, Spenato U, Stingelin S, Gabay C. CD14 Is an Acute-Phase Protein. The Journal of Immunology. 2004 Apr 1;172(7):4470–9.
130. Price TK, Hilt EE, Thomas-White K, Mueller ER, Wolfe AJ, Brubaker L. The urobiome of continent adult women: a cross-sectional study. BJOG. 2020 Jan 1;127(2):193–201.
131. Onderdonk AB, Delaney ML, Fichorova RN. The human microbiome during bacterial vaginosis. Clin Microbiol Rev. 2016 Feb 10;29(2):223–38.
132. Gilbert NM, O'Brien VP, Waller C, Batourina E, Mendelsohn CL, Lewis AL. Gardnerella Exposures Alter Bladder Gene Expression and Augment Uropathogenic Escherichia coli Urinary Tract Infection in Mice. Front Cell Infect Microbiol. 2022 Jun 16;12.
133. Merino-Ribas A, Araujo R, Bancu I, Graterol F, Vergara A, Noguera-Julian M, et al. Gut microbiome in hemodialysis patients treated with calcium acetate or treated with sucroferric oxyhydroxide: a pilot study. Int Urol Nephrol. 2022 Aug 1;54(8):2015–23.
134. Muckenthaler MU, Rivella S, Hentze MW, Galy B. A Red Carpet for Iron Metabolism. Cell [Internet]. 2017;168(3):344–61. Available from: http://dx.doi.org/10.1016/j.cell.2016.12.034
135. Paganini D, Zimmermann MB. The effects of iron fortification and supplementation on the gut microbiome and diarrhea in infants and children: A review. American Journal of Clinical Nutrition. 2017;106(C):1688S-1693S.
136. Kortman GAM, Dutilh BE, Maathuis AJH, Engelke UF, Boekhorst J, Keegan KP, et al. Microbial metabolism shifts towards an adverse profile with supplementary iron in the TIM-2 in vitro model of the human colon. Front Microbiol. 2016;6(JAN):1–18.
137. Wu PH, Liu PY, Chiu YW, Hung WC, Lin YT, Lin TY, et al. Comparative gut microbiome differences between ferric citrate and calcium carbonate phosphate binders in patients with end-stage kidney disease. Microorganisms. 2020;8(12):1–14.
138. Iguchi A, Yamamoto S, Oda A, Tanaka K, Kazama JJ, Saeki T, et al. Effect of sucroferric oxyhydroxide on gastrointestinal microbiome and uremic toxins in

patients with chronic kidney disease undergoing hemodialysis. Clin Exp Nephrol [Internet]. 2020;24(8):725–33. Available from: https://doi.org/10.1007/s10157-020-01892-x

139. Abdelbary MMH, Kuppe C, Michael SSY, Krüger T, Floege J, Conrads G. Impact of sucroferric oxyhydroxide on the oral and intestinal microbiome in hemodialysis patients. Sci Rep. 2022 Dec 1;12(1).
140. Floege J. Phosphate binders in chronic kidney disease: an updated narrative review of recent data. J Nephrol [Internet]. 2020;33(3):497–508. Available from: https://doi.org/10.1007/s40620-019-00689-w
141. Palmer SC, Gardner S, Tonelli M, Mavridis D, Johnson DW, Craig JC, et al. Phosphate-Binding Agents in Adults With CKD: A Network Meta-analysis of Randomized Trials. American Journal of Kidney Diseases [Internet]. 2016;68(5):691–702. Available from: http://dx.doi.org/10.1053/j.ajkd.2016.05.015
142. Kendrick J, Parameswaran V, Ficociello LH, Ofsthun NJ, Davis S, Mullon C, et al. One-Year Historical Cohort Study of the Phosphate Binder Sucroferric Oxyhydroxide in Patients on Maintenance Hemodialysis. Journal of Renal Nutrition [Internet]. 2019;29(5):428–37. Available from: https://doi.org/10.1053/j.jrn.2018.11.002
143. Covic AC, Floege J, Ketteler M, Sprague SM, Lisk L, Rakov V, et al. Iron-related parameters in dialysis patients treated with sucroferric oxyhydroxide. Nephrology Dialysis Transplantation. 2017;32(8):1363–72.
144. Rastogi A. Sevelamer revisited: Pleiotropic effects on endothelial and cardiovascular risk factors in chronic kidney disease and end-stage renal disease. Ther Adv Cardiovasc Dis. 2013;7(6):322–42.
145. Azcarate-Peril MA, Roach J, Marsh A, Chey WD, Sandborn WJ, Ritter AJ, et al. A double-blind, 377-subject randomized study identifies Ruminococcus, Coprococcus, Christensenella, and Collinsella as long-term potential key players in the modulation of the gut microbiome of lactose intolerant individuals by galacto-oligosaccharides. Gut Microbes. 2021 Jan 1;13(1):1957536.
146. Hughes RL, Kable ME, Marco M, Keim NL. The Role of the Gut Microbiome in Predicting Response to Diet and the Development of Precision Nutrition Models. Part II: Results. Vol. 10, Advances in Nutrition. Oxford University Press; 2019. p. 979–98.
147. Jones RB, Alderete TL, Kim JS, Millstein J, Gilliland FD, Goran MI. High intake of dietary fructose in overweight/obese teenagers associated with depletion of Eubacterium and Streptococcus in gut microbiome. Gut Microbes. 2019 Nov 2;10(6):712–9.
148. Mahowald MA, Rey FE, Seedorf H, Turnbaugh PJ, Fulton RS, Wollam A, et al. Characterizing a model human gut microbiota composed of members of its two dominant bacterial phyla [Internet]. Available from: http://genome.wustl.edu/hgm/
149. Zhou X, Li J, Guo J, Geng B, Ji W, Zhao Q, et al. Gut-dependent microbial translocation induces inflammation and cardiovascular events after ST-elevation myocardial infarction. Microbiome. 2018 Apr 3;6(1):66.
150. Peh A, O'Donnell JA, Broughton BRS, Marques FZ. Gut Microbiota and Their Metabolites in Stroke: A Double-Edged Sword. Vol. 53, Stroke. Lippincott Williams and Wilkins; 2022. p. 1788–801.

151. Ko CY, Liu QQ, Su HZ, Zhang HP, Fan JM, Yang JH, et al. Gut microbiota in obstructive sleep apnea–hypopnea syndrome: Disease-related dysbiosis and metabolic comorbidities. Clin Sci. 2019 Apr 15;133(7):905–17.
152. Rastogi S, Singh A. Gut microbiome and human health: Exploring how the probiotic genus Lactobacillus modulate immune responses. Vol. 13, Frontiers in Pharmacology. Frontiers Media S.A.; 2022.
153. Liu S, Zhao W, Liu X, Cheng L. Metagenomic analysis of the gut microbiome in atherosclerosis patients identify cross-cohort microbial signatures and potential therapeutic target. FASEB Journal. 2020 Nov 1;34(11):14166–81.
154. Nie X, Chen J, Ma X, Ni Y, Shen Y, Yu H, et al. A metagenome-wide association study of gut microbiome and visceral fat accumulation. Comput Struct Biotechnol J. 2020 Jan 1;18:2596–609.
155. Luo M, Cai J, Luo S, Hong X, Xu L, Lin H, et al. Causal effects of gut microbiota on the risk of chronic kidney disease: a Mendelian randomization study. Front Cell Infect Microbiol. 2023;13:1142140.
156. Bay L, Barnes CJ, Fritz BG, Thorsen J, Restrup MEM, Rasmussen L, et al. Universal dermal microbiome in human skin. mBio. 2020 Jan 1;11(1).
157. De R, Mukhopadhyay AK, Dutta S. Metagenomic analysis of gut microbiome and resistome of diarrheal fecal samples from Kolkata, India, reveals the core and variable microbiota including signatures of microbial dark matter. Gut Pathog. 2020 Jul 7;12(1).
158. Rudney JD, Xie H, Rhodus NL, Ondrey FG, Griffin TJ. A metaproteomic analysis of the human salivary microbiota by three-dimensional peptide fractionation and tandem mass spectrometry. Mol Oral Microbiol. 2010 Feb;25(1):38–49.
159. Zhang H, Chang Y, Zheng Q, Zhang R, Hu C, Jia W. Altered intestinal microbiota associated with colorectal cancer. Front Med. 2019 Aug 1;13(4):461–70.
160. Bai X, Shi Y, Tang L, Chen L, Fan H, Wang H, et al. Heat Stress Affects Faecal Microbial and Metabolic Alterations of Rabbits. Front Microbiol. 2022 Feb 28;12.
161. Durham AL, Speer MY, Scatena M, Giachelli CM, Shanahan CM. Role of smooth muscle cells in vascular calcification: Implications in atherosclerosis and arterial stiffness. Vol. 114, Cardiovascular Research. Oxford University Press; 2018. p. 590–600.
162. Visseren FLJ, MacH F, Smulders YM, Carballo D, Koskinas KC, Bäck M, et al. 2021 ESC Guidelines on cardiovascular disease prevention in clinical practice. Vol. 42, European Heart Journal. Oxford University Press; 2021. p. 3227–337.
163. Park SK, Kim WJ, Kim HJ, Kim HW, Kim B, Lee HJ, et al. High dose haemodialysis and haemodiafiltration parameters and the relationship with advanced vascular calcification. BMC Nephrol. 2020 Mar 6;21(1).
164. Wolfe RA, Ashby VB, Ma JT, Daugirdas LYC, Agodoa CA, Jones FK, et al. Body Size, Dose of Hemodialysis, and Mortality. Vol. 35, American Journal of Kidney Diseases. 2000.
165. Dimkovic N, Schlieper G, Jankovic A, Djuric Z, Ketteler M, Damjanovic T, et al. Prognostic value of cardiovascular calcifications in hemodialysis patients: a longitudinal study. Int Urol Nephrol. 2018 May 1;50(5):939–46.
166. Floege J, Ketteler M. Vascular calcification in patients with end-stage renal disease. Vol. 19, Nephrology Dialysis Transplantation. 2004.
167. Cozzolino M, Ciceri P, Galassi A, Mangano M, Carugo S, Capelli I, et al. The key role of phosphate on vascular calcification. Vol. 11, Toxins. MDPI AG; 2019.

168. Singh A, Tandon S, Tandon C. An update on vascular calcification and potential therapeutics. Vol. 48, Molecular Biology Reports. Springer Science and Business Media B.V.; 2021. p. 887–96.
169. Wang J, Zhou JJ, Robertson GR, Lee VW. Vitamin D in vascular calcification: A double-edged sword? Vol. 10, Nutrients. MDPI AG; 2018.
170. Mathew S, Lund RJ, Chaudhary LR, Geurs T, Hruska KA. Vitamin D receptor activators can protect against vascular calcification. Journal of the American Society of Nephrology. 2008 Aug;19(8):1509–19.
171. Ramirez-Sandoval JC, Casanova I, Villar A, Enrique Gomez F, Cruz C, Correa-Rotter R. Biomarkers associated with vascular calcification in peritoneal dialysis. Peritoneal Dialysis International. 2016 May 1;36(3):262–8.
172. Ghorbanihaghjo A, Argani H, Golmohamadi Z, Rashtchizadeh N, Abbasi MM, Bargahi N, et al. Linkage of Fibroblast Growth Factor 23 and Phosphate in Serum: Phosphate and Fibroblast Growth Factor 23 Reduction by Increasing Dose of Sevelamer. J Bone Metab. 2018;25(3):153.
173. Zhang H, Li G, Yu X, Yang J, Jiang A, Cheng H, et al. Progression of Vascular Calcification and Clinical Outcomes in Patients Receiving Maintenance Dialysis. JAMA Netw Open. 2023 May 1;6(5):e2310909.
174. Balla J, Balla G, Zarjou A. Ferritin in kidney and vascular related diseases: Novel roles for an old player. Pharmaceuticals. 2019 Jun 1;12(2).
175. Choi SR, Lee YK, Jin Cho A, Park HC, Han CH, Choi MJ, et al. Malnutrition, inflammation, progression of vascular calcification and survival: Interrelationships in hemodialysis patients. PLoS One. 2019 May 1;14(5).
176. Henze LA, Luong TTD, Boehme B, Masyout J, Schneider MP, Brachs S, et al. Impact of C-reactive protein on osteo-/chondrogenic transdifferentiation and calcification of vascular smooth muscle cells [Internet]. Vol. 11. 2019. Available from: www.aging-us.com
177. Reiner AP, Lange EM, Jenny NS, Chaves PHM, Ellis J, Li J, et al. Soluble CD14: Genomewide association analysis and relationship to cardiovascular risk and mortality in older adults. Arterioscler Thromb Vasc Biol. 2013 Jan;33(1):158–64.
178. Longenecker CT, Jiang Y, Orringer CE, Gilkeson RC, Debanne S, Funderburg NT, et al. Soluble CD14 is independently associated with coronary calcification and extent of subclinical vascular disease in treated HIV infection. AIDS. 2014 Apr 24;28(7):969–77.
179. Poesen R, Ramezani A, Claes K, Augustijns P, Kuypers D, Barrows IR, et al. Associations of soluble CD14 and endotoxin with mortality, cardiovascular disease, and progression of kidney disease among patients with CKD. Clinical Journal of the American Society of Nephrology. 2015 Sep 4;10(9):1525–33.
180. Raj DSC, Shah VO, Rambod M, Kovesdy CP, Kalantar-Zadeh K. Association of Soluble Endotoxin Receptor CD14 and Mortality Among Patients Undergoing Hemodialysis. American Journal of Kidney Diseases. 2009 Dec;54(6):1062–71.
181. Seabra VF, Thomas G, Jaber BL. Soluble CD14 and Endotoxin Levels in Hemodialysis Patients: A Tale of 2 Molecules. Vol. 54, American Journal of Kidney Diseases. 2009. p. 990–2.

10. ANNEXES

10.1. Scientific articles

Merino-Ribas A, Araujo R, Bancu I, Graterol F, Vergara A, Noguera-Julian M, Paredes R, Bonal J, Sampaio-Maia B. Gut microbiome in hemodialysis patients treated with calcium acetate or treated with sucroferric oxyhydroxide: a pilot study. Int Urol Nephrol. 2022 Aug;54(8):2015-2023. doi: 10.1007/s11255-021-03091-3. Epub 2021 Dec 19. PMID: 34923600; PMCID: PMC9262763.

International Urology and Nephrology
https://doi.org/10.1007/s11255-021-03091-3

NEPHROLOGY - ORIGINAL PAPER

Gut microbiome in hemodialysis patients treated with calcium acetate or treated with sucroferric oxyhydroxide: a pilot study

Ana Merino-Ribas[1,2,3,4] · Ricardo Araujo[4] · Ioana Bancu[1,2] · Fredzzia Graterol[1,2] · Andrea Vergara[5] · Marc Noguera-Julian[5] · Roger Paredes[5] · Jordi Bonal[2] · Benedita Sampaio-Maia[4,6]

Received: 23 June 2021 / Accepted: 8 December 2021

Abstract

Purpose It has been proved that the gut microbiome is altered in patients with chronic kidney disease. This contributes to chronic inflammation and increases cardiovascular risk and mortality, especially in those undergoing hemodialysis. Phosphate binders may potentially induce changes in their microbiome. This trial aimed to compare the changes in the gut microbiome of hemodialysis patients treated with calcium acetate to those treated with sucroferric oxyhydroxide.

Methods Twelve hemodialysis patients were distributed to receive calcium acetate or sucroferric oxyhydroxide for 5 months. Blood samples (for biochemical analysis) and stool samples (for microbiome analysis) were collected at baseline, 4, 12, and 20 weeks after treatment initiation. Fecal DNA was extracted and a 16S rRNA sequencing library was constructed targeting the V3 and V4 hypervariable regions.

Results Regarding clinical variables and laboratory parameters, no statistically significant differences were observed between calcium acetate or sucroferric oxyhydroxide groups. When analyzing stool samples, we found that all patients were different ($p=0.001$) among themselves and these differences were kept along the 20 weeks of treatment. The clustering analysis in microbial profiles grouped the samples of the same patient independently of the treatment followed and the stage of the treatment.

Conclusion These results suggest that a 5-month treatment with either calcium acetate or sucroferric oxyhydroxide did not modify baseline diversity or baseline bacterial composition in hemodialysis patients, also about the high-variability profiles of the gut microbiome found among these patients.

Keywords Gut microbiome · Chronic kidney disease · Hemodialysis · Phosphate binders · Sucroferric oxyhydroxide · Calcium acetate

Introduction

Chronic kidney disease (CKD) is a worldwide public health problem, with an increasing prevalence, a high economic burden, and elevated morbidity and mortality [1].

In CKD patients, cardiovascular pathology plays an important role. These patients present an increased risk of developing cardiovascular disease (CVD) and a cardiovascular mortality rate 30 times higher than the general population [2]. Besides the traditional cardiovascular risk factors that most of the time are more prevalent in patients affected by CKD than in the general population, the interconnection between CKD and CVD could be explained by the presence of bone and mineral disorders, hydration status, and inflammation that our patients develop.

✉ Ana Merino-Ribas
amerinor.girona.ics@gencat.cat

1 Universitat Autònoma de Barcelona, Barcelona, Spain
2 Nephrology Department, Hospital Universitari Germans Trias i Pujol, Badalona, Spain
3 Nephrology Department, Hospital Universitari de Girona Doctor Josep Trueta, Avinguda de França S/N, 17007 Girona, Spain
4 i3S—Instituto de Investigação e Inovação em Saúde, Universidade Do Porto, Porto, Portugal
5 IrsiCaixa AIDS Research Institute, Hospital Universitari Germans Trias i Pujol, Badalona, Barcelona, Spain
6 Faculty of Dental Medicine, University of Porto, Porto, Portugal

Published online: 19 December 2021

Under normal conditions, inflammation is a protective and physiological response to various inimical stimuli. However, in several debilitating disorders, such as CKD, inflammation becomes harmful and persistent [3]. It is well known that CKD is accompanied by a persistent inflammatory status [4, 5]. Inflammation is likely the consequence of a multifactorial etiology and interacts with several factors that emerge when uremic toxins accumulate and has been described as a predictor of cardiovascular and total mortality [6]. Moreover, there is mounting evidence supporting the presence of intestinal barrier dysfunction and alterations in the gut microbiota composition in CKD, commonly referred to as gut dysbiosis [7–9]. This dysbiotic state concomitantly generates toxic by-products and contributes to the chronic status of oxidative stress and inflammation in these patients [10–12].

Several factors contribute to gut microbial dysbiosis in patients with advanced CKD. The accumulation of urea in body fluids and its diffusion to the gastrointestinal tract lead to the expansion of urease-possessing bacteria. Also, the hydrolysis of urea generates products that degrade the epithelial tight junction, thereby facilitating translocation of endotoxin and microbial fragments into the systemic circulation [7, 8, 10, 13–15]. Dietary recommendations in CKD including restricted intake of potassium, phosphate, sodium, and proteins result in a low intake of fermentable carbohydrates and this may lead to an expansion of proteolytic species and an increased generation of bacterial toxins [12, 13, 16]. Moreover, patients with CKD are commonly associated with other comorbid conditions, such as diabetes, autoimmune diseases, and hypertension. All these comorbidities result per se in gut microbiota alterations [17, 18].

A very important factor which favors dysbiosis are drugs. It is well known that patients with advanced CKD are usually poly-medicated. Iron supplementation or antibiotics, frequently used in our patients, have been demonstrated to alter the gut microbiome [19–21]. However, the effects on the gut microbiome of other widely used drugs in CKD patients remain unknown.

Most hemodialysis patients tend to present hyperphosphatemia and they need high doses of different types of phosphate binders to correct this condition. Phosphate binders can be classified as calcium and non-calcium-based phosphate binders. It has been described that both groups of phosphate-binding agents can potentially produce changes in the composition of the microbiome [22–25].

Recently, new non-calcium-based phosphate-binding agents have been approved for the treatment of hyperphosphatemia in hemodialysis subjects. Some of these new agents, such as sucroferric oxyhydroxide (SFO) and ferric citrate, hold iron in their compositions. It is believed that, given the critical role of iron in microbial growth and virulence, the large iron load administrated with these drugs, may alter gut microbiome composition [26, 27]. Nevertheless, there is still little evidence about the effects of these new phosphate binders on the gut microbiome [25].

Given the importance of the altered gut microbiome in CKD patients and its contribution to their inflammatory state, and the lack of information about the effects on the gut microbiome of these nowadays widely used drugs, we decided to monitor and compare the changes on the gut microbiome of patients undergoing hemodialysis taking SFO or calcium-based phosphate binder calcium acetate (CA).

Materials and methods

Recruitment

Twelve patients on hemodialysis in Hospital Universitari Germans Trias i Pujol were invited to participate in our study with a 5-month follow-up. All the subjects were recruited from the Hemodialysis Department of the Hospital Universitari Germans Trias i Pujol, in Badalona, Spain. All patients were aged above 18 years old and had been on hemodialysis for at least 1 year (4 h sessions, 3 sessions per week). This study was approved by the Clinical Research Ethics Committee of the Hospital Universitari Germans Trias i Pujol (PI-16-169, NCT5551048) and conformed to the principles outlined in the Declaration of Helsinki. All participants were recruited voluntarily after receiving detailed information on the study protocol. Written informed consent was obtained from all patients.

Exclusion criteria included inability to give informed consent, history of gastrointestinal disease, hospitalization, and antibiotics intake in the last 3 months.

Relevant clinical and demographic information was gathered for each individual at baseline. Clinical characteristics collected were: gender, age, CKD etiology, history of high blood pressure, diabetes mellitus, dyslipidemia, cardiovascular disease (peripheral vascular disease, ischemic cardiomyopathy or stroke), and cancer.

We also collected information regarding their vascular access, and their previous phosphate-binder treatment at the beginning of the study (nine received calcium acetate, one received calcium carbonate, and two were not previously treated for hyperphosphatemia).

We divided patients into two groups, and we changed their treatment for hyperphosphatemia: 5 patients were placed in CA group (4 continuing CA therapy and 1 patient changing from calcium carbonate therapy) and 7 were switched to SFO (5 changing from CA therapy and 2 starting phosphate-binding treatment).

Sample collection

Fecal samples were collected from 12 hemodialysis patients receiving phosphate binders, 5 in the CA group and 7 in the SFO group. We collect also blood samples from the routine checks realized in our hemodialysis unit. The samples (blood and fecal samples) were collected in a 5-month follow-up: at baseline, 4, 12, and 20 weeks after treatment initiation.

In blood samples, we analyzed the following parameters: hemoglobin, ferritin, transferrin saturation index, calcium, phosphate, parathyroid hormone, C-reactive protein, sedimentation velocity, and albumin.

DNA extraction, library construction, and sequencing

Fecal DNA was extracted by Powersoil DNA Isolation Kit MoBio, and a 16S rRNA sequencing library was constructed targeting the V3 and V4 hypervariable regions.

Sequencing was performed on a MiSeq platform (2×300). OTU table construction, taxonomic assignment, and descriptive and statistical analyses were performed using R version 3.4.2. and different packages (DADA 2, vega, ggplot, phyloseq) and the Greengenes rRNA database.

Data and statistical analysis

Primer v7 (PRIMER-e, Auckland, New Zealand) was used for calculation of the diversity indices, similarity percentages (SIMPER) analysis, and multivariate analysis, mainly analysis of similarities (ANOSIM) one-way analysis and permutational multivariate analysis of variance (PERMANOVA; using squared root transformed data, Bray–Curtis similarities and 4999 permutations of residuals under a reduced model) used to test the significance of Beta-diversity. The percentage of OTU data per sample was used for these analyses, followed by squared root transformed data, resemblance matrices of similarity data types, using Bray–Curtis similarities, adding dummy value, and testing 4999 permutations. STAMP was used for analyzing taxonomic profiles among groups of samples and calculation of statistical differences [28].

For statistical treatment of the clinical data, the statistical analysis software Statistical Package for Social Sciences (SPSS) 26.0 for MAC OS was used. The categorical variables were described through relative frequencies (%) whereas continuous variables were described using mean ± standard deviation (SD). We applied when appropriate Chi-square independence test to analyze hypotheses regarding the categorical variables and Student's t test concerning continuous variables. A level of 0.05 was considered significant.

Results

The main clinical parameters were not different between patients assigned to CA or SFO groups at baseline (Table 1). We have observed that in the CA group, there was an increased prevalence, but not statistically significant, in history of arterial hypertension, dyslipidemia, peripheral vascular disease, stroke, ischemic cardiomyopathy than in the SFO group. The patients assigned to the SFO group presented a greater incidence of a catheter as vascular access, but also not statistically significant.

At baseline, no patient was treated with SFO, some were treated with CA in both groups of treatment, and 2 patients in the SFO group have no phosphate-binding treatment. At this time point, we found no statistically significant differences regarding laboratory parameters, such as hemoglobin, ferritin, transferrin saturation index, calcium, phosphate, parathyroid hormone, C-reactive protein, sedimentation velocity, and albumin (Table 2). Collectively, we observed in the SFO group at baseline higher transferrin saturation indexes, and lower values of C-reactive protein than the CA group, but those differences were not statistically significant. We also noted in patients assigned to the CA group, an increased trend to hyperphosphatemia at baseline, but this was also not statistically significant.

In Table 2, we present the evolution of the laboratory parameters over the different time points. We found that in the CA group, the 20-week calcium was higher than in the SFO group with statistical significance ($p=0.02$). Sedimentation velocity was increased in the CA group at week 12 of treatment when compared with the SFO group, with

Table 1 Clinical characterization of patients undergoing calcium acetate (CA) or sucroferric oxyhydroxide (SFO) as phosphate-binding agent

Clinical parameter	CA	SFO
Age, years	66.8 ± 13.9	61.1 ± 16.7
Women, %	40.0%	42.9%
Arterial hypertension, %	100.0%	85.7%
Dyslipidaemia, %	60.0%	42.9%
Diabetes mellitus, %	40.0%	42.9%
Peripheral vascular disease, %	40.0%	14.3%
Stroke, %	40.0%	14.3%
Ischemic cardiomyopathy, %	40.0%	14.3%
Cancer, %	20.0%	28.6%
Catheter as a vascular access, %	60.0%	71.4%

Values are means ± SD or relative frequencies (%). No statistically differences found between CA vs SFO

Table 2 Laboratory clinical data of patients undergoing calcium acetate (CA) or sucroferric oxyhydroxide (SFO) as phosphate-binding agent

Laboratory parameter	CA	SFO
Ferritin, ng/ml		
Basal	1451.8 ± 1299.3	1185.1 ± 268.2
4 weeks	1670.4 ± 1326.9	1166.0 ± 187.2
12 weeks	1722.2 ± 1622.0	1056.8 ± 327.9
20 weeks	1691 ± 1557.1	1149.8 ± 360.0
Transferrin saturation, %		
Basal	29 ± 8.9	53.1 ± 29.4
4 weeks	43.4 ± 19.7	46.7 ± 28.9
12 weeks	38.8 ± 19.3	41.4 ± 18.6
20 weeks	32.4 ± 14.0	42.0 ± 15.6
Calcium, mg/dl		
Basal	9.34 ± 0.3	9.1 ± 0.3
4 weeks	9.24 ± 0.3	8.9 ± 0.4
12 weeks	9.02 ± 0.5	8.7 ± 0.7
20 weeks	10.06 ± 0.7	8.9 ± 0.5*
Phosphate, mg/dl		
Basal	5.16 ± 2.1	4.4 ± 2.2
4 weeks	4.88 ± 1.4	4.4 ± 1.8
12 weeks	4.42 ± 1.7	5.5 ± 2.7
20 weeks	3.26 ± 0.8	4.7 ± 2.9
Parathormone, pg/ml		
Basal	242.1 ± 182.7	216.9 ± 259.2
4 weeks	309.4 ± 211.2	244.1 ± 267.7
12 weeks	327.1 ± 196.9	254.5 ± 380.9
20 weeks	181.5 ± 136.8	134.4 ± 108.8
C-reactive protein, mg/ml		
Basal	16.9 ± 20.6	4.2 ± 2.6
4 weeks	12.02 ± 6.0	5.6 ± 7.0
12 weeks	12.54 ± 7.3	4.4 ± 3.9
20 weeks	7.78 ± 6.3	4.2 ± 1.7
Sedimentation velocity, mm		
Basal	51.4 ± 24.2	45.3 ± 19.1
4 weeks	46.8 ± 24.2	39.2 ± 18.3
12 weeks	61.6 ± 26.9	26.4 ± 17.6*
20 weeks	47.6 ± 7.2	38.4 ± 19.4
Albumin, g/l		
Basal	39.04 ± 2.2	35.7 ± 3.0
4 weeks	37.54 ± 1.6	35.4 ± 2.4
12 weeks	37 ± 3.7	33.9 ± 4.0
20 weeks	39.5 ± 1.8	32.8 ± 2.3*
Hemoglobin, g/dl		
Basal	11.26 ± 0.9	10.9 ± 1.4
4 weeks	11.42 ± 0.8	10.1 ± 2.9
12 weeks	10.14 ± 1.3	11.8 ± 1.3
20 weeks	10.55 ± 0.8	10.7 ± 1.2

Values are means ± SD. *Values in SFO are significantly different from CA

statistical significance ($p = 0.04$). Also, a statistically significant lower albumin was observed in the SFO group at 20-week treatment when we compare it with the CA group ($p < 0.01$). The ferritin levels in both groups at baseline and after 20 weeks of treatment were high in the two groups, and both groups get normal levels of phosphate at 20 week of treatment, with no statistically significant differences. The levels of transferrin saturation indexes, parathormone, C-reactive protein, sedimentation velocity, and albumin, at 20 weeks of treatment were similar in both groups.

The samples of all time points (baseline, week 4, week 12, and week 20) were collected in eight out of the total twelve individuals, in a total of 38 stool and blood samples. From the initial set of 12 patients, patient 7 (SFO group) dropped out because he was derived to another hospital due to clinical reasons and we could no longer monitor all the variables relevant for the study, patient 3 (SFO group) received a kidney transplant before the collection of 20-week samples, patient 9 (SFO group) died before the collection of 12-week samples, and we only could get good-quality samples for gut microbiome from week 12 and week 20 on patient 8 (SFO group).

The set of 38 fecal samples showed over 2 million reads, then classified using the Greengenes database. A high number of ASVs (33,734) were found among the tested samples and classified as belonging to the kingdom Bacteria. Shannon diversity was measured in each sample and the group of 38 samples showed values for Shannon diversity ranging from 6.2 to 7.7.

Interestingly, we found that all patients were very different among themselves ($p = 0.001$) when comparing one patient with another patient at baseline (Fig. 1A). These differences among the patients were kept along the 20 weeks of treatment; there were no significant differences ($p > 0.05$) when the samples were grouped by week of treatment (baseline, 4, 12, or 20 weeks). It is important to note that the gut microbiome was found stable throughout the 20 weeks of study in patients that were on CA before the study and maintained that therapeutic within the study protocol, and also in patients who changed phosphate-binding therapeutics (from no treatment, CA or calcium carbonate to CA or SFO).

When we compared the microbial profiles of the patients treated with CA versus SFO considering all time points, we found statistical differences (Fig. 1B); and these differences were confirmed by ANOSIM ($p = 0.002$) and PERMANOVA ($p = 0.001$). This statistical analysis was done independently of the differences observed at baseline.

The bacterial communities were studied and Bacteroidetes and Firmicutes were the most common phyla found in the fecal samples, followed by Proteobacteria, Actinobacteria, and Verrucomicrobia. Looking for more specific compositional differences, we compared multiple taxonomical levels among these samples. When analyzing the bacterial

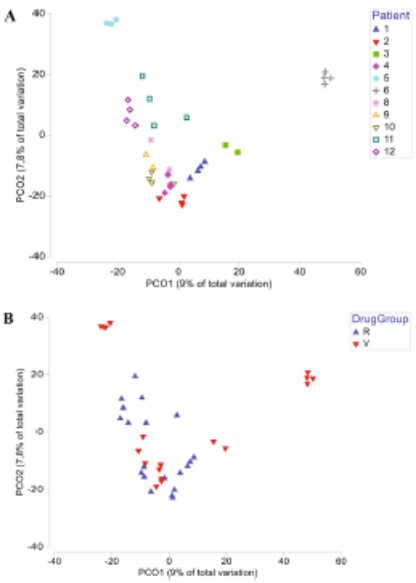


Fig. 1 **A** Principal Co-ordinate Analysis (PCO) of the microbiome profiles for multiple patients. **B** Principal Co-ordinate Analysis (PCO) of the microbiome profiles for drug treatments (calcium acetate versus sucroferric oxyhydroxide)

composition at the genus level, *Bacteroides* was the most prevalent in both groups of patients, independently if they were treated with either CA or SFO (Fig. 2). The microbial profiles were very distinct among patients and, once again, the clustering analysis grouped the samples of the same patient independently of the treatment followed and the stage of the treatment (baseline, 4, 12, or 20 weeks).

When we consider all the time points (all patients and all weeks), it was possible to find statistical differences ($p < 0.05$) for the microbial communities when comparing the samples for multiple variables, including gender, ischemic cardiomyopathy, the use of a catheter as vascular access, or age (the patients were organized in three groups: under 45, range 61–69, above 71). Such statistical differences could not be observed when each treatment stage (baseline, 4, 12, or 20 weeks) was considered separately; therefore, no differences were observed for the variables gender, age, ischemic cardiomyopathy, catheter use, and drug treatments (CA versus SFO).

Discussion

This is the first study comparing the changes in the gut microbiome of hemodialysis patients taking CA versus SFO. In our study, there were no consistent differences in the bacterial composition of the gut microbiome between these different phosphate-binding agents. Although we found different microbiome profiles when both groups of treatment were compared, this different profile was present already at baseline, and long-term treatment did not modify this diversity in any of the two groups. So, no significant changes

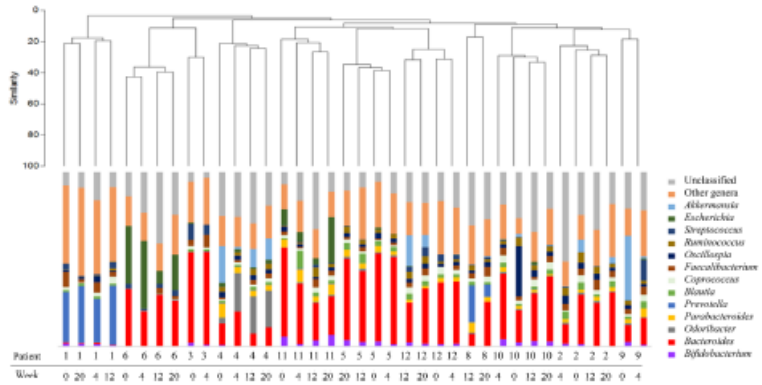


Fig. 2 Clustering analysis and microbiome profiles (at genus level) for the samples considered in this study

were observed over different time points (baseline, week 4, week 12, week 20) in hemodialysis subjects gut microbiome treated with CA versus those treated with SFO.

The gut microbiome of our patients is in accordance with previous reports, dominated by Bacteroidetes and Firmicutes phyla, being Actinobacteria, Proteobacteria, and Verrucomicrobia in the second line of colonization [29]. As previously extensively discussed, CKD patients have numerous intrinsic factors that promote gut dysbiosis besides the pharmacological therapies, namely reduced colonic transit, altered digestive capacity, metabolic acidosis, intestinal wall edema, and one of the most important, the high intestinal availability of uremic toxins. In comparison to healthy controls, patients undergoing hemodialysis present an increased Bacteroidetes abundance [30], also corroborating with the results of our study.

There is little evidence about the effect of phosphate binders on the gut microbiome [25]. Studies assessing the effects of calcium-based phosphate binders, including CA, on the CKD patients gut microbiome, analyzing fecal samples, are lacking [31]. Trautvetter et al. [32], observed an increase of fecal total short-chain fatty acids and a higher relative abundance of the genus *Clostridium* XVIII in healthy individuals taking calcium carbonate. Navarro-Gonzalez et al. [22], analyzed hemodialysis patients serum samples taken either the non-calcium-based phosphate binder sevelamer or the calcium-based phosphate binder CA and concluded that treatment with sevelamer was associated with a significant decrease in high-sensitive C-reactive protein, IL-6, serum endotoxin, and soluble CD14 concentrations independent predictors of mortality in hemodialysis patients.

SFO is an iron-based phosphate binder, and data suggested that the iron contained in the compound may switch gut microbiota because some gut bacteria use iron to increase relative abundance [26, 33, 34]. Moreover, it has been shown that an increase in the amount of iron reaching the colon may promote virulence of some pathogenic bacteria and a pro-inflammatory environment [20, 35]. But despite this evidence, our study shows that SFO treatment in hemodialysis patients does not seem to modify the gut microbiome, nor CA treatment.

Ling Lau et al. [27], compared fecal microbiome and uremic toxins in serum samples between CKD rats (who underwent 5/6 nephrectomy) and normal rats, randomly assigned to a regular diet or a diet containing 4% ferric citrate for 6 weeks. They observed that CKD rats had lower relative abundances of some Firmicutes and *Lactobacillus* and a lower gut microbial diversity compared to normal rats, but they also described that ferric citrate treatment in CKD rats increased bacterial diversity almost to levels observed in control rats and that this treatment did not increase uremic toxins. In a recent study, Wu et al. [36], compared hemodialysis patients gut microbiome treated with either calcium carbonate or ferric citrate. They observed a significantly increased microbial diversity in the group treated with ferric citrate, with an increased abundance of Bacteroidetes and a decreased abundance of Firmicutes.

To our knowledge, there is only one study performed in humans regarding SFO effect on the gut microbiome. Iguchi et al. [37], compared 3 months' changes in the gut microbiome and uremic toxins of hemodialysis patients treated with either SFO versus no treatment for hyperphosphatemia. They also found no changes in the gut microbiome in patients treated with SFO throughout time. So, our study confirms this long-term stability of the gut microbiome in hemodialysis patients treated with SFO for 5 months.

Another important point to discuss is that in our study, we observed differences in hemodialysis patients gut microbiome compared by age or gender, but we have not found differences when we compare them by group of treatment (CA versus SFO). In accordance, some alterations have been demonstrated in the gut microbiome by aging [38]. Elderly patients, especially those with high frailty scores, present relative proportions of Bacteroidetes predominating, less microbial diversity, and decreases in *Bifidobacteria*, *Bacteroides*/*Prevotella*, *Lactobacillus*, and *Clostridium* cluster IV, when compared with young individuals, which present more microbial diversity and higher proportions of Firmicutes, among others [39, 40]. There is also mounting evidence supporting that there are alterations in the gut microbiome if comparing women and men [41, 42]. In our study, some differences were observed in the gut microbiome according to gender and age, but the differences found among each patient were much more pronounced.

Regarding laboratory findings, as expected, patients treated with the calcium-based phosphate binder CA presented higher calcium levels than those treated with SFO. We observed, although not statistically significant, increased levels of inflammatory parameters, such as sedimentation velocity, C-reactive protein, and ferritin in the CA group when compared with the SFO group; such pleiotropic effect on diminishing inflammation was described for some phosphate binders other than calcium-based binders [43].

It is essential to consider that our study presents some limitations. On the one hand, the size of the patient sample is small, so it is difficult to draw solid conclusions, especially on the effects of the clinical and biochemical variables analyzed. To validate our results, a larger study, with an increased number of patients is needed. On the other hand, our patients display different backgrounds, with distinctive comorbidities which can influence the gut microbiome. So, our study alerts about the high variability of profiles found on the gut microbiome of patients receiving phosphate binders. Such differences limit any

additional conclusions and differences found among the patients receiving different phosphate binders.

As we reported, to search if a specific clinical variable could influence this differentiated microbiome profile, we analyzed the main clinical parameters at baseline of our patients and we found not statistically significant differences between both groups of treatment. Patients 5 and 6 were a little bit out of order and it can be stated that patient 5 received vancomycin and tobramycin for 3 weeks, while patient 6 presented chronic diarrhea with repeatedly negative cultures and a possible wasting syndrome associated.

In our study, we observed no changes in the gut microbiome of our hemodialysis patients after 20 weeks of treatment, independently of the phosphate binder. For the moment, when choosing a phosphate binder, we should rely on their power on the reduction of serum phosphate, the pill burden, the association to the vascular calcification progress, the adverse events, or the gastrointestinal tolerance [44–47]; although the influence of these phosphate binders on gut microbiome was expected, and still remains possible, for now, there is no evidence that this aspect should influence our approach when treating hyperphosphatemia.

In conclusion, our study observed that 5-month treatment with either CA or SFO did not modify baseline diversity nor baseline bacterial composition in hemodialysis patients, but alerts about the high variability of profiles found on the gut microbiome of CKD patients.

Acknowledgements Not applicable.

Author contributions IB, FG, and JB conceived and contributed to the study design; AMR, IB, and FG contributed to the patient inclusion and clinical management; AMR, IB, and FG. contributed to sampling and data collection, AV, MNJ., RP contributed to the extraction of fecal DNA and the sequencing of 16S rRNA; RA, AV, MNJ, RP, and BSM contributed to the construction of the OTU table, the taxonomic assignment and the data and statistical analysis; AMR, RA, IB, FG, BSM contributed to the drafting of the manuscript and provided critical revisions of the manuscript. The authors had full access to the data, and the corresponding author had the final responsibility for submitting the manuscript for publication.

Funding This research was funded by FEDER—Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020—Operational Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT/MCTES in the framework of the project MicroMOB "POCI-01-0145-FEDER-029777 / PTDC/MEC-MCI/29777/2017".

Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest A.M.R., R.A., I.B., F.G., A.V, M.N.J., R.P., J.B., B.S.M. have no conflicts of interest to declare.

Ethics approval This study was approved by the Clinical Research Ethics Committee of the Hospital Universitari Germans Trias i Pujol (PI-16–169, NCT5551048) and conformed to the principles outlined in the Declaration of Helsinki.

Consent to participate All participants were recruited voluntarily after receiving detailed information on the study protocol. Written informed consent was obtained from all patients.

Consent for publication Not applicable.

References

1. Saran R, Robinson B, Abbott KC, Bragg-Gresham J, Chen X, Gipson D et al (2020) US Renal data system 2019 annual data report: epidemiology of kidney disease in the United States. Am J Kidney Dis 75(1):A6-7. https://doi.org/10.1053/j.ajkd.2019.09.003
2. Sharma S, Sarnak MJ. Epidemiology: The global burden of reduced GFR: ESRD, CVD and mortality. Nat Rev Nephrol. 2017;13(8):447–448. https://doi.org/10.1038/nrneph.2017.84.
3. Cobo G, Lindholm B, Stenvinkel P. Chronic inflammation in end-stage renal disease and dialysis. Nephrol Dial Transplant. 2018;33:iii35–iii40. https://doi.org/10.1093/ndt/gfy175.
4. Chmielewski M, Cohen G, Wiecek A, Jesús Carrero J. The Peptidic Middle Molecules: Is Molecular Weight Doing the Trick?. Semin Nephrol. 2014;34(2):118–34. https://doi.org/10.1016/j.semnephrol.2014.02.005.
5. Mihai S, Codrici E, Popescu ID, Enciu AM, Albulescu L, Necula LG et al (2018) Inflammation-related mechanisms in chronic kidney disease prediction, progression, and outcome. J Immunol Res 2018:2180373. https://doi.org/10.1155/2018/2180373
6. Amdur RL, Feldman HI, Dominic EA, Anderson AH, Beddhu S, Rahman M et al (2019) Use of measures of inflammation and kidney function for prediction of atherosclerotic vascular disease events and death in patients With CKD: findings from the CRIC study. Am J Kidney Dis 73(3):344–353. https://doi.org/10.1053/j.ajkd.2018.09.012
7. Vaziri ND, Yuan J, Rahimi A, Ni Z, Said H, Subramanian VS (2012) Disintegration of colonic epithelial tight junction in uremia: a likely cause of CKD-associated inflammation. Nephrol Dial Transplant 27(7):2686–2693. https://doi.org/10.1093/ndt/gfr624
8. Vaziri ND, Yuan J, Norris K (2013) Role of urea in intestinal barrier dysfunction and disruption of epithelial tight junction in

chronic kidney disease. Am J Nephrol 37(1):1–6. https://doi.org/10.1159/000345969
9. Vaziri ND, Wong J, Pahl M, Piceno YM, Yuan J, Desantis TZ et al (2013) Chronic kidney disease alters intestinal microbial flora. Kidney Int 83(2):308–315. https://doi.org/10.1038/ki.2012.345.E
10. Vaziri ND (2012) CKD impairs barrier function and alters microbial flora of the intestine: a major link to inflammation and uremic toxicity. Curr Opin Nephrol Hypertens 21(6):587–592. https://doi.org/10.1097/MNH.0b013e328358c8d5
11. Andersen K, Kesper MS, Marschner JA, Konrad L, Ryu M, Kumar SVR et al (2017) Intestinal dysbiosis, barrier dysfunction, and bacterial translocation account for CKD-related systemic inflammation. J Am Soc Nephrol 28(1):76–83. https://doi.org/10.1681/ASN.2015111285
12. Evenepoel P, Poesen R, Meijers B. The gut–kidney axis. Pediatr Nephrol. 2017;32(11):2005–2014. https://doi.org/10.1007/s00467-016-3527-x.
13. Lau WL, Kalantar-Zadeh K, Vaziri ND (2015) The gut as a source of inflammation in chronic kidney disease. Nephron 130(2):92–98. https://doi.org/10.1159/000381990
14. Vaziri ND, Zhao YY, Pahl MV (2016) Altered intestinal microbial flora and impaired epithelial barrier structure and function in CKD: the nature, mechanisms, consequences and potential treatment. Nephrol Dial Transpl 31(5):737–746. https://doi.org/10.1093/ndt/gfv095
15. Wong J, Piceno YM, DeSantis TZ, Pahl M, Andersen GL, Vaziri ND (2014) Expansion of urease- and uricase-containing, indole- and p-cresol-forming and contraction of short-chain fatty acid-producing intestinal microbiota in ESRD. Am J Nephrol 39(3):230–237. https://doi.org/10.1159/000360010
16. Kieffer DA, Piccolo BD, Vaziri ND, Liu S, Lau WL, Khazaeli M et al (2016) Resistant starch alters gut microbiome and metabolomic profiles concurrent with amelioration of chronic kidney disease in rats. Am J Physiol—Ren Physiol 310(9):F857–F871. https://doi.org/10.1152/ajprenal.00513.2015
17. Yang T, Richards EM, Pepine CJ, Raizada MK. The gut microbiota and the brain-gut-kidney axis in hypertension and chronic kidney disease. Nat Rev Nephrol. 2018;14(7):442–456. https://doi.org/10.1038/s41581-018-0018-2.
18. Kanbay M, Onal EM, Afsar B, Dagel T, Yerlikaya A, Covic A, et al. The crosstalk of gut microbiota and chronic kidney disease: role of inflammation, proteinuria, hypertension, and diabetes mellitus. Int Urol Nephrol. 2018 ;50(8):1453–1466.https://doi.org/10.1007/s11255-018-1873-2.
19. Nazzal L, Roberts J, Singh P, Jhawar S, Matalon A, Gao Z et al (2017) Microbiome perturbation by oral vancomycin reduces plasma concentration of two gut-derived uremic solutes, indoxyl sulfate and p -cresyl sulfate, in end-stage renal disease. Nephrol Dial Transpl 32(11):1809–1817. https://doi.org/10.1093/ndt/gfx029
20. Kortman GAM, Reijnders D, Swinkels DW (2017) Oral iron supplementation: Potential implications for the gut microbiome and metabolome in patients with CKD. Hemodial Int 21(Suppl 1):S28–S36. https://doi.org/10.1111/hdi.12553
21. Lee T, Clavel T, Smirnov K, Schmidt A, Lagkouvardos I, Walker A et al (2016) Oral versus intravenous iron replacement therapy distinctly alters the gut microbiota and metabolome in patients with IBD. Gut 66(5):863–871. https://doi.org/10.1136/gutjnl-2015-309940
22. Navarro-González JF, Mora-Fernández C, de Fuentes MM, Donate-Correa J, Cazaña-Pérez V, García-Pérez J (2011) Effect of phosphate binders on serum inflammatory profile, soluble CD14, and endotoxin levels in hemodialysis patients. Clin J Am Soc Nephrol 6(9):2272–2279. https://doi.org/10.2215/CJN.01650211
23. Guida B, Cataldi M, Riccio E, Grumetto L, Pota A, Borrelli S, et al. Plasma p-cresol lowering effect of sevelamer in peritoneal dialysis patients: evidence from a cross-sectional observational study. PLoS One. 2013;8(8). https://doi.org/10.1371/journal.pone.0073558.
24. Lin C, Pan C, Chuang C, Liu H, Huang S-F, Chen H-H et al (2017) Effects of sevelamer hydrochloride on uremic toxins serum indoxyl sulfate and P-cresyl sulfate in hemodialysis patients. J Clin Med Res 9(9):765–770. https://doi.org/10.14740/jocmr1803e
25. Biruete A, Hill Gallant KM, Lindemann SR, Wiese GN, Chen NX, Moe SM. Phosphate Binders and Nonphosphate Effects in the Gastrointestinal Tract. J Renal Nutr. 2020 ;30(1):4–10. https://doi.org/10.1053/j.jrn.2019.01.004.
26. Wilson BR, Bogdan AR, Miyazawa M, Hashimoto K, Tsuji Y. Siderophores in Iron Metabolism : From Mechanism to Therapy Potential. Trends Mol Med. 2016;22(12):1077–1090. https://doi.org/10.1016/j.molmed.2016.10.005.
27. Lau WL, Vaziri ND, Nunes ACF, Comeau AM, Langille MGI, England W et al (2018) The phosphate binder ferric citrate alters the gut microbiome in rats with chronic kidney disease. J Pharmacol Exp Ther 367(3):452–460. https://doi.org/10.1124/jpet.118.251389
28. Parks DH, Beiko RG (2010) Identifying biologically relevant differences between metagenomic communities. Bioinformatics 26(6):715–721. https://doi.org/10.1093/bioinformatics/btq041
29. Sampaio-Maia B, Simões-Silva L, Pestana M, Araujo R, Soares-Silva IJ (2016) The role of the gut microbiome on chronic kidney disease. Adv Appl Microbiol 96:65–94. https://doi.org/10.1016/bs.aambs.2016.06.002
30. Simões-Silva L, Araujo R, Pestana M, Soares-Silva I, Sampaio-Maia B. The microbiome in chronic kidney disease patients undergoing hemodialysis and peritoneal dialysis. Pharmacol Res. 2018;130:143–151. https://doi.org/10.1016/j.phrs.2018.02.011.
31. Rahbar Saadat Y, Niknafs B, Hosseiniyan Khatibi SM, Ardalan M, Majdi H, Bahmanpoor Z, et al. Gut microbiota; an overlooked effect of phosphate binders. Eur J Pharmacol. 2020;868:172892. https://doi.org/10.1016/j.ejphar.2019.172892.
32. Trautvetter U, Camarinha-Silva A, Jahreis G, Lorkowski S, Glei M (2018) High phosphorus intake and gut-related parameters—results of a randomized placebo-controlled human intervention study. Nutr J 17(1):1–11. https://doi.org/10.1186/s12937-018-0331-4
33. Muckenthaler MU, Rivella S, Hentze MW, Galy B. A Red Carpet for Iron Metabolism. Cell. 2017;168(3):344–61. https://doi.org/10.1016/j.cell.2016.12.034.
34. Paganini D, Zimmermann MB. The effects of iron fortification and supplementation on the gut microbiome and diarrhea in infants and children: A review. Am J Clin Nutr. 2017;106(C):1688S-1693S. https://doi.org/10.3945/ajcn.117.156067.
35. Kortman GAM, Dutilh BE, Maathuis AJH, Engelke UF, Boekhorst J, Keegan KP et al (2016) Microbial metabolism shifts towards an adverse profile with supplementary iron in the TIM-2 in vitro model of the human colon. Front Microbiol 6:1418. https://doi.org/10.3389/fmicb.2015.01481
36. Wu PH, Liu PY, Chiu YW, Hung WC, Lin YT, Lin TY et al (2020) Comparative gut microbiome differences between ferric citrate and calcium carbonate phosphate binders in patients with end-stage kidney disease. Microorganisms 8(12):1–14. https://doi.org/10.3390/microorganisms8122040
37. Iguchi A, Yamamoto S, Oda A, Tanaka K, Kazama JJ, Saeki T, et al. Effect of sucroferric oxyhydroxide on gastrointestinal microbiome and uremic toxins in patients with chronic kidney disease undergoing hemodialysis. Clin Exp Nephrol. 2020;24(8):725–33. https://doi.org/10.1007/s10157-020-01892-x.

38. Buford TW. (Dis)Trust your gut: the gudoi: https://doi.org/10.1186/s40168-017-0296-0.t microbiome in age-related inflammation, health, and disease. Microbiome. 2017;5(1):80.
39. Zwielehner J, Liszt K, Handschur M, Lassl C, Lapin A, Haslberger AG. Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of Bacteroides, bifidobacteria and Clostridium cluster IV in institutionalized elderly. Exp Gerontol. 2009;44(6–7):440–6. https://doi.org/10.1016/j.exger.2009.04.002.
40. Claesson MJ, Jeffery IB, Conde S, Power SE, O'connor EM, Cusack S et al (2012) Gut microbiota composition correlates with diet and health in the elderly. Nature 488(7410):178–184. https://doi.org/10.1038/nature11319
41. Santos-Marcos JA, Rangel-Zuñiga OA, Jimenez-Lucena R, Quintana-Navarro GM, Garcia-Carpintero S, Malagon MM et al (2018) Influence of gender and menopausal status on gut microbiota. Maturitas 116:43–53. https://doi.org/10.1016/j.maturitas.2018.07.008
42. Rizzetto L, Fava F, Tuohy KM, Selmi C (2018) Connecting the immune system, systemic chronic inflammation and the gut microbiome: the role of sex. J Autoimmun 92:12–34. https://doi.org/10.1016/j.jaut.2018.05.008
43. Rastogi A (2013) Sevelamer revisited: Pleiotropic effects on endothelial and cardiovascular risk factors in chronic kidney disease and end-stage renal disease. Ther Adv Cardiovasc Dis 7(6):322–342. https://doi.org/10.1177/1753944713513061
44. Floege J. Phosphate binders in chronic kidney disease: an updated narrative review of recent data. J Nephrol. 2020;33(3):497–508. https://doi.org/10.1007/s40620-019-00689-w.
45. Palmer SC, Gardner S, Tonelli M, Mavridis D, Johnson DW, Craig JC, et al. Phosphate-binding agents in adults with CKD: a network meta-analysis of randomized trials. Am J Kidney Dis. 2016;68(5):691–702. https://doi.org/10.1053/j.ajkd.2016.05.015.
46. Kendrick J, Parameswaran V, Ficociello LH, Ofsthun NJ, Davis S, Mullon C, et al. One-year historical cohort study of the phosphate binder sucroferric oxyhydroxide in patients on maintenance hemodialysis. J Ren Nutr. 2019;29(5):428–37. https://doi.org/10.1053/j.jrn.2018.11.002.
47. Covic AC, Floege J, Ketteler M, Sprague SM, Lisk L, Rakov V et al (2017) Iron-related parameters in dialysis patients treated with sucroferric oxyhydroxide. Nephrol Dial Transpl 32(8):1363–1372. https://doi.org/10.1093/ndt/gfw242

Merino-Ribas A, Araujo R, Pereira L, Campos J, Barreiros L, Segundo MA, Silva N, Costa CFFA, Quelhas-Santos J, Trindade F, Falcão-Pires I, Alencastre I, Dumitrescu IB, Sampaio-Maia B. Vascular Calcification and the Gut and Blood Microbiome in Chronic Kidney Disease Patients on Peritoneal Dialysis: A Pilot Study. Biomolecules. 2022 Jun 21;12(7):867. doi: 10.3390/biom12070867.
PMID: 35883423; PMCID: PMC9313079.

biomolecules

MDPI

Article

Vascular Calcification and the Gut and Blood Microbiome in Chronic Kidney Disease Patients on Peritoneal Dialysis: A Pilot Study

Ana Merino-Ribas [1,2,3], Ricardo Araujo [1], Luciano Pereira [1,4], Joana Campos [1], Luisa Barreiros [5], Marcela A. Segundo [5], Nádia Silva [4], Carolina F. F. A. Costa [1,6], Janete Quelhas-Santos [7], Fábio Trindade [7], Inês Falcão-Pires [7], Ines Alencastre [1], Ioana Bancu Dumitrescu [2,8] and Benedita Sampaio-Maia [1,9,*]

1 Nephrology & Infectious Diseases R & D Group, i3S—Instituto de Investigação e Inovação em Saúde, INEB—Instituto de Engenharia Biomédica, Universidade do Porto, 4200-135 Porto, Portugal; anamerinoribas@gmail.com (A.M.-R.); ricardo.araujo@i3s.up.pt (R.A.); lucianoarturpereira@hotmail.com (L.P.); joanaaraujocampos@gmail.com (J.C.); carolina.costa@i3s.up.pt (C.F.F.A.C.); ines.alencastre@ineb.up.pt (I.A.)
2 Departament de Medicina, Universitat Autonoma de Barcelona, 08035 Barcelona, Spain; ioana_bancu@yahoo.com
3 Nephrology Department, Hospital Universitari de Girona Doctor Josep Trueta, 17007 Girona, Spain
4 Nephrology Department, Centro Hospitalar Universitário de São João, 4200-319 Porto, Portugal; nadiaraquel77@gmail.com
5 LAQV, REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, 4050-313 Porto, Portugal; lbarreiros78@gmail.com (L.B.); msegundo@ff.up.pt (M.A.S.)
6 Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, 4050-313 Porto, Portugal
7 UniC@RISE- Cardiovascular Research and Development Centre, Department of Surgery and Physiology, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal; sjanete@med.up.pt (J.Q.-S.); ftrindade@med.up.pt (F.T.); ipires@med.up.pt (I.F.-P.)
8 Fresenius Nephrocare, 110372 Pitesti, Romania
9 Faculdade de Medicina Dentária, Universidade do Porto, 4200-393 Porto, Portugal
* Correspondence: bmaia@fmd.up.pt; Tel.: +351-220-901-100

Abstract: Vascular calcification (VC) is a frequent condition in chronic kidney disease (CKD) and a well-established risk factor for the development of cardiovascular disease (CVD). Gut dysbiosis may contribute to CVD and inflammation in CKD patients. Nonetheless, the role of gut and blood microbiomes in CKD-associated VC remains unknown. Therefore, this pilot study aimed to explore the link between gut and blood microbiomes and VC in CKD patients on peritoneal dialysis (CKD-PD). Our results showed relative changes in specific taxa between CKD-PD patients with and without VC, namely *Coprobacter*, *Coprococcus 3*, *Lactobacillus*, and *Eubacterium eligens* group in the gut, and *Cutibacterium*, *Pajaroellobacter*, *Devosia*, *Hyphomicrobium*, and *Pelomonas* in the blood. An association between VC and all-cause mortality risk in CKD-PD patients was also observed, and patients with higher mortality risk corroborate the changes of *Eubacterium eligens* in the gut and *Devosia* genus in the blood. Although we did not find differences in uremic toxins, intestinal translocation markers, and inflammatory parameters among CKD-PD patients with and without VC, soluble CD14 (sCD14), a nonspecific marker of monocyte activation, positively correlated with VC severity. Therefore, gut *Eubacterium eligens* group, blood *Devosia*, and circulating sCD14 should be further explored as biomarkers for VC, CVD, and mortality risk in CKD.

Keywords: chronic kidney disease; vascular calcification; gut microbiome; blood microbiome; mortality risk; sCD14

check for updates

Citation: Merino-Ribas, A.; Araujo, R.; Pereira, L.; Campos, J.; Barreiros, L.; Segundo, M.A.; Silva, N.; Costa, C.F.F.A.; Quelhas-Santos, J.; Trindade, F.; et al. Vascular Calcification and the Gut and Blood Microbiome in Chronic Kidney Disease Patients on Peritoneal Dialysis: A Pilot Study. *Biomolecules* 2022, *12*, 867. https://doi.org/10.3390/biom12070867

Academic Editor: Csaba Szabo

Received: 21 April 2022
Accepted: 16 June 2022
Published: 21 June 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Chronic kidney disease (CKD) is a major public health problem carrying a high socio-economic burden with elevated morbidity and mortality [1]. It is expected that CKD will

become the fifth global cause of death by 2040 [2]. Cardiovascular disease (CVD) is the leading cause of death among CKD patients with a mortality rate 30 times higher than the general population [3]. The increased CVD risk in CKD patients is only partially explained by traditional cardiovascular risk factors such as diabetes, hypertension, dyslipidaemia, smoking, obesity, among others. Non-traditional risk factors such as inflammation, oxidative stress, endothelial dysfunction, and vascular calcification (VC) have been identified as key players in the development of CVD in these patients [4].

Under normal conditions, inflammation can arise as a protective physiological response to various inimical stimuli. However, in several debilitating disorders, such as CKD, the inflammatory process becomes persistent and contributes to the aggravation of the disease [5]. In CKD, inflammation is likely a consequence of multifactorial aetiology and interacts with several factors that emerge in response to the accumulation of uremic toxins due to renal function impairment, contributing significantly to the higher CVD risk in CKD [6].

A disturbed or unbalanced gut microbiota, described as gut dysbiosis, is currently recognised as a key factor in the pathogenesis or progression of CKD. This CKD dysbiotic ecosystem is characterized by a shift towards proteolytic metabolism mostly due to an increased number of bacteria that possess urease, uricase, and p-cresol, and indole-forming enzymes, and by a decline in saccharolytic fermentation, leading to a reduction of short-chain fatty acids (SCFA) production [7–9]. CKD-associated gut dysbiotic state leads to an increase of uremic toxins derived from the microbial metabolism (such as trimethylamine N-oxide (TMAO), p-cresol sulfate (PCS), indoxyl sulfate (INDS), and indole-3-acetic acid (3-IAA) further contributing to the chronic status of oxidative stress and inflammation, and the consequent increase in CVD risk [10–12]. Moreover, CKD-related gut dysbiosis is also associated with an impaired epithelial barrier, a condition commonly referred to as leaky gut, which allows the translocation of living bacteria, endotoxins (lipopolysaccharides (LPS), bacterial DNA, and gut-derived uremic toxins into the systemic circulation [13], eliciting or further aggravating the inflammatory state [14]. Together, these data highlight the potential role of gut microbes in CKD and associated CVD.

Beyond the gut, an increasing body of evidence supports the existence of a human blood microbiome with relevance in health and disease, although its origin, structure, and function remain unrevealed [15,16]. Different reports suggested that blood owns a unique microbiome and that a dysbiotic blood microbiome is associated with different pathologies such as atherosclerosis, CVD, ischaemic stroke, and liver fibrosis [17–19]. Specifically, in CKD, a recent study showed a blood microbiome profile with lower alpha diversity and significant taxonomic variations when compared with healthy controls [20].

VC and its severity have long been recognized as an important factor in CVD development in CKD patients [21]. VC is an active and highly regulated cellular process defined by the deposition of calcium-phosphate crystals within the intima and media layers of the vasculature and/or heart valves. Several factors have been related with VC, such as biomarkers of inflammation (for example high-sensitivity C-reactive protein (PCR), interleukin (IL)- 6, Tumour necrosis factor-α (TNF-α), and of monocyte activation (for example soluble CD14 and CD163) [22]. In fact, the mineral bone disorder associated with CKD is characterised by one or more abnormalities in circulating minerals and their regulating hormones, bone abnormalities, and VC [21]. Mounting evidence indicates that the gut dysbiosis associated with CKD may be involved in the pathogenesis of bone–vascular axis [8,23]. Recent data suggest that an increased protein fermentation, and consequent uremic toxins production, decreased carbohydrate fermentation, vitamin K deficiency, and gut-derived inflammation may, alone or together, drive to a vascular and skeletal pathobiology in CKD patients [8,23]. Still, to our knowledge, there are currently no data on the putative association between blood microbiome and vascular calcification.

Given the importance of VC in CKD and the associated increased risk of CVD in these patients, the aim of our study was to explore the link between VC, all-cause mortality risk, and the gut and blood microbiome in CKD patients on peritoneal dialysis (CKD-PD).

2. Materials and Methods

2.1. Study Design, Subjects, and Sample Collection

This cross-sectional observational study included 44 CKD patients undergoing peritoneal dialysis in Centro Hospitalar Universitário de São João in Porto, Portugal, between 2018 and 2019. This study was approved by the local Ethics Committee (approval references 200/18), in accordance with the 1964 Helsinki declaration and its later amendments. All participants were recruited voluntarily after receiving detailed information on the study protocol. Written informed consent was obtained from all patients. Exclusion criteria included age under 18 years old, inability to give informed consent, history of infection in the last 3 months, and antibiotic intake in the last 3 months.

Relevant clinical and demographic information was gathered for each participant. Clinical characteristics collected were gender, age, CKD aetiology, history of high blood pressure, diabetes mellitus, dyslipidaemia, obesity (defined as body mass index of 30 kg/m^2 and higher), and history of cardiovascular disease (peripheral vascular disease, ischemic cardiomyopathy, or cerebrovascular disease). Their pharmacological treatment and infection history was also gathered.

VC was estimated in all patients using Adragao score through hands and pelvic radiographies [24]. The Charlson Comorbidity Index was also calculated predicting 10-years survival in patients with multiple comorbidities [25,26].

Blood samples were collected in the peritoneal dialysis unit, and the self-collected stool specimens were brought refrigerated by the patient within 48 h after collection. Whole blood and stool samples were collected in DNA-free sterile containers and were immediately frozen and stored at −80 °C for microbiome analysis. Plasma was obtained after blood centrifugation (1500× *g*, 15 min, 4 °C) and stored at −80 °C for biochemical analysis.

2.2. Sample Processing and Microbiome Analysis

Genomic DNA was isolated in a strictly controlled environment at Vaiomer SAS (Labège, France) as previously described [20]. Total DNA was extracted from whole blood (100 µL) using a specific Vaiomer protocol carefully designed to minimise any risk of contamination between samples from the experimenters or the environment. Negative controls (molecular grade water added in an empty tube, the same used for sample storage and peritoneal dialysis solution) were extracted, amplified, and sequenced at the same time as the samples. PCR amplification was performed using universal primers targeting the V3-V4 region of the bacterial 16S rRNA gene (340F-781R). Illumina sequencing length, by use of the 2 × 300 paired-end MiSeq kit V3, was designed to encompass the 476-base pair amplicons. Sample multiplexing and sequencing library generation were conducted, as previously described [27]. qPCR was used to quantify the DNA concentration in the pool employing a 7900HT Fast Real-Time PCR System (Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA) and KAPA Library Quantification Kits for Illumina Platform (Kapa Biosystems, Inc., Wilmington, NC, USA). The final pool, at a concentration after dilution between 5 and 20 nM, was used for sequencing as suggested previously [27]. The sequencing steps were performed using a paired-end sequencing run in a MiSeq Illumina device.

2.3. 16S rRNA Gene Sequence Analysis

The targeted gene regions were analysed using the FROGS bioinformatics pipeline established by Vaiomer SAS (Labège, France) [28]. The following filters were applied as previously suggested [27]: (1) amplicons with a length < 350 nt or a length > 480 nt were removed; (2) amplicons without the two PCR primers were removed (10% of mismatches were authorised); (3) amplicons with at least one ambiguous nucleotides ('N') were removed; (4) operational taxonomic units (OTU) identified as chimera (with search v1.9.5) in all samples in which they were presented were removed; (5) OTU with an abundance lower than 0.005% of the whole dataset abundance were removed, and (6) OTU with a strong similarity (coverage and identity ≥ 80%) with the phiX (library used as a control

for Illumina sequencing runs) were removed. OTU were produced via single-linkage clustering, and taxonomic assignment was performed by Blast+ v2.2.30+ with the databank RDP v11.4.

2.4. Biochemical Analysis

Routine clinical analyses were collected from our patients' clinical records, namely, urea, proteinuria, albumin, haemoglobin, cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, phosphorus (P), calcium (Ca), calcium phosphate product, ferritin, B-type natriuretic peptide (BNP), parathyroid hormone (PTH), sedimentation velocity (SV), CRP, creatinine clearance (Ccreat), residual renal function, and Kt/V (urea). Kt/V (urea) is a parameter that measures adequacy to PD using urea weekly clearance normalised by urea estimated distribution volume. Tumour necrosis factor α (TNF-α), IL-1, IL-6, IL-10 were determined in plasma by Luminex Multiplex Assay (Millipore Corporation, Billerica, MA, USA). ELISA kits were used to evaluate Lipopolysaccharide-binding protein (LPS-BP, Cloud-clone Corp.®, Katy, TX, USA), Toll-like receptor 4 (TLR4, Cloud-clone Corp.®, Katy, TX, USA), and soluble CD14 (sCD14, Quantikine® ELISA, R&D Systems, Inc., Minneapolis, MN, USA), and TMAO (MyBiosource®, San Diego, CA, USA) whereas endotoxins were evaluated by Traditional Kinetic Limulus Amebocyte Lysate (LAL) Assay (Lonza Walkersville, Inc., Walkersville, MA, USA).

Uremic toxins were quantified following the method described by [29] with modifications. p-Cresol sulfate (PCS), 3-indoxyl sulfate (3-INDS), and indole-3-acetic acid (3-IAA) were detected by high-performance liquid chromatography (HPLC) with fluorescence detection (275 and 330 nm). Elution was performed in gradient mode using as mobile phase a mixture of (A) aqueous NaH_2PO_4 buffer (20 mM, pH 4.6), containing tetrabutyl ammonium iodide (TBAI, 5 mM), and (B) acetonitrile, at a flow rate of 1.5 mL/min, and injection volume of 20 µL. Prior to HPLC analysis, 100 µL of each plasma standard or sample was added to 300 µL of ethanol containing 0.22 mg/L of internal standard 4-ethylphenol. After vortexing during 30 s, 100 mg of NaCl were added and mixed vigorously. After 10 min, 700 µL of component (A) of mobile phase was further added following centrifugation at 18,000× *g* for 10 min at 4 °C and supernatant analysis by HPLC.

2.5. Statistics

All the results are represented as mean ± standard deviation (SD) or in percentage (%). Statistical analysis was performed using SPSS Statistics version 27 (IBM). The categorical variables were described through absolute or relative frequencies (%) and analysed using the Pearson's chi-square test or Fisher's exact test when more than 1 cell displayed expected counts less than 5. Continuous variables were described using mean ±SD and analysed by Student's *t* test for independent samples when following a normal distribution, or by Mann-Whitney U test when there was no normality of the data. Normality was assessed by the Shapiro-Wilk test. A partial correlation between vascular calcification and all-cause mortality risk, while controlling of the effect of age and sex, was performed using JASP-stats software. For all analysis, statistical significance was assumed when p values were less than 0.05.

Primer v7 (PRIMER-e, Auckland, New Zealand) was used for the calculation of diversity indices, non-metric multidimensional scaling (NMDS) and principal coordinate analyses (PCO), and other multivariate analyses, mainly ANOSIM and PERMANOVA, were used to test the significance of Beta-diversity. The percentage of OTU data per sample was used for these analyses, followed by squared root transformed data, resemblance matrices of similarity data types using Bray-Curtis similarities, adding dummy value and testing 4999 permutations. The reads in each sample were converted into percentage values according to the total number of sequences in the sample to eliminate the effect of the final number of reads [30]. Post-hoc analyses were done in STAMP 2.1.3 [31] for multiple groups using one-way analysis of variance (ANOVA), Tukey-Kramer (0.95) and Eta-squared for

effect size, while, with two groups, analysis using Welch's *t*-test was conducted (two-sided, Welch's inverted for confidence interval method).

3. Results

Our 44 CKD-PD patients presented an Adragao score mean of 2.98 ± 2.74, included 26.1% patients without VC (Adragao score = 0); 30.4% with moderate VC (Adragao score of 1 or 2) and 39.1% with severe VC (Adragao score higher than 2). In our study, we compared CKD-PD patients with moderate or severe VC versus patients with no VC. Demographic and clinical characteristics of the studied CKD-PD population with and without VC are shown in Table 1.

CKD-PD patients with moderate or severe VC were older and included more males than CKD-PD patients without VC. Concerning the comorbidities, no differences were found in terms of arterial hypertension (present in 95.5% of the studied population), obesity (11.4% of the studied population, with all obese patients presenting VC), or CVD (25.0% of the studied population). A significantly higher prevalence of patients with diabetes mellitus was observed in the group with VC in comparison to the group without VC (43.8% vs. 8.3%, $p = 0.035$).

Most PD technical parameters did not differ significantly between patients with and without VC, except total Kt/V (urea), which was lower in CKD-PD patients with VC (Table 1). In addition, this parameter was inversely correlated with VC severity (Spearman correlation, correlation coefficient = −0.437, $p < 0.01$).

The analysis of the mean values of Charlson Index showed that CKD-PD patients with VC presented a significant increase in all-cause mortality risk compared with CKD-PD patients without VC (5.6 ± 2.2 vs. 3.92 ± 3.0, $p < 0.05$). Accordingly, CKD-PD patients with VC included twice as many patients with severe Charlson Index than patients without VC (Table 1). When VC severity was correlated with all-cause mortality risk, we observed a significant positive correlation (spearman correlation, correlation coefficient (r) = 0.538, $p < 0.001$), meaning that patients with more severe VC present higher mortality risk. Moreover, by multivariable analysis, we found that vascular calcification correlates with the all-cause mortality risk, independently of sex and age.

Pharmacological therapies did not differ significantly between patients with or without VC regarding iron supplementation, erythropoietin, laxatives, hypouricemic agents, statins, calcimimetics, calcium-based phosphate binders, non-calcium-based phosphate binders, and vitamin D. However, the percentage of CKD-PD patients on vitamin D analogues and activators of vitamin D receptor (including alpha D, calcitriol, paricalcitol, and vitamin D receptor selective activators) was 100% in patients without VC whereas it was only ~72% in patients with VC, representing a statistically significant difference ($p < 0.05$). Further, two patients were on chronic anti-inflammatory drugs (prednisolone), both with severe VC (Adragao score of 8), and only three patients were not on anti-hypertensive drugs, all with VC.

Regarding biochemical parameters, only phosphorous plasma levels were significantly lower in CKD-PD patients with VC than patients without VC. Moreover, markers of inflammation (IL-1β, IL-6, TNF-α, and the anti-inflammatory IL-10), markers of intestinal translocation (endotoxins, LPS-binding protein, TLR4, and sCD14), and uremic toxins of microbial origin (T-MAO, PCS, 3-INDS, and 3-IAA) did not differ significantly between patients with or without VC. Regarding sCD14, although no statistically significant differences were found between CKD-PD patients with and without VC, a positive correlation was observed between sCD14 levels and VC severity (r = 0.338, $p < 0.05$). So, CKD-PD patients with more severe VC presented higher plasma values of sCD14.

Table 1. Demographic and clinical characterization of chronic kidney disease patients on peritoneal dialysis (CKD-PD) with and without vascular calcification (VC).

	CKD-PD (n = 44)	CKD-PD With no VC (n = 12)	CKD-PD with VC (n = 32)	p-Value
Demographic data				
Age, years	56.1 ± 10.9	47.7 ± 11.5	59.4 ± 8.8	**<0.001** [a]
Sex, % male	65.9%	33.3%	78.1%	**0.011** [d]
PD parameters				
PD duration, months	33.4 ± 30.0	36.3 ± 43.4	30.9 ± 23.8	0.668 [b]
PD type, %				>0.999 [d]
APD	52.3%	50.0%	53.1%	
CAPD	47.7%	50.0%	46.9%	
Ccreat, L/week	114.8 ± 56.8	105.7 ± 45.1	118.2 ± 60.8	0.668 [b]
Residual renal function, mL/min	5.6 ± 4.0	5.8 ± 3.8	5.6 ± 4.1	0.706 [b]
Kt/V (urea)	2.2 ± 0.5	2.6 ± 0.6	2.1 ± 0.4	**0.004** [b]
Charlson Index, %				**0.003** [c]
Low (≤2)	18.2%	50.0%	6.3%	
Moderate (3–4)	31.8%	25.0%	34.4%	
Severe (>5)	50.0%	25.0%	59.4%	
Biochemical parameters				
Urea, mg/dL	125.0 ± 37.0	127.6 ± 20.1	124.0 ± 41.8	0.780 [a]
Proteinuria mg/24 h	1.0 ± 1.2	0.9 ± 1.0	1.0 ± 1.2	0.342 [b]
Albumin, g/L	37.1 ± 3.3	37.0 ± 2.6	37.1 ± 3.6	0.944 [a]
Hemoglobin, g/dL	11.5 ± 1.4	11.0 ± 0.9	11.7 ± 1.6	0.133 [a]
Cholesterol, mg/dL	171.0 ± 56.8	169.9 ± 42.8	171.4 ± 61.8	0.825 [b]
LDL, mg/dL	95.7 ± 42.6	99.9 ± 33.7	94.0 ± 46.1	0.547 [b]
HDL, mg/dL	45.6 ± 10.7	47.4 ± 9.3	45.0 ± 11.3	0.267 [b]
Triglycerides, mg/dL	158.6 ± 68.4	129.8 ± 42.9	169.4 ± 73.5	0.169 [b]
P, mg/dL	5.0 ± 1.1	5.72 ± 1.05	4.73 ± 1.02	**0.011** [a]
Ca, mg/dL	9.02 ± 0.89	9.39 ± 0.85	8.84 ± 0.89	0.073 [a]
Ca • P product	43.83 ± 10.63	52.08 ± 9.32	40.67 ± 9.70	**0.002** [b]
Ferritin, ng/mL	361.3 ± 222.9	316.1 ± 221.3	378.3 ± 224.6	0.419 [a]
BNP, pg/mL	143.1 ± 119.2	87.0 ± 36.6	163.1 ± 131.9	0.124 [b]
PTH, pg/mL	462.5 ± 280.0	485.5 ± 366.4	453.9 ± 246.7	0.866 [b]
SV, mm	64.2 ± 25.6	67.2 ± 18.7	63.1 ± 27.9	0.644 [a]
CRP, mg/L	5.3 ± 8.5	4.8 ± 7.7	5.5 ± 8.9	0.907 [b]
TNF-α, pg/mL	11.4 ± 4.3	10.4 ± 2.8	11.7 ± 4.7	0.524 [b]
IL-1β, pg/mL	1.3 ± 0.93	1.3 ± 1.0	1.3 ± 0.9	0.969 [b]
IL-10, pg/mL	17.7 ± 14.7	17.5 ± 16.7	17.8 ± 14.2	0.825 [b]
IL-6, pg/mL	2.9 ± 6.3	5.4 ± 10.3	2.0 ± 3.8	0.687 [b]
Endotoxins, EU/mL	3.8 ± 0.8	3.8 ± 0.4	3.7 ± 0.8	0.978 [a]
LPS-BP, µg/mL	39.9 ± 17.1	32.2 ± 13.4	41.2 ± 18.3	0.442 [b]
TLR-4, pg/mL	624.4 ± 439.2	699.1 ± 464.5	596.4 ± 433.7	0.630 [b]
sCD14, µg/mL	5.0 ± 2.1	4.4 ± 2.0	5.3 ± 2.1	0.224 [b]
T-MAO	0.52 ± 0.62	0.47 ± 0.40	0.57 ± 0.70	0.854 [b]
PCS, mg/L	33.5 ± 19.1	36.4 ± 18.0	32.3 ± 19.7	0.341 [b]
3-INDS, mg/L	23.7 ± 14.6	24.1 ± 9.6	23.5 ± 16.22	0.442 [b]
3-IAA, mg/L	1.1 ± 1.2	1.0 ± 0.5	1.1 ± 1.4	0.169 [b]

Results are shown in absolute or relative frequencies (%) or mean ± standard deviation (SD). CKD, chronic kidney disease; PD, peritoneal dialysis; APD, Automated Peritoneal Dialysis; CAPD, continuous ambulatory peritoneal dialysis; Ccreat, creatinine clearance; residual renal function; Kt/V (urea); LDL, low-density lipoprotein; HDL, High-density lipoprotein; P, phosphorous; Ca, calcium; Ca·P product, calcium phosphate product, BNP, B-type natriuretic peptide; PTH, Parathyroid hormone; SV, sedimentation velocity, CRP, C reactive protein; TNF-α, Tumour necrosis factor-α; IL, Interleukin; LPS-BP, Lipopolysaccharide-binding protein; TLR-4, Toll-like receptor 4; sCD14, soluble CD14; TMAO, trimethylamine N-oxide; PCS, p-cresol sulphate; 3-INDS, 3-indoxyl sulfate; 3-IAA, indole-3-acetic acid. p values were calculated using the following statistical analysis: [a] Student's t-test, [b] Mann-Whitney U test, [c] Pearson Chi-square test, and [d] Fisher test.

The bacterial microbiome was evaluated in stool samples and whole blood samples. Stool samples displayed a median of 32,370 reads (range: 15,879–41,566). A median of 105 OTUs was observed per sample, with samples presenting between 39 and 216 OTUs. Blood samples displayed a median of 43,131 reads (range: 17,494–50,646). A median of 39 OTUs was observed per sample, with samples presenting between 25 and 56 OTUs. Alpha-diversity analysis was calculated by Shannon index; gut samples showed an average of 4.2 (values ranging from 3.03 to 4.89), while blood samples showed an average of 2.9 (values from 2.3 to 3.3). Similar values of diversity were observed in both groups of patients (with or without VC) separately regarding gut and blood samples. Beta-diversity assessment did not show differences in the gut and blood microbial communities when comparing PD patients with and without VC (Figure 1).

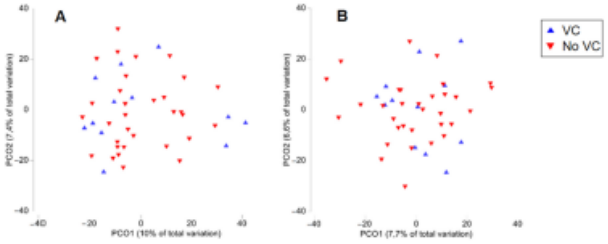


Figure 1. Principal coordinates analysis (PCO) of gut (**A**) and blood (**B**) microbiome in chronic kidney disease patients on peritoneal dialysis with vascular calcification (VC) or without vascular calcification (No VC).

ANOSIM and PERMANOVA confirmed the PCO observations, as the groups for both analyses were not significantly different ($p > 0.1$). Therefore, the taxonomic profiles of the gut and blood microbiome were similar at phylum and family taxonomic levels within each group of patients with or without VC (Figure 2).

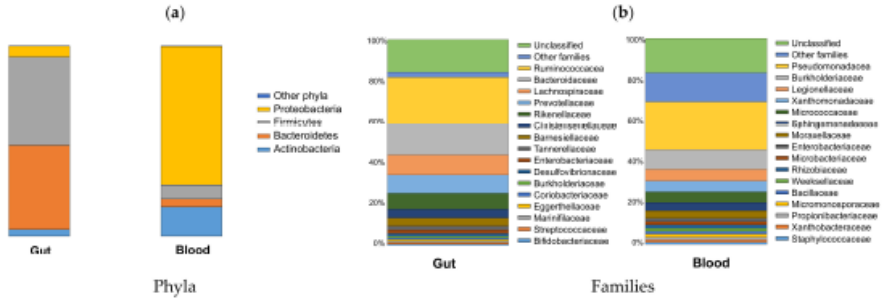


Figure 2. Relative abundance of bacteria phyla (**a**) and family (**b**) in the gut and blood microbiome in chronic kidney disease patients on peritoneal dialysis.

Gut microbiome was dominated by Firmicutes and Bacteroidetes at the phylum level, and by Ruminococcaceae, Bacteroidaceae, Lachnospiraceae, and Prevotellaceae at family level. The blood microbiome was dominated by Proteobacteria and Actinobacteria at the phylum level, and by Pseudomonadaceae, Burkholderiaceae, and Legionellaceae at family level. Nonetheless, relative changes of specific rare and/or less abundant taxa were

observed between CKD-PD patients with and without VC, namely *Coprobacter*, *Coprococcus 3*, *Lactobacillus*, and *Eubacterium eligens* group in gut microbiome, and *Cutibacterium*, *Pajaroellobacter*, *Devosia*, *Hyphomicrobium*, and *Pelomonas* in blood microbiome (Figure 3).

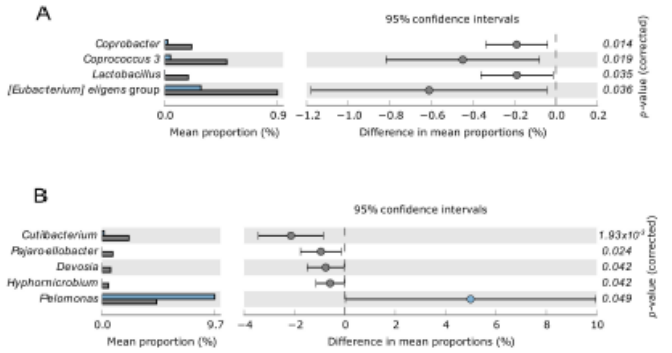


Figure 3. Relative changes of gut (**A**) and blood (**B**) bacterial taxa at the genus/family level in chronic kidney disease patients on peritoneal dialysis comparing patients with vascular calcification (grey bars) with patients without vascular calcification (blue bars).

Given the correlation between VC and all-cause mortality risk, we explored the gut and blood microbiome differences between CKD-PD patients with low and high mortality risk (Figure 4).

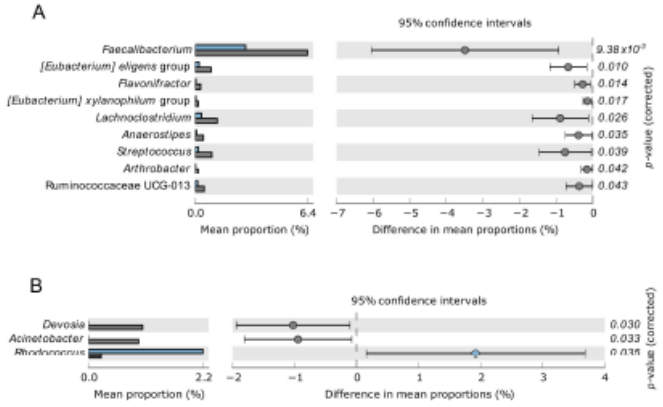


Figure 4. Relative changes of gut (**A**) or blood (**B**) bacterial taxa at the genus/family level in chronic kidney disease patients on peritoneal dialysis comparing patients with low all-cause mortality risk (Charlson Index scores of 2 or less, blue bars) with patients with moderate or severe all-cause mortality risk (Charlson Index scores of 3 or more, grey bars).

Among the taxonomic differences observed in CKD-PD patients with and without VC, patients with high mortality risk presented higher relative abundance in *E. eligens* group in

the gut microbiome and *Devosia* in the blood microbiome when compared to patients with low mortality risk.

Given that patients with VC included more male and older participants, we further investigate if sex and age would play a role in the relative changes of gut or blood microbiome (Figures S1 and S2). We found that male participants also have higher levels of *E. eligens* group in the gut in comparison to females. Although *Hyphomicrobium* was elevated in patients with VC in comparison to patients without VC, we found that *Hyphomicrobium* was present in adult participants but not in senior participants. Therefore, except for *E. eligens* group, the results suggest that the variation of the specific taxa in Figures 3 and 4 are mostly explained by vascular calcification in CKD-PD patients.

4. Discussion

Our results showed relative changes in specific taxa between CKD-PD patients with and without VC, namely *Coprobacter*, *Coprococcus 3*, *Lactobacillus*, and *E. eligens* group in the gut, and *Cutibacterium*, *Pajaroellobacter*, *Devosia*, *Hyphomicrobium*, and *Pelomonas* in the blood. An association between VC and all-cause mortality risk in CKD-PD patients was also observed, and patients with higher mortality risk corroborate the changes of *E. eligens* in the gut and *Devosia* genus in the blood. Although we did not find differences in uremic toxins, intestinal translocation markers, and inflammatory parameters among CKD-PD patients with and without VC, sCD14, a nonspecific marker of monocyte activation, was positively correlated with VC severity, suggesting its association with inflammation. Collectively, these results open new avenues for biomarkers discovery in CKD-PD patients.

The gut microbiome of our CKD-PD population was dominated by Firmicutes and Bacteroidetes at the phylum level, as described in healthy individuals, and by Ruminococcaceae, Bacteroidaceae, Lachnospiraceae, and Prevotellaceae at the family level, following other studies describing the gut microbiome of CKD-PD patients [32–34]. Despite only a few taxa differed between CKD-PD patients with and without VC, these taxa represent relevant groups among the gut microbiome, such as Coprobacter, *Coprococcus*, *Lactobacillus* or *Eubacterium*, which were more abundant in CKD-PD patients with VC. Some of these taxa are key players in the gut microbiome [35–38] and may be altered when the gut microbiome becomes dysbiotic, for example, in CKD patients [11]. Among the taxonomic differences observed in the gut microbiome for CKD-PD patients with or without VC, patients with higher mortality risk also demonstrated higher relative abundance in *E. eligens* group, highlighting a potential critical role of this taxon in CKD-PD patients. However, the microbiome differences associated to the sex may have contributed to this result, given that participants with VC include more males, and male participants also presented higher *E. eligens* group prevalence in comparison to females. The increase in the relative abundance of *E. eligens* group is most frequently associated with a healthy status [39–41]. For example, *E. eligens* were depleted in stool samples from atherosclerotic patients from Sweden and China cohorts and were appointed as promising probiotics and potential therapeutic targets for atherosclerosis [40]. However, the relative abundance of *E. eligens* group in the gut has also been found, occasionally, associated with disease [42]. Taking our results into account, the increase in *E. eligens* may not always constitute a protective factor as has been reported in previous studies.

Although still controversial, there is evidence supporting the existence of a healthy non-infectious human blood-microbiome [15,17,43]. In our CKD-PD patients, the blood microbiome was dominated by Proteobacteria and Actinobacteria at the phylum level and by Pseudomonadaceae, Burkholderiaceae, and Legionellaceae at the family level. Similarly, Shah et al. [20] observed that Pseudomonadaceae and Enterobacteriaceae families were significantly higher in the blood microbiome of non-dialysis CKD patients than in healthy controls. They demonstrated higher Proteobacteria and Actinobacteria predominance in the blood in contrast to Bacteroidetes and Firmicutes predominance in the gut. Proteobacteria is a major phylum of Gram-negative bacteria, which includes a wide variety of pathogens such as *Escherichia*, *Salmonella*, *Vibrio*, *Yersinia*, *Pseudomonas*, *Burkholderia*,

Legionella, and many other genera. Proteobacteria are higher both in the gut and blood in many chronic inflammatory diseases, including inflammatory bowel disease, metabolic syndrome, cardiovascular diseases, and chronic lung diseases. They have also been detected in atherosclerotic plaques and been related to the progression of CKD [17,20,44]. The correlation of all these diseases with gut dysbiosis, intestinal bacterial translocation, and endotoxaemia-related inflammation, as well as the clinical association between one and the other, suggests a common mechanism underlying these diseases associated with inflammation arising from the gut.

It is also relevant to note that families found in the blood microbiome of our CKD-PD patients include serious clinical pathogens, such as Pseudomonadaceae, Burkholderiaceae, and Legionellaceae. When evaluating the infection history of these patients, five presented previous *Pseudomonas aeruginosa* infections (between 4 months to 2 years before), with this pathogen being isolated from the catheter exit-site in four of these five patients, and in the respiratory tract in the remaining patient. However, it is important to highlight that the blood microbiome was evaluated through the detection of short sequences of bacterial genetic material, specifically the V3–V4 variable regions of the 16S rRNA gene. Therefore, these genetic sequences may result from circulating microbial DNA derived from phagocyted microbial cells of microorganisms translocated from the gut, the oral cavity, the PD catheter biofilm, or even from PD solutions [18,45,46]. Notwithstanding, the hypothesis that some of these DNA sequences may originate from living microbes should not be discarded, given the fact that viable bacteria have been found in blood from donors reported as medically healthy [47].

We observed in the blood microbiome of CKD-PD patients with VC an increase in *Cutibacterium*, *Pajaroellobacter*, *Devosia*, and *Hyphomicrobium*, and a decrease in relative abundance of *Pelomonas* when compared to CKD-PD patients without VC. Most of these groups appear sporadically in different areas of the human microbiome (skin, oral, gut) [48–51], but the real role of these genera remains unknown. An increase in the relative abundance of *Devosia* genus was found both in CKD-PD patients with VC when compared with CKD-PD patients without VC, as well as in CKD-PD patients with a higher mortality risk. To our knowledge, *Devosia* has not been reported previously in the blood microbiome but has been found to be increased in the gut microbiota of colorectal cancer patients [51] and in rabbits with heat stress [52], suggesting its possible translocation from the gut into the systemic circulation.

In our work, we also measured markers of intestinal translocation (endotoxins, LPS-binding protein, TLR4, and sCD14), inflammatory parameters (C-reactive protein, ferritin, sedimentation velocity, IL-1β, IL-6, TNF-α, and the anti-inflammatory IL-10), uremic toxins (PCS, 3-INDS, 3-IAA, and TMAO), and other routine laboratory parameters (such as urea, proteinuria, albumin, haemoglobin, cholesterol and its different fractions, triglycerides, calcium, parathormone, BNP), but no statistical significant differences were found between CKD-PD patients with or without VC. Although markers of intestinal translocation, uremic toxins, or inflammatory parameters are known to be increased in CKD patients [53–55], it should be noted that our study population included only end-stage kidney disease patients, and not healthy controls for comparison. The absence of differences between CKD-PD patients with or without VC could be associated with the relatively small number of patients included in this study.

Interestingly, we found that sCD14, a human monocyte differentiation antigen that acts as a pattern recognition receptor and is a TLR co-receptor for the detection of pathogen-associated molecular patterns such as lipopolysaccharides [56], was positively correlated with VC severity. In accordance, plasma sCD14 levels have been independently associated with myocardial infarction, coronary heart disease, and all-cause mortality among men and women above 65 years old in the Cardiovascular Health Study [57]. Longenecker et al. [22] observed that sCD14 was independently associated with coronary artery calcification measured by computed tomography and also predicted the extent of subclinical disease in other vascular beds in HIV patients. Poesen et al. [58] demonstrated that sCD14 was

elevated in patients with decreased kidney function and was associated with mortality and CVD in patients with CKD not yet on dialysis during a median follow-up of 52–54 months. Other studies positively related higher levels of sCD14 level to markers of inflammation and negatively to nutritional status and concluded sCD14 to be an independent predictor of all-cause mortality in long-term haemodialysis patients [59,60]. Together, these findings support a putative role of sCD14 in VC that should be explored in future studies in CKD-PD population.

When comparing CKD-PD patients with and without VC we observed higher estimated mortality risk in patients with VC, corroborating previous reports [61]. In our study, the CKD-PD patients with VC included more males, older patients, and a higher prevalence of diabetes in comparison with CKD-PD patients without VC. In fact, these three factors were previously recognised as major contributors to VC [62,63]. Moreover, we also observed lower Kt/V (urea) values in CKD-PD patients with VC when compared with patients without VC. In accordance, lower Kt/V values have been associated with VC and CVD in dialysis patients, including PD and haemodialysis patients [61,64].

When comparing phosphorous levels between CKD-PD patients with or without VC, we unexpectedly found higher phosphorous levels in patients without VC. We also found higher levels of calcium-phosphate product in patients without VC when compared with patients with VC but below the cut-off established for higher risk of VC and CVD in end-stage CKD patients [65]. According to KDIGO guidelines [66] and previously published articles [67,68], VC is marked by hyperphosphataemia and higher levels of calcium-phosphate product. Perhaps our results could be explained by some peculiarities in our study population. We performed a unique blood test and we did not collect samples in different time-points, so it is possible our CKD-PD patients with VC presented higher phosphorous levels in the past. Another argument is that PD patients on vitamin D analogues and activators of vitamin D receptor (including alpha D, calcitriol, paricalcitol, and vitamin D receptor selective activators) represented 100% of patients without VC, and only ~72% of patients with VC, denoting a significant difference ($p < 0.05$). The relationship between vitamin D and VC is complex. Moderate activation of vitamin D receptor (VDR) signaling protects against VC, but a deficient or excessive activation of VDR has been associated to VC [69]. As some studies proved that clinically relevant dosages of calcitriol and paricalcitol may protect against VC [70], others found no differences in the presence of VC in PD patients treated with calcitriol or calcium-based phosphate binders [71]. Vitamin D analogues and activators of vitamin D receptor promote an increase in phosphate levels through different mechanisms, so the higher intake of these drugs in the group without VC may collaborate on the higher levels of phosphorous in that group. Another argument to be looked at with caution is that, although not statistically significant, in our study we observed in the group with VC a higher calcium-based phosphate binders intake and lower non-calcium-based phosphate binders intake, resulting in better phosphorous control in that group. In clinical trials for the pharmacological management of phosphate imbalance, phosphate binders, especially non-calcium-based phosphate binders, were reported to low serum phosphorous levels by decreasing fibroblast growth factor-23 (FGF-23), which has been shown to stimulate phosphorous excretion and reduce VC [68] with protective effects on VC [72].

Lastly, it would be more accurate to evaluate VC using coronary computed tomography instead of Adragao score; however, the simplicity of the used method is of great advantage in clinical studies [24]. Adragao score measures VC and therefore may estimate CVD risk in CKD patients through hands and pelvic radiographies. Charlson Comorbidity Index predicts 10-year survival in patients with multiple comorbidities and has been useful in the prediction of mortality risk in CKD patients [73].

5. Conclusions

Vascular calcification is a highly frequent condition in CKD and a well-established risk factor for the development of CVD in CKD patients. Traditional factors fall short

in explaining the high prevalence of VC and CVD in kidney disease, suggesting the involvement of a CKD-specific pathological pathway that remains unknown. In recent years, gut dysbiosis has been shown to contribute to CVD, inflammation, and VC in CKD patients, but nothing was so far known regarding the role of gut microbiome in CKD-associated VC and CVD. Moreover, the information regarding blood microbiome and its putative relevance in health and disease is still very scarce.

Our results showed relative changes of specific taxa between CKD-PD patients with and without VC, namely regarding *Coprobacter*, *Coprococcus 3*, *Lactobacillus*, and *E. eligens* group in gut, and *Cutibacterium*, *Pajaroellobacter*, *Devosia*, *Hyphomicrobium*, and *Pelomonas* in the blood. Relative changes in the *E. eligens* group may also be associated with higher male prevalence in the group of participants with vascular calcification. An association between VC and all-cause mortality risk in CKD-PD patients was also observed, and patients with higher mortality risk corroborated the changes of *E. eligens* in the gut and *Devosia* genus in the blood. Although we did not find differences in uremic toxins, intestinal translocation markers, and inflammatory parameters among CKD-PD patients with and without VC, sCD14, a nonspecific marker of monocyte activation, was positively correlated with VC severity, suggesting an association with low grade inflammation. Figure 5 shows a schematic view of our results.

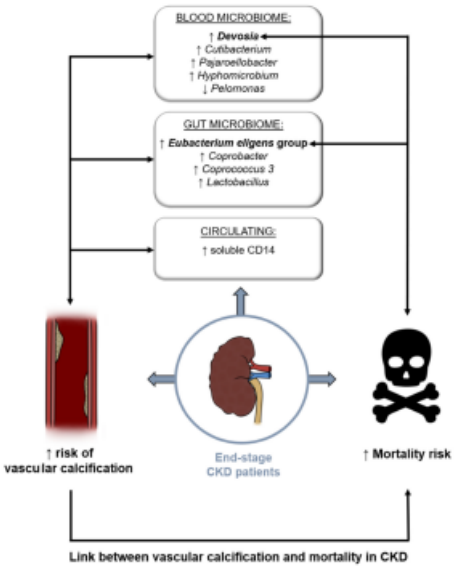


Figure 5. Our results suggest that specific taxa in the gut microbiome (*Coprobacter*, *Coprococcus 3*, *Lactobacillus*, and *Eubacterium eligens* group) and in the blood microbiome (*Cutibacterium*, *Pajaroellobacter*, *Devosia*, *Hyphomicrobium*, and *Pelomonas*) are different between CKD-PD patients with and without VC. sCD14 (a nonspecific marker of monocyte activation) correlated with vascular calcification (VC) severity in CKD-PD patients. An association between VC and all-cause mortality risk in CKD-PD patients was observed and patients with higher mortality risk corroborate the changes of *Eubacterium eligens* in the gut and *Devosia* genus in the blood.

In conclusion, our results suggest a role as biomarkers of gut *E. eligens* group, blood *Devosia*, and circulating sCD14 in CKD-VC, CVD, and mortality risk that should be further explored.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biom12070867/s1. Figure S1: Relative changes of gut (**A**) or blood (**B**) bacterial taxa at the genus/family level in chronic kidney disease patients on peritoneal dialysis comparing male (yellow bars) with female (blue bars) patients. Figure S2: Relative changes of gut (**A**) or blood (**B**) bacterial taxa at the genus/family level in chronic kidney disease patients on peritoneal dialysis comparing adulthood (until 65 years old, grey bars) with seniorhood (>65 years old, green bars) patients.

Author Contributions: Conceptualization, B.S.-M., I.B.D. and A.M.-R.; methodology, B.S.-M., A.M.-R., M.A.S. and I.A.; formal analysis, B.S.-M. and R.A.; investigation, A.M.-R., L.P., N.S., J.C., L.B., C.F.F.A.C., J.Q.-S., F.T. and I.F.-P.; resources, B.S.-M., L.P., M.A.S., I.F.-P. and I.A.; data curation, B.S.-M., A.M.-R. and R.A.; writing—original draft preparation, A.M.-R., R.A. and B.S.-M.; writing—review & editing, All authors; visualization, C.F.F.A.C.; supervision, B.S.-M. and I.B.D.; project administration, B.S.-M.; funding acquisition, B.S.-M. All authors have read and agreed to the published version of the manuscript.

Funding: This work is a result of the project POCI-01-0145-FEDER-029777 (PTDC/MEC-MCI/29777/2017), co-financed by Competitiveness and Internationalization Operational Program (POCI), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF) and through national funds by the FCT – Fundação para a Ciência e a Tecnologia. PT national funds (FCT/MCTES) also supported this work through grants UIDB/50006/2020. The Ph.D. fellowships of C.F.F.A.C. was supported by FCT/MCTES scholarships with the reference 2020.08540.BD. R.A. was supported by Individual Call to Scientific Employment Stimulus—Second Edition (grant number CEECIND/01070/2018). ISA was supported by FCT/MCTES contract DL 57/2016/CP1360/CT0007. F.T. is a recipient of a postdoctoral research grant by UnIC (UIDP/00051/2020).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Centro Hospitalar Universitário de São João (approval references 200/18).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data supporting reported results can be found at NCBI under BioProject: PRJNA819486.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Johansen, K.L.; Chertow, G.M.; Foley, R.N.; Gilbertson, D.T.; Herzog, C.A.; Ishani, A.; Israni, A.K.; Ku, E.; Kurella Tamura, M.; Li, S.; et al. US renal data system 2020 annual data report: Epidemiology of kidney disease in the United States. *Am. J. Kidney Dis.* **2021**, *77*, A7–A8. [CrossRef] [PubMed]
2. Foreman, K.J.; Marquez, N.; Dolgert, A.; Fukutaki, K.; Fullman, N.; McGaughey, M.; Pletcher, M.A.; Smith, A.E.; Tang, K.; Yuan, C.W.; et al. Forecasting life expectancy, years of life lost, and all-cause and cause-specific mortality for 250 causes of death: Reference and alternative scenarios for 2016-40 for 195 countries and territories. *Lancet* **2018**, *392*, 2052–2090. [CrossRef]
3. Sharma, S.; Samak, M.J. Epidemiology: The global burden of reduced GFR: ESRD, CVD and mortality. *Nat. Rev. Nephrol.* **2017**, *13*, 447–448. [CrossRef] [PubMed]
4. Kendrick, J.; Chonchol, M.B. Nontraditional risk factors for cardiovascular disease in patients with chronic kidney disease. *Nat. Clin. Pract. Nephrol.* **2008**, *4*, 672–681. [CrossRef] [PubMed]
5. Mihai, S.; Codrici, E.; Popescu, I.D.; Enciu, A.M.; Albulescu, L.; Necula, L.G.; Mambet, C.; Anton, G.; Tanase, C. Inflammation-related mechanisms in chronic kidney disease prediction, progression, and outcome. *J. Immunol. Res.* **2018**, *2018*, 2180373. [CrossRef]
6. Amdur, R.L.; Feldman, H.I.; Dominic, E.A.; Anderson, A.H.; Beddhu, S.; Rahman, M.; Wolf, M.; Reilly, M.; Ojo, A.; Townsend, R.R.; et al. Use of Measures of inflammation and kidney function for prediction of atherosclerotic vascular disease events and death in patients with CKD: Findings from the CRIC study. *Am. J. Kidney Dis.* **2019**, *73*, 344–353. [CrossRef]
7. Canyelles, M.; Tondo, M.; Cedo, L.; Farras, M.; Escola-Gil, J.C.; Blanco-Vaca, F. Trimethylamine N-oxide: A link among diet, gut microbiota, gene regulation of liver and intestine cholesterol homeostasis and HDL function. *Int. J. Mol. Sci.* **2018**, *19*, 3228. [CrossRef]

8. Evenepoel, P.; Dejongh, S.; Verbeke, K.; Meijers, B. The role of gut dysbiosis in the bone-vascular axis in chronic kidney disease. *Toxins* **2020**, *12*, 285. [CrossRef]
9. Jovanovich, A.; Isakova, T.; Stubbs, J. Microbiome and cardiovascular disease in CKD. *Clin. J. Am. Soc. Nephrol.* **2018**, *13*, 1598–1604. [CrossRef]
10. Koeth, R.A.; Wang, Z.; Levison, B.S.; Buffa, J.A.; Org, E.; Sheehy, B.T.; Britt, E.B.; Fu, X.; Wu, Y.; Li, L.; et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat. Med.* **2013**, *19*, 576–585. [CrossRef]
11. Sampaio-Maia, B.; Simoes-Silva, L.; Pestana, M.; Araujo, R.; Soares-Silva, I.J. The role of the gut microbiome on chronic kidney disease. *Adv. Appl. Microbiol.* **2016**, *96*, 65–94. [CrossRef]
12. Shimizu, H.; Hirose, Y.; Nishijima, F.; Tsubakihara, Y.; Miyazaki, H. ROS and PDGF-beta [corrected] receptors are critically involved in indoxyl sulfate actions that promote vascular smooth muscle cell proliferation and migration. *Am. J. Physiol. Cell Physiol.* **2009**, *297*, C389–C396. [CrossRef]
13. Meijers, B.; Evenepoel, P.; Anders, H.J. Intestinal microbiome and fitness in kidney disease. *Nat. Rev. Nephrol.* **2019**, *15*, 531–545. [CrossRef]
14. Lau, W.L.; Kalantar-Zadeh, K.; Vaziri, N.D. The gut as a source of inflammation in chronic kidney disease. *Nephron* **2015**, *130*, 92–98. [CrossRef] [PubMed]
15. Castillo, D.J.; Rifkin, R.F.; Cowan, D.A.; Potgieter, M. The healthy human blood microbiome: Fact or fiction? *Front. Cell Infect. Microbiol.* **2019**, *9*, 148. [CrossRef]
16. D'Aquila, P.; Giacconi, R.; Malavolta, M.; Piacenza, F.; Burkle, A.; Villanueva, M.M.; Dolle, M.E.T.; Jansen, E.; Grune, T.; Gonos, E.S.; et al. Microbiome in blood samples from the general population recruited in the MARK-AGE project: A pilot study. *Front. Microbiol.* **2021**, *12*, 707515. [CrossRef] [PubMed]
17. Amar, J.; Lange, C.; Payros, G.; Garret, C.; Chabo, C.; Lantieri, O.; Courtney, M.; Marre, M.; Charles, M.A.; Balkau, B.; et al. Blood microbiota dysbiosis is associated with the onset of cardiovascular events in a large general population: The D.E.S.I.R. study. *PLoS ONE* **2013**, *8*, e54461. [CrossRef] [PubMed]
18. Potgieter, M.; Bester, J.; Kell, D.B.; Pretorius, E. The dormant blood microbiome in chronic, inflammatory diseases. *FEMS Microbiol. Rev.* **2015**, *39*, 567–591. [CrossRef]
19. Schierwagen, R.; Alvarez-Silva, C.; Madsen, M.S.A.; Kolbe, C.C.; Meyer, C.; Thomas, D.; Uschner, F.E.; Magdaleno, F.; Jansen, C.; Pohlmann, A.; et al. Circulating microbiome in blood of different circulatory compartments. *Gut* **2019**, *68*, 578–580. [CrossRef]
20. Shah, N.B.; Allegretti, A.S.; Nigwekar, S.U.; Kalim, S.; Zhao, S.; Lelouvier, B.; Servant, F.; Serena, G.; Thadhani, R.I.; Raj, D.S.; et al. Blood microbiome profile in CKD: A pilot study. *Clin. J. Am. Soc. Nephrol.* **2019**, *14*, 692–701. [CrossRef]
21. Evenepoel, P.; Opdebeeck, B.; David, K.; D'Haese, P.C. Bone-vascular axis in chronic kidney disease. *Adv. Chronic Kidney Dis.* **2019**, *26*, 472–483. [CrossRef] [PubMed]
22. Longenecker, C.T.; Jiang, Y.; Orringer, C.E.; Gilkeson, R.C.; Debanne, S.; Funderburg, N.T.; Lederman, M.M.; Storer, N.; Labbato, D.E.; McComsey, G.A. Soluble CD14 is independently associated with coronary calcification and extent of subclinical vascular disease in treated HIV infection. *AIDS* **2014**, *28*, 969–977. [CrossRef]
23. Rodrigues, F.G.; Ormanji, M.S.; Heilberg, I.P.; Bakker, S.J.L.; de Borst, M.H. Interplay between gut microbiota, bone health and vascular calcification in chronic kidney disease. *Eur. J. Clin. Invest.* **2021**, *51*, e13588. [CrossRef] [PubMed]
24. Adragao, T.; Pires, A.; Lucas, C.; Birne, R.; Magalhaes, L.; Goncalves, M.; Negrao, A.P. A simple vascular calcification score predicts cardiovascular risk in haemodialysis patients. *Nephrol. Dial. Transpl.* **2004**, *19*, 1480–1488. [CrossRef] [PubMed]
25. Charlson, M. Charlson Comorbidity Index (CCI). Available online: https://www.mdcalc.com/charlson-comorbidity-index-cci (accessed on 7 August 2020).
26. Charlson, M.E.; Pompei, P.; Ales, K.L.; MacKenzie, C.R. A new method of classifying prognostic comorbidity in longitudinal studies: Development and validation. *J. Chronic. Dis.* **1987**, *40*, 373–383. [CrossRef]
27. Simoes-Silva, L.; Araujo, R.; Pestana, M.; Soares-Silva, I.; Sampaio-Maia, B. Peritoneal microbiome in end-stage renal disease patients and the impact of peritoneal dialysis therapy. *Microorganisms* **2020**, *8*, 173. [CrossRef] [PubMed]
28. Escudie, F.; Auer, L.; Bernard, M.; Mariadassou, M.; Cauquil, L.; Vidal, K.; Maman, S.; Hernandez-Raquet, G.; Combes, S.; Pascal, G. FROGS: Find, rapidly, OTUs with galaxy solution. *Bioinformatics* **2018**, *34*, 1287–1294. [CrossRef]
29. Calaf, R.; Cerini, C.; Genovesio, C.; Verhaeghe, P.; Jourde-Chiche, N.; Berge-Lefranc, D.; Gondouin, B.; Dou, L.; Morange, S.; Argiles, A.; et al. Determination of uremic solutes in biological fluids of chronic kidney disease patients by HPLC assay. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2011**, *879*, 2281–2286. [CrossRef] [PubMed]
30. Araujo, R.; Dunlap, C.; Franco, C.M.M. Analogous wheat root rhizosphere microbial successions in field and greenhouse trials in the presence of biocontrol agents Paenibacillus peoriae SP9 and Streptomyces fulvissimus FU14. *Mol. Plant Pathol.* **2020**, *21*, 622–635. [CrossRef]
31. Parks, D.H.; Beiko, R.G. Identifying biologically relevant differences between metagenomic communities. *Bioinformatics* **2010**, *26*, 715–721. [CrossRef]
32. Jiang, N.; Zhang, C.; Feng, H.; Yuan, J.; Ding, L.; Fang, W.; Gu, A.; Huang, J.; Li, N.; Gu, L.; et al. Clinical characteristics associated with the properties of gut microbiota in peritoneal dialysis patients. *Perit. Dial. Int.* **2021**, *41*, 298–306. [CrossRef] [PubMed]
33. Lun, H.; Yang, W.; Zhao, S.; Jiang, M.; Xu, M.; Liu, F.; Wang, Y. Altered gut microbiota and microbial biomarkers associated with chronic kidney disease. *Microbiologyopen* **2019**, *8*, e00678. [CrossRef] [PubMed]

34. Luo, D.; Zhao, W.; Lin, Z.; Wu, J.; Lin, H.; Li, Y.; Song, J.; Zhang, J.; Peng, H. The effects of hemodialysis and peritoneal dialysis on the gut microbiota of end-stage renal disease patients, and the relationship between gut microbiota and patient prognoses. *Front. Cell Infect. Microbiol.* **2021**, *11*, 579386. [CrossRef]
35. Azcarate-Peril, M.A.; Roach, J.; Marsh, A.; Chey, W.D.; Sandborn, W.J.; Ritter, A.J.; Savaiano, D.A.; Klaenhammer, T.R. A double-blind, 377-subject randomized study identifies Ruminococcus, Coprococcus, Christensenella, and Collinsella as long-term potential key players in the modulation of the gut microbiome of lactose intolerant individuals by galacto-oligosaccharides. *Gut Microbes* **2021**, *13*, 1957536. [CrossRef] [PubMed]
36. Hughes, R.L.; Kable, M.E.; Marco, M.; Keim, N.L. The role of the gut microbiome in predicting response to diet and the development of precision nutrition models. Part II: Results. *Adv. Nutr.* **2019**, *10*, 979–998. [CrossRef]
37. Jones, R.B.; Alderete, T.L.; Kim, J.S.; Millstein, J.; Gilliland, F.D.; Goran, M.I. High intake of dietary fructose in overweight/obese teenagers associated with depletion of Eubacterium and Streptococcus in gut microbiome. *Gut Microbes* **2019**, *10*, 712–719. [CrossRef]
38. Mahowald, M.A.; Rey, F.E.; Seedorf, H.; Turnbaugh, P.J.; Fulton, R.S.; Wollam, A.; Shah, N.; Wang, C.; Magrini, V.; Wilson, R.K.; et al. Characterizing a model human gut microbiota composed of members of its two dominant bacterial phyla. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 5859–5864. [CrossRef]
39. Li, Y.; Wang, D.D.; Satija, A.; Ivey, K.L.; Li, J.; Wilkinson, J.E.; Li, R.; Baden, M.; Chan, A.T.; Huttenhower, C.; et al. Plant-based diet index and metabolic risk in men: Exploring the role of the gut microbiome. *J. Nutr.* **2021**, *151*, 2780–2789. [CrossRef]
40. Liu, S.; Zhao, W.; Liu, X.; Cheng, L. Metagenomic analysis of the gut microbiome in atherosclerosis patients identify cross-cohort microbial signatures and potential therapeutic target. *FASEB J.* **2020**, *34*, 14166–14181. [CrossRef]
41. Nie, X.; Chen, J.; Ma, X.; Ni, Y.; Shen, Y.; Yu, H.; Panagiotou, G.; Bao, Y. A metagenome-wide association study of gut microbiome and visceral fat accumulation. *Comput. Struct. Biotechnol. J.* **2020**, *18*, 2596–2609. [CrossRef]
42. Kossenas, K.; Constantinou, C. Epidemiology, molecular mechanisms, and clinical trials: An update on research on the association between red meat consumption and colorectal cancer. *Curr. Nutr. Rep.* **2021**, *10*, 435–467. [CrossRef]
43. Paisse, S.; Valle, C.; Servant, F.; Courtney, M.; Burcelin, R.; Amar, J.; Lelouvier, B. Comprehensive description of blood microbiome from healthy donors assessed by 16S targeted metagenomic sequencing. *Transfusion* **2016**, *56*, 1138–1147. [CrossRef] [PubMed]
44. Rizzatti, G.; Lopetuso, L.R.; Gibiino, G.; Binda, C.; Gasbarrini, A. Proteobacteria: A common factor in human diseases. *Biomed. Res. Int.* **2017**, *2017*, 9351507. [CrossRef] [PubMed]
45. Bossola, M.; Sanguinetti, M.; Scribano, D.; Zuppi, C.; Giungi, S.; Luciani, G.; Torelli, R.; Posteraro, B.; Fadda, G.; Tazza, L. Circulating bacterial-derived DNA fragments and markers of inflammation in chronic hemodialysis patients. *Clin. J. Am. Soc. Nephrol.* **2009**, *4*, 379–385. [CrossRef]
46. Mair, R.D.; Sirich, T.L. Blood microbiome in CKD: Should we care? *Clin. J. Am. Soc. Nephrol.* **2019**, *14*, 648–649. [CrossRef] [PubMed]
47. Damgaard, C.; Magnussen, K.; Enevold, C.; Nilsson, M.; Tolker-Nielsen, T.; Holmstrup, P.; Nielsen, C.H. Viable bacteria associated with red blood cells and plasma in freshly drawn blood donations. *PLoS ONE* **2015**, *10*, e0120826. [CrossRef]
48. Bay, L.; Barnes, C.J.; Fritz, B.G.; Thorsen, J.; Restrup, M.E.M.; Rasmussen, L.; Sorensen, J.K.; Hesselvig, A.B.; Odgaard, A.; Hansen, A.J.; et al. Universal dermal microbiome in human skin. *mBio* **2020**, *11*, e02945-19. [CrossRef]
49. De, R.; Mukhopadhyay, A.K.; Dutta, S. Metagenomic analysis of gut microbiome and resistome of diarrheal fecal samples from Kolkata, India, reveals the core and variable microbiota including signatures of microbial dark matter. *Gut Pathog.* **2020**, *12*, 32. [CrossRef]
50. Rudney, J.D.; Xie, H.; Rhodus, N.L.; Ondrey, F.G.; Griffin, T.J. A metaproteomic analysis of the human salivary microbiota by three-dimensional peptide fractionation and tandem mass spectrometry. *Mol. Oral Microbiol.* **2010**, *25*, 38–49. [CrossRef]
51. Zhang, H.; Chang, Y.; Zheng, Q.; Zhang, R.; Hu, C.; Jia, W. Altered intestinal microbiota associated with colorectal cancer. *Front. Med.* **2019**, *13*, 461–470. [CrossRef]
52. Bai, X.; Shi, Y.; Tang, L.; Chen, L.; Fan, H.; Wang, H.; Wang, J.; Jia, X.; Chen, S.; Lai, S. Heat stress affects faecal microbial and metabolic alterations of rabbits. *Front. Microbiol.* **2021**, *12*, 817615. [CrossRef] [PubMed]
53. Balla, J.; Balla, G.; Zarjou, A. Ferritin in kidney and vascular related diseases: Novel roles for an old player. *Pharmaceuticals* **2019**, *12*, 96. [CrossRef] [PubMed]
54. Choi, S.R.; Lee, Y.K.; Cho, A.J.; Park, H.C.; Han, C.H.; Choi, M.J.; Koo, J.R.; Yoon, J.W.; Noh, J.W. Malnutrition, inflammation, progression of vascular calcification and survival: Inter-relationships in hemodialysis patients. *PLoS ONE* **2019**, *14*, e0216415. [CrossRef] [PubMed]
55. Henze, L.A.; Luong, T.T.D.; Boehme, B.; Masyout, J.; Schneider, M.P.; Brachs, S.; Lang, F.; Pieske, B.; Pasch, A.; Eckardt, K.U.; et al. Impact of C-reactive protein on osteo-/chondrogenic transdifferentiation and calcification of vascular smooth muscle cells. *Aging* **2019**, *11*, 5445–5462. [CrossRef]
56. Bas, S.; Gauthier, B.R.; Spenato, U.; Stingelin, S.; Gabay, C. CD14 is an acute-phase protein. *J. Immunol.* **2004**, *172*, 4470–4479. [CrossRef]
57. Reiner, A.P.; Lange, E.M.; Jenny, N.S.; Chaves, P.H.; Ellis, J.; Li, J.; Walston, J.; Lange, L.A.; Cushman, M.; Tracy, R.P. Soluble CD14: Genomewide association analysis and relationship to cardiovascular risk and mortality in older adults. *Arter. Thromb. Vasc. Biol.* **2013**, *33*, 158–164. [CrossRef]

58. Poesen, R.; Ramezani, A.; Claes, K.; Augustijns, P.; Kuypers, D.; Barrows, I.R.; Muralidharan, J.; Evenepoel, P.; Meijers, B.; Raj, D.S. Associations of soluble CD14 and endotoxin with mortality, cardiovascular disease, and progression of kidney disease among patients with CKD. *Clin. J. Am. Soc. Nephrol.* **2015**, *10*, 1525–1533. [CrossRef]
59. Raj, D.S.; Shah, V.O.; Rambod, M.; Kovesdy, C.P.; Kalantar-Zadeh, K. Association of soluble endotoxin receptor CD14 and mortality among patients undergoing hemodialysis. *Am. J. Kidney Dis.* **2009**, *54*, 1062–1071. [CrossRef] [PubMed]
60. Seabra, V.F.; Thomas, G.; Jaber, B.L. Soluble CD14 and endotoxin levels in hemodialysis patients: A tale of 2 molecules. *Am. J. Kidney Dis.* **2009**, *54*, 990–992. [CrossRef]
61. Niu, Q.; Zhao, H.; Wu, B.; Tsai, S.; Wu, J.; Zhang, M.; Lu, L.; Qiao, J.; Men, C.; Zuo, L.; et al. Study on the prevalence of vascular calcification in different types of arteries and influencing factors in maintenance peritoneal dialysis patients. *Blood Purif.* **2019**, *47*, 8–16. [CrossRef]
62. Durham, A.L.; Speer, M.Y.; Scatena, M.; Giachelli, C.M.; Shanahan, C.M. Role of smooth muscle cells in vascular calcification: Implications in atherosclerosis and arterial stiffness. *Cardiovasc. Res.* **2018**, *114*, 590–600. [CrossRef] [PubMed]
63. Visseren, F.L.J.; Mach, F.; Smulders, Y.M.; Carballo, D.; Koskinas, K.C.; Back, M.; Benetos, A.; Biffi, A.; Boavida, J.M.; Capodanno, D.; et al. 2021 ESC Guidelines on cardiovascular disease prevention in clinical practice. *Eur. Heart J.* **2021**, *42*, 3227–3337. [CrossRef] [PubMed]
64. Dimkovic, N.; Schlieper, G.; Jankovic, A.; Djuric, Z.; Ketteler, M.; Damjanovic, T.; Djuric, P.; Marinkovic, J.; Radojcic, Z.; Markovic, N.; et al. Prognostic value of cardiovascular calcifications in hemodialysis patients: A longitudinal study. *Int. Urol. Nephrol.* **2018**, *50*, 939–946. [CrossRef] [PubMed]
65. Floege, J.; Ketteler, M. Vascular calcification in patients with end-stage renal disease. *Nephrol. Dial. Transpl.* **2004**, *19*, V59–V66. [CrossRef] [PubMed]
66. Kidney Disease: Improving Global Outcomes (KDIGO) Transplant Work Group. KDIGO clinical practice guideline for the care of kidney transplant recipients. *Am. J. Transpl.* **2009**, *9*, S1–S155. [CrossRef]
67. Cozzolino, M.; Ciceri, P.; Galassi, A.; Mangano, M.; Carugo, S.; Capelli, I.; Cianciolo, G. The Key Role of Phosphate on Vascular Calcification. *Toxins* **2019**, *11*, 213. [CrossRef]
68. Singh, A.; Tandon, S.; Tandon, C. An update on vascular calcification and potential therapeutics. *Mol. Biol. Rep.* **2021**, *48*, 887–896. [CrossRef]
69. Wang, J.; Zhou, J.J.; Robertson, G.R.; Lee, V.W. Vitamin D in vascular calcification: A double-edged sword? *Nutrients* **2018**, *10*, 652. [CrossRef]
70. Mathew, S.; Lund, R.J.; Chaudhary, L.R.; Geurs, T.; Hruska, K.A. Vitamin D receptor activators can protect against vascular calcification. *J. Am. Soc. Nephrol.* **2008**, *19*, 1509–1519. [CrossRef]
71. Ramírez-Sandoval, J.C.; Casanova, L.; Villar, A.; Gomez, F.E.; Cruz, C.; Correa-Rotter, R. Biomarkers associated with vascular calcification in peritoneal dialysis. *Perit. Dial. Int.* **2016**, *36*, 262–268. [CrossRef]
72. Ghorbanihaghjo, A.; Argani, H.; Golmohamadi, Z.; Rashtchizadeh, N.; Abbasi, M.M.; Bargahi, N.; Vatankhah, A.M.; Sanajou, D. Linkage of fibroblast growth factor 23 and phosphate in serum: Phosphate and fibroblast growth factor 23 reduction by increasing dose of sevelamer. *J. Bone Metab.* **2018**, *25*, 153–159. [CrossRef] [PubMed]
73. Xia, X.; Qiu, Y.; Yu, J.; Lin, T.; Lu, M.; Yi, C.; Lin, J.; Ye, H.; Chen, W.; Mao, H.; et al. Ten-year survival of patients treated with peritoneal dialysis: A prospective observational cohort study. *Perit. Dial. Int.* **2020**, *40*, 573–580. [CrossRef] [PubMed]

10.2. Book chapter

Merino-Ribas, A, Costa, C.C.F, Azevedo, M.J, Alencastre, I, Pestana, M, Araujo, R, Sampaio-Maia B. The gut microbiome in chronic kidney disease. Gut microbiome in chronic kidney disease. Publicat al llibre: *Human-Gut Microbiome: Establishment and Interactions* (View series: Developments in Microbiology), Gunjan Goel, Teresa Requena, Saurabh Bansal, Ed.; Elsevier: 2022. ISBN: 9780323913133.

10.3. Communications in congresses or posters

Merino-Ribas A, Bancu I, Graterol F, Vergara A, Noguera-Julian M, Paredes R, Bonal J.
"Gut microbiome in hemodialysis patients treated with different types of phosphate binders"
Comunicació oral, percentil 75.58/100
56th ERA-EDTA Congress
Budapest (Hungria), 13-16/06/2019

Costa, C, Campos C, Merino A, Silva N, Mesquita R, Rangel A, Sampaio-Maia B.
"Urease-positive Bacteria Assessment in Chronic Kidney Disease"
Comunicació pòster
CED-IADR/NOF Oral Health Research Congress
Madrid (Espanya), 19-21/09/2019

Merino-Ribas A, Bancu I, Graterol F, Vergara A, Noguera-Julian M, Paredes R, Bonal J.
"Microbiota intestinal en paciente en hemodiálisis tratados con diferentes tipos de quelantes del fósforo"
Comunicació oral tipus e-poster
XLIX Congreso Nacional de la Sociedad Española de Nefrología y X Congreso Iberoamericano de Nefrología
A Coruña (Espanya), 5-8/10/2019.

Costa C, Campos C, Merino-Ribas A, Silva N, Mesquita R, Rangel A, Sampaio-Maia B.
"Raoultella ornithinolytica: an opportunistic pathogen in the oral cavity of chronic kidney disease patients"
Comunicació pòster
Microbiotec 19, Congrés Nacional de la Societat Portuguesa de Microbiologia
Coimbra (Portugal), 5-7/12/2019

Sampaio-Maia, B, Sampaio S, Araujo R, Merino-Ribas A, Lelouvier B, Servant F, Quelhas dos Santos J, Barreiros L, Segundo M, Pestana M.
"Blood, Gut, and Oral Microbiome in Kidney Transplant Recipients"
Comunicació pòster
World Microbe 2021, Congrés organizat per les Societat americana de Microbiologia i la Federació de les Societats Europees de Microbiologia.
Online, 20-24/06/2021

Costa, C, Merino-Ribas A, Campos C, Silva N, Pereira L, Mesquita R, Rangel A, Sampaio-Maia B.
"Oral Enterobacteriaceae in Chronic Kidney Disease Patients"
Comunicació pòster
World Microbe 2021, Congrés organizat per les Societat americana de Microbiologia i la Federació de les Societats Europees de Microbiologia.
Online, 20-24/06/2021

Sampaio S, Araujo R, Merino-Ribas A, Quehlhas-Santos J, Barreiros L, Segundo M, Pestana M, Sampaio-Maia B.
"Uremic toxin levels are associated with Gut Microbiome changes in Kidney Transplant Recipients"
Comunicació poster
20th Congress of the European Society for Organ Transplantation (ESOT2021) Milà (Itàlia), 29/08-01/09/2021

Sampaio-Maia, B, Sampaio S, Merino-Ribas A, Campos J, Pestana M, Araujo R.
"Oral Microbiome and Recovery of Renal Function After Kidney Transplantation"
Comunicació pòster
CED-IADR/NOF Oral Health Research Congress
On-line, 16-18/09/2021

Sampaio S, Araujo R, Merino-Ribas A, Quehlhas-Santos J, Barreiros L, Segundo M, Pestana M, Sampaio-Maia B.
"Níveis circulantes de p-sulfato de cresol e sulfato de indoxilo associam-se a alterações do Microbioma Intestinal em Recetores de Transplante Renal" Comunicació oral
XV Congresso Português de Transplantação
Online, 24-26/03/2021

Merino-Ribas A, Araujo R, Bancu I, Pereira L, Campos J, Barreiros L, Segundo MA, Silva N, Costa CFFA, Quelhas-Santos J, Trindade F, Falcão-Pires I, Alencastre I, Sampaio-Maia B.
“Vascular Calcification and the Gut and Blood Microbiome in Chronic Kidney Disease Patients on Peritoneal Dialysis”
Comunicació mini-oral, percentil 59.24/100
59th ERA-EDTA Congress
Paris (França), 19-22/05/2022

Merino-Ribas A, Araujo R, Bancu I, Pereira L, Campos J, Barreiros L, Segundo MA, Silva N, Costa CFFA, Quelhas-Santos J, Trindade F, Falcão-Pires I, Alencastre I, Sampaio-Maia B.
“Calcificación vascular y microbiota intestinal y en sangre en diálisis peritoneal”
Comunicació oral
52 Congreso Nacional de la Sociedad Española de Nefrología
Granada (Espanya), 12-14/11/2022