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ADVANCES IN THE ROLE
OF THE MICROBIOME IN
PERITONEAL DIALYSIS

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LILIANA CARINA SIMÕES DA SILVA
TESE DE DOUTORAMENTO APRESENTADA
À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO EM
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SILVA

**ADVANCES IN THE ROLE OF THE MICROBIOME
IN PERITONEAL DIALYSIS**

Liliana Carina Simões da Silva

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“Tenho em mim todos os sonhos do mundo.”

Fernando Pessoa

À minha família e amigos

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RESUMO

As infeções são uma das principais complicações da técnica de diálise peritoneal (DP), estando associadas à morbilidade e mortalidade dos doentes renais crónicos (DRC) em tratamento de DP. Apesar de os doentes em DP adotarem rigorosos procedimentos de higiene, um baixo mas persistente número de peritonites é ainda registado anualmente. A contaminação no momento da troca de dialisante é considerada como uma das principais causas de peritonite, no entanto, a existência de outras vias de infeção é hoje uma hipótese emergente. Presentemente, os fatores responsáveis pela ocorrência de infeções associadas à técnica de DP, nomeadamente as peritonites e as infeções do orifício de saída do cateter peritoneal, não são totalmente compreendidos. Com a recente descoberta do papel do microbioma humano, a comunidade científica debruçou-se sobre o seu estudo como uma possível fonte de agentes infecciosos, especialmente em populações com imunidade reduzida como é o caso de doentes com DRC. A existência de estudos que descrevem a existência de ADN bacteriano no efluente peritoneal e em células do peritoneu, bem como estudos que revelam que a bactéria *Staphylococcus aureus* mantém a viabilidade intracelular após internalização em células mesoteliais, vem reforçar esta perspetiva. Estes factos sugerem ainda que o peritoneu poderá ter o seu próprio microbioma. Por outro lado, a cavidade oral tem sido apontada como um ponto de partida para a disseminação de microrganismos patogénicos. A proximidade dos microrganismos orais da corrente sanguínea, torna a bacteremia com origem na cavidade oral um evento comum, embora transitório, que pode resultar da rotina das atividades diárias ou de procedimentos dentários invasivos. Apesar de ter sido reportada a existência de uma associação entre as doenças orais e uma sobrevivência reduzida de doentes com DRC, as evidências científicas que associam esta via de infeção ao desenvolvimento de peritonite são escassas.

Neste trabalho, procuramos estudar o papel do microbioma humano na população em DP e a sua possível contribuição para o desenvolvimento de infeções associadas à DP. No capítulo I, apresentamos uma revisão bibliográfica onde descrevemos o papel do microbioma intestinal na DRC. A disbiose deste microbioma é um factor de risco não tradicional para o risco cardiovascular e progressão da DRC. No capítulo II, descrevemos o microbioma de doentes com DRC submetidos a diferentes técnicas de diálise, nomeadamente a hemodiálise (HD) e a DP. A terapia de substituição renal parece influenciar não só o microbioma intestinal, melhor caracterizado, mas também microbiomas de outros locais.

Nos capítulos seguintes, apresentamos estudos em que a microbiota de doentes em DP foi caracterizada, incidindo em diferentes tipos de microrganismos. No capítulo III avaliamos a colonização da cavidade oral pelos principais agentes responsáveis por infeções associadas à DP, as bactérias do género *Staphylococcus* sp.. No Capítulo IV, apresentamos os resultados de estudos sobre a colonização pela levedura *Candida* sp., uma vez que as infeções fúngicas, apesar de serem menos frequentes, são de mais difícil resolução. O último grupo de microrganismos avaliado, os protozoários, encontra-se descrito no Capítulo V. As metodologias usadas na identificação microbiana diferiram nos diferentes microrganismos analisados: crescimento em meio de cultura Mannitol-Salt agar e sequenciação do gene *DnaJ* dos isolados de *Staphylococcus*; crescimento em meio de cultura CHROMagar-Candida™ e sequenciação do gene 18S rRNA/ITS para identificação das leveduras *Candida* sp.; na identificação de protozoários foi realizada uma avaliação microscópica de esfregaços diretos corados por Lugol e por técnicas de coloração específicas (Giemsa, Trichrome e Kinyoun). No último capítulo, apresentamos os resultados de estudos em que o microbioma do peritoneu foi caracterizado em doentes com DRC, por sequenciação de nova geração e em que foi avaliado o efeito da DP na composição do microbioma peritoneal.

Os resultados apresentados sugerem que a colonização por *Staphylococcus* da cavidade oral pode atuar como uma fonte de agentes infecciosos em doentes em DP. Identificámos a existência de espécies comuns à microbiota oral dos doentes em DP e aos agentes patogénicos de infeções ocorridas nestes mesmos doentes. Por outro lado, os doentes em DP apresentaram menor diversidade de *Staphylococcus* spp. na cavidade oral. Neste trabalho foram ainda testados diferentes métodos para a identificação de isolados de *Staphylococcus* spp.: API 32 STAPH, sequenciação do gene *DnaJ* e MALDI-TOF (VITEK MS). Pudemos concluir que os métodos moleculares devem ser utilizados sempre que possível, pois apesar de a técnica de MALDI-TOF ser usada por rotina na prática clínica, não atinge os níveis de precisão necessários para uma correta identificação microbiana.

Relativamente à colonização oral por *Candida* sp., verificamos que esta representa um risco limitado para o desenvolvimento de infeções fúngicas em doentes em DP. Ao comparar doentes em DP e HD, observamos que a HD tem um impacto mais adverso na saúde oral, em particular no desenvolvimento da doença periodontal. Verificamos também que a colonização por leveduras do género *Candida* é maior em doentes em HD. O impacto da HD na saúde oral parece ser reduzido quando os doentes transitam da HD para a DP.

Nos estudos em que foi avaliada a colonização por protozoários na população de DP, os nossos resultados demonstraram que 10,2% dos efluentes de doentes em DP apresentavam colonização por protozoários, sem no entanto esses doentes apresentarem sinais clínicos ou sintomas de peritonite no momento da colheita. Este facto sugere que o efluente peritoneal pode ser suscetível de uma colonização assintomática por protozoários, sendo necessário um estudo mais aprofundado do possível impacto clínico desta colonização. No que se refere à colonização oral por protozoários, esta foi muito baixa tanto nos doentes em DP como nos controlos, sugerindo que a colonização oral por protozoários da população portuguesa é baixa e não se altera na presença de DRC.

A última parte deste trabalho foi dedicada à caracterização do microbioma do peritoneu de doentes com DRC. Os nossos resultados revelaram que o peritoneu destes doentes apresenta um microbioma específico, dominado por Proteobactérias, Firmicutes, Actinobactérias e Bacteroidetes. A DP parece ter um efeito na composição do microbioma do peritoneu, diminuindo a sua riqueza em termos de diversidade microbiana, verificando-se o predomínio das famílias Pseudomonadaceae e Prevotellaceae.

Na sua globalidade, este trabalho contribuiu para uma melhor caracterização do microbioma dos doentes em DP e poderá ajudar a uma melhor compreensão do papel do microbioma nas infeções associadas à DP.

ABSTRACT

One of the major complications of the peritoneal dialysis (PD) technique is the infection-related morbidity and mortality. Despite the standard hygiene procedures adopted by PD patients, there is still a persistent low number of peritonitis every year. Although the contamination at the time of the PD exchange is assumed to be a major cause of peritonitis the factors influencing the occurrence of PD-related infections, namely peritonitis and catheter exit-site infections, are still far from being fully understood. The recent discovery of tissue specific microbiomes is changing researcher's perception on possible sources of infection, especially in populations with impaired immunity like end-stage renal disease (ESRD) patients. Corroborating this hypothesis are the previous reports describing bacterial DNA in PD effluent or peritoneum cells as well as studies revealing intracellular viability of *Staphylococcus aureus* after sequestration by mesothelial cells. These facts suggest that the peritoneum may harbor its own microbiome. On the other hand, the oral cavity has been pointed out as a starting point for dissemination of pathogenic organisms. The proximity of oral microorganisms to the blood stream makes bacteremia from the oral cavity a common, but transient event that may result from routine daily activities or from invasive dental procedures. Despite the possible association between oral diseases and an adverse survival of renal diseases patients, the scientific evidence linking this route of infection to peritonitis is still scarce.

In this work, we aimed to understand the role of the human microbiome in the PD population and its possible contribution for the development of PD-related infections. In chapter I, we present a bibliographical review on the role of the gut microbiome on Chronic Kidney Disease (CKD). Gut dysbiosis is being unraveled in CKD and pointed as a non-traditional risk factor for cardiovascular risk and CKD progression. In chapter II, we focused on the microbiome of ESRD patients undergoing different renal replacement therapies, namely HD and PD. These appear to influence not only the most studied microbiome, the gut microbiome, but also microbiomes in other body habitats. In the following chapters, we characterized the microbiota of PD patients, focusing on different types of microorganisms. In chapter III we evaluated the colonization of the oral cavity by the most prevalent PD-infectious agent, *Staphylococcus* sp. In Chapter IV the same approach was carried out for the yeast *Candida* sp.. Despite being less frequent, *Candida* infections represent a major problem once its resolution is more difficult. The last group of microorganisms evaluated, the protozoa is presented in Chapter V. The microbial identification was microorganism-dependent: growth in Mannitol-Salt agar and sequencing of *DnaJ* gene for

Staphylococcus species; growth in CHROMagar-Candida™ and sequencing of 18S rRNA/ITS for *Candida* species; and microscopic evaluation by direct mounts with Lugol's direct smear and specific staining techniques (Giemsa, Trichrome and Kinyoun) for protozoa identification. In the last chapter, we studied the peritoneum of ESRD patients to evaluate its microbiome by next-generation sequencing, as well as the influence of peritoneal catheter insertion in the peritoneum microbiome composition.

The results of our study suggests that the oral cavity could act as a potential reservoir of *Staphylococci* infectious agents in PD patients, as in this study we identified the existence of common agents between the oral microbiota and PD-related pathogens. Additionally, PD patients presented less diversity of *Staphylococcus* species in the oral cavity. During this work, we tested different methods for *Staphylococcus* identification, API 32 STAPH, *DnaJ* sequencing and MALDI-TOF (VITEK MS). We suggest that molecular methods should be used when possible, since even though MALDI-TOF mass spectrometry is widely used in the clinical setting, we demonstrate that it did not achieve the established levels of accuracy that are necessary for clinical practice.

On the other hand, oral yeast colonization represents a limited risk for the development of fungal infections in PD patients. When comparing PD and hemodialysis (HD) patients, we observed that HD has a more adverse impact on oral health, in particular periodontal disease. Also, *Candida* colonization is also higher in HD patients. The impact on oral health of HD appears to be reduced or ameliorated when patients change from HD to PD therapy.

In another study, we also searched for protozoa colonization in the PD population. Our data showed that 10.2% of effluent of PD patients had protozoa, however none of these patients presented clinical signs or symptoms of peritonitis at the time of the screening. This demonstrated that PD effluent may be susceptible to asymptomatic protozoa colonization, and the clinical impact of this finding should be further investigated. Concerning oral protozoa colonization, it was very low in both PD patients and controls, suggesting that the oral protozoa colonization of Portuguese population is low and not significantly modified by the presence of ESRD.

The last part of this work was dedicated to the characterization of the peritoneum microbiome of ESRD patients. Our data demonstrates that the peritoneum of these patients presents a unique low-abundance microbiome dominated by Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes. The PD therapy appears to have an impact in this microbiome, decreasing species richness with a dominance by Pseudomonadaceae and Prevotellaceae families.

Together this work increases the existing knowledge on the microbiome of PD patients. We expect that in the future it will contribute for the better understanding of the role of the microbiome in PD-related infections.

ABBREVIATIONS

APD – Automated peritoneal dialysis

AVF – native arteriovenous fistula

CAPD – Continuous ambulatory peritoneal dialysis

CCPD – Continuous cyclic peritoneal dialysis

CKD - Chronic kidney disease

CVC – ventral venous catheter

CVD - Cardiovascular disease

ESRD - End-stage renal disease

GFR - Glomerular filtration rate

HD – Haemodialysis

HOMD - Human Oral Microbiome Database

IPD – Intermittent peritoneal dialysis

ISPD – International Society of Peritoneal Dialysis

K/DOQI - Kidney Disease Outcomes Quality Initiative

NIPD – Nocturnal intermittent peritoneal dialysis

NKF - National Kidney Foundation

PD - Peritoneal dialysis

RRT - Renal replacement therapy

TPD – Tidal peritoneal dialysis

INTRODUCTION AND GOALS

Introduction

The human microbiome, defined by the NIH human microbiome project (<http://hmpdacc.org/>) as the collection of all the microorganisms living in association with the human body, includes eukaryotes, archaea, bacteria and viruses. In the last years, after the discovery that the human body harbours 10 times more microbial cells than human cells (Alberts, B *et al.* 2002), the study of the human microbiome and the existing host-microbe interactions became a priority. Diverse studies have characterized the microbial communities existing in the different body locations and its association with health and disease conditions. However, much is yet to be uncovered with regards to mapping extant microbiome biodiversity.

The recent discovery of microbiomes in body sites previously considered sterile, together with the need to further understand infection occurrence in chronic kidney disease (CKD) patients undergoing Peritoneal Dialysis (PD), triggered the need to explore the existence of a peritoneal microbiome and understand its role in PD-related infections. Additionally, the role played by other microbiomes, specially by the oral microbiome whose relation with CKD was previously suggested, was also assessed. In this work, we focused in different microbial groups, namely bacteria, in particular *Staphylococcus*, fungi and protozoa.

Chronic kidney disease

The definition of chronic kidney disease (CKD) has undergone readjustments over time. It is usually defined by a progressive loss of renal function and is a general term for heterogeneous disorders affecting the structure and function of the kidney. According to the Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines from National Kidney Foundation (NKF) CKD is defined by the decrease of kidney function detected by decreased GFR (less than 60 mL/min per 1.73 m²), by increased rates of urinary albumin excretion or by abnormal kidney structure detected by imaging occurring for at least three months, independently of the cause (National Kidney, Foundation 2002, Webster, A. C. *et al.* 2016). Chronic Kidney Disease is classified in five different stages according to the glomerular filtration rate (GFR): more than 90 mL/min per 1.73 m² (stage 1), 60-89 mL/min per 1.73 m² (stage 2), 30–59 mL/min per 1.73 m² (stage 3), 15–29 mL/min per 1.73 m² (stage 4), and less than 15 mL/min per 1.73 m² (stage 5), being this last the most severe form of CKD.

As a consequence of renal failure, the kidney is unable to excrete toxic substances, which accumulate in the plasma. The uremic syndrome is manifested by elevated plasma urea and creatinine levels and

is associated to symptoms as fatigue, nausea, loss of appetite, vomiting, pruritis, and neurological changes (Chikotas, N. *et al.* 2006). The progression of renal failure is evaluated by the combination of GFR, clinical history and physical signs and symptoms.

When patients progress to stage 5 CKD, called end stage-renal disease (ESRD), renal replacement therapy (RRT) may be required in the form of hemodialysis (HD), peritoneal dialysis (PD) or renal transplant (RTx).

Due to increasing numbers of patients, CKD is currently considered a public health problem. The incidence and prevalence of this disease is affected by ethnicity, social health determinants, and possibly by epigenetic factors (Webster, A. C. *et al.* 2016). In 2016, Hill and colleagues concluded that CKD has a high prevalence world-wide, being the global CKD prevalence between 11 to 13%, approximately 18% in Europe (Hill, N. R. *et al.* 2016). In Portugal, it is estimated that 800,000 individuals have CKD; more than 2,000 new cases of renal failure occur every year. In the end of 2015, 18,928 patients were submitted to a RRT: 11,514 were on HD, 751 on PD and 6,663 received a RTx. A report from the Portuguese Nephrology Society (2016) reveals that, in comparison to the majority of European countries, Portugal has a higher incidence and prevalence of ESRD patients, however the factors responsible for these increases are not well clarified. Overall, the incidence and prevalence of ESRD is variable, but the majority of patients being treated reside in countries with an aging population with access to health care (Webster, A. C. *et al.* 2016).

There are several causes of CKD, however, the most common causes worldwide are diabetes and hypertension (Levey, A. S. *et al.* 2012). On the other hand, cardiovascular (CV) disease is the major cause of death in CKD patients. The CV risk increases exponentially during CKD progression, and CV-related mortality rate may be up to 500-fold higher in CKD patients than the general population (Sarnak, M. J. *et al.* 2003). This is the reason why CKD patients are five to ten times more likely to die prematurely than progress to ESRD (Webster, A. C. *et al.* 2016). Also, ESRD patients present a high burden of infectious complications, having an annual mortality rate secondary to sepsis 100 to 300-fold higher than in the general population (Li, P. K. *et al.* 2011). Interestingly, septicemia was recognized as a risk factor for the development of CV events and death in HD or PD patients (Foley, R. N. *et al.* 2004).

In comparison to the general population, CKD patients present not only premature mortality but also a considerably lower quality of life (De Nicola, L. *et al.* 2016). Also, with the progression of CKD to ESRD there is a significant increase in social and financial costs (De Nicola, L. *et al.* 2016).

Renal replacement therapy

The life expectancy of patients with ESRD has improved since the introduction of dialysis as a RRT in the 1960s, although it is still below that of the general population (Mailloux, L.U. *et al.* 2016). As previously mentioned the therapeutic modalities of CKD in stage 5 are kidney transplantation, dialysis in the form of chronic HD or chronic PD.

The clinical relevant outcomes of kidney transplant and dialysis were assessed in a systematic review, where it was concluded that kidney receptors presented reduced cardiovascular events and mortality and had a superior life quality, in comparison to dialysis patients (Tonelli, M. *et al.* 2011). Although the prevalence of treated ESRD patients with a functioning kidney transplant in Portugal is increasing progressively, it is still far below the national needs (Sociedade Portuguesa de Nefrologia 2016). The transplanted kidney can be from a cadaver or a living donor that usually is a familiar member. Since kidney transplantation cannot be performed in all cases, due to the lack of suitable donors, most patients with ESRD are submitted to dialysis during their lifetime. The global prevalence of people who have a functioning kidney transplant is 65 per million, compared with 280 per million people of ESRD patients on dialysis (Webster, A. C. *et al.* 2016). Dialysis can occur in the form of HD or one of the two forms of PD, continuous ambulatory PD (CAPD) or automated PD (APD) (Teixeira, J. P. *et al.* 2015). There are many factors that influence the choice of dialysis type; they range from the patient's motivation/education, underlying medical problems, age, distance to HD unit, the physician/nurse's influence to cost-effectiveness. PD presents some advantages over HD, as it is associated with higher survival rate at the start of dialysis, together with higher patient satisfaction and lower costs (Sinnakirouchenan, R. *et al.* 2011). A population-based cohort study from Sweden reported that patients on HD incurred the highest healthcare cost, 45 times more than general population and 50% higher than patients on PD (Eriksson, J. K. *et al.* 2016). However, these PD advantages do not directly translate into increased use of PD, since it is possible to verify that the option for dialysis modality varies significantly across countries (Li, P. K. *et al.* 2016). In China, USA and Thailand, PD is increasing, whereas in Japan and most European countries it is decreasing.

According to the data provided by the Portuguese Society of Nephrology for the year 2015, in Portugal the incidence of patients starting RRT decreased 4.9%, contradicting the tendency of the previous two years; the incidence of patients initiating RRT with HD decreased significantly (5.6%), whereas the prevalence of patients on HD slightly increased (1.4%) (Sociedade Portuguesa de Nefrologia 2016). Regarding PD, there was a slight increase in both incidence (1.06%) and prevalence (2.6%). PD was the first treatment option of 9.8% of patients and corresponds to 6.1% of prevalent patients on dialysis.

Regarding transplantation, an increase in renal transplantation from both cadaver and living donors was observed.

Haemodialysis

Technically, HD consists in the process of purifying the blood of ESRD patients, through the extracorporeal removal of waste products (for example creatinine, urea and potassium) and free water, with a dialysis machine that pumps the patient's blood and the dialysate through the dialyzer; for this process an extensive water purification system is absolutely critical. For these reasons, HD is usually performed in a dialysis outpatient facility, either a hospital or a dedicated clinic, three or more times a week. Rarely HD is done at home.

The HD procedure require a well-functioning vascular access, which can be of three types: native arteriovenous fistula (AVF), arteriovenous graft, and central venous catheter (CVC). AVF is the most used access since is the best regarding longevity, morbidity and mortality association (Santoro, D. *et al.* 2014). Arteriovenous grafts are the second-best option followed by CVCs. CVCs can be inserted in different body sites, preferably in the internal jugular and femoral veins, since the subclavian vein is associated to a high risk of thrombosis. Infections are the cause of 30% to 60% of CVC's removal and, in comparison to patients with AVF, patients with CVC present higher hospitalization rates.

As previously described, fistulas present great advantages, such as best longevity, morbidity and mortality association. These are recommended by international guidelines instead of CVCs, however they require a long time of maturation, being this the reason for increased catheter-dependence for vascular access (Recommendations, K/DOQI Clinical Practice Guidelines and Clinical Practice 2006). In our country, tunnel cuffed catheters (TCC) are the permanent vascular access in more than 20% of prevalent HD patients and is used for the initial dialysis session for 50% of incident HD patients (<http://www.spnephro.pt/>). Catheter design improved in these last years, but in comparison to AVF the TCC still presents more risk of sepsis, hospitalization and even mortality (Zelenitsky, S. A. *et al.* 2016). Accordingly, it has been estimated that the relative risk of bacteremia is 10 times higher in HD patients with TCC than with AVF (Power, A. *et al.* 2009). In HD patients with TCC, catheter-related bacteremia can have two origins: the skin (migration from the skin along the outside of the catheter into the bloodstream) and/or catheter biofilm (direct inoculation from a biofilm containing pathogenic microorganisms on the inner surface of the catheter) (Allon, M. 2004). A study performed in the Hospital S. João Nephrology Department, showed that, in comparison to both HD patients with AVF and PD

patients, incident HD patients with TCC experienced a significantly higher mortality rate at one year of dialysis, being infection the most common cause of death (Coentrão, L. *et al.* 2012). Also 43% of all deaths in these patients were related to dialysis access complications, which were all caused by infections.

Peritoneal dialysis

PD consists in the removal from the body of soluble substances and water by transfer across a self-dialysis membrane, the peritoneum. This is a serous membrane, composed by a layer of mesothelium supported by a thin layer of connective tissue that supports many of the abdominal organs and serves as a conduit for their blood vessels, lymphatic vessels, and nerves. The space that exists between the peritoneum that surrounds the abdominal wall and the peritoneum that surrounds the internal organs is the peritoneal cavity. In this RRT, a dialysis solution (dialysate) is introduced into the peritoneal cavity through a long-term catheter. Basically, solute diffusion and ultrafiltration occurs between blood in the peritoneal capillaries and dialysate, across the peritoneum. The dialysate is drained from the peritoneal cavity and is exchanged several times during the day (Gülcan, E. *et al.* 2014). The ultrafiltration depends not only of fluid removal but also of solute clearance, essential to PD success (Teitelbaum, I. 2015).

Over the years, the PD technique has undergone several improvements in the different elements of the system (PVC bag, dialysate, transfer set, and catheter access to the peritoneal cavity); for example, the connection technology (Y-shaped connector), and the addition of a “flush before fill” system between bags exchange (Mehrotra, R. *et al.* 2009, Castledine, C. I. *et al.* 2011). These have contributed to the decrease in the risk of PD-related infections, however different results are still obtained among centers which may be related with unequal application of the available knowledge and experience. These technical innovations now used in PD allow the maintenance of patients on PD for long periods, with a survival rate equivalent to HD (Mehrotra, R. *et al.* 2016).

One of the advantages of this technique is that it is a home-based dialysis technique in which the patients are trained to perform the treatment themselves, both in case of continuous ambulatory peritoneal dialysis (CAPD) and automated peritoneal dialysis (APD) (Mehrotra, R. *et al.* 2009). CAPD implies 3 to 5 exchanges during the day and one during the night. APD implies the use of cycler machines performing dialysis exchanges and includes the following methods: continuous cyclic peritoneal dialysis (CCPD), intermittent peritoneal dialysis (IPD), nocturnal intermittent peritoneal

dialysis (NIPD), and tidal peritoneal dialysis (TPD). In some cases, the patients need the help of an assistant, a nurse or a trained family member, to perform the PD exchanges or to operate de PD cyclers, in order to ensure the continuation of PD (assisted PD). This happens especially in physically dependent patients.

One of the main advantages of APD is that it gives patients more freedom, since dialysis exchanges can be performed during the night, allowing patients or patients' helpers to work during the day (Castledine, C. I. *et al.* 2011).

As already described PD treatment has many advantages over HD, however it is still underused. This may be related with diverse factors such as the development of recurrent peritonitis, failure to attain efficient dialysis, technical troubles with PD catheter, low literacy level of patients, and familial and occupational reasons (Gülcan, E. *et al.* 2014). A nationwide survey in Japan concluded that to prolong the use of PD by CKD patients, it is necessary to develop solutions that better preserve peritoneal function, to educate PD patients regarding salt and fluid restriction, and also to improve the early diagnosis and treatment of peritonitis (Kawaguchi, Y. *et al.* 2003).

According to the data provided by the Portuguese Society of Nephrology, in Portugal the number of incident and prevalent PD patients increased in 2015 and APD was less commonly used than CAPD. The first cause of PD abandonment is peritoneal access infection, followed by inadequate ultrafiltration or dialysis failure. Regarding the death causes of PD patients in 2015, cardiovascular diseases were the leading cause corresponding to approximately 40% of deaths in this population. Also, there was a decrease in infections related to peritoneal access (2.6%) (Sociedade Portuguesa de Nefrologia 2016).

Although the incidence of peritonitis is decreasing, this complication persists as the Achilles' heel of PD. Prevention of infection thus is a critical step to the success of every PD-program once PD-related infections are responsible for catheter loss, transfer to HD, prolonged hospitalization and in more serious cases death. These infections are not only a cause of PD failure but also an important contributor to morbidity and mortality in this population.

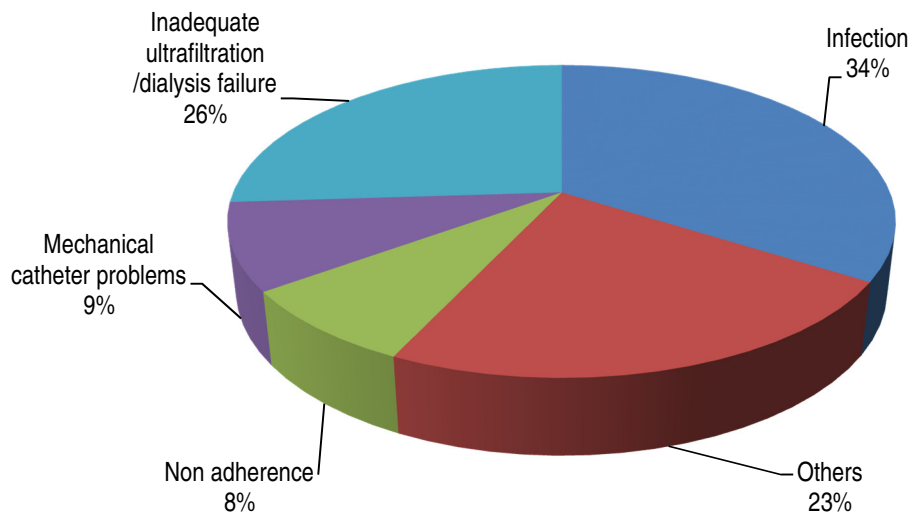


Figure 1: Reasons for discontinuation of PD in 2015 (N = 187). Adapted from SPN Registration Office, Fernando Macário 2015.

PD-related infections

Peritonitis and exit-site infections (ESI) are the more relevant and more common PD-related infections. Recently Kim and colleagues (2017) reported that peritonitis and exit-site/tunnel infections were responsible for 45.7% of technique failure, and that lower education of PD patients is associated to the development of infections and higher risk of PD failure.

Despite the importance of PD-related infections, the factors responsible for development of these infections are still not fully understood. It is assumed that contamination at the time of PD fluid exchange remains a major cause of peritonitis, occurring mainly by an external route (Barracough, K. A. *et al.* 2009). Thus, high standard hygiene procedures during the PD exchange are promoted in PD patients. According to the data provided by the Portuguese Society of Nephrology the peritonitis rate was in 2015 of 0.37 episodes/patient and in the same year 34% interrupted the technique due to infection (Figure 1). The International Society for Peritoneal Dialysis (ISPD) guidelines/recommendations state that the peritonitis rate should be at most 0.5 episodes per year at risk (Li, P. K. *et al.* 2016). This rate corresponds to the number of infections by organism for a time period, divided by dialysis years' time at risk, and expressed as episodes per year. Ideally each PD patient shouldn't have more than one infection episode every two years on PD.

For an improved resolution of PD-related infections it is very important to conduct a thorough microbiological study of these infections. Some observational studies have described a decrease of PD-related infections rates in recent years, especially in the case of Gram-positive but also in Gram-

negative infections (Rocha, A. *et al.* 2012, Cho, Y. *et al.* 2014, Ozisik, L. *et al.* 2015). This reduction has been associated to diverse factors as implementation of twin bag disconnection system, mupirocin administration in *Staphylococcus aureus* carriers, chemoprophylaxis protocol, topical application of gentamicin in catheter exit site and improved training of patients, among others.

In a study conducted in Turkey over a six-year period with 440 PD patients, Ozisik and colleagues (2015) reported that coagulase-negative staphylococci were the most frequent microorganisms identified as infectious agents. Additionally, the peritonitis episodes were caused in the majority by gram-positive, followed by gram-negative and in a minority of the cases by fungi (Ozisik, L. *et al.* 2015). In another study, Zelenitsky and colleagues (2016) reported the microbiological etiology and antimicrobial susceptibilities regarding PD-related peritonitis during a 9 year period (2005-2014). They observed that, in monomicrobial peritonitis 71% are caused by gram-positive bacteria, including coagulase-negative staphylococci, *Streptococcus* spp. and *Staphylococcus aureus*; whereas in polymicrobial infections a mixed gram-positive/gram-negative bacteria and multiple gram-positive bacteria was observed, being coagulase-negative staphylococci and *Streptococcus* spp. the most prevalent. Also in the relapse cases, coagulase-negative staphylococci and *S. aureus* were the most common infectious agents. Coagulase-negative staphylococci reported as PD-related infectious agents included *Staphylococcus haemolyticus*, *Staphylococcus warneri*, *Staphylococcus hominis*, *Staphylococcus simulans*, *Staphylococcus capitis*, and *Staphylococcus saprophyticus* (Wilcox, M. H. *et al.* 1991, Shin, J. H. *et al.* 2011). An infectious agent that is frequently associated with a poor response to antibiotics as well as with a high rate of catheter removal is *Pseudomonas* species. In a study by Lu and colleagues (2018) 8.3% of peritonitis episodes were caused by this specie.

In accordance with the data available in recent years in the PD outpatient clinic of the Nephrology Department of the S. João Hospital Center, the most common peritonitis agents were *Streptococcus* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Escherichia coli* and *Klebsiella pneumonia* (unpublished data). Also, catheter exit-site infections are frequent in these patients, and the most common agents responsible for this type of infections were *Staphylococcus* spp., *Pseudomonas* spp. and *Corynebacterium* spp. (unpublished data).

Regarding fungal infections, these are usually less frequent, however with high clinical importance since its resolution is more difficult. In the majority of fungal peritonitis episodes, the patients need to replace or even remove the catheter, and consequently they are transferred to HD. *Candida* spp. is the principal infectious fungal agent (Rex, J. H. *et al.* 2000). In 2010, Restrepo and co-workers described for the first time that the administration of prophylactic fluconazole to patients receiving antibiotics

(bacterial peritonitis) successfully reduced the appearance of secondary fungal peritonitis and other complications (Restrepo, C. *et al.* 2010).

In the study of Zelenitsky, only 5% of culture-positive peritonitis were from fungal origin (Zelenitsky, S. A. *et al.* 2016). There are several *Candida* species that colonize specific body locations, as the skin, oropharynx, gastrointestinal tract and vagina, and that in the presence of a microbiota disruption may be responsible for opportunistic infections (Kauffman, C.A. 2017). Fungal infections are usually associated with immunosuppression, malnutrition and previous antibiotherapy. Additionally in PD patients, fungal infections are associated with previous bacterial infections (Matuszkiewicz-Rowinska, J. 2009).

Although fungal peritonitis have already been the subject of several studies, and international guidelines reflect the results obtained in the procedures adopted for its diagnosis and treatment, fungal ESI are less explored. In a study conducted in the 90's the majority of the etiology of ESI episodes was *S. aureus*, followed by CNS, *S. epidermidis* and the less frequent fungi (Luzar, M. A. *et al.* 1990). In another study, the authors compared the use of a prophylactic antiseptic against mupirocin, and described a greater rate of infection in the first group (Findlay, A. *et al.* 2013). They also described the etiology of ESI episodes in both groups, being gram-negative bacteria the only identified agent in the mupirocin group contrasting with the antiseptic group that presented ESI episodes caused by gram-negative, namely *Pseudomonas*, followed by gram-positive mostly by *S. aureus* (Findlay, A. *et al.* 2013). In a recent study of the PD unit from São João Hospital, including 26 patients that developed ESI, 5.6% were from fungal origin. In these ESI from fungal origin, 13% had been exposed to immunosuppressive therapy in the previous 6 months, 75% had been exposed to antibiotherapy in the previous 3 months (the majority for PD-related infections) and almost half received a prophylactic treatment with fluconazol (Coelho, S. *et al.* 2016). The most frequent *Candida* species identified as ESI infectious agents were: *Candida parapsilosis* (67%), *Candida glabrata* (10%), *Candida zeylanoides* (6.6%), *Candida famata* (6.6%) and *Candida tropicalis* (3.3%). Antifungal therapy was efficient in 80% of the patients, and the remaining patients were subjected to catheter replacement or removal (Coelho, S. *et al.* 2016).

Another important aspect regarding the management of PD-related infections is the existence of culture negative peritonitis. Zelenitsky and colleagues (2016) described a rate of culture-negative peritonitis of 0.11 episodes per patient-year and in another study more than 20% of PD-related peritonitis were reported as culture-negative (Li, P. K. *et al.* 2010). These cases frequently result from the presence of uncultivable microorganisms, low pathogen number or previous antibiotic administration. These facts

outline the need for improvement in diagnosis, since in these situations, treatment with a specific antibiotic is not possible and an empirical treatment with broad spectrum antibiotics has to be performed. This lack of a targeted and specific treatment contributes to the development of antibiotic resistance, drug toxicity and to microbiota imbalance (Pereira, B. *et al.* 2005). Notwithstanding, these problems can also occur in culture-positive peritonitis, since traditional microbiology results take several days. Misidentifications of microorganisms in PD-related infections can also pose a problem, namely when the diagnosis of recurrent peritonitis and the possibility of catheter replacement are at stake. Thus, a fast and accurate diagnosis of microbial infections in PD is a fundamental step to improve patient management and to implement infection control procedures. Until recently, microbial identification relied on the characterization of phenotypic markers; however, these techniques are hardly applied to slow growing or uncultivable microorganisms. With the development of molecular biology, new strategies should be adopted (Petinaki, E. *et al.* 2006). Molecular assays can either be designed specifically for one organism or be broad-range assays. Several groups have studied the usefulness of such techniques, such as 16SrRNA sequencing, DGGE, PCR-RFLP and PFGE for bacterial diagnosis in clinical infections (Ratanarat, R. *et al.* 2007, Woo, P. C. *et al.* 2008, Cho, C. H. *et al.* 2009, Reier-Nilsen, T. *et al.* 2009), but their application to PD-related infections is still necessary.

The human microbiome

Microorganisms play an important role in the human body. It is now known that some microorganisms play a role in digestion, appetite regulation, and in modulation of the immune system (Alarcon, P. *et al.* 2016, Andoh, A. 2016, Fetissov, S. O. 2017, Fung, T. C. *et al.* 2017). The majority of microorganisms are harmless, but they can cause disease if for some reason, they are in an unusual habitat or in increased levels. The Human Microbiome Project (HMP) Consortium described the diversity of the microbiome across multiple body sites (Human Microbiome Project, Consortium 2012). The HMP analysed samples collected from five major body sites: oral cavity, nasal cavity, skin, gastrointestinal tract and urogenital tract.

The microbiome of skin, gastrointestinal tract and oral cavity are constituted by four principal phyla: Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria, but with different proportions. In the skin the most prevalent are Actinobacteria while in gastrointestinal tract are Firmicutes and Bacteroidetes (Grice, E. A. *et al.* 2011). Regarding the oral cavity, and considering the diverse oral samples analysed,

the dominant phyla are Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria, TM7 and Spirochaetes (Lazarevic, V. *et al.* 2009).

The definition of a healthy microbiome is not an easy task, since variation is present across individuals. The human microbiome of diverse locations differs in healthy individuals, and this diversity is not yet fully explained. However, some reasons have been pointed for its diversity, namely host genetics, early microbial exposure, environment and diet (Human Microbiome Project, Consortium 2012).

Lloyd-Price and colleagues (2016) made such an attempt taking into account not only the composition, but also the function, dynamics, and ecology of the microbial species that compose the human microbiome. Accordingly, a “core” healthy microbiome might include specific microbial gene family combinations, metabolic modules and regulatory pathways that together promote a stable host-associated ecology.

The definition of a “healthy microbiome” based on sets of taxa that might be expected to be found prevalently in healthy people (Lloyd-Price, J. *et al.* 2016), led to the identification of typical genera or families in healthy populations namely:

- Skin: *Propionibacterium*, *Staphylococcus*, *Corynebacterium*, *Moraxella* and *Malassezia*;
- Oral cavity: *Streptococcus*, *Rothia*, *Neisseria*, *Candida*, *Veillonella* and *Actinomyces*;
- Vagina: *Lactobacillus*, *Gardnerella* and *Mycoplasma/Ureaplasma*;
- Gut: Bacteroidaceae, Clostridiaceae, Prevotellaceae, Eubacteriaceae, Ruminococcaceae, Bifidobacteriaceae, Lactobacillaceae, Enterobacteriaceae, Saccharomycetaceae and Methanobacteriaceae.

Taking into account the ecological patterns, several aspects affect the microbial composition, such as the order of colonization, that is affected by geography and early exposures, the prolonged availability of each microbe in the host’s local environment, and host selection that is influenced by genetics or diet (Lloyd-Price, J. *et al.* 2016).

In terms of dynamics, an unstable infant microbiome that gradually became a healthy adult state, could be subjected to perturbations. The two possible results are: resistance or a healthy state microbiome alteration that could result in a resilient microbiome that will return to a healthy state (possibly different from the original healthy state) or result in an unhealthy state (Montebugnoli, L. *et al.* 2004).

With the acquired knowledge in recent years, the human microbiome is now considered by some authors as a metabolically active endogenous “organ” in itself (Human Microbiome Project, Consortium 2012, Ramezani, A. *et al.* 2014, Nallu, A. *et al.* 2017). It influences the well-being of the host by contributing to its nutrition, metabolism, physiology, and immune function (Dethlefsen, L. *et al.* 2007, Costello, E. K. *et al.* 2009, Human Microbiome Project, Consortium 2012, Rautava, S. *et al.* 2012), maintaining the balance between health and disease states.

The role of disturbances of normal gut microbiome in the pathogenesis of several chronic diseases has been reported, such as obesity, insulin resistance, diabetes, inflammatory bowel disease, liver cirrhosis, myocardial ischemia and CKD (Frank, D. N. *et al.* 2007, Wang, Z. *et al.* 2011, Qin, J. *et al.* 2012, Le Chatelier, E. *et al.* 2013, Qin, N. *et al.* 2014, Sampaio-Maia, B. *et al.* 2016). Other dysbiosis of microbiomes were associated with chronic diseases, as is the case of the lower airway dysbiosis that has been shown to contribute to the pathophysiology of asthma (Lu, J. *et al.* 2017). Also, skin microbiome dysbiosis was reported as one of the contributing factors to atopic dermatitis, the most common chronic inflammatory skin disease (Weidinger, S. *et al.* 2018). In the oral cavity, the ecological imbalance was already associated to diverse local infectious diseases (dental caries, periodontitis, periodontal diseases) (Costalonga, M. *et al.* 2014), but also with several systemic diseases, such as cancer, diabetes, cardiovascular diseases and preterm birth (Herzberg, M. C. *et al.* 1996, Wu, T. *et al.* 2000, Campus, G. *et al.* 2005, Farrell, J. J. *et al.* 2012). In the specific case of rheumatoid arthritis, beyond the environmental factor disturbances, the microbiome of the intestine, lung and oral cavity were described as being involved its pathogenesis (Branimir, Anic *et al.* 2014).

Recent studies have described the existence of specific microbiomes in tissues previously considered sterile, such is the case of placenta and breast tissue (McLaughlin, R. W. *et al.* 2002, Aagaard, K. *et al.* 2014, Damgaard, C. *et al.* 2015, Potgieter, M. *et al.* 2015, Hieken, T. J. *et al.* 2016, Paise, S. *et al.* 2016). Still many other studies are necessary to fully characterize the human microbiome, and its role in health and disease development.

The hypothesis that peritoneum may harbor its own microbiome will be explored in the chapter VI.

The human oral microbiome

The human oral microbiome constitutes one of the most diverse microbiomes (bacteria, fungi, protozoa, viruses and archaea) of human body (Wade, W. G. 2013), being the second most abundant after the gastrointestinal tract (Verma, D. *et al.* 2018).

In 2010, the Human Oral Microbiome Database (HOMD) included 619 taxa in 13 phyla (*Actinobacteria*, *Bacteroidetes*, *Chlamydiae*, *Chloroflexi*, *Euryarchaeota*, *Firmicutes*, *Fusobacteria*, *Proteobacteria*, *Spirochaetes*, SR1, *Synergistetes*, *Tenericutes*, and TM7) (Dewhirst, F. E. *et al.* 2010) identified by 16S rRNA gene sequences. Recently, the expanded Human Oral Microbiome Database (eHOMD, www.homd.org) was created. This includes comprehensive information on the bacterial species present in the human aerodigestive tract (oral cavity, pharynx, nasal passages, sinuses and esophagus). Currently, eHOMD includes genomes for 475 taxa. In addition, to allow access to bacterial taxa, this resource contains a repository of bacterial genome sequences and an identification tool based on 16S rRNA sequencing. 16S rDNA profiling of the healthy oral cavity categorized the inhabitant bacteria (96% of total oral bacteria) into six broad phyla: Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria, Bacteroidetes and Spirochaetes (Verma, D. *et al.* 2018).

The first studies on the Human oral microbiome were done with traditional methods based on microbial cultures. The introduction of culture-independent methods revealed that approximately half of the oral microbiome was composed by uncultured species. These studies enabled a more complete identification of the oral bacterial community and revealed that the individuals' oral microbiome is highly specific concerning the species level (Wade, W. G. 2013). Recently Lloyd-Price and colleagues (2016) reported that overall the oral microbial community is more similar within a certain population than with the microbial communities from other habitats of the same person. However, intra-individual variability over time is lower than inter-individual differences within habitats (Human Microbiome Project, Consortium 2012, Monteiro-da-Silva, F. *et al.* 2014, Lloyd-Price, J. *et al.* 2016).

The two bacterial diseases most common in man are dental caries and periodontal diseases. They are the result of different factors interactions, namely host susceptibility, commensal microbiota and environmental factors. In order to understand host-microbiome interactions the focus should be not only on the microbiome composition but also on the function of each microorganism within oral community (Wade, W. G. 2013). Some studies point out the oral cavity as a starting point for dissemination of pathogenic organisms (Li, X. *et al.* 2000); the oral cavity is an entry way, since food and air pass through the mouth and follow the respective pathways probably spreading oral microorganisms to other

body habitats (Dewhirst, F. E. *et al.* 2010). Also, it is known that oral microorganisms are frequently responsible for bacteraemia due to routine daily hygiene activities, such as tooth brush, dental tape use, or invasive dental procedures, reason why patients at risk are prophylactically treated with antibiotic therapy before dental interventions (Bahrani-Mougeot, F. K. *et al.* 2008, Sampaio-Maia, B. *et al.* 2016). Moreover, several studies reported that oral bacteria are associated to different systemic diseases, as cardiovascular disease, stroke, diabetes and pneumonia (Joshiyura, K. J. *et al.* 2003, Montebugnoli, L. *et al.* 2004, Beck, J. D. *et al.* 2005, Genco, R. J. *et al.* 2005, Seymour, G. J. *et al.* 2007, Awano, S. *et al.* 2008). A preliminary study by Kshirsagar and colleagues (2009) described that a moderate-to-severe periodontal disease was strongly associated with CVD mortality in CKD patients on dialysis, despite the possible association between oral diseases and an adverse survival of renal diseases patients the scientific evidence linking this route of infection to peritonitis is still scarce (Strippoli, G. F. *et al.* 2013). In the particular case of PD-related infections, an evaluation of possible alternative routes of infection would help to improve the knowledge on infection development and to implement preventive strategies to control them. Oral microbiota could represent a reservoir of infectious agents that could reach the peritoneum by hematogenous route.

Microbiome and CKD

As previously mentioned, the existence of an imbalanced human microbiome was already reported in CKD. Most of the existing studies have focused on the gut microbiome and is now a well described phenomenon (Wang, F. *et al.* 2012, Barrios, C. *et al.* 2015, Barros, A. F. *et al.* 2015). CKD associated factors may play a role in the promotion of gut microbiome imbalance, such as increasing the intestinal availability of uremic toxins, metabolic acidosis, intestinal wall edema and reducing the colonic transit and digestive capacity. Pharmacological therapies (e.g., antibiotics and iron delivery) and dietary restrictions (e.g. reduced fiber intake) also alter gut microbiome and may play a significant role on gut dysbiosis. Within the intestinal epithelia, commensal bacteria compete with pathogens for epithelial adhesion sites and nutrients. In a dysbiotic state this natural defense is reduced, consequently increasing CKD patients' vulnerability to pathogen invasion.

The intestinal microbial imbalance occurs either quantitatively and qualitatively being frequently associated with the overgrowth of Enterobacteriaceae, Lachnospiraceae and some Ruminococcaceae, and with the reduction of some Bacteroidaceae, Prevotellaceae and particular *Bifidobacterium* and *Lactobacillus* species (Sampaio-Maia, B. *et al.* 2016).

The pathophysiological mechanisms underlying the role of gut dysbiosis in CKD are presented in chapter I. However, the characterization of other human microbiomes and its relationship with disease progression is still poorly explored in CKD.

The midstream urine microbiome was recently described in CKD (Kramer, H. *et al.* 2018) reporting that the microbial diversity decreases with the progression of CKD. However, it was not possible to determine whether it is the kidney disease that influences the midstream urinary microbiome or vice versa, and thus further studies are still necessary to understand this relationship.

In 2012 another study compared the oral microbiome in CKD patients with periodontitis to the one of individuals without CKD and with periodontitis. The periodontitis-associated subgingival microbiome was equivalent in both groups in terms of composition, but the subgingival microbiome of patients with CKD presented increased levels of pathogenic species after mechanical non-surgical periodontal treatment (Artese, H. P. *et al.* 2012). Recently, Hu and colleagues (2018) described the association between the oral microbiome of CKD patients and the presence of inflammatory kidney biomarkers, suggesting that the oral microbiome has a potential role in CKD pathogenesis. Bossola and colleagues (2009) showed that 20.7% of blood samples from HD patients presented bacterial DNA (*E. coli*, *S. aureus*, *P. aeruginosa*, *S. epidermidis*, *E. faecalis*, *Proteus mirabilis* and *Staphylococcus haemolyticus*), in contrast to the absence of blood microbiome in healthy controls. In a study of 2015 lower α -diversity levels were observed in the periodontal microbiome of some ESRD patients when compared to controls; suggesting that increasing dialysis vintage was associated to less diversity and smaller microbial community (Araujo, M. V. *et al.* 2015). Among CKD patients the dialysis therapies have been associated with different modifications of the gut microbiome, such as a decrease in Proteobacteria relative abundance in HD patients and increase in PD patients. However, both therapies may have an impact on the human microbiome beyond the gut. Chapter II presents the impact of HD and PD therapies on the human microbiome.

Aim of the Study

This thesis emerged from the need to further characterize and understand the role of the human microbiome in the development of infection in the PD population. Although the number of peritonitis has decreased in the last years, there is still a considerable number of patients that abandon this technique due to peritonitis episodes that result in catheter loss, transfer to HD and, in severe cases, death.

Our goal was to study the relationship between the patients' microbiota, infection development and PD-related infectious agents. In the future, this could lead to a decrease in PD-related infection occurrence, by identification of novel infection routes and implementation of newly devised preventive strategies in this population.

For this purpose, we evaluated a group of ESRD adult patients undergoing PD, followed up in the outpatient clinic of the Nephrology Department of the "Centro Hospitalar de S. João".

The major goals of this study, subdivided in the following six chapters, were:

- Chapter I - Review of the literature focused on the role of gut dysbiosis in CKD;
- Chapter II - Review of the literature on the characterization of the human microbiome in CKD patients undergoing HD and PD;
- Chapter III – Colonization of the oral cavity by *Staphylococcus* and its correlation to PD-infection development; and comparison between different methodologies for *Staphylococcus* identification;
- Chapter IV - The oral colonization by yeasts and the development of PD-related infections; and comparison between yeast colonization of CKD patients on HD and PD;
- Chapter V - Protozoa colonization of the peritoneal effluent and oral cavity of PD patients;
- Chapter VI - Characterization of the peritoneum microbiome in ESRD adult patients and evaluation of the effect of PD on its composition.

CHAPTER I

“The role of the Gut microbiome on Chronic Kidney Disease”

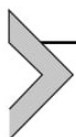
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In the last years, the role of the gut microbiome has been evaluated in diverse chronic diseases. In CKD, gut dysbiosis has been pointed out as a nontraditional risk factor for CV risk and CKD progression. In this chapter, we review the state-of-the-art concerning gut dysbiosis in CKD and its role in pathophysiological mechanisms of CKD, particularly in relation with CV risk. The strategies towards the prevention and treatment of gut dysbiosis in CKD progression are also described.

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L. Simões-Silva participated in the review of the literature and writing of the manuscript.



The Role of the Gut Microbiome on Chronic Kidney Disease

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Abstract

Chronic kidney disease (CKD) is estimated to affect nearly 500 million people worldwide and cardiovascular (CV) disease is a major cause of death in this population. However, therapeutic interventions targeting traditional CV risks are not effective at lowering the incidence of CV events or at delaying the progression of the disease in CKD patients. In recent years, disturbances of normal gut microbiome were recognized in the pathogenesis of diverse chronic diseases. Gut dysbiosis is being unraveled in CKD and pointed as a nontraditional risk factor for CV risk and CKD progression. The most often reported changes in gut microbiome in CKD are related to the lower levels of Bifidobacteriaceae and Lactobacillaceae and to higher levels of Enterobacteriaceae. Although metagenomics brought us an amplified vision on the microbial world that inhabits the human host, it still lacks the sensitivity to characterize the microbiome up to species level, not revealing alterations that occur within specific genus. Here, we review the current state-of-the-art concerning gut dysbiosis in CKD and its role in pathophysiological mechanisms in CKD, particularly in relation with CV risk. Also, the strategies towards prevention and treatment of gut dysbiosis in CKD progression will be discussed.



1. INTRODUCTION

Chronic kidney disease (CKD) is a general term for heterogeneous disorders affecting the structure and function of the kidney (Levey & Coresh, 2012). The definition of CKD is based on the presence of kidney damage (i.e., albuminuria) or decreased kidney function (i.e., glomerular filtration rate [GFR] <60 mL/min/1.73 m²) for 3 months or more, irrespective of the clinical diagnosis (Bailie, Uhlig, & Levey, 2005). According to the National Kidney Foundation Clinical Practice Guidelines for Chronic Kidney Disease (Eknoyan & Levin, 2002), CKD is classified into five stages on the basis of GFR, from normal kidney function (stage 1) to end-stage kidney disease (stage 5).

CKD is a global health problem with high rates of morbidity and mortality. A recent study indicated that in 2010 over 497 million adults in the world had CKD from which, 236 million had moderate or severe decreases in kidney function presenting CKD stages 3–5 (Mills, Xu, et al., 2015).

One potential outcome of CKD is end-stage renal disease (ESRD), requiring costly renal replacement therapy. The number of patients requiring renal replacement therapy in the form of hemodialysis (HD), peritoneal dialysis, or kidney transplant, increases 10–15% each year (Lozano et al., 2012). The progressive loss of renal function leads to the accumulation of organic waste products, in particular derivatives of nitrogen metabolism normally cleared by the kidneys. It is under these circumstances that the “uremic state” is established. Nonetheless, some adverse outcomes of CKD can be prevented through early detection and treatment (Berns, 2014; Jha et al., 2013).

Diabetes and hypertension are the most common causes of CKD worldwide. Other causes can include glomerulonephritis, pyelonephritis, and polycystic kidney disease (Meyer & Hostetter, 2012). One of the major causes of death in CKD patients is cardiovascular (CV) disease. Patients with CKD carry a high CV burden by the time they commence renal replacement therapy, as CV risk increases exponentially with disease progression. CV-related mortality may be up to 500-fold higher in patients with ESRD than in the general population (Sarnak et al., 2003). Therapeutic interventions targeting traditional CV risk factors (e.g., hypertension, diabetes, dyslipidemia) are not effective at lowering the incidence of CV events or delaying the progression of the disease (Alani, Tamimi, & Tamimi, 2014). Therefore nontraditional CKD-related risk factors that increase in

prevalence as kidney function declines (e.g., chronic inflammation, vascular calcification, sympathetic hyperactivity, and anemia, among others) were suggested to contribute both to the increased CV risk and the accelerated progression of the disease (Gansevoort et al., 2013; Sarnak, 2003).

In recent years, it has become increasingly clear that the human microbiome has a far-reaching impact on human physiology and is now considered by some authors a metabolically active endogenous “organ” in itself, whose metabolic capacity exceeds that of the liver (Huttenhower et al., 2012; Nallu, Sharma, Ramezani, Muralidharan, & Raj, 2016; Ramezani & Raj, 2014). The human microbiome, defined by the NIH human microbiome project (<http://hmpdacc.org/>) as the collection of all the microorganisms living in association with the human body, influences the well-being of the host by contributing to its nutrition, metabolism, physiology, and immune function (The Human Microbiome Project Consortium, 2012). Although the human microbiome includes bacteria, archaea, fungi, protozoa, and viruses, the majority of the studies focused on bacteria (bacteriome). Disturbances of normal gut microbiome are currently recognized in the pathogenesis of diverse chronic diseases, such as obesity (Le Chatelier et al., 2013; Ley, Turnbaugh, Klein, & Gordon, 2006), insulin resistance, diabetes (Brown et al., 2011; Kriegel et al., 2011; Qin et al., 2012), inflammatory bowel disease (Frank et al., 2007), liver cirrhosis (Qin et al., 2014), and myocardial ischemia (Wang et al., 2011). In addition, recent reports described disturbances of normal gut microbiome (dysbiosis) in CKD and suggested that gut dysbiosis may represent a nontraditional factor for CV risk and progression of CKD (Aron-Wisniewsky & Clement, 2016; Ramezani & Raj, 2014; Vaziri, Wong et al., 2013). On the other hand, the human microbiome may also be endowed with beneficial effects on the host, for example, on the treatment of recurrent *Clostridium difficile* infection (van Nood et al., 2013) and of autism spectrum disorder (Hsiao et al., 2013), suggesting the human microbiome as a potential target for therapeutic interventions. Additionally, studies in animal models of human disease with germ-free and gnotobiotic mice revealed the importance of the gut microbiota in the balance between health and disease (Tlaskalova-Hogenova et al., 2011).

In the view of the above stated, this chapter focuses on the recent advances in the study of the gut microbiome in CKD patients and its relation with CV risk and with disease progression. Given that the studies available in the literature refer only bacteria, our review will only focus on the role of the

gut bacteriome in CKD. Possible strategies towards prevention and treatment of intestinal dysbiosis associated with CKD, such as pre- or probiotics will also be addressed.



2. GUT DYSBIOSIS IN CHRONIC KIDNEY DISEASE

The gut microbiome is constituted by more than 50 bacterial phyla, with Bacteroidetes and Firmicutes being the most prevalent (Wing, Patel, Ramezani, & Raj, 2015). The abundance and diversity of the bacteria in the gut increases from the proximal to distal parts of the intestine reaching a maximum of 10^{12} bacterial cells/mL within the colonic lumen (Wing et al., 2015).

In health, the gut microbiome coexists with the host in a symbiotic manner, conferring trophic and protective functions. In disease, this equilibrium is altered and dysbiosis occurs. In this dysbiotic state, intestinal microbiota develops qualitative and quantitative changes in its metabolic activity and local distribution, when compared with a “normal” functioning gut, producing harmful effects (Holzapfel et al., 1998). Many questions and doubts arise from the interpretation of all available metagenomics information and complex microbial relationships. It is now important to understand which microbial alterations are direct consequences of human nutrition and host environment and which may be associated with human well-being.

Despite its stability throughout host's life, it is known that the human microbiome adapts dynamically to changes in the ecosystem and changes in the host's health (Brown et al., 2011; Frank et al., 2007; Kriegel et al., 2011; Le Chatelier et al., 2013; Ley et al., 2006; Monteiro-da-Silva, Araujo, & Sampaio-Maia, 2014; Monteiro-da-Silva, Sampaio-Maia, Pereira Mde, & Araujo, 2013; Qin et al., 2012, 2014; Wang et al., 2011). As stated before, human microbiome dysregulation has been observed in CKD in both humans and animal models, revealed by either quantitative and qualitative changes of the gut microbiota (Barrios et al., 2015; Barros et al., 2015; De Angelis et al., 2014; Hida et al., 1996; Ranganathan et al., 2009; Vaziri, Wong, et al., 2013; Wang, Jiang, et al., 2012; Wang, Zhang, Jiang, & Cheng, 2012). This microbiota imbalance in CKD may be due to intestinal availability of uremic toxins, metabolic acidosis, intestinal wall edema, slow colonic transit, decreases in digestive capacity, pharmacological therapies (e.g., antibiotics and iron deliver), and dietary restrictions (e.g., reduced fiber intake). All these previously mentioned factors may disturb the symbiotic

relationship that prevails between gut microbiome and the host under normal conditions, leading to the production and absorption of pro-inflammatory and otherwise harmful by-products, while simultaneously limiting the beneficial functions and products conferred by the normal and “healthy” microbiota. Such events can further contribute to uremic toxicity, inflammation, and CV risk in CKD patients (Vaziri, Wong, et al., 2013).

More than 80 uremic toxins are known (Meyer & Hostetter, 2012) and probably most of them are secreted into the gut altering intestinal milieu, inducing changes in the structure, composition, and function of the gut microbiome. A clear example of uremic toxin mechanism is the increased secretion of urea and uric acid into the gut. Once in the gut lumen, urea hydrolysis occurs resulting in the formation of large quantities of ammonia that in turn are converted to ammonium hydroxide. This compound dynamically alters the gut pH, inducing a change in the proportion of pH-sensitive bacteria, causing mucosal damage, and irritation (Kang, 1993; Vaziri, Dure-Smith, Miller, & Mirahmadi, 1985). Also, in the gut of ESRD patients, secreted uremic toxins serve as alternative substrates for gut microbiota, which normally utilize indigestible complex carbohydrates. Since, unlike other mammals, humans lack the enzyme uricase that converts uric acid to allantoin in the gut (Oda, Satta, Takenaka, & Takahata, 2002), bacterial families possessing urease, uricase, p-cresol and indole-forming enzymes are expanded, whereas short-chain fatty acid (SCFA)-forming bacteria are contracted in ESRD patients (Wong et al., 2014).

Moreover, a study conducted by Wikoff et al. (2009) demonstrated that a significantly large number of chemical species found in systemic circulation arise due to the presence of the microbiome, revealing the role of the colonic microbiota as a significant contributor to the metabolome (collection of small molecule metabolites) in patients with CKD. Thus, uremic toxins have implications not only at the intestinal level, but also in the human plasma metabolome, that ultimately results from a combination of endogenous metabolites and metabolites originating from the colonic microbiota.

In ESRD patients, restricted consumption of fruits and vegetables, a rich source of potassium, is invariably recommended to prevent hyperkalemia. Since fruits and vegetables are also the main source of dietary fiber, their limited consumption in CKD may have a direct impact on the composition, function, and metabolism of the gut microbiome. For example, a daily diet based on fibers, fruits, and vegetables (agrarian diet) in young children is associated with an increase of Actinobacteria and Bacteroidetes in fecal

samples, while a “Western” diet based in lower fiber and higher fat/sugar content is associated with an increase of Proteobacteria (including the most common enteric pathogens) (Simpson & Campbell, 2015). Some other studies conducted with samples obtained from children living in Burkina Faso, Malawi, Venezuela, and Bangladesh versus children living in Italy and the United States suggested similar results and dynamics in the human gut microbiome according to the diet regimen (De Filippo et al., 2010; Lin et al., 2013; Yatsunenkov et al., 2012). Moreover, the use of various phosphate-binding products, i.e., anion-exchange resins, iron-based products, calcium acetate, calcium carbonate, and aluminum hydroxide which are commonly prescribed for patients with advanced CKD, will certainly have yet unrecognized effects on the gut microbiome. Finally, the frequent use of antibiotics can significantly affect the microbiome in CKD patients.

The first studies describing gut dysbiosis in CKD patients with intermediary renal insufficiency stages (3–4) (Ranganathan et al., 2009) and ESRD patients undergoing HD (stage 5) (Hida et al., 1996) employed traditional microbiological methods, using nonselective and selective culture media. These studies reported a decrease on the gut colonization by *Bifidobacterium* spp. and an increase of colonization by Enterobacteriaceae. More recent studies evaluated gut dysbiosis in CKD using molecular technologies, namely the evaluation by next-generation sequencing of the DNA region coding for the 16S rRNA (De Angelis et al., 2014; Wang, Jiang, et al., 2012), microarray (Vaziri, Wong, et al., 2013; Wong et al., 2014), or Denaturing Gradient Gel Electrophoresis (DGGE) (Barros et al., 2015). Interestingly, De Angelis et al. (2014) evaluated not only the DNA coding for the 16S rRNA, but also the RNA itself to explore both total and metabolically active bacteria. The target population of these studies included CKD patients in stages 3–4 (Barros et al., 2015; De Angelis et al., 2014; Vaziri, Wong, et al., 2013) and in stage 5 (Vaziri, Wong, et al., 2013; Wang, Jiang, et al., 2012; Wong et al., 2014). The study of De Angelis et al. (2014) only included patients with immunoglobulin A nephropathy. Each study presented a list of bacterial phyla, family, genera, or species altered in CKD in comparison to a healthy control population. Fig. 1 depicts the studies that reported increases (to the right in green) or decreases (to the left in red) in each family or species of the gut microbiota in CKD. The studies conducted by Vaziri and Wong (Vaziri, Wong, et al., 2013; Wong et al., 2014) analyzed the same sample of patients, and, therefore, the results from both studies were treated as a single study in Fig. 1.

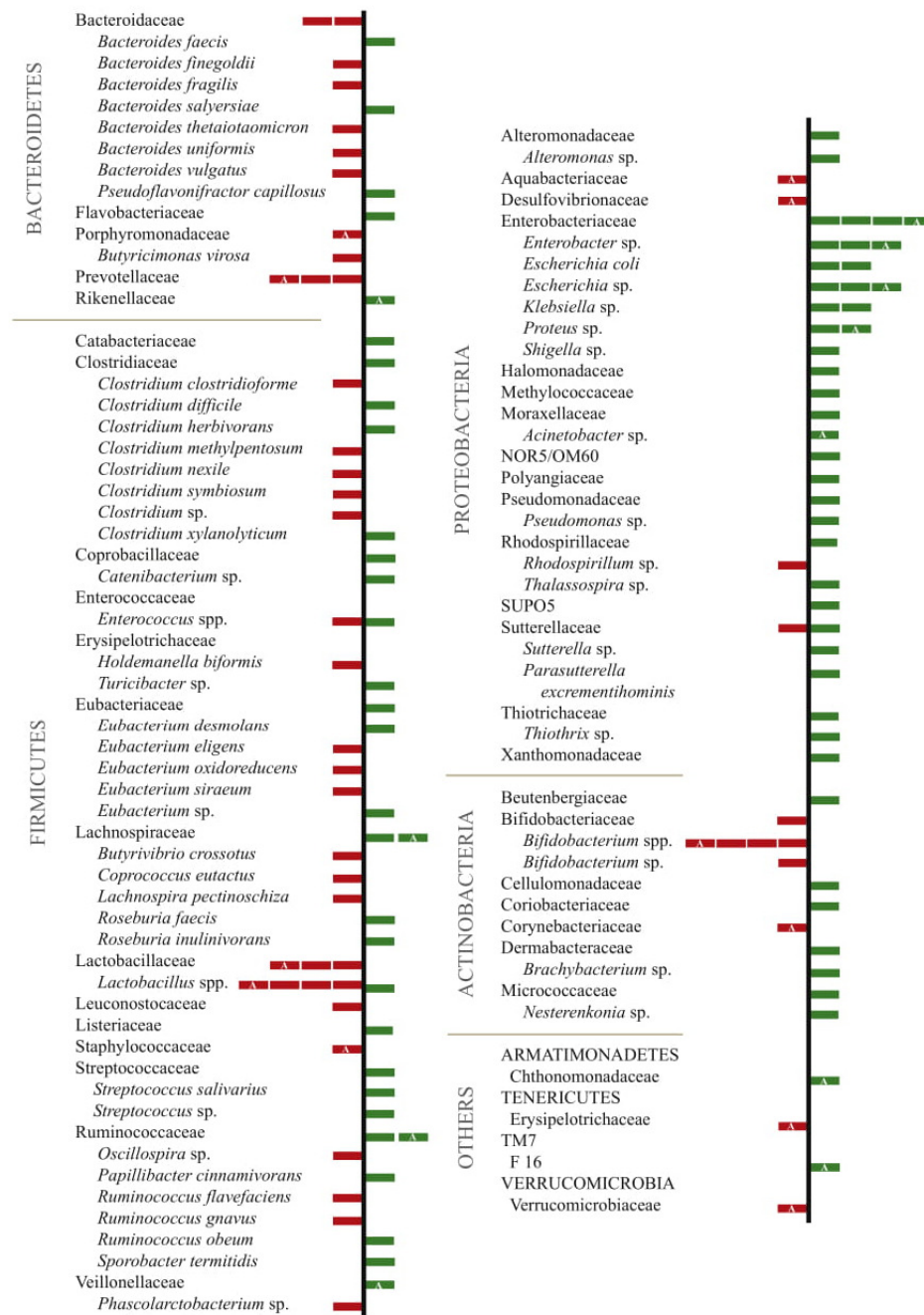


Figure 1 Description and systematization of the quantity of studies that reported gut dysbiosis in chronic kidney disease (CKD): increases (to the right in green (light gray in print versions)) or decreases (to the left in red (gray in print versions)) for each family or species of the gut microbiota (A — results obtained in animal models).

Herein, we summarize the relevant emerging results that were in agreement in two or more studies: CKD is associated with lower intestinal levels of the families Bifidobacteriaceae, specially the genus *Bifidobacterium* (De Angelis et al., 2014; Hida et al., 1996; Ranganathan et al., 2009), Lactobacillaceae (De Angelis et al., 2014; Hida et al., 1996; Vaziri, Wong, et al., 2013), Bacteroidaceae (De Angelis et al., 2014; Vaziri, Wong, et al., 2013), and Prevotellaceae (De Angelis et al., 2014; Wong et al., 2014) and with higher intestinal levels of Enterobacteriaceae, specially the genera *Escherichia*, *Enterobacter*, and *Klebsiella* (De Angelis et al., 2014; Hida et al., 1996; Vaziri, Wong, et al., 2013; Wang, Jiang, et al., 2012). In general, the variations more often described in those studies were increases in Enterobacteriaceae and decreases in both *Bifidobacterium* spp. and *Lactobacillus* spp.

To isolate the effect of uremia from interindividual variations, comorbid conditions, and dietary and pharmacological interventions, rats submitted to 5/6 nephrectomy were evaluated in two different studies (Vaziri, Wong, et al., 2013; Wang, Zhang, et al., 2012). Interestingly, the studies using CKD animal models corroborate the results regarding the groups presented above: decreases in *Bifidobacterium* spp., Lactobacillaceae and Prevotellaceae and increases in Enterobacteriaceae (Vaziri, Wong, et al., 2013; Wang, Zhang, et al., 2012).

Gathering the information from human patients and animal models, CKD appears to be associated with gut dysbiosis evidenced by overgrowth of Enterobacteriaceae family members and probably Ruminococcaceae and Lachnospiraceae families, along with a reduction in the growth of species within *Bifidobacterium* and *Lactobacillus* genera and probably members of Prevotellaceae and Bacteroidaceae families (Fig. 2).

As shown in Fig. 1, most of the alterations were observed in a single study and therefore it is still not possible to associate such changes with CKD. Interestingly, the most detailed study conducted by De Angelis et al. (2014) showed that within each taxonomic family there were species increasing and others decreasing its colonization in the gut of CKD patients, e.g., *Bacteroides faecis* and *Bacteroides salyersiae* increased in CKD patients, whereas *Bacteroides fnegoldii*, *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Bacteroides uniformis*, and *Bacteroides vulgatus* decreased its colonization in these population. Distinct results were also found, namely for *Enterococcus* spp.

Although metagenomics brought us an amplified vision about the microbial world that inhabits the human host, it still lacks the sensitivity to characterize the microbial population up to species level. It is important to

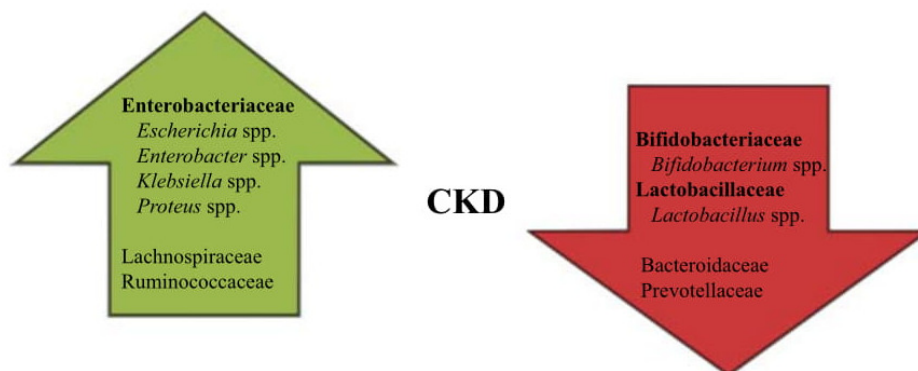


Figure 2 Overview of the relevant emerging results (in agreement in at least two studies) regarding bacterial family or genera altered in chronic kidney disease (CKD) in comparison to a healthy control population. CKD is associated with lower levels of the families described in the *downward red arrow* (gray in print versions) and with higher levels of the families described in the *upward green arrow* (light gray in print versions).

note that despite the results for bacteria taxonomic families as a whole, individual bacterial genera within a family may have a self-dynamic response to milieu alterations. In fact, each taxonomic family may comprise numerous genus and species, with specific characteristics. Thus, additional studies are needed to fully characterize gut dysbiosis in CKD and to understand its physiological impact. Also, it is important to conduct other studies where the remaining members of the microbiome are evaluated, namely, fungi, archaea, virus, and protozoa. Only then we will be able to fully characterize the microbiome on CKD. Nevertheless, regarding the bacteriome some major differences have been systematically described by multiple studies on particular taxa such as Bifidobacteriaceae, Lactobacillaceae, or Enterobacteriaceae that strongly suggest a direct association to CKD (De Angelis et al., 2014; Hida et al., 1996; Ranganathan et al., 2009; Vaziri, Wong, et al., 2013; Wang, Zhang, et al., 2012).

Two types of microbial fermentation occur in the colon: saccharolytic and proteolytic fermentation (Hamer, De Preter, Windey, & Verbeke, 2012; Leclercq et al., 2014). Saccharolytic fermentation is generally considered to be beneficial to the host, for example, the production of SCFAs, whereas proteolytic fermentation is presumed to be detrimental and might be involved in the etiology of some human pathologies, such as colon cancer and ulcerative colitis (Nicholson et al., 2012; Windey, De Preter, & Verbeke, 2012). Increased protein fermentation results in generation of potentially toxic metabolites such as ammonia, phenols, amines, indoles,

and thiols (Gibson, Mcfarlan, Hay, & Macfarlane, 1989). Interestingly in CKD, lower levels of recognized saccharolytic gut bacteria are observed, including Bifidobacteriaceae and Lactobacillaceae.

The declining populations of species of *Bifidobacterium* and *Lactobacillus* facilitate the overgrowth of other microbial groups and also the intestinal translocation of some pathobionts. Bacterial overgrowth is one of the main factors promoting bacterial translocation through the intestinal barrier, contributing to microinflammation in uremic rats (Wang, Zhang, et al., 2012). Animals with advanced CKD tend to show disintegration of the colonic tight junction apparatus what contributes to systemic inflammation and accounts for defective intestinal barrier function (Vaziri, Goshtasbi, et al., 2012). The transition in the intestinal environment from healthy to CKD resulted in a shift of the microbiota from a more evenly distributed and complex community to one that was simpler and apparently more dominated (Vaziri, Yuan, & Norris, 2013).

It is also important to refer the study conducted by Barrios et al. (2015) where a population with minimal renal function decline was analyzed. The authors identified changes in the intestinal microbiota associated with the production of uremic toxins indoxyl sulfate, p-cresyl sulfate, and phenylacetylglutamine, suggesting that these uremic toxins may represent early markers of renal function decline.

2.1 Bifidobacteriaceae and Lactobacillaceae

It is known since long that *Bifidobacterium* spp. and *Lactobacillus* spp. colonize the human gut and their role on competitive protection against pathogenic Gram-negative and facultative anaerobic bacteria is relevant. These beneficial bacteria act by preventing Gram-negative and facultative anaerobic bacteria from adhering to enterocytes (this role is frequently known as colonization resistance) and limits the growth and spread of harsh bacteria on the human gut by competing for nutrients, iron, and other essential ions (Kedia, Rampal, Paul, & Ahuja, 2016). Generally, bifidobacteria are characterized by producing acetate following the fermentation of glucose and fructose, fermenting and colonizing mucin for obtaining energy, while *Lactobacillus* spp. fortify the epithelial barrier, secrete mucin, enhance tight-junction function, upregulate cytoprotective heat shock proteins, and prevent apoptosis of epithelial cells. In fact, it is well known that intestinal bacteria, especially facultative anaerobic bacteria, adhere and invade intestinal epithelial cells, being, then, more resistant to phagocytic killing when compared with other bacteria such as anaerobic bacteria (Wenzl, Schimpl,

Feierl, & Steinwender, 2001). Therefore, by reducing the number of facultative anaerobic bacteria at the intestine, *Bifidobacterium* spp. and *Lactobacillus* spp. bacteria help reducing the translocation of opportunistic bacteria through the intestinal epithelium.

Bifidobacterium spp. are Gram-positive, nonmotile, anaerobic bacteria that inhabit the gastrointestinal and fraction of urinary tracts in humans and other mammals. Bifidobacteria are one of the major genera of bacteria present at the colon and are frequently used as probiotics (El Enshasy et al., 2016). Numerous species and strains of bifidobacteria are responsible for a wide range of advantageous effects in the human body, especially in the intestine, such as the inhibition of pathogen growth; competition for nutrients, energy sources and habitat on the gut mucosa therefore contributing to the maintenance of microbial balance and diversity; the regulation of immune responses; the repression of harsh enzymatic activities caused by some microbes; the conversion of some dietary foods into bioactive molecules; and the production of vitamins and other bioproducts that are valuable for the human body (Kedia et al., 2016; Pinzone, Celesia, Di Rosa, Cacopardo, & Nunnari, 2012).

Lactobacillus spp. are Gram-positive facultative anaerobic rod-shaped bacteria capable of fermenting glucose and other sugars primarily to lactic acid (homofermentative metabolism) or to lactic acid, carbon dioxide, and ethanol (heterofermentative metabolism) (Patten & Laws, 2015). As described above for bifidobacteria, lactobacilli can also restore physiological and microbial balance in distinct human ecosystems. Some studies suggest that lactobacilli produce low-molecular weight compounds capable of inhibiting microorganisms and protect intestinal and vaginal epithelia. In addition, lactobacilli can maintain an acidic environment that clearly limits the growth of other microorganisms and are capable of producing hydrogen peroxide during central carbon and energy metabolism by oxidases, such as pyruvate oxidase, lactate oxidase, and NADH oxidases (Badel, Bernardi, & Michaud, 2011; Hertzberger et al., 2014; Vaughan, Heilig, Ben-Amor, & de Vos, 2005). This represents another strategy for limiting the development of pathogens in human habitats.

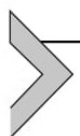
Thus, the reduced bifidobacteria and lactobacilli populations in the gut of CKD patients may allow the overgrowth of pathobionts and facilitate the translocation of gut microorganisms through epithelial intestinal barrier.

2.2 Enterobacteriaceae

The Enterobacteriaceae are Gram-negative, facultative anaerobic, rod-shaped non-spore-forming bacteria. This family is characterized by

possessing flagella for motility (some exceptions are reported), catalase reaction is variable and generally lack cytochrome C oxidase, although few exceptions can be found (Janda, Abbott, & McIver, 2016). These bacteria present very diverse metabolic pathways: can ferment sugars to produce lactic acid and various other products, being as well known as hydrogen-consuming sulfate-reducing bacteria, with many species being able to reduce nitrate to nitrite. As Gram-negative, enterobacteria produce endotoxins responsible for inflammatory and vasodilatory immune processes in the human body when released to the bloodstream. This family is very diverse with individuals commonly found in the environment (soil, water, plants) and are usually called “enteric bacteria,” as several members of the family grow and live in the intestine of humans and animals (van Elsas, Semenov, Costa, & Trevors, 2011; Trosvik et al., 2010).

Enterobacteriaceae includes many harmless symbionts and relevant pathogens, such as *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonella enterica*, *Serratia marcescens*, *Shigella dysenteriae*, and *Yersinia pestis*. In intestinal lumen, some members of Enterobacteriaceae are considered pathobionts due to their competitive efficiency and capacity to opportunistically adhere and invade host tissues (Servin, 2014). Thus, the overgrowth of Enterobacteriaceae in the gut of CKD patients may further promote a metabolic shift in intestinal lumen and potentiate the emergence of pathobionts. Additionally, as Gram-negative bacteria, its translocation to the blood can result in an increased risk of endotoxemia.



3. UREMIC TOXINS AND THE GUT MICROBIOME

The uremic syndrome is characterized by the retention of various solutes that under normal conditions are excreted by the healthy kidneys. In recent years there has been an increasing interest in the identification of these uremic toxins that may be inefficiently removed by dialysis, despite the advances in renal replacement therapies (Dhondt et al., 2000; Vanholder et al., 2015). Identifying toxic compounds and devising new strategies for their removal are, therefore, crucial strategies for the improvement of dialysis efficacy and consequently for the patient's health.

The importance of uremic toxicity led to the creation of the European Uremic toxin (EUTox) work group (<http://uremic-toxins.org/index.html>) devoted to the identification of yet unknown uremic toxins, its

Table 1 Classification of uremic toxins

	Number of members (EUTox)	Mw	Compounds	Pathological associations
Small water- soluble compounds	68	<500 Da	1-Methyladenosine	CV
			Asymmetric dimethylarginine (ADMA)	CV
			Dimethylamine	RNL
			Phenylacetic acid	CV
			S-adenosylhomocysteine	CV
			Symmetric dimethylarginine (SDMA)	CV
			Trimethylamine	RNL
			Urea	CV
			Xantine	CV
			Adiponectin	CV
Middle molecules	32	>500 Da	Adrenomedullin	CV
			Atrial natriuretic peptide (ANP)	CV
			Hyaluronic acid	RNL
			Monomethylamine	RNL
			Neuropeptide Y	CV
			Parathyroid hormone	CV
			Resistin	CV
			Homocysteine	CV
Protein bound	30		Indoxyl sulfate	RNL, CV
			Leptin	RNL
			<i>p</i> -Cresyl sulfate	RNL, CV

Members of each category are presented as well as their association with cardiovascular and renal pathologies according to EUTox. CV, cardiovascular; RNL, renal.

characterization, and the development of new therapeutic approaches for the treatment of CKD.

Uremic toxins can be classified in three types of solutes, according to their physicochemical characteristics, small water soluble compounds, middle molecules, and protein-bound molecules (Table 1). The group of protein-bound molecules is particularly relevant since they are resistant to removal by dialysis (Wing et al., 2015). Alternatively, uremic toxins can be classified according to their origin: endogenous metabolism, microbial metabolism, or exogenous intake (Evenepoel et al., 2009).

The first publication in Pubmed related to uremic toxins is from the year 1960 (Desi, Feher, & Szold, 1960). Although only recently has the predominant role of the intestine in the generation of some uremic solutes been highlighted (Aronov et al., 2011; Fukuuchi et al., 2002; Lekawanvijit, 2015; Yatsunenkov et al., 2012), back in 1966 Einheber and Carter (Einheber & Carter, 1966) already reported that in mice with bilateral nephrectomy the absence of microbiome prolonged the mice life expectancy. Also, in 1982, another group reported in weanling pigs with normal kidney function the decreased fecal and urinary excretion of phenolic and aromatic bacterial metabolites after depletion of gut microbiome by antibiotics (Yokoyama, Tabori, Miller, & Hogberg, 1982). In 2009, Wikoff and colleagues showed an association between gut microbiota and some protein-bound uremic toxins, namely indoxyl sulfate, hippuric acid, and phenylacetic acid. Later on, Aronov et al. (2011) analyzed the plasma from HD patients with and without colons to identify and characterize colon-derived uremic solutes. In this study, they identified by high-resolution mass spectrometry more than 30 individual features in patients with colons that were either absent or present in lower concentrations in patients without colons. These features were also more prominent in the plasma of HD patients when compared to control subjects, suggesting that they represent uremic solutes produced by the colon in CKD. More recently, Poesen, Windey, et al. (2016) compared the stool metabolome between HD patients, not related healthy subjects, and related household contacts on the same diet as well as between sham and 5/6th nephrectomized rats, 6 weeks after surgery. The conclusions of this study were that CKD associates with a distinct colonic microbial metabolism, although the effect of renal function loss per se in humans may be inferior to the effects of dietary and other CKD-related factors. Thus, although more studies are needed to exclude non-CKD factors, nowadays it is well established that colonic microbes may produce an important portion of uremic solutes, mostly still unidentified.

The most extensively studied colon-derived uremic toxins are indoxyl sulfate and p-cresol sulfate (Meyer & Hostetter, 2012; Wing et al., 2015). P-cresol is a protein-bound solute that results from the fermentation of the amino acids tyrosine and phenylalanine (Cummings, 1983). Most of the p-cresol generated is conjugated to p-cresyl sulfate in the intestine and to p-cresyl glucuronide in the liver. Several studies reported the association between p-cresol and CV outcomes in CKD (Dou et al., 2004; Liabeuf et al., 2010; Meijers, Claes, et al., 2010). P-cresol sulfate

levels increased with decreasing estimated GFR (Meijers, Claes, et al., 2010); in addition the baseline concentration of p-cresol was an independent predictor for CV events, and was associated with increased risk of death in ESRD patients on HD (Bammens, Evenepoel, Keuleers, Verbeke, & Vanrenterghem, 2006).

The metabolization of dietary tryptophan by bacterial tryptophanase generates indole, which is subsequently absorbed and metabolized to indoxyl sulfate in the liver (Cummings, 1983). The baseline concentration of indoxyl sulfate was also considered a predictor of CKD progression (Wu et al., 2011). In animal models, indoxyl sulfate was associated to the renal expression of genes related to tubulointerstitial fibrosis, such as TGF- β 1 and tissue inhibitor of metalloproteinases (Miyazaki, Ise, Seo, & Niwa, 1997), as well as podocytes proinflammatory phenotype, decreased expression of podocyte-specific genes, and decreased cell viability (Ichii et al., 2014). Moreover, the elevated levels of indoxyl sulfate were associated with vascular stiffness, aortic calcification, and higher CV mortality (Barreto et al., 2009).

Together indoxyl sulfate and p-cresol sulfate induced changes in the epithelial mesenchymal phenotypic of renal proximal tubular cells, activated the intrarenal renin—angiotensin—aldosterone system, promoted interstitial fibrosis and glomerulosclerosis (Nallu et al., 2016; Sun, Chang, & Wu, 2012).

Another important uremic toxin is trimethylamine N-oxide (TMAO). This compound is generated by gut bacteria, by conversion of choline and betaine present in food into trimethylamine, which is then oxidized into TMAO. The levels of TMAO are increased in patients with CKD and have been associated with tubulointerstitial fibrosis, atherosclerosis, and increased risk for major CV events (Tang et al., 2015; Wang et al., 2011). A recent publication by Missailidis and coworkers (Missailidis et al., 2016) demonstrated that elevated TMAO levels are strongly associated with a decrease of renal function and normalize after renal transplantation. In addition, the levels of this metabolite correlate with increased systemic inflammation and are an independent predictor of mortality in CKD patients included in stages 3–5.

Interestingly, after renal transplantation a substantially decrease of colonic microbiota-derived uremic retention solutes was observed, suggesting that renal solute handling may differ between transplant recipients and CKD patients (Poesen, Evenepoel, et al., 2016).



4. INTESTINAL TRANSLOCATION, INFLAMMATION, AND CARDIOVASCULAR RISK IN CHRONIC KIDNEY DISEASE PATIENTS

Systemic inflammation is a persistent condition in CKD patients, and it is linked to other common sequels in CKD, such as acquired immune dysfunction, protein-energy wasting, and accelerated vascular aging that promote premature CV disease and infections, the two leading causes of death in CKD patients (Machowska, Carrero, Lindholm, & Stenvinkel, 2016). Gupta et al. (2012) demonstrated that the elevated plasma levels of high-sensitivity C-Reactive protein (hs-CRP) and interleukin-6 in CKD patients are associated with left ventricular hypertrophy and systolic dysfunction.

The microbiota plays an important role in immune system stimulation. In fact, germ-free mice display severe immune disorders, and gut dysbiosis in man was associated to the development and progression of numerous chronic diseases, such as inflammatory bowel diseases, diabetes, dyslipidemia, obesity, cancer, allergic disorders, and IgA nephropathy (Backhed, Manchester, Semenkovich, & Gordon, 2007; Brugman et al., 2006; Coppo, 2015; Frank et al., 2007; Huycke & Gaskins, 2004; Isolauri, Kalliomaki, Laitinen, & Salminen, 2008; Vaziri, Zhao, & Pahl, 2015). Also, it was recently shown that expansion of the regulatory T cells induced by microbial SCFA (propionate, acetate, and butyrate) helps to attenuate inflammation by suppressing the activity of inflammatory cells (Krishnamurthy et al., 2012). These facts reveal the importance of the gut microbiota in the host immune system regulation. *Bifidobacterium* spp. and *Lactobacillus* spp. play an important role in the maintenance of intestinal epithelial barrier as previously discussed under Section 2.1. A reduction in the levels of these two groups of protective bacteria as a result of gut dysbiosis in CKD may potentiate a disruption of the intestinal epithelial barrier. In agreement, previous reports suggested that an impairment of the intestinal epithelial barrier structure and function occurs in CKD, allowing entry of gut-derived uremic toxins in to the systemic circulation (Magnusson, Magnusson, Sundqvist, & Denneberg, 1990, 1991; Nallu et al., 2016; Vaziri, Goshtasbi, et al., 2012; Vaziri, Yuan, Nazertehrani, Ni, & Liu, 2013; Vaziri, Yuan, & Norris, 2013; Vaziri, Yuan, et al., 2012). Ammonia, a product of urea metabolism by gut microbiota, was shown to be a major mediator of uremia-induced intestinal barrier disruption, causing a massive depletion of the gastrointestinal epithelial

tight junction proteins in CKD animals and a significant depletion of the tight junction proteins and reduction of *trans*-epithelial electrical resistance in cultured human colonocytes in vitro (Vaziri, Goshtasbi, et al., 2012; Vaziri, Yuan, Khazaeli, et al., 2013; Vaziri, Yuan, Nazertehrani, et al., 2013; Vaziri, Yuan, & Norris, 2013; Vaziri, Yuan, et al., 2012). The toxic effect of phenol on intestinal epithelial cells has been also demonstrated in in vitro studies (Hughes et al., 2008), suggesting that phenol is a potential driver of gut-barrier alterations. On the other hand, indolic compounds were shown to improve intestinal cell barrier function and to decrease pro-inflammatory IL-8 expression.

The impairment of intestinal epithelial barrier structure and function has been described in other pathologies, such as inflammatory bowel diseases, celiac disease, food allergy, irritable bowel syndrome, obesity, and other metabolic diseases (Bischoff et al., 2014).

In CKD, the impairment of the intestinal barrier function may enable the translocation of intestinal microorganisms; endotoxin, antigens, and other microbial products to the intestinal wall; systemic circulation; and the internal milieu, thus suggesting that the presence of the gastrointestinal barrier dysfunction may play a role in the pathogenesis of systemic inflammation in uremic humans and animals. Accordingly, ESRD patients commonly exhibit histological evidence of chronic inflammation throughout the gastrointestinal tract (Vaziri et al., 1985) and consistently present endotoxemia in the absence of clinical infection (Feroze et al., 2012; Goncalves et al., 2006; Szeto et al., 2008). Endotoxemia in CKD patients was associated with systemic inflammation, markers of malnutrition, cardiac injury, and reduced survival (McIntyre et al., 2011). Moreover, plasma levels of the endotoxin-soluble receptor sCD14 were considered an independent predictor of mortality in patients with ESRD (Raj et al., 2009). Curiously, the presence of microbiota in the blood has been implicated in atherosclerosis, type II diabetes, and CV disease and was suggested to represent the first step in the disease kinetics, and ultimately serve as biomarkers for CV disease risk (Amar et al., 2013, 2011; Potgieter, Bester, Kell, & Pretorius, 2015; Sato et al., 2014). Moreover, colonic bacterial DNA has been detected in the mesenteric lymph nodes, blood, liver, and spleen of animals with experimental CKD (Wang, Zhang, et al., 2012).

The mechanisms underlying the relationship between gut dysbiosis and increased CV risk in CKD are depicted in Fig. 3.

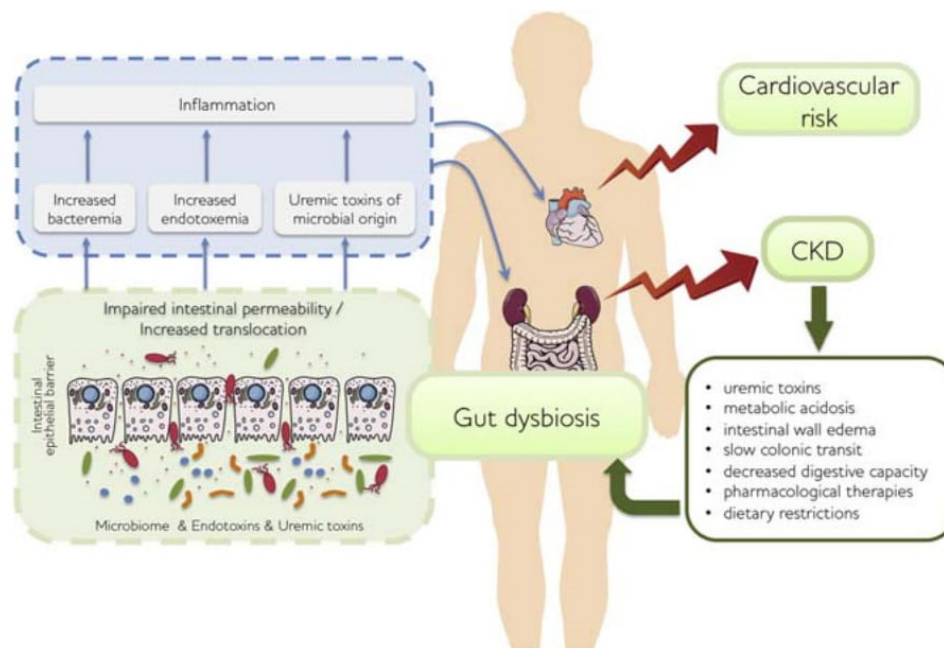


Figure 3 Mechanisms underlying the relation between gut dysbiosis and increased cardiovascular (CV) risk in chronic kidney disease (CKD). *Figure was produced using Servier Medical Art, <http://www.servier.com/Powerpoint-image-bank>.*

5. PREVENTION STRATEGIES OF GUT DYSBIOSIS IN CHRONIC KIDNEY DISEASE

The human microbiome itself may have protective effects at the kidney level. Studies conducted in animal models showed that germ-free mice present an increased susceptibility to acute kidney injury (Wing et al., 2015). This can occur through the production of beneficial metabolites by intestinal bacteria, such as SCFAs that serve as a major source of energy for colonocytes, since it was shown that treatment with the SCFAs acetate, propionate, and butyrate improved renal dysfunction and reduced local and systemic inflammation (Andrade-Oliveira et al., 2015). Also, mice treated with acetate-producing bacteria had better outcomes after acute kidney injury (Wing et al., 2015).

Understanding the role of gut dysbiosis in the pathogenesis of CKD may contribute to develop new strategies to prevent the increased CV risk and disease progression in CKD. Several approaches have been attempted in animal models and humans with CKD, such as administration of oral adsorbents to limit absorption of the toxins of microbial origin, probiotics,

prebiotics, and a combination of prebiotics and probiotics (synbiotics) (Vaziri, 2016).

Oral adsorbents, namely oral-activated charcoal (AST-120), were used to reduce the systemic gut-derived uremic toxin absorption through gastrointestinal sequestration. Currently, the results are not clear about the real effectiveness of this strategy (Schulman et al., 2006, 2015; Wu et al., 2014). Notwithstanding, Vaziri, Yuan, Khazaeli, et al. (2013) showed a significant reduction of endotoxemia as well as in plasma concentration of inflammatory cytokines, chemokines, and adhesion molecules in CKD animals treated with oral-activated charcoal. Other investigators reported that the oral administration of AST-120 to predialysis patients may diminish the levels of indoxyl sulfate and delay the time to dialysis initiation (Hatakeyama et al., 2012; Sato et al., 2015).

Other targeted interventions focused on the modulation of the gut microbiome to revert gut dysbiosis in CKD. Active ingredients that modify the gut microbiota, including probiotics, prebiotics, or synbiotics, are being scrutinized in in vitro models, in animals, and in human CKD studies trying to identify potential therapeutic strategies that modulate gut microbiome.

The results obtained so far with probiotics alone were not very conclusive (Ando et al., 2003; Natarajan et al., 2014; Ranganathan et al., 2009, 2005, 2010). Ranganathan et al. (2006) attempted to use the alkalophilic urease-positive bacterium *Sporosarcina pasteurii* (Sp) in CKD rats to decrease the blood urea—nitrogen levels. Although a slight reduction in blood urea—nitrogen levels was observed, the resulting concomitant increase in ammonia levels may represent a significant harmful side effect that impairs the intestinal barrier and contributes to systemic inflammation (Vaziri, Goshtasbi, et al., 2012). Also, as explained in a review by Vaziri (2016), ammonia generated in the gut is transported via portal vein to the liver, where it is converted to urea, thus explaining the lack of significant reduction in urea level in the treated subjects.

In uremia, the intestinal lumen presents a very specific environment that interferes with bacterial growth, in particular probiotics. If this intestinal adverse milieu remains unaltered, probiotics will not grow effectively, justifying its ineffectiveness in the attenuation of CKD gut dysbiosis. The modification of intestinal milieu can occur with the use of prebiotics. Several oligosaccharides tested in CKD were found to reduce the serum levels of p-cresol sulfate and in some cases indoxyl sulfate in HD patients and CKD animals (Furuse et al., 2014; Koppe et al., 2013; Meijers, De Preter, Verbeke, Vanrenterghem, & Evenepoel, 2010). Also, a diet with a high

content in amylose-resistant starch was shown to slow CKD progression and attenuate oxidative stress and inflammation in rats (Vaziri et al., 2014), as well as to reduce the plasma levels of the colon-derived solutes, indoxyl sulfate, and possibly p-cresol sulfate, without the need to intensify dialysis treatments in HD patients (Sirich, Plummer, Gardner, Hostetter, & Meyer, 2014).

The combination of prebiotics with probiotics—synbiotics—appears to be the most efficient strategy. So far, three clinical trials showed significant reductions in both p-cresol sulfate and indoxyl sulfate in patients with CKD or ESRD with the administration of synbiotics (Guida et al., 2014; Rossi et al., 2016).

Other strategies were also described, such as, the treatment with Acarbose. This compound is an inhibitor of α -glucosidase enzymes in the intestinal brush border. It blocks the hydrolysis of poly- and oligosaccharides increasing its availability for gut microbiome and promotes a reduction in the colonic generation of p-cresol in healthy persons (Evenepoel, Bammens, Verbeke, & Vanrenterghem, 2006). Another possibility is treatment with Sevelamer, a large cationic polymer phosphate binder that also binds endotoxins and reduces endotoxin and sCD14 levels in HD patients (Navarro-Gonzalez et al., 2011). Lubiprostone, a synthetic derivative of prostaglandin, was associated with attenuated inflammation in the kidney, improvement in microbiome profile with proliferation of saccharolytic bacteria, and decrease in microbiome-derived uremic toxins (Mishima et al., 2015). Also, 3, 3-dimethyl-1-butanol (DMB), a microbial trimethylamine (TMA) formation inhibitor, reduced plasma TMAO levels and atherosclerotic lesions in mice (Wang et al., 2015).

Ramezani and Raj (2014), in their comprehensive review, propose future targeted interventions to treat intestinal dysbiosis in CKD, such as gut microbiome transplantation.



6. CONCLUSION

In the last few years a number of researchers are unraveling the role of the gut and its microbiome on CKD progression and the associated increased CV risk. However, a more interdisciplinary approach is needed to further clarify the pathogenic role of the intestinal microbiota in kidney disease.

Gut dysbiosis is starting to be characterized in CKD. So far, lower levels of Bifidobacteriaceae and Lactobacillaceae and higher levels of

Enterobacteriaceae were consistently described in several studies; however, the characterization of the gut dysbiosis in CKD up to species level is desired. Also, information about the impact of CKD in gut archaea, fungi, and virus communities may be relevant to fully understand and further characterize the gut dysbiosis in CKD. The ultimate goal is to understand if gut dysbiosis or uremic toxins of microbial origin can be used as CKD biomarkers as well as if the manipulation of gut microbiota can represent a preventive measure for CV risk and CKD progression.

GLOSSARY

Chronic kidney disease CKD is characterized by abnormalities of kidney structure or function for three or more months, irrespective of the cause. CKD may be detected by decreased glomerular filtration rate (GFR, <60 mL/min/1.73 m²), by increased rates of urinary albumin excretion or by abnormal kidney structure detected by imaging.

Endotoxemia Presence of endotoxins in the blood, which may result in shock. Endotoxins refer to the lipid component (lipid A) of the lipopolysaccharides (LPS) present in the outer wall of most Gram-negative bacteria that may be released in the blood when the bacterial cell wall is disrupted. Endotoxins may elicit strong immune responses.

End-stage renal disease Patients in stage 5 of CKD, who have severe loss of renal function (GFR <15 mL/min/1.73 m²) and require renal replacement therapy in the form of hemodialysis, peritoneal dialysis, or renal transplantation.

Glomerular filtration rate GFR refers to the flow rate of filtered blood through renal glomerulus. GFR is usually estimated (eGFR) using serum creatinine and one of several available equations and is useful for staging CKD from normal kidney function to end-stage kidney disease: >90 (stage 1); 60–89; 45–59; 30–44; 15–29; <15 mL/min/1.73 m² (stage 5).

Gut dysbiosis Imbalance of the intestinal microbiota that results in alterations of gastrointestinal tract activity producing deleterious effects.

Human microbiome The collection of all symbiotic, commensal, and pathogenic microorganisms living in association with the human body, composed of bacteria, archaea, fungi, protozoa, and viruses.

Pathobionts Symbiotic microorganisms that present the potential to become pathogenic to the host under specific circumstances.

Prebiotics Nondigestible fiber compounds used as substrate for selective gut microbiota. Prebiotics induce specific changes, both in the composition and/or activity of the gastrointestinal microbiota, conferring benefit to the host.

Probiotics Live microorganisms that, when administered in adequate amounts, may confer benefits to the host.

Synbiotics Mixture of pre- and probiotics.

Uremia Uremia or uremic state is a clinical state that occurs in patients with kidney failure and cannot be explained by disturbances in extracellular volume, inorganic ion concentrations (for example, hyperkalemia), or lack of known renal synthetic products (for example, erythropoietin). It is presumed that uremia is established by the accumulation of uremic toxins.

Uremic toxins Organic waste products that are normally cleared by the kidneys and accumulated in the plasma and/or tissues of CKD patients. More than 80 uremic toxins are known, including urea, D-amino acids, low molecular weight proteins, guanidines, aromatic compounds, tryptophan metabolites, aliphatic amines, among others.

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CHAPTER II

“The microbiome in chronic kidney disease patients undergoing hemodialysis and peritoneal dialysis.”

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The increasing knowledge on the human microbiome and its role in health or disease state has changed our view regarding the relevance of microorganisms as an ecological community and not only as potential infectious agents. In this chapter, we review the existent literature reporting the microbiome of ESRD patients undergoing RRT, in specific HD and PD. These appear to influence the gut microbiome composition, and also other microbiomes.

“The microbiome in chronic kidney disease patients undergoing hemodialysis and peritoneal dialysis.”

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L. Simões-Silva participated in the review of the literature and writing of the manuscript.



Review

The microbiome in chronic kidney disease patients undergoing hemodialysis and peritoneal dialysis

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ABSTRACT

Chronic kidney disease (CKD) is associated with an imbalanced human microbiome due not only to CKD-associated factors such as uremia, increased inflammation and immunosuppression, but also to pharmacological therapies and dietary restrictions. End-stage renal disease patients require renal replacement therapies commonly in the form of hemodialysis (HD) or peritoneal dialysis (PD). HD implies the existence of a vascular access, such as an arteriovenous fistula/graft or a venous catheter, whereas PD implies a long-term peritoneal catheter and the constant inflow of peritoneal dialysate. Also, dietary adaptations are mandatory in both therapies. This revision explores the impact of HD or PD therapies on human microbiome.

HD and PD appear to be associated with different changes in the gut microbiome, for example a decrease in Proteobacteria relative abundance in HD patients and increase in PD patients. Both therapies may also have an impact on the human microbiome beyond the gut, leading to increased relative abundance of specific bacteria in the blood microbiome of HD patients and increased relative abundance of other bacteria in the peritoneal microbiome of PD patients. HD and PD catheter biofilms may also play an important role in the changes observed in these microbiomes.

A more interdisciplinary approach is needed to further clarify the role of microbial groups other than bacteria in all body habitats to allow the complete understanding of the impact of HD or PD on the microbiome of CKD patients. Moreover, strategies that promote a healthy balance of the human microbiome on these patients should be explored.

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1. Introduction

The term human microbiome was firstly defined by Lederberg & McCray [1] as the collection of all symbiotic, commensal and pathogenic microorganisms living in association with the human body. It is currently estimated that human microbiome encompasses 10 times more cells and 100 times more genes than the host, and includes a range of organisms, such as bacteria, archaea, fungi, protozoa and viruses [2]. The human microbiome is considered by some authors a metabolically active endogenous “organ” in itself [2–4] that influences the well-being of the host by contributing to its nutrition, metabolism, physiology and immune function [2,5–7]. The microbiome is now described as a central part of human health. Therefore, it is essential to study its composition and take advantage of the continuous improvement of technology to increase the existing knowledge on microorganisms-host interaction and its role in disease development.

Each individual harbors a unique microbiome with differences in species, abundance and diversity of the microbial communities in various body sites [8]. Much of this diversity found between individuals has been attributed to differences in host genetics, geographical origin, age, lifestyle, dietary habits, and exposure to antibiotics [8,9]. In healthy individuals, Bacteroidetes and Firmicutes contribute to 49% of all species of the so far characterized human microbiome, being Actinobacteria, Proteobacteria and Verrucomicrobia in a second line of colonization [10].

The homeostasis of the human microbiome is largely dependent on environmental conditions and, therefore, this complex system is greatly influenced by health and disease states and the subsequent therapeutic strategies used. One of the most well studied microbiome is the gut microbiome. Disturbances of the normal gut microbiome, known as gut dysbiosis, are currently recognized in the pathogenesis of diverse chronic diseases, such as obesity [11,12], insulin resistance and diabetes [13–15], inflammatory bowel disease [16], liver cirrhosis [17], myocardial ischemia [18], cancer [19] and neuronal disorders [20–22]. Gut dysbiosis has also been reported in chronic kidney disease (CKD), in either humans or animal models [23–31].

The effect of some therapeutics on microbial composition has been intensively studied. For instance antibiotics induce long-term decreases in bacterial diversity and their effects may be hard to reverse [10,32] and corticosteroids and immunosuppressive agents were also shown to disrupt microbial networks in allograft recipients [33,34]. Moreover, diet strongly influences the composition of human microbiome in a way we are just now starting to understand [18].

End-stage renal disease (ESRD) patients are a complex group of patients with many comorbidities, such as diabetes and hypertension, subjected to life-long medications including frequently antibiotherapy and appropriate diet recommendations, as the avoidance of sodium and phosphate-rich foods [35]. The most common renal replacement (RRT) therapies, hemodialysis (HD) and peritoneal dialysis (PD), present particularities that may have a further impact on the human microbiome. Hemodialysis implies the existence of a vascular access, in the form of an arteriovenous fistula/graft or a venous catheter, and strict dietary restrictions, as limitation of potassium intake. Peritoneal dialysis implies a long-term peritoneal catheter and the constant inflow of peritoneal dialysate containing a primary microbial nutrient, glucose, as well

as sodium, chloride, calcium and lactate (or bicarbonate) [36]. Therefore, in this review, we will explore the influence of the RRTs, HD and PD, on the human microbiome of ESRD patients.

2. Chronic kidney disease and renal replacement therapies

Due to the increasing number of patients, CKD is currently considered a public health problem worldwide [37]. CKD has a complex etiology involving both an inherited predisposition and exposure to environmental factors [38,39]. Within health determinants, the most common causes of CKD worldwide are diabetes and hypertension [37]. Cardiovascular (CV) disease is the major cause of death in CKD patients. Although the life expectancy of patients with ESRD markedly improved since the introduction of RRT in the 1960s, it remains lower than that of the general population [40]. When compared to patients on HD or PD, kidney transplant recipients present reduced CV events and mortality as well as a superior quality of life. However, kidney transplantation as a RRT cannot be performed in all ESRD patients due associated comorbidities and lack of suitable donors. Therefore most patients with ESRD are submitted to dialysis during their lifetime, either HD or PD [41].

Several nutritional guidelines are followed by CKD patients depending on the existence and type of RRT and are mainly focused on protein, energy and ion requirements [42,43]. The dietary intake recommendations for CKD patients, with and without dialysis therapy are summarized in Table 1 [42,44].

In patients undergoing HD, the incidence of vascular access-related infections varies among medical facilities and is closely related to the type of vascular access used, being lowest in HD patients with arteriovenous fistulas or arteriovenous grafts and highest in patients with tunneled and non-tunneled Central Venous Catheters (CVCs) [45,46]. In HD patients, catheter-related bacteremia can have two sources: 1) migration from the skin to the exterior of the catheter and from there to the bloodstream, and 2) direct inoculation from a biofilm growing on the inner surface of the catheter to the bloodstream [47]. The most common agent responsible for CVC-related bloodstream infections is *Staphylococcus aureus*, followed by coagulase-negative staphylococci [45,48]. Together they account for 40–80% of cases in most studies [49]. In addition, *S. aureus* bacteremia is associated with significant morbidity and mortality. Non-staphylococcal dialysis CVC-related bacteremia is due predominantly to enterococci and Gram-negative rods [49]. HIV-positive dialysis patients are more likely to develop polymicrobial infections and infections due to Gram-negative and fungal pathogens [50]. Gram-negative organisms account for 30–40% of all episodes of catheter-related bacteremia and 10–20% of episodes were polymicrobial in several case series [51]. Besides septicemia, other infections also frequently occur in HD patients such as pulmonary, gastrointestinal, genitourinary and soft-tissue infections [52].

Peritonitis and exit-site/tunnel infections are the most relevant PD-related infections and these complications remain the Achilles' heel of the technique, being responsible for catheter loss, transfer to HD, prolonged hospitalization and, in more serious cases, death [53]. Thus, prevention of PD-related infections is critical to the success of the program. Peritonitis in PD patients are frequently caused by single microorganisms, being Gram-positive bacteria responsible for 65% of the cases followed by Gram-negative bacteria (15–24%) and fungi (1.5%–5%) [49,54–57]. Within Gram-positive

Table 1
Dietary intake recommendations for CKD patients, with and without dialysis therapy [42,44].

	Non-dialysis patients ^a	HD	PD
Protein ^b	0.60–0.8 g/kg/d (Low-protein diet)	>1.2 g/kg/d	1.2–1.3 g/kg/d
Energy		35 kcal/kg/d (<60 years old) ^c 30–35 kcal/kg/d (≥60 years old) ^c	
Mineral:			
- Sodium		80–100 mmol/d	
- Phosphorus		800–1000 mg and binders if elevated	
- Potassium	<1 mmol/kg if elevated		Not usually an issue

^a GFR <30 ml/min/1.73 m.^b At least 50% of high biological value.^c PD - including kcal from dialysate.

bacteria, the most common are coagulase-negative staphylococci, *S. aureus* and *Streptococcus* spp. Gram-negative organisms are more likely to promote more severe infections with poor outcomes [45]. *Pseudomonas* sp. is probably the most important cause of severe Gram-negative peritonitis in patients undergoing PD [45]. Although fungal peritonitis is less frequent, they represent a clinical challenge due to its difficult resolution. In addition, a fraction of 11–21% of PD-related peritonitis may be caused by multiple organisms [49,54,56,57].

Few studies described the microorganisms responsible for exit-site infections. A study by Scalapogna et al. [58] reported the following organisms, in diminishing order of frequency from 102 exit-site infection episodes: *S. aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Enterobacter cloacae*. A more recent study evaluating 34 exit-site infection episodes reported *P. aeruginosa* as the most common infective organism, responsible for 58.8% of the cases. *S. aureus* was isolated in 29.4% of the cases, and *Klebsiella*, *E. coli* and *Serratia* species in 12% of the cases [59]. Topical application of antimicrobial agents such as mupirocin, gentamicin and polysporin triple ointment have been successfully used to prevent infections [60,61].

The complete list of factors responsible for PD-related infections remains not fully understood, nevertheless the most critical factor appears to be the contamination of the peritoneal catheter with skin-related bacteria [62–64]. Some pathogens may colonize the catheter's exit-site and tunnel during the connection and disconnection procedures of the dialysis transfer-set.

The existence of biofilms on peritoneal catheters was demonstrated long ago from skin-associated bacteria [65,66]. PD-catheter biofilms are formed by common Gram-positive and Gram-negative bacteria and are frequently associated with clinical peritonitis. Microbial biofilms may persist on the catheter surface, even in the absence of infection, suggesting that it may act as a reservoir of infectious agents; for a review see [67].

Microorganisms colonizing the catheter exit-site can also be associated with peritonitis, but curiously only in a very low percentage of patients [68–70]. Catheter replacement can be sometimes ineffective in preventing subsequent episodes of peritonitis, suggesting other sources of infection [45]. Therefore, alternative routes and factors have been recognized as responsible for peritonitis in PD patients, such as transvisceral microbial migration through the impaired intestinal barrier (e.g., bowel leak), hematogenous and vaginal leak [71]. Previous reports described bacterial DNA in PD effluent or peritoneum cells what supports this hypothesis [72–75]. In addition, the intracellular viability of *S. aureus* after sequestration by mesothelial cells was revealed in different studies [76,77]. Lately, the description of the blood microbiome in a non-infectious state added more clues to the limitations affecting PD technique [78–82]. Together these studies reinforce the need to explore the human microbiome in PD patients. This is not only necessary to fully understand the impact of microorganisms in the correct implemen-

tation of this RRT technique, but also to identify and limit all routes of infection in these patients.

3. The human microbiome and renal disease

3.1. The microbiome in CKD

As already stated, gut dysbiosis is well described in CKD [23–31]. This intestinal microbial imbalance occurs either quantitative and qualitatively, being frequently associated with the overgrowth of Enterobacteriaceae, Lachnospiraceae and some Ruminococcaceae, and with the reduction of some Bacteroidaceae, Prevotellaceae and particular *Bifidobacterium* and *Lactobacillus* species [31]. CKD associated factors may play a role in the promotion of gut microbiota imbalance, such as increasing the intestinal availability of uremic toxins, metabolic acidosis, intestinal wall edema and reducing the colonic transit and digestive capacity. Pharmacological therapies (e.g., antibiotics and iron deliver) and dietary restrictions (e.g. reduced fiber intake) also alter gut microbiome and may play a significant role on gut dysbiosis [31]. Within the intestinal epithelia, commensal bacteria compete with pathogens for epithelial adhesion sites and nutrients. In a dysbiotic state this natural defense is reduced, consequently increasing CKD patients' vulnerability to pathogen invasion.

Gut dysbiosis itself plays a role in CKD progression and it is starting to be recognized as a non-traditional factor for CV risk in CKD patients [31]. For example, gut microbiome-produced metabolic compounds, such as indoxyl sulfate, p-cresol sulfate, and trimethylamine N-oxide (TMAO), are associated with the promotion of CV events [3,18,83–86]. Uremia is also associated to immune dysfunction characterized by immunodepression in CKD patients, contributing to a high prevalence of infections, increased inflammation and CV risk; for a review see [87]. Moreover, ammonia derived from metabolism of urea by microbial urease, was shown to cause a massive disruption of the intestinal epithelial barrier structure and function [88–92], allowing the translocation of gut derived uremic toxins, endotoxin, antigens and intestinal microorganisms or other microbial products into circulation [3,88,90–94]. This 'atopobiosis' (phenomenon describing the microbes that appear in places other than their normal location) is for long recognized as an important route of endogenous infections, but more recently has been associated to the dynamics of a variety of inflammatory diseases [78]. The pathophysiological mechanisms underlying the role of gut dysbiosis in CKD were deeply explored in a previous review [31].

Although several researchers studied the role of the gut microbiome on CKD progression and increased CV risk, a more interdisciplinary approach is still needed in order to clarify the role of each microorganism in the development and progression of kidney diseases. Information regarding other microbial habitats in human body, as well as better characterization of other micro-

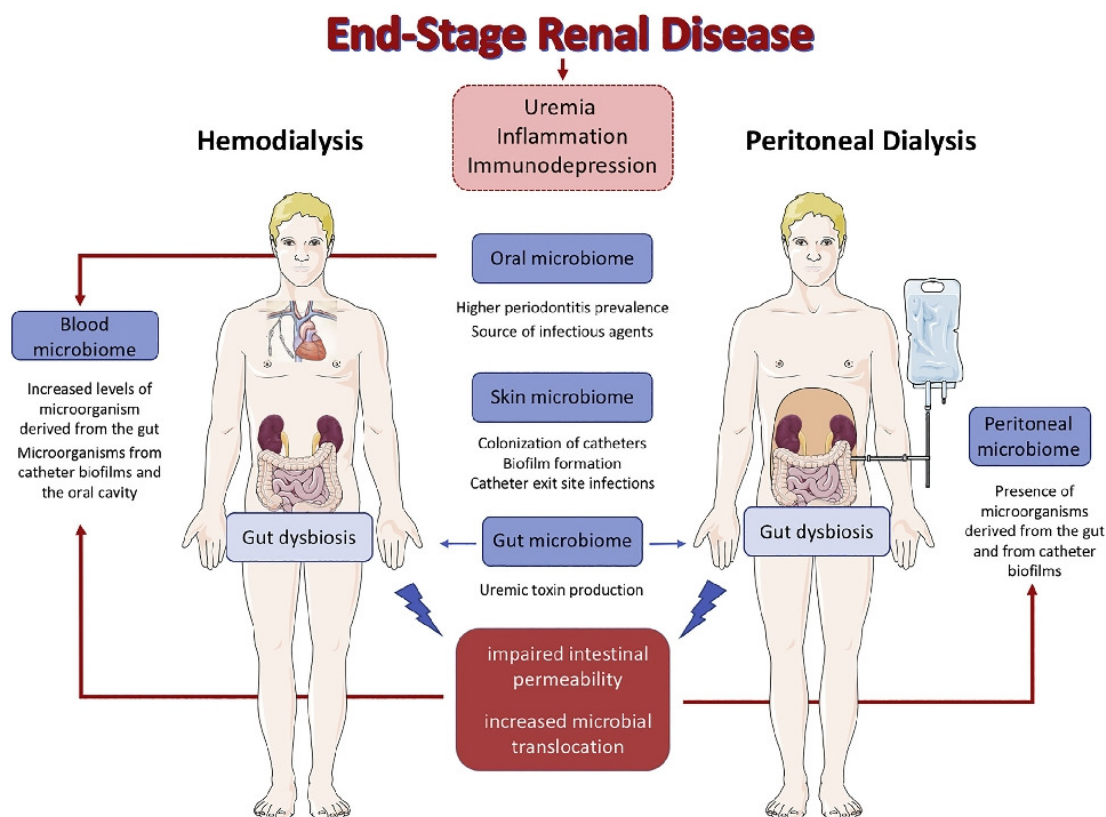


Fig. 1. Factors, complexity and diversity of microbial communities related to end-stage renal disease.

bial groups such as archaea, fungi and virus, may also be relevant to understand the complete role of microorganisms in CKD pathophysiology.

3.2. The microbiome in HD

Until now, very few studies compared the gut microbiome in HD and other RRT. However, it can be assumed that the existing specificities of this dialysis type naturally influence the intestinal microbiota of ESRD (Fig. 1). Besides the obvious influence of the vascular access, a portal of entrance of microorganisms, HD patients have specific nutritional restrictions as potassium in comparison to PD patients, as previously described. Corroborating this hypothesis is the study conducted recently by Crespo-Salgado et al. [95] that compared the gut microbiome of pediatric patients undergoing PD, HD and post-kidney transplant and healthy controls. According to the authors, the relative abundance of Bacteroidetes was significantly increased in HD patients compared to healthy controls. In addition, Proteobacteria was significantly decreased in HD patients when compared to PD patients. Regarding the α -diversity levels, the gut microbiome of HD patients was similar to controls, having higher diversity than PD patients. Although HD patients have significantly increased levels of the uremic toxins indoxyl and p-cresyl sulfates, no differences were observed in the taxa usually associated to the production of these toxins, namely Bifidobacteriaceae, Clostridiaceae, Enterobacteriaceae and Lactobacillaceae [95].

Other study analyzed the gut microbiome of ESRD patients during HD therapy and compared it with healthy persons, reporting an increase of Proteobacteria (primarily Gammaproteobacteria), Actinobacteria and Firmicutes (especially subphylum Clostridia) by phylogenetic microarrays [28]. However, because there was no comparison to CKD patients not undergoing HD, the findings can be a consequence of HD or CKD itself (or both).

A recent study carried out on HD patients analyzed the effect of a single dose of oral vancomycin (250 mg) in the gut microbiome of these patients, together with the plasma levels of uremic toxins indoxyl and p-cresyl sulfates [96]. As expected, the microbial taxonomic richness of fecal samples decreased after vancomycin administration and the effect persisted for 4 weeks. Enterobacteriaceae, Lachnospiraceae and particularly the genus *Blautia* decreased on 7th day and returned to or above baseline at 28th day after vancomycin administration, coinciding with the decline and subsequent rebound of plasma indoxyl sulfate concentrations. Regarding p-cresyl sulfate levels, vancomycin also induced its decrease in the plasma. This result could be explained by suppression of specific tryptophan or tyrosine degrading microbiota and/or by an increase in competing taxa that bloomed in the vancomycin-perturbed gut [96]. However, the recovery of uremic toxin levels 28 days after vancomycin administration suggests resilience of the taxa responsible for its generation in ESRD patients. Enterococcaceae, Bacteroidales and the genus *Bilophila* also diminished significantly following treatment (7 days after), while Veillonellaceae increased. There was a continued significant

decrease in Clostridiales and Lachnospiraceae (*Roseburia* sp.) even 28 days after the vancomycin administration.

Besides the gut, other microbiomes have been characterized in the HD population. Bossola and colleagues [97] analyzed blood DNA by 16S rRNA gene PCR amplification, followed by amplicons purification and sanger sequencing and showed that 20.7% of 58 HD patients presented bacterial DNA in their whole blood samples obtained either from the peripheral vein or from the central venous catheter or the arteriovenous fistula, in contrast to the absence of blood microbiome in healthy controls. The following species were found in HD patient's blood: *E. coli*, *S. aureus*, *P. aeruginosa*, *S. epidermidis*, *E. faecalis*, *Proteus mirabilis*, and *Staphylococcus haemolyticus*. Moreover, blood cultures of HD patients and healthy controls were all negative for bacterial growth, suggesting very low levels of microbial load (not detected by this methodology) or lack of microbial viability. In addition, these authors associated the circulating bacterial-derived DNA found in HD patients with higher levels of inflammatory markers such as high-sensitivity C-reactive protein (CRP) and IL-6. More recently, Shi et al. [98] explored the blood microbiome of 22 HD patients and compared to 30 ESRD non-dialysis patients and 10 healthy controls using next-generation sequencing. Blood microbiome was found in 27%, 20% and 0% of HD, ESRD non-dialysis and controls, respectively. The bacterial blood colonization within ESRD patients (HD and non-dialysis patients) was similar, being Firmicutes, Bacteroidetes and Proteobacteria the dominating bacteria phylum. At the genus level, the prevalent bacteria common in both groups were *Escherichia-Shigella*, *Prevotella*, *Faecalibacterium*, *Bacteroides*, and *Ruminococcus*. However, in comparison to ESRD non-dialysis patients, HD patients showed greater complexity at the species level that was correlated with higher levels of CRP and endotoxins, and elevated gut permeability. The authors suggested that the bloodstream bacteria mainly originate from the ESRD dysbiotic gut microbiome and the HD, to some extent, aggravates microinflammation in these patients via promotion of gut microbiota translocation due to the gut barrier impairment. Although with a limited number of patients, other studies showed bacterial DNA in the whole blood of dialysis patients without signs of active infection [99–101].

The periodontal microbiome of HD patients was characterized in a 2015 pilot study focused on the high prevalence of periodontitis in these patients [102]. No major differences were observed in the subgingival microbiome between ESRD and control individuals, but *Prevotella gingivalis* and *Tannerella forsythia* appeared among the most abundant periodontitis-related phylotypes in a subgroup of patients with greater periodontitis extent. The authors highlighted the negative correlation between dialysis vintage and microbial diversity within the ESRD group. In addition, it was suggested that widespread and localized forms of periodontitis could be associated with different microbiomes, particularly if comparing some specific OTUs [102]. Moreover, Kshirsagar et al. [103] described a moderate-to-severe periodontal disease strongly associated with CV mortality in HD patients. Other studies have associated the oral microbiome to the onset of systemic diseases, such as atherosclerosis [104], insulin resistance and CV risk [105,106].

3.3. The microbiome in PD

As PD is not a frequent option of RRT, studies in these patients' population are also scarce. To our knowledge, there are only two studies evaluating the gut microbiome in PD patients. As mentioned above, the gut microbiome of pediatric patients (from 2 to 18 years old) undergoing PD or HD and age-matched healthy controls was compared, showing the α -diversity significantly decreased in PD patients using both phylogenetic and non-phylogenetic diversity measures [95]. The loss of bacterial biodiversity is the most frequent result following intestinal dysbiosis [107] and it has been

described for infants with necrotizing enterocolitis [108], inflammatory bowel disease [109], colorectal cancer [110] and CKD [31].

Pediatric PD patients showed relative lower abundance of gut bacteria within the Firmicutes and Actinobacteria, whereas the Proteobacteria were significantly increased [95]. The higher levels of Proteobacteria (iron oxidizing bacteria) could be associated with the oral iron supplementation of PD patients (HD patients receive it directly through the bloodstream). Also, PD patients have increased intestinal absorption of glucose from the PD dialysate that promotes glucose fermentable bacteria, such as the Enterobacteriaceae [95]. Taking into consideration the translocation of gut microbiota to the peritoneal cavity, the increase in intestinal Enterobacteriaceae may be responsible for the development of peritonitis in PD patients. Note that members of the Enterobacteriaceae family account for up to 12% of all peritonitis episodes in these patients [111].

In another study, Wang et al. [112] compared adult PD patients and control individuals by species-specific real-time PCR. They described a decrease of gut Firmicutes and Actinobacteria, especially *Bifidobacterium catenulatum*, *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Lactobacillus plantarum* and *Lactobacillus paracasei* in adult PD patients [112]. In general, CKD patients showed lower intestinal colonization by *Lactobacillus* and *Bifidobacterium* species [31]. These bacteria are normal colonizers of the human gut and play a critical and beneficial role by inhibiting the growth of pathogens through the production of acetic acid and lactic acid, lowering the pH in the intestinal milieu and competing with pathogens that could colonize the gut mucosa for epithelial adhesion sites and nutrients [113,114]. Both groups participate actively on the regulation of intestinal microbial homeostasis and may help to reduce the rate of constipation – these bacteria are rarely isolated on adults with chronic constipation [115]. So, reduced populations and diversity of *Lactobacillus* and *Bifidobacterium* in PD patients may be associated to several adverse effects.

Another interesting result from Wang et al. study [112] was the higher prevalence of *P. aeruginosa* in the fecal samples from PD patients. *Pseudomonas* is not a very frequent agent of gram-negative peritonitis, but it is probably one of the most important causes of severe peritonitis in PD patients and is responsible for around 40% of catheter-related infection removals [45,116–118]. However, due to the lack of CKD controls in the study we cannot clarify if the alterations in microbial species reported by Wang and colleagues [112] are exclusively related to CKD or may be associated to the dialysis therapy.

As described for HD patients, for PD patients the oral cavity may also represent a starting point for dissemination of pathogens through the human body due to the proximity of oral microorganisms to the bloodstream [119]. The oral cavity represents a common but transient source of microorganisms that may be associated to some routine daily activities or invasive dental procedures [119,120]. Preliminary studies suggest that the oral environment of PD patients in comparison to healthy controls may induce changes in oral microbial colonization [121].

As previously stated, gut dysbiosis may impair the intestinal barrier in PD patients promoting 'atopobiosis', namely the translocation of enteric organisms or bacteria-derived toxins to the peritoneal cavity by migration through epithelial intestinal barrier. This mechanism may represent an important cause of peritonitis in PD patients. The detection of bacterial DNA in PD effluent corroborates the hypothesis of 'atopobiosis' in PD patients [74,75,77]. Moreover, our group recently reported the asymptomatic protozoa colonization in peritoneal dialysate of PD patients [122], highlighting the magnitude of intestinal translocations in PD patients. And so, an additional route for PD-related infections that needs further research is the peritoneal microbiome (Fig. 1). Recent studies are unravelling microbiome in body sites that were previously consid-

ered sterile, as the peritoneum. Other examples are the recently revealed placenta microbiome [7,123] and breast tissue microbiome [124]. These studies suggest that microorganisms reach these tissues by spreading through hematogenous route. Reinforcing this view are the studies recognizing blood microbiome in a non-infectious state [78–82]. Preliminary results from our group demonstrated that the peritoneum tissue of CKD patients harbours a unique low-abundance microbiome (data not published).

4. Perspectives on patient nutrition and supplements

The microbiome protection and recovery of HD and PD patients should be an emerging focus on future research. Many different strategies are being implemented with variable outcomes. For example, bionics (pre, pro and/or post) are reemerging as potential modulators of the human microbiome. Prebiotics refer to non-digestible food components that induce the growth or activity of beneficial microorganisms in the gut (e.g. bacteria and fungi); probiotics refer to the ingestion of living microorganisms and post-biotics refer to the ingestion of the bioactive microbial produced molecules.

The intake of vegetable fibers and some yogurt and cheeses have been described as problematic for ESRD patients. Nevertheless, if these foods are supplemented with potassium binding resins and phosphate binders they may result in a more balanced gut microbiome in ESRD patients [125]. A small trial with HD patients in Belgium evaluated circulating p-cresyl sulfate levels following oligofructose-enriched inulin supplementation [126]. The oligofructose-enriched inulin administration started with 10 g once daily during the first week, at day 8 the dose was escalated to 10 g twice daily, and stopped at day 28 after the midweek dialysis session. These patients were followed for another 4-week run-out period. Interestingly in these HD patients, the oligofructose-enriched inulin supplementation induced a decrease in circulating p-cresyl sulfate levels up to the run-out period. Moreover, prebiotic amylose maize resistant starch can be metabolized by colon bacteria and produce short-chain fatty acids [127]. A study in a CKD rat model showed that a high-fiber diet containing 59% high amylose maize resistant starch for 3 weeks retards CKD progression and attenuates oxidative stress and inflammation [128]. Fiber supplementation and phytochemical-rich foods can modify the composition of gut microbiota, while inulin, fructo-oligosaccharides, galacto-oligosaccharides, soya-oligosaccharides, xylo-oligosaccharides and pyrodextrins are prebiotics that have been tested in CKD patients [125,126,129].

Probiotics appear to balance intestinal microbiome following massive changes of the initial biodiversity and reduce the production of uremic toxins. For example, *Lactobacillus acidophilus* (strains NCFM and BG2F04) [26] and Lebenin® (from Wakamoto Pharmaceutical, Tokyo, Japan, includes *Bifidobacterium longum* subsp. *infantis*, *Enterococcus faecalis* and *L. acidophilus*) showed to improve health in HD patients by reducing the levels of uremic toxins [26,130]. A formulation of *Streptococcus thermophilus* (KB19), *Lactobacillus acidophilus* (KB27) and *Bifidobacterium longum* (KB31) over 3 months was shown to improve blood urea nitrogen and quality-of-life in patients with CKD stages 3 and 4 [131]. Data is still missing to completely consider the role of synbiotics, a combination of pre and probiotics, in CKD patients but a preliminary study showed a reduction of p-cresyl sulfate blood levels and *Bifidobacterium* enrichment and Ruminococcaceae depletion in stools using a symbiotic constituted by a combination of high-molecular weight inulin, fructo-oligosaccharides, and galacto-oligosaccharides together with nine different strains from *Lactobacillus*, *Bifidobacteria*, and *Streptococcus* genera [132].

Recently, the first reports on fecal microbiota transplantation were available showing an effective and safe alternative in patients post *Clostridium difficile* infection [133] but its role in the gut of CKD patients still needs to be clarified.

Other strategies are being tested in CKD patients, as the AST-120 therapy, an adsorbent consisting of porous carbon particles capable to adsorb indole in the intestine [134]; sevelamer, a large cationic polymer phosphate binder that also binds endotoxins and reduces endotoxin and CD14 levels in HD patients [135]; acarbose, an inhibitor of α -glucosidase enzymes in the intestinal brush border; among others. However, the particular efficiency of these strategies on HD or PD patients still needs to be explored.

More recently, the microbiome is looked as a therapeutic tool in it-self. “Smart” bacteria [136] may guide our immune system, metabolism, and other commensal microorganisms, envisioning that targeting and modeling specific microbiota will be the design of personal medicine programs in a near future.

With the constant development of new technologies, one day it will be possible to fully understand the function, as well as the existing interactions between microorganisms that compose the human microbiota. It can be envisioned that targeting and modeling specific microbiota will play a central role in the design of more personalized medicine programs in a near future, and vast improvements will be done for disease prevention, diagnosis and therapy.

5. Conclusion

The ultimate goal of any treatment is to improve the patient's well-being while preventing the development of disease. As they interfere with the human microbiome, some frequent therapies may need to be carefully used and/or complemented with additional protective strategies. Manipulations of the microbiota can result on changes of the host's susceptibility to diseases and/or affect its severity.

The specific impact of RRT such as HD or PD on the microbiota imbalance of CKD patients is still underexplored. HD and PD have unique characteristics that may contribute to changes in the human microbiome, not only restricted to the gut, but also affecting the blood microbiome (HD) and the peritoneal microbiome (PD) (Fig. 1). Factors promoting these changes may be related to specific dietary restrictions and different types of dialysis access (vascular vs. peritoneal), as well as the continuous inflow of peritoneal dialysate, containing microbial nutrients. Notwithstanding, understanding the impact of HD or PD on the human microbiome is still in its infancy. A more interdisciplinary approach is still needed in order to better clarify the role of microbial groups other than bacteria and expand the existing research to other human microbial habitats. Moreover, strategies to promote a healthier human microbiome in these patients should be delineated.

Conflict of interest

None (for all the authors).

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CHAPTER III

**“Oral colonization of *Staphylococcus* species in a Peritoneal Dialysis population:
a possible reservoir for PD-related infections?”**

In press in Can J Infect Dis Med Microbiol

**“What is your method of choice? Importance of methodologies in *Staphylococcus*
isolate identification”**

Submitted for publication

Staphylococcus species are the most common agents responsible for PD-related infections, causing either peritonitis or exit-site infections. In this chapter, we characterize the colonization of oral cavity of PD patients by *Staphylococcus*, in comparison to a healthy population, and study its relation to the clinical history of infections. Also, we compared the performance of different methodologies for the identification of *Staphylococcus* isolates: API STAPH 32 methodology, sequencing of the *DnaJ* gene and MS VITEK2.

“Oral colonization of *Staphylococcus* species in a Peritoneal Dialysis population: a possible reservoir for PD-related infections?”

In press Can J Infect Dis Med Microbiol

L. Simões-Silva participated in the experimental design, conducted the experiments, participated in analysis and interpretation of the data and wrote the manuscript.

Research Article

Oral Colonization of *Staphylococcus* Species in a Peritoneal Dialysis Population: A Possible Reservoir for PD-Related Infections?

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Peritoneal dialysis-related infections are important morbidity/mortality causes, being *Staphylococcus* the most prevalent agents. Since *Staphylococcus aureus* nasopharynx carriage is a known risk factor for PD infections and the oral cavity is a starting point for systemic diseases development, we aimed at comparing the oral staphylococci colonization between PD patients and controls and studying the association with PD-related infections. Saliva samples were plated in Mannitol salt, and isolates were identified by *DnaJ* gene sequencing. Staphylococci PD-related infections were recorded throughout the 4-year period following sample collection. *Staphylococcus* colonization was present in >90% of the samples from both groups (a total of nine species identified). PD patients presented less diversity and less prevalence of multispecies *Staphylococcus* colonization. Although all patients presenting *Staphylococcus epidermidis* PD-related infections were also colonized in the oral cavity by the same agent, only 1 out of 7 patients with ESI caused by *S. aureus* presented *S. aureus* oral colonization. Staphylococci are highly prevalent in the oral cavity of both groups, although PD patients presented less species diversity. The association between oral *Staphylococcus* carriage and PD-related infections was present for *S. epidermidis* but was almost nonexistent for *S. aureus*, so, further studies are still necessary to evaluate the infectious potential of oral *Staphylococcus* carriage in PD.

1. Introduction

Peritoneal dialysis (PD) is a home-based renal replacement therapy for end-stage chronic kidney disease (CKD) patients, presenting significantly lower costs and higher satisfaction levels in comparison to hemodialysis (HD) [1]. Nonetheless, infection remains an important cause of morbidity and mortality among PD patients [1, 2]. Peritonitis and exit-site infections (ESI) are the more relevant and

common PD-related infections, and *Staphylococcus* species are the most frequent etiological agents responsible for both types of infection [3, 4]. Worldwide, *Staphylococcus epidermidis* and other coagulase-negative *Staphylococcus* (CNS) are the most prevalent PD-related infectious agents. On the contrary, *Staphylococcus aureus* is associated with more severe PD-related infections leading to increased risks of hospitalization, death, and catheter removal [2, 5, 6]. *Staphylococcus haemolyticus*, *Staphylococcus warneri*, *Staphylococcus hominis*,

Staphylococcus simulans, *Staphylococcus capitis*, and *Staphylococcus saprophyticus* are staphylococci species also reported as PD-related infectious agents [7, 8].

It is assumed that contamination at the time of PD fluid exchange is a major cause of peritonitis, occurring mainly by an external route [9]. For this reason, general measures concerning exit-site care, such as meticulous hand hygiene and face-mask wear are recommended during the dialysis exchange by the International Society for Peritoneal Dialysis (ISPD) [10]. Other measure is the prophylactic use of topical antibiotic treatment of PD catheter exit-site, known to reduce ESI [4, 11], although its effect on peritonitis rate is not clear [4, 11, 12]. Also, the screening for nasal *S. aureus* carriage prior to PD catheter insertion is recommended and, if positive, treatment with topical nasal application of mupirocin is recommended [13]. The efficacy of this prophylactic intranasal antibiotic treatment has been showed in several prospective studies [14–16].

Another less explored route of infection in PD patients is the haematogenous pathway. It is known that oral microorganisms are frequently responsible for bacteraemia due to routine daily hygiene activities, such as tooth brushing and dental tape use or invasive dental procedures [17, 18]. For that reason, risk patients including those on PD therapy should receive prophylactic antibiotic treatment before dental interventions [17]. Although it is recognized that the oral cavity can be a starting point for dissemination of pathogenic organisms, by both external and haematogenous routes, to our knowledge, there are no studies linking the oral microbiome and PD-related infections. Thus, the aim of the present study was to assess the oral colonization with *Staphylococcus* spp. in PD patients in comparison with healthy controls and explore in patients on PD therapy the association between oral colonization with *Staphylococcus* spp. and PD-related infections.

2. Materials and Methods

Twenty-one end-stage CKD adult patients undergoing PD therapy followed up at the outpatient clinic of the Nephrology Department of the “Centro Hospitalar de S. João” for more than one month were invited to participate in the study. A convenience sample was obtained related with the attendance of patients to the outpatient clinic during a period of 6 months. The control group included 14 adult healthy subjects recruited among family members of PD patients, to gather individuals with the same socioeconomic and environmental conditions as PD patients. The exclusion criteria were recent history of infection (less than 1 month), inability to give informed consent, pregnancy, and severe acute illness. The study protocol was approved by the Ethics Committee for Health and Institutional Review Board of São João Hospital Centre.

Demographic and relevant clinical information was gathered for each patient including age, gender, smoking habits, education level, blood pressure, aetiology of renal disease, residual renal function, time on PD, and infectious complications during PD. Demographic information was also gathered for the control healthy population, namely, age, gender, smoking habits, and education level.

2.1. Oral Evaluation. Oral hygiene was assessed in both groups using the VPI in four sites of each tooth (mesio-buccal, midbuccal, distobuccal, and midlingual); the percentage of the examined sites with visible plaque ranged from 0% to 100%.

Additionally, before the oral clinical evaluation, unstimulated whole saliva was collected from both groups into a sterile container for 5 minutes in a quiet room, between 9:00 to 11:00 AM to minimize the circadian rhythm effects and at least 2 h after eating, tooth brushing, mouth washing, or smoking. Immediately after collection, the saliva was stored at -20°C for biochemical analysis or mixed 1:1 in Brain Heart Infusion with 20% glycerol for microbial analysis and cryopreserved at -80°C . The total volume collected over a 5 min period was registered, for saliva flow rate (mL/min) determination. The saliva pH was also measured immediately after collection using pH strips (5.0–8.0, Duotest, Germany). The saliva urea concentration was determined by an enzymatic UV test (method “urease-GLDH”) and quantified by an automatic analyser, Pentra C200 (Horiba ABX Diagnostics, Switzerland).

2.2. *Staphylococcus* spp. Isolation. *Staphylococcus* was isolated from saliva from both groups and quantified. Saliva samples were serially diluted with 0.9% sterile NaCl solution and plated in triplicate in a selective and differential culture medium, Mannitol salt agar (Liofilchem, Italy). Plates were incubated aerobically for 48 h at 37°C . The total number of colonies was determined, and the quantification results were expressed in a logarithmic scale of colony-forming units per mL of saliva ($\log_{10}\text{CFU/mL}$). The lower limit of detection was 10^2 CFU/mL . The Gram-positive and catalase-positive isolates were purified by reisolation in BHI (Biolab Inc, Budapest, Hungary) agar and stored in BHI containing 10% glycerol at -80°C . The isolated colonies were identified using the *DnaJ* gene amplification and sequencing approach.

2.3. *Staphylococcus* Identification by *DnaJ* Gene Amplification. DNA was extracted using the boiling method; briefly, 5 colonies were suspended in $100\text{ }\mu\text{L}$ of molecular grade water and incubated at 100°C for 10 minutes. The diluted suspension (1 : 10) was centrifuged at 12,000 rpm for 10 min, and the supernatant was used as a DNA template. *DnaJ* gene was amplified with the DFS Master Mix (Bioron, Germany) according to the manufacturer’s instructions using the primers Staph_forward, 5'-GCCAAAAGAGACTATTATGA-3', and Staph_reverse, 5'-ATTGYTTACCYGTTTGTGTACC-3' and the amplification conditions previously described, with MyCycler Thermal Cycler (Bio-Rad, California, USA) [19]. PCR products were visualized by electrophoresis on a 1% agarose gel (SeaKem® LE Agarose; Lonza, Cologne, Germany) with GelRed™ (GeneON, Ludwigshafen am Rhein, Germany) and then visualized in ChemiDoc XR (Bio-Rad, California, USA). PCR amplification products were identified by sequencing (StabVida, Caparica, Portugal). The identification by sequencing of *DnaJ* gene was only considered when the sequencing identity was 98% or higher to *DnaJ* sequences or

sequences resulting from whole genome sequencing deposited at GenBank [20].

2.4. PD-Related Infection Episodes. The PD-related infections by *Staphylococcus* spp. occurring from the start of PD therapy up to 4 years after sample collection were registered in all 21 studied PD patients. However, due to PD technique dropout, 20 patients remained in the first follow-up year, 19 in the second, 14 in the third, and 11 in the fourth.

2.5. Data Analysis. Data were analysed using IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp, NY, USA). The categorical variables were described through relative frequencies (%) and analysed by the chi-square independence test or Fisher Exact test when more than 1 cell had expected counts less than 5. Continuous variables were described using mean $\pm$ standard deviation (SD) and analysed by Student's *t*-test. A *P* value of less than 0.05 was assumed to denote a statistically significant difference.

3. Results

Clinical information of PD patients is included in Table 1, namely, aetiology of CKD, PD vintage, residual renal function, and blood pressure. The most prevalent aetiologies of CKD were glomerular disease, including diabetic nephropathy, and tubulointerstitial disease, namely, polycystic kidney disease. At sample collection, the average time on PD therapy was 15.45 ± 16.90 months, ranging from 1 to 72 months.

Demographic information, namely, age, gender, educational level, smoking habits, parameters regarding oral hygiene, and saliva biochemistry of both groups is included in Table 2. The two groups presented similar demographic characteristics. Also, there were no significant differences between the two groups in the education level and smoking habits. Both groups had high visual plaque indexes (VPI) values, revealing a low oral hygiene status. Regarding saliva biochemical parameters, the only significant differences were in the saliva pH and urea concentration, being both parameters higher in PD patients when compared to the control group.

The PD patients and healthy controls presented similar total load (CFU/mL) of *Staphylococcus* in saliva (Table 3). Nine *Staphylococcus* species were identified in the saliva from both groups, namely, *S. epidermidis*, *S. aureus*, *S. capitis*, *S. saprophyticus*, *S. hominis*, *Staphylococcus cohnii*, *Staphylococcus pasteuri*, *Staphylococcus lugdunensis*, and *S. warneri* (Supplementary Table 1).

No significant differences were observed in the prevalence of the different *Staphylococcus* species between the two groups. However, the prevalence of more than one *Staphylococcus* species (multispecies) in saliva was significantly higher in the control group than in PD patients (Table 4). When analyzing the colonization status of the family members of PD patients (10 pairs), we found that 6 patient-family member pairs presented oral colonization by *S.*

TABLE 1: Aetiology of chronic kidney disease (CKD), peritoneal dialysis (PD) vintage, residual renal function, and blood pressure of PD patients.

	PD patients
Aetiology of CKD	
Glomerular disease	52.38%
Diabetic nephropathy	19.05%
Other glomerular diseases	33.33%
Tubulointerstitial disease	23.81%
Autosomal dominant polycystic kidney disease	14.29%
Other tubulointerstitial diseases	9.52%
Unknown	23.81%
PD vintage (months)	15.45 ± 16.90
Residual renal function (mL/min)	6.98 ± 4.52
Blood pressure	
Systolic	130.19 ± 19.65
Diastolic	78.29 ± 10.96

Results are shown in prevalence (%); CKD, chronic kidney disease; PD, peritoneal dialysis.

TABLE 2: Demographic information and parameters regarding oral hygiene of peritoneal dialysis (PD) patients and healthy controls.

	PD patients	Controls	<i>P</i> value
Age (years)	46.8 ± 9.7	42.2 ± 14.5	0.282
Sex (male %)	42.9%	28.6%	0.392
Education level			>0.999
Basic/elementary school	85.7%	83.3%	
High school/university	14.3%	16.7%	
Smoking habits			
Past	58.3%	33.3%	0.387
At sample collection	16.7%	11.1%	>0.999
Visual plaque index (%)	59.7 ± 27.7	48.4 ± 23.1	0.254
Saliva biochemistry			
Flow rate (mL/min)	0.40 ± 0.26	0.41 ± 0.30	0.871
pH	7.72 ± 0.53	7.06 ± 0.39	<0.001
Urea (mg/dL)	110.41 ± 36.64	28.79 ± 5.90	<0.001

Results are shown in prevalence (%) or mean $\pm$ SD; PD, peritoneal dialysis.

epidermidis. One of these pairs also presented colonization by *S. saprophyticus*.

The occurrence of PD-related *Staphylococcus* infectious episodes was retrieved from clinical records from the date of entrance in the PD program until PD dropout or the end of the 4th year after oral sample collection, accounting for an average of 53.9 ± 27.8 months. The total number of patients that had at least one infection episode, peritonitis, and/or ESI is presented in Table 5, whereas in Table 6, the total number of infection episodes and the *Staphylococcus* species detected are presented.

The association between the PD-related infections by staphylococci and the oral colonization with the same *Staphylococcus* species was examined (Figure 1). All PD

TABLE 3: Prevalence and quantification of *Staphylococcus* in the oral cavity of PD patients and controls.

	PD patients	Controls	P value
Prevalence	90.5%	92.9%	>0.999
Counts (log ₁₀ CFU/mL)	2.79 ± 0.60	2.43 ± 0.64	0.120

Results are prevalence (%) or mean ± SD; PD, peritoneal dialysis; CFU, colony-forming units.

TABLE 4: Prevalence of *Staphylococcus* species identified in isolates from saliva of peritoneal dialysis (PD) patients and controls.

	PD patients	Controls	P value
<i>Staphylococcus</i> species			
<i>S. epidermidis</i>	89.5%	92.3%	>0.999
<i>S. aureus</i> spp. <i>aureus</i>	21.1%	23.1%	>0.999
<i>S. capitis</i>	15.8%	7.7%	0.629
<i>S. saprophyticus</i>	10.5%	15.4%	>0.999
<i>S. hominis</i>	5.3%	23.1%	0.279
<i>S. cohnii</i> spp. <i>cohnii</i>	5.3%	0%	>0.999
<i>S. pasteurii</i>	5.3%	7.7%	>0.999
<i>S. lugdunensis</i>	0%	7.7%	0.406
<i>S. warneri</i>	0%	15.4%	0.157
Multispecies	36.8%	76.9%	0.026*

Results are prevalence (%); PD, peritoneal dialysis; * $P < 0.05$.

TABLE 5: Total number of PD patients with one or more infectious episodes, peritonitis, and/or exit-site infections (ESI), in particular caused by *Staphylococcus* spp.

	Peritonitis		ESI	
	Total	<i>Staphylococcus</i> spp.	Total	<i>Staphylococcus</i> spp.
PD patients with infectious episodes (n = 19)	10 (47.6%)	5 (23.8%)	19 (90.5%)	12 (57.1%)

TABLE 6: Total number of infectious episodes and *Staphylococcus* species identified in peritonitis and exit-site infections (ESI) of PD patients.

Infectious agent	PD-related infections (n = 114)	
	Peritonitis (n = 23)	ESI (n = 91)
Others	16 (69.6%)	63 (69.2%)
<i>Staphylococci</i>	7 (30.4%)	28 (30.8%)
<i>S. epidermidis</i>	5	9
<i>S. aureus</i>	1	16
<i>S. warneri</i>	1	1
<i>S. hominis</i>	0	1
Nonidentified CNS	0	1

CNS, coagulase-negative *Staphylococcus*.

patients with peritonitis or ESI caused by *S. epidermidis* were colonized by the same species in the oral cavity (Table 7). Among the PD patients with ESI caused by *S. aureus*, only 1 was colonized with the same species in the oral cavity

(Table 7). Moreover, the only patient with *S. aureus* peritonitis was also not orally colonized with the same species. Oral colonization of PD patients with multispecies staphylococci was not associated to a higher prevalence of PD-related infections ($P > 0.05$).

4. Discussion

In the Human Oral Microbiome Database (<http://www.homd.org/>), few *Staphylococcus* species are described, namely, *S. aureus*, *S. epidermidis*, *S. warneri*, and *Staphylococcus caprae*. Our results provide evidence for the presence of six other *Staphylococcus* species, namely, *S. saprophyticus*, *S. capitis*, *S. cohnii*, *S. pasteurii*, *S. hominis*, and *S. lugdunensis*. Taking in consideration that we found a prevalence close to 100% of *Staphylococcus* in both PD patients and control groups, we suggest that this genus is a common member of the oral microbiome. In agreement, other authors also described *Staphylococcus* species as frequent oral colonizers of adults, in health and oral disease [21, 22]. Nonetheless, our study was the first to report *S. pasteurii*, considered a rare CNS clinical isolate [23], as a member of the oral microbiome.

Although staphylococci are major pathogens of PD-related infections, there are no studies exploring the oral microbiome as a source of infection in PD patients. The likelihood that these microorganisms could act as opportunistic infectious agents to distant body locations triggered our need to better understand the oral colonization status of PD patients. Overall, we did not observe differences between the two groups in specific species oral colonization, although the oral microbiota of PD patients presented lower staphylococci diversity. This lower diversity in PD patients may be justified by more frequent antibiotic therapy of these patients, as well as by changes in the oral biochemical milieu, including higher pH and urea content observed in patients on PD therapy.

Although infections are a major concern to PD patients, risk factors and transmission routes are far from being fully understood. Given that many preventive measures are in place to prevent PD-related infections by external route, such as mask use and prophylactic use of topical antibiotic treatment of PD catheter exit-site [4, 10, 11], staphylococci transmission by a haematogenous route should be considered. In line with this, recent studies demonstrate that the uterine microbiome is more similar to the oral microbiome than to the vaginal, intestinal, or skin microbiome [24, 25], which reinforces the role of the haematogenous route in the transmission of oral microorganisms.

In agreement with previous reports, the *Staphylococcus* species identified as agents of PD-related infections in our group of PD patients were *S. aureus*, *S. epidermidis*, *S. warneri*, and *S. hominis* [7, 8, 26]. In the present study, all the patients with *S. epidermidis* infections, either peritonitis or ESI, were colonized orally by the same species suggesting that the oral cavity may have the potential to behave as a reservoir of PD-related infections induced by these agents. However, *S. epidermidis* is a highly ubiquitous microorganism in human skin and mucosa, and so, the phylogenetic relatedness between common species would be an important

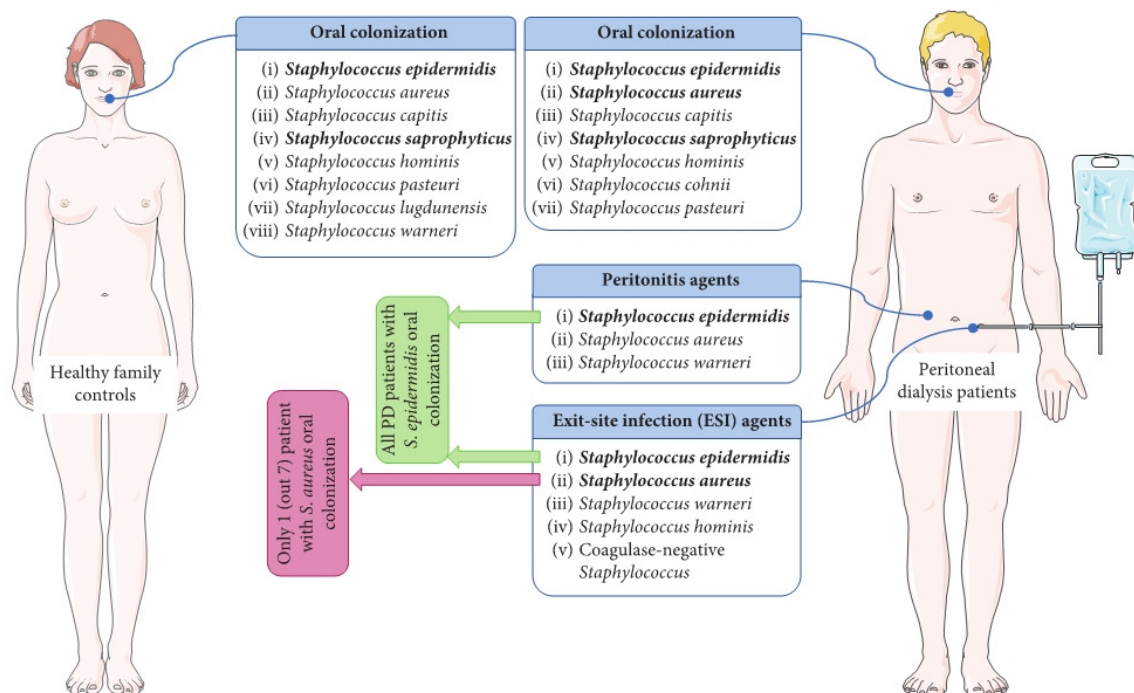


FIGURE 1: *Staphylococcus* oral colonization of PD patients and healthy controls and *Staphylococcus* agents responsible for PD-related infections. Common species are in bold. Figure was produced using Servier Medical Art, <http://www.servier.com/Powerpoint-image-bank>.

TABLE 7: Comparison between peritoneal infections, peritonitis, or exit-site infections (ESI) caused by *Staphylococcus* species and oral colonization of PD patients.

	Infectious agent		Oral colonization	
	ESI	Peritonitis	ESI	Peritonitis
<i>Staphylococcus epidermidis</i>	6	3	6	3
<i>Staphylococcus aureus</i>	7	1	1	0

asset to determine the real transmission route. Moreover, only 1 out of 7 patients with *S. aureus* ESI was colonized orally by *S. aureus* thus suggesting that the oral cavity may not behave as a potential reservoir of PD-related infections induced by *S. aureus* specifically. These partial contradictory results, together with the small sample size restricted to a single-centre, hinder us to draw a conclusion about the potential of the oral cavity as a reservoir of PD-related pathogens. Further studies are thus necessary to clarify this hypothesis.

5. Conclusion

Staphylococci are highly prevalent in the oral cavity of both groups, although PD patients presented less diversity of species in comparison to controls. Given that the association between oral *Staphylococcus* carriage and PD-related infections was present for *S. epidermidis* but was almost

inexistent for *S. aureus*, further studies are necessary to evaluate the relationship between oral *Staphylococcus* carriage and PD-related infections development.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee at which the studies were conducted (Ethics Committee for Health and Institutional Review Board of São João Hospital Centre approval number 159/11) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Consent

Informed consent was obtained from all individual participants included in the study.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Acknowledgments

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Supplementary Materials

Supplementary Table 1 presents the *Staphylococcus* species identified in PD patients and controls, as well as the number of individuals where each of the *Staphylococcus* species was identified. (*Supplementary Materials*)

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Supplementary Material 1: *Staphylococcus* species identified in peritoneal dialysis (PD) patients and controls. n reports to the number of individuals, PD patients and controls, where each of the *Staphylococcus* specie was identified.

		<i>Staphylococcus</i> species (n)	Acession number
Controls	PD patients	<i>S. saprophyticus</i> subsp. <i>bovis</i> (1)	AB234088.1
		<i>S. capitis</i> subsp. <i>urealyticus</i> (3)	AB234061.1
		<i>S. cohnii</i> subsp. <i>cohnii</i> (1)	AB234066.1
		<i>S. epidermidis</i> (30)	AB234069.1
		<i>S. aureus</i> subsp. <i>aureus</i> (8)	AB234058.1
		<i>S. pasteuri</i> (2)	CP004014.1
		<i>S. saprophyticus</i> subsp. <i>saprophyticus</i> (4)	AB234089.1
		<i>S. hominis</i> subsp. <i>novobiosepticus</i> (2)	AB234076.1
	Controls	<i>S. capitis</i> subsp. <i>capitis</i> (1)	AB234060.1
		<i>S. hominis</i> subsp. <i>hominis</i> (2)	AB234075.1
		<i>S. lugdunensis</i> (1)	AB234081.1
		<i>S. warneri</i> (2)	AB234328.1

**“What is your method of choice? Importance of methodologies in
Staphylococcus isolate identification”**

Submitted for publication

L. Simões-Silva participated in the experimental design, conducted the experiments of *Staphylococcus* identification, by API/ID32 and by *DnaJ* gene sequencing, participated in analysis and interpretation of the data and wrote the manuscript.

What is your method of choice?

Importance of molecular methods in the identification of *Staphylococcus* isolates

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Declarations of interest: none.

Abstract

Presently, there is an extensive set of methods available for microbial identification based on their phenotypic and/or molecular characteristics. Although many use MALDI-TOF-based methodologies, in less equipped laboratories phenotypic and API methodologies are still widely used. In this study, we compared the performance of phenotypic and molecular tools for the identification of *Staphylococcus* isolates, growing in Mannitol Salt agar. The API STAPH 32 methodology, based on biochemical tests, and sequencing of the *DnaJ* gene were applied to 52 isolated strains. The API method resulted in 23% unidentified isolates vs. 4% by DNA sequencing. A third MALDI-TOF-based methodology, VITEK MS, was applied when discrepancies were observed. In this subset of samples, only 33% of the identifications were in accordance with API or *DnaJ* sequencing, 67% were ambiguous or different from both methods. Also, we verified that *Staphylococcus aureus* can represent a minority of the gram positive, catalase positive cocci, isolated from yellow colonies grown in Mannitol Salt agar. Here, we highlight the importance of the method of choice for *Staphylococcus* identification. Molecular methods are in general more accurate than phenotype- based methods and should be used when possible. Even though MALDI-TOF mass spectrometry is considered a very accurate, rapid, high-throughput proteomic technique for bacteria identification, we demonstrate that it did not achieve the established levels of accuracy in a subpopulation of clinical isolates.

Keywords: API 32 STAPH; *DnaJ*; identification; *Staphylococcus*; VITEK MS.

1. Introduction

Staphylococci are regarded as one of the most important group of pathogens, being frequently associated with human and animal infections (Archer, 1998, Becker et al., 2014, Widerstrom et al., 2012). The genus *Staphylococcus* is characterized by gram- positive, catalase-positive cocciform bacteria. There are currently 52 established *Staphylococcus* species (<http://www.bacterio.net/>), 12 of which contain different subspecies (28 in total). Staphylococci are usually divided into two groups, coagulase-positive and coagulase negative (CoNS), based on the presence or absence of the secreted coagulase polypeptide that promotes the fibrin of blood plasma to clot (Becker, et al., 2014). Very few staphylococci

are coagulase-positive, although *Staphylococcus aureus*, the most extensively studied species and an important human pathogen, belongs to this group. *Staphylococcus aureus* is frequently isolated from community and hospital infections, being the leading cause of skin and soft tissue infections, foreign body infections, and infective endocarditis (Archer, 1998). Its well-studied mechanisms of pathogenicity, such as antibiotic resistance and biofilm-forming capacity, immune evasion and toxin production contribute to *S. aureus*'s success as human pathogen (McCarthy et al., 2015, Otto, 2014a). The majority of the *Staphylococcus* species are CoNS, and are regarded as saprophytes with pathogenic potential. CoNS species most frequently associated with human infections include *Staphylococcus epidermidis* (Otto, 2014b),

Staphylococcus haemolyticus (Czekaj et al., 2015) and *Staphylococcus saprophyticus* (Widerstrom, et al., 2012). Other strains are also recognized as opportunistic pathogens, including *S. Staphylococcus warneri*, *Staphylococcus capitis*, *Staphylococcus cohnii*, *Staphylococcus hominis*, *Staphylococcus simulans*, *Staphylococcus xylosus*, *Staphylococcus lugdunensis*, and *Staphylococcus saccharolyticus* (Becker, et al., 2014). Infections by CoNS have increased in recent years, possibly deriving from changes in patient population structure and the increasing use of inserted foreign bodies, i.e. catheters, stents or prosthesis (Becker, et al., 2014). Thus, it is important to correctly identify *Staphylococcus* species, since accurate identification is the basis for a better understanding of the pathogenic potential of its various species (Dai et al., 2012, Heikens et al., 2005). Before the development of molecular methods, microbial identification relied solely on characterization by phenotypic-based methods. Such is the case of the API methodology that depends on the detection of metabolic activities of the analysed microorganisms. A common drawback of this methodology is that without the existence of the optimal conditions for the expression of metabolic activities, these assays may result in the misidentification of isolates (Sheraba et al., 2010). Recently the MALDI-TOF MS was introduced in the clinical setting. This technology is based on protein fingerprinting and has successfully reduced the time required to identify pathogens (Tan et al., 2012). MALDI-TOF MS's high diagnostic accuracy makes it an attractive option to be used for direct detection of pathogens from clinical specimens (Wattal and Oberoi, 2016). Molecular methods are reliable alternatives to phenotypic methods. The 16S rRNA

gene is the most frequently used gene for identification and classification of bacteria. However, in recent years other candidate genes, such as *hsp60*, *rpoB* and *sodA* have appeared, since closely related species have high degrees of sequence similarity for the 16SrRNA gene (Drancourt and Raoult, 2002, Ghebremedhin et al., 2008, Kwok et al., 1999, Poyart et al., 2001). Some of these are species specific as they are not ubiquitous, such is the case of the *MecA* gene present in *Staphylococci* (Rolo et al., 2017). Ezaki and his team (Shah et al., 2007) have developed a sequence-based assay using the *DnaJ* gene. This assay displayed a greater resolving power for the genus *Staphylococcus*, resulting in the identification and phylogenetic characterization of almost all staphylococci at both the species and, with some exceptions, the subspecies levels. In the present work, we compared the identification of *Staphylococcus* spp. isolated from yellow colonies grown in Mannitol Salt agar, by API 32 STAPH and sequencing of *DnaJ* gene. Isolates with disagreeing identifications were further characterized by the Vitek MS system.

2. Material and Methods

2.1. Biological material

Samples from saliva, nose and skin surrounding the peritoneal catheter exit-site were obtained from Peritoneal Dialysis (PD) patients, followed up in the outpatient clinic of the Nephrology Department of "Hospital S. João" (Porto, Portugal); saliva samples were also collected from controls. Written informed consent was obtained from both PD patients and controls (approved by the Ethics Committee of

Centro Hospitalar de S. João). Saliva samples were collected by spitting the whole-mouth saliva under resting conditions for 5 min. Saliva was mixed in a 1:1 ratio with Brain Heart Infusion containing 20% glycerol, and subsequently cryopreserved at -80°C until microbial analysis. Epithelial cell swabs from the peritoneal catheter exit-site and nasal cavity were collected only from PD patients without previous topic antibiotic application. Swabs were cryopreserved at -80°C in Brain Heart Infusion with 10% glycerol, until microbial analysis. Staphylococci were isolated on Mannitol Salt agar (Liofilchem, Italy) and sub-cultured in BHI agar (Biolab, Hungary). Samples were serially diluted with 0.9% sterile NaCl solution up to 10⁻². Plates were incubated aerobically for 48h at 37°C. Staphylococci were first screened with Gram-stain and catalase tests. The Gram-positive, catalase-positive cocci isolated from yellow colonies grown in Mannitol Salt agar were submitted to further characterization.

2.2. *Staphylococcus* identification by API/ID32

The multi-test system, API 32 STAPH (Biomérieux) was performed according to the manufacturer's instructions. The Apiweb software was used to calculate the probability of identification within a 10 to 100% range (<https://apiweb.biomerieux.com>). Only identifications with >90% of probability were accepted (Sampimon et al., 2009).

2.3. DNA extraction and amplification of the *DnaJ* gene

DNA was extracted with the boiling method. Briefly, 5 colonies were resuspended in 100 µL of molecular grade water and incubated at 100°C for 10 min. The diluted suspension (1:10) was

centrifuged at 12.000 rpm for 10 min and the supernatant used as DNA template. *DnaJ* gene was amplified as previously described (Shah, et al., 2007). PCR amplification products were identified by Sanger sequencing (StabVida, Caparica, Portugal). The identification by sequencing of *DnaJ* gene was only accepted when sequence identity was 98% or higher to *DnaJ* sequences resulting from whole genome sequencing deposited at GenBank® (Benson et al., 2013).

2.4. *Staphylococcus* identification by the Vitek MS system

Identification with the Vitek 2 MS system (Biomérieux) was performed according to the manufacturer's instructions and based on the clinical protocol. Four spots were analysed with the recommended matrix. Before measurement, cultures were checked for purity and the colony used for analysis was obtained from fresh agar plates (24h incubation). The strains did not changed colony morphology during storage.

2.5. Phylogenetic comparison

Staphylococcus isolates were classified according to the phylogenetic analysis conducted by Lamers et al. (2012), where staphylococci were classified into 6 “species groups” (based on phenotypic variance) and 15 “cluster groups” (based on molecular data). In the disagreeing results, we verified if the identified species belonged to the same cluster group, the same species group, different species groups or different genera.

2.6. Data analysis

The reference method for staphylococci species identification was *DnaJ* gene sequencing. Isolates

were considered to be correctly identified by API Staph ID 32 and VITEK MS if the identification retrieved the same species as the reference method. A misidentified isolate was considered when a different species was found, compared to the reference method. For API STAPH 32, sensitivity and specificity were calculated (Sampimon, et al., 2009). Sensitivity was calculated as the proportion of true positive isolates that were correctly identified. Specificity was calculated as the proportion of true negatives that were correctly identified; for a given species it is the proportion of isolates other than that species, based on sequencing data, which were identified as something other than that species by API. An ambiguous identification was considered when the obtained result included more than one species. Results are presented as percentages. Data was analysed using IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp, NY, USA). The agreement

test, Kappa value, was applied when appropriate. A P value of less than 0.05 was assumed to denote a statistically significant difference.

3. Results

In the present study, 52 staphylococci strains were isolated and analysed by both *DnaJ* sequencing and API 32 STAPH. Fifty *Staphylococcus* clinical isolates were successfully identified with the reference method, *DnaJ* sequencing (Shah, et al., 2007). Of the two isolates not identified by sequencing, no amplification of *DnaJ* was obtained in one isolate, whereas in the other, sequence identity was inferior to 98%. Regarding analysis by API 32 STAPH, 10 unidentified results were obtained, whose probability score was <90%. A total of 42 isolates were identified by both methods (Fig. 1A).

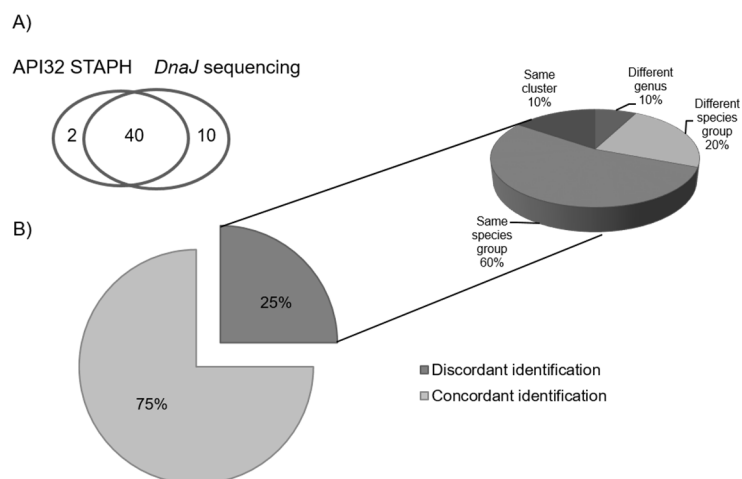


Figure 1: A) number of isolates (n=52) identified by API32 STAPH and *DnaJ* sequencing and both techniques. B) Phylogenetic relationships for disagreeing results obtained between API32 STAPH and *DnaJ* sequencing. Species were organized according to Lamers et al. (2012) as belonging to the same cluster group, the same species group, different species group or different genus.

The *Staphylococcus* species identified by the two methods are described in Table 1.

Table 1: *Staphylococcus* species identified by *DnaJ* sequencing, API32 STAPH, and 202 VITEK MS.

	Species identification		
	<i>DnaJ</i> sequencing (n=50)	API32 STAPH (n=42)	VITEK MS (n=6)
<i>S. aureus</i>	12	9	-
<i>S. epidermidis</i>	14	11	2
<i>S. capitis</i>	3	2	1
<i>S. saprophyticus</i>	7	4	-
<i>S. warneri</i>	3	6	1
<i>S. cohnii</i>	5	3	-
<i>S. xylosus</i>	1	3	-
<i>S. hominis</i>	3	1	1
<i>S. pasteurii</i>	2	-	-
<i>S. equorum</i>	-	1	-
<i>S. kloosii</i>	-	1	-
<i>S. haemolyticus</i>	-	-	1
<i>Kocuria varians</i>	-	1	-

In isolates characterized by both methods, the comparison between API 32 STAPH and *DnaJ* sequencing revealed a disagreeing identification in 10 (25%) of the isolates (Fig. 1B). To further extend the characterization of the isolates with disagreeing species identification, these were subsequently subjected to analysis by the automated microbial identification system VITEK MS. We were unable to

recover growth from one of the isolates, and, three isolates had ambiguous identifications, presenting more than one species in its identification report, resulting in six de facto identifications (Table 1). In 2 isolates the identification was identical to the obtained by *DnaJ* sequencing, and one to API (Table 2). However, 3 isolates had a new identification, different from the two previous.

Table 2: Species identification of a subpopulation of staphylococci by VITEK® MS. Percentage of results obtained by VITEK® MS similar to the reference method *DnaJ* sequencing, similar to API32 STAPH, different from both methods or unable to discriminate between more than one species.

	VITEK MS identification (n=9)
Identical to <i>DnaJ</i> sequencing	2
Identical to API32 STAPH	1
Different from both methods	3
Unable to discriminate between more than one species	3

We used the sensitivity and specificity to evaluate the API performance for *Staphylococcus* identification. The best sensitivity was found for the species *S. capitis*, *S. xylosus* and *S. aureus*, as each presented 100% of sensitivity; the specificity of detection for these species was of 100, 98 and 98%,

respectively (Table 3). The other species presented sensitivities that ranged from 50% to 80%, with the exception of *S. pasteurii* that displayed 0% sensitivity, although only two isolates were tested. Nevertheless, the specificity of detection of this specie was high (100%) (Table 3).

Table 3: Sensitivity and specificity of the API Staph ID 32 multi-test system for the identification of *Staphylococcus* species, using *DnaJ* sequencing as a reference method.

<i>Staphylococcus</i> spp.	API Staph ID 32	
	Sensitivity	Specificity
<i>S. capitis</i>	100%	100%
<i>S. xylosus</i>	100%	98%
<i>S. aureus</i>	100%	98%
<i>S. saprophyticus</i>	80%	100%
<i>S. epidermidis</i>	70%	95%
<i>S. warneri</i>	67%	94%
<i>S. cohnii</i>	60%	100%
<i>S. hominis</i>	50%	100%
<i>S. pasteurii</i>	0%	100%

The Kappa agreement test was applied to measure the correlation between the results obtained with the two methodologies. This test revealed a Kappa value of 0.700 ($p < 0.001$) that according to McHugh et al (2012) corresponds to a moderate agreement between the two methods used. To further analyse

the inconsistent results obtained with the two methods we investigated the phylogenetic relationships of the *Staphylococcus* isolates identified by the two methods, based on the analysis conducted by Lamers et al. (2012) (Fig. 1). In the disagreeing results obtained with API 32

STAPH vs *DnaJ* sequencing, we observed that approximately 20% of inconsistent identifications belonged to different species groups, 60% to the same species group, and only 10% to the same cluster group, which is the closest phylogenetic order.

4. Discussion

One of the most widely used methods for the isolation of staphylococci is the growth in Mannitol salt agar, a selective medium for *Staphylococcus*, followed by identification based on phenotypic or molecular methods. *Staphylococcus aureus*, the most pathogenic *Staphylococcus* species, is described as developing yellow colonies in this medium, due to its capacity to ferment mannitol producing acid and consequently turning the phenol red pH indicator from red to yellow. Most manufacturers also state that coagulase-positive staphylococci are expected to produce yellow colonies that may have a yellow halo around the colony, whereas coagulase-negative staphylococci will produce small, colourless-to-pink colonies with no colour change to the medium (Neogen, UK; BD, Germany). Other manufacturers refer that yellow colonies are only presumed to be coagulase-positive and that a coagulase test is necessary to confirm this phenotype (Oxoid Limited, UK). Nonetheless, some authors claim that the coagulase test is not always effective and if the coagulase test is inaccurate, conventional testing methods may wrongly identify *S. aureus* as coagulase-negative staphylococci (CoNS) (Dai, et al., 2012). In the present work, we compared different methodologies for the identification of staphylococci, having as the starting point the

detection of yellow colonies in Mannitol Salt agar. In our study only 23.7% of yellow colonies grown in Mannitol salt agar were identified as *S. aureus*. No other described coagulase-positive *Staphylococcus* was presently identified, namely *Staphylococcus delphini*, *Staphylococcus hyicus*, *Staphylococcus intermedius*, *Staphylococcus lutrae*, *Staphylococcus pseudintermedius*, and *Staphylococcus schleiferi* subsp. *coagulans*. Given this low percentage of *S. aureus* growing as yellow colonies in Mannitol salt agar it is of outmost importance to identify the isolates obtained by this methodology, given that a serious pathogen may be overestimated. When comparing API 32 STAPH and *DnaJ* sequencing methodologies, we observed that the former identified a wider range of species than the latter. In parallel, there was an increased number of unidentified isolates by the API 32 STAPH method, representing more than 23% of total isolates vs 4% by *DnaJ* sequencing. Two isolates were not identified with the *DnaJ* sequencing approach. In one of the isolates no amplification was obtained and in the other the identity of *DnaJ* sequence was lower than 98%. The primers used to amplify the *DnaJ* gene were designed for the genus *Staphylococcus* and the isolate could belong to another genus and thus no amplification was obtained. Alternatively, the isolate was from a *Staphylococcus* species that was not included in the original study and thus no *DnaJ* sequence is available (Shah, et al., 2007). Having an identity of *DnaJ* sequence lower than 98% suggests the presence of another specie of *Staphylococcus* since in the original study, only 45 *Staphylococcus* type strains were analysed (Shah, et al., 2007). All the species here identified by DNA sequencing have already been associated with

human disease on a higher or lesser extent (Rupp and Fey, 2014). Assuming that DNA sequencing is the most accurate method for *Staphylococcus* identification (Shah, et al., 2007), the percentage of isolates correctly identified by API 32 STAPH was 74%. We further characterized disagreeing results based on the phylogenetic relationships among *Staphylococcus* (Lamers, et al., 2012). When analysing inconsistent results obtained with API and *DnaJ* sequencing, we verified that the majority belonged to the same species group and only 10% to the same cluster group, which is the closest phylogenetically. This could be linked to the fact that the species group classification is based on phenotypic characteristics, and API 32 STAPH identification is based on phenotypic tests. The existence of *Staphylococcus* misidentifications not even belonging to the same species group, and thus phylogenetically most distant, is noteworthy. In fact, one of the isolates was misidentified for species from different genus (*Kocuria varians* and *Staphylococcus epidermidis*). In the past, other methods have led to the erroneous identification of coagulase-negative staphylococci as *Kocuria* spp., namely the VITEK 2 system (Ben-Ami et al., 2005), later identified as CoNS by 16S rRNA gene sequencing. This emphasises the importance of careful consideration of the laboratory and clinical procedures when uncommon pathogenic microorganisms are identified. As stated, disagreeing isolates were also identified by the VITEK MS system. Results obtained with this method were somewhat unexpected, since only 22% were in agreement with the reference method of *DnaJ* sequencing. In addition, only 11% were in accordance with API and 67% of the analysed isolates were either ambiguous or different from

both techniques tested (API and *DnaJ* sequencing). These values may be biased by the fact that this is a small subsample of the original group, enriched in isolates whose identification was already inconsistent with the two initial techniques. As such, isolates may represent atypical strains that do not have the phenotypic traits characteristics of the reference strains and thus are difficult to identify by these technologies. Nevertheless, a study by Loonen and co-workers (2012) reported a correct identification rate of 99.3% for the identification of CoNS when using MALDI-TOF MS technology, although a different system was used in this study. Thus, considering the accuracy of DNA sequencing and MALDI-TOF MS (Ksiazczyk et al., 2016) the results of these two methodologies would be expected to be more identical.

5. Conclusions

With the current strategy, we wanted to highlight the importance of the method-of-choice for the identification of bacteria, using as a model the *Staphylococcus* genus. Phenotypic methods may be useful in preliminary identification of bacteria, as they lead to important data concerning phenotypic or physiological traits in the absence of more sophisticated analysis. However, the present report reinforces the notion that molecular methods are more accurate and should be preferably used, especially when dealing with atypical isolates. Even though MALDI-TOF MS methodologies, such as VITEK MS, are considered very accurate, rapid and high-throughput techniques for bacteria identification, we demonstrate that in a sub-group of isolates it did not achieve the described levels of accuracy necessary for the clinical setting, and

should be supplemented by molecular methods when strains difficult to identify are encountered.

Competing interests

The authors declare that they have no competing interests.

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CHAPTER IV

“Oral yeast colonization and fungal infections in Peritoneal Dialysis patients: a pilot study”

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“Influence of dialysis therapies on oral health - a pilot study”

Submitted for Publication

Despite being rare, fungal peritonitis are associated with significant morbidity and mortality in PD patients. In this chapter, we characterize the colonization of oral cavity of PD patients by yeasts, and study the relation between yeast colonization and fungal infection development in these patients. Additionally, we evaluated the influence of HD and PD in the oral yeast colonization.

**“Oral yeast colonization and fungal infections in Peritoneal
Dialysis patients: a pilot study”**

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L. Simões-Silva participated in the experimental design, conducted the experiments of microbiological analysis, collected the data regarding the history of infection, participated in analysis and interpretation of the data and writing of the manuscript.

Research Article

Oral Yeast Colonization and Fungal Infections in Peritoneal Dialysis Patients: A Pilot Study

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Peritonitis and exit-site infections are important complications in peritoneal dialysis (PD) patients that are occasionally caused by opportunistic fungi inhabiting distant body sites. In this study, the oral yeast colonization of PD patients and the antifungal susceptibility profile of the isolated yeasts were accessed and correlated with fungal infection episodes in the following 4 years. Saliva yeast colonization was accessed in 21 PD patients and 27 healthy controls by growth in CHROMagar-Candida® and 18S rRNA/ITS sequencing. PD patients presented a lower oral yeast prevalence when compared to controls, namely, *Candida albicans*. Other species were also isolated, *Candida glabrata* and *Candida carpophila*. The antifungal susceptibility profiles of these isolates revealed resistance to itraconazole, variable susceptibility to caspofungin, and higher MIC values of posaconazole compared to previous reports. The 4-year longitudinal evaluation of these patients revealed *Candida parapsilosis* and *Candida zeylanoides* as PD-related exit-site infectious agents, but no correlation was found with oral yeast colonization. This pilot study suggests that oral yeast colonization may represent a limited risk for fungal infection development in PD patients. Oral yeast isolates presented a variable antifungal susceptibility profile, which may suggest resistance to some second-line drugs, highlighting the importance of antifungal susceptibility assessment in the clinical practice.

1. Introduction

Peritoneal dialysis (PD) is a home-based and widely used renal replacement therapy for patients with end-stage renal disease (ESRD). In PD patients, infectious complications, namely, peritonitis and exit-site infections, account for a significant percentage of catheter loss, transfer to haemodialysis, prolonged hospitalization, or even death, making

prevention of infection a critical step to the success of a PD program [1]. Although a rare event, fungal peritonitis is associated with significant morbidity and mortality in PD patients [2]. Fungal exit-site infections are more frequent than peritonitis, but more easily resolved, although they may potentiate the development of a subsequent peritonitis [3]. Fungal infections are primarily caused by opportunistic fungal pathogens, such as *Candida* species, that take

advantage of a locally or systemically debilitated immune system to proliferate in the human host and cause disease. In a recent study where fungal exit-site infections of PD patients were evaluated, the most frequently isolated species were *Candida parapsilosis* (67%), followed by *Candida glabrata* (10%), *Candida famata* (7%), and *Candida zeylanoides* (7%) [4].

Factors that influence the occurrence of PD-related infections are still not completely understood. Some authors have highlighted the importance of the oral microbiome as a starting point for dissemination of pathogens to distant body sites [5, 6]. Despite the association between oral pathology and adverse outcomes of renal patients [7], so far, existing studies have neither evaluated the oral yeast colonization of PD patients nor evaluated its relation with the development of fungal infections. This topic is particularly relevant when considering the opportunistic character of *Candida* species, a frequent colonizer of the oral cavity [6] and the immune impairment of ESRD patients and knowing that chronic kidney disease itself, and PD therapy in particular, alters significantly the oral milieu [8].

Therefore, in the present study, the oral yeast colonization and the oral health of PD patients were characterized and compared with a healthy population. The antifungal susceptibility profile of yeasts isolated from the oral cavity was also assessed. Additionally, the clinical history of fungal infections was evaluated and related with the oral yeast colonization of PD patients.

2. Material and Methods

Patients followed up for at least 1 month in the PD outpatient clinic of the Nephrology Department of "Centro Hospitalar de S. João," over 18 years of age and with no recent history of infection (less than 1 month) were invited to participate in the study. A convenience sample was obtained related with the attendance of patients to the outpatient clinic during a period of 6 months. A group of 21 PD patients accepted to participate and were included in the study. The control group consisted of 27 adult healthy subjects, including 10 PD family members (in order to select individuals living in similar environment and conditions as the patients) and 17 nonfamily members of PD patients. The exclusion criteria were: inability to give informed consent, pregnancy, and severe acute illness. The study protocol was approved by the Ethics Committee for Health and Institutional Review Board of "Centro Hospitalar de S. João," and all recruited patients and controls were asked to give their written informed consent. This work is constituted by a cross-sectional study, regarding the comparison of *Candida* oral colonization in PD patients and controls, followed by a longitudinal study, in which the history of *Candida* spp. infections was analysed during 4 years to establish a comparison between the *Candida* species present in the oral cavity and the *Candida* species responsible for subsequent PD-related fungal infections. Regarding the longitudinal evaluation of PD-related fungal infections, the study began with 21 patients, and due to PD technique dropout, 20, 19, 14, and 11 patients remained at the end of the first, second, third, and fourth follow-up year, respectively.

Patients' clinical information was gathered including age, gender, smoking habits, blood pressure, aetiology of renal disease, residual renal function, PD vintage, infectious complications during PD, and PD-related fungal infection episodes and agents. Demographic information was gathered for control population, namely, age, gender, and smoking habits.

A noninvasive intraoral examination was performed in both groups in order to evaluate the oral hygiene by visible plaque index (VPI) in four sites of each tooth (mesiobuccal, midbuccal, distobuccal, and midlingual); the percentage of the examined sites with visible plaque ranged from 0% to 100%. Whole saliva was collected in both groups before oral examination for microbial analysis and pH evaluation. The patients were instructed not to eat, drink, and perform the normal mouth hygiene at least two hours before the procedure. Samples of nonstimulated saliva were collected in one time point for each patient under resting conditions. The patients were asked to spit the whole-mouth saliva after 5 min. The volume was quantified gravimetrically, and the salivary flow rate was determined (mL min^{-1}). The pH of saliva was determined immediately after collection using pH strips (5.0–8.0, Duotest, Germany). The saliva was mixed 1 : 1 in Brain Heart Infusion with 20% glycerol and cryopreserved at -80°C until microbial analysis.

Saliva samples were unfrozen for yeast isolation and quantification. The samples were serially diluted with 0.9% sterile NaCl solution and plated in triplicate in a selective and differential culture medium, CHROMagar-Candida. Plates were incubated aerobically for 48 h at 37°C . Total number of colonies was determined, and quantification results were expressed in logarithmic scale of colony forming units per ml of saliva ($\text{Log}_{10} \text{CFU mL}^{-1}$). Identification of *Candida albicans* was possible due to the specific colour of the colonies. Isolates were identified by 18S rDNA and internal transcribed spacer (ITS) region DNA sequencing approach as previously described [9]. PCR amplification was performed using a group of specific primers: EF3 (5'-TCCTCTAAATGACCAAGTTTG-3'), EF4 (5'-GGAAGGG [G/A]TGTATTTATTAG-3'), fung5 (5'-GTAAAAGTCC-TGGTTC-3'), ITS1 (5'-TCCGTAGGTGAACCTTGC-GG-3'), and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') in a Thermo-Hyaid-PX2 thermal cycler. Amplification products were visualized in a polyacrilamide gel followed by silver-staining. Sequence analysis was performed in a genetic analyser ABI-Prism-3100 (Applied Biosystems). Genomic data were compared with a database that comprises a large collection of yeast sequences of 18S rDNA and ITS regions obtained from GenBank.

Antifungal susceptibility testing was performed by the determination of minimum inhibitory concentration (MIC) and according to clinical breakpoints (CBP) defined in the M27-A3 and M27-S4 protocols of the Clinical and Laboratory Standards Institute (CLSI) (<http://clsi.org/>). Due to the loss of viability of some isolates, antifungal susceptibility was performed in 2 isolates from the PD group and 4 out of 10 from the controls. The following antifungals were tested: voriconazole (Pfizer, Groton, CT), posaconazole (Schering-Plough, Summit, NJ), fluconazole (Pfizer, Groton, CT),

amphotericin B (Bristol-Myers Squibb, New York, NY, USA), caspofungin (Merck, Rahway, NJ, USA), anidulafungin (Pfizer, Groton, CT, USA), and micafungin (Astellas Pharma, Inc., Tokyo, Japan). For species whose clinical breakpoints are not defined, the phenotype was characterized based on epidemiological cutoff values (ECVs) according to Pfaller and Diekema [10].

Saliva biochemical parameters were quantified by an automatic analyser, Pentra C200 (Horiba ABX Diagnostics, Switzerland). In brief, phosphate was detected by UV using phosphomolybdate, whereas α -amylases were detected by an enzymatic photometric test, using the substrate 4,6-ethylidene-(G7)-p-nitrophenyl-(G1)- α -D6 maltoheptaoside (EPS-G7). In addition, salivary IgA was determined by immunoturbidimetry and urea by enzymatic UV test (method "Urease-GLDH").

Statistical analyses were performed using IBM® SPSS® version 23.0 (Statistical Package for Social Sciences). The categorical variables were described through relative frequencies (%) and analysed by the chi-square independence test or Fisher exact test when more than 1 cell had expected counts less than 5. The normality test was performed with Shapiro-Wilk. When normally distributed, continuous variables were described using mean $\pm$ standard deviation (SD) and analysed by student's *t*-test, whereas when not normally distributed, continuous variables were described using median (min, max) and analysed by the Mann-Whitney *U* test. $P < 0.05$ was assumed to denote a significant difference.

3. Results

PD patients and controls presented similar demographic characteristics (Table 1).

The clinical history of PD patients, such as the most prevalent aetiologies of chronic kidney disease, and the most relevant clinical data, such as PD vintage, residual renal function, and blood pressure determined at the day of sample collection, are presented in Table 2. Additionally, this table also presents the prevalence of patients on specific medication reported to be associated with altered susceptibility to fungal infections, namely, calcium channel blockers, statins, vitamin D, and iron supplementation [11–14].

The study was initiated by an oral clinical evaluation and saliva collection. At this point, the average time on PD therapy was 15.5 ± 16.9 months, ranging from 1 to 72 months (Table 2).

PD patients presented a lower prevalence of yeasts in saliva compared to the healthy controls; however, the difference did not attain statistical significance (Table 3). Three *Candida* species were identified, namely, *C. albicans* and *C. glabrata* in PD patients and *C. albicans* and *C. carpophila* in controls. The prevalence of *C. albicans* was significantly lower in PD patients than in controls. One control was colonized by two different species: *C. albicans* and *C. glabrata*. Despite the low oral yeast prevalence in PD patients, the quantification of total yeast number (Log_{10} CFU mL^{-1}) in individuals colonized with yeast did not differ between PD patients and the control group (Table 3).

TABLE 1: Age and sex of peritoneal dialysis (PD) patients and controls.

	PD patients ($n = 21$)	Controls ($n = 27$)	<i>P</i> value
Age (years)	46.8 ± 9.7	43.2 ± 11.9	0.273
Sex (male, %)	42.9%	18.5%	0.066

Results are shown in prevalence (%) or mean $\pm$ SD. PD, peritoneal dialysis.

TABLE 2: Aetiology of chronic kidney disease (CKD), time on peritoneal dialysis, residual renal function, and blood pressure of peritoneal dialysis (PD) patients.

	PD patients ($n = 21$)
Aetiology of CKD	
Glomerular disease	52.3%
Diabetic nephropathy	19.0%
Other glomerular disease	33.3%
Tubulointerstitial disease	23.8%
Autosomal dominant polycystic kidney disease	14.3%
Other tubulointerstitial disease	9.5%
Unknown	23.8%
PD vintage (months)	15.5 ± 16.9
Residual renal function (mL min^{-1})	7.0 ± 4.5
Blood pressure	
Systolic	130.2 ± 19.7
Diastolic	78.3 ± 11.0
Therapy	
Calcium channel blockers	47.6%
Statins	57.1%
Vitamin D supplementation	71.4%
Iron supplementation	90.5%

Results are shown in prevalence (%). CKD, chronic kidney disease; PD, peritoneal dialysis.

TABLE 3: Prevalence and quantification of yeast colonizers in peritoneal dialysis (PD) patients and controls.

	PD patients ($n = 21$)	Controls ($n = 27$)	<i>P</i> value
Yeast prevalence	9.6% (2/21)	33.3% (9/27)	0.083
Yeasts (Log_{10} CFU mL^{-1})	2.39 ± 0.80	2.55 ± 0.82	0.803
Species prevalence			
<i>Candida albicans</i>	4.8% (1/21)	33.3% (9/27)	0.029*
<i>Candida glabrata</i>	0%	3.7% (1/27)	>0.999
<i>Candida carpophila</i>	4.8% (1/21)	0%	0.438

Results are prevalence (%) or mean $\pm$ SD. PD, peritoneal dialysis; CFU, colony-forming units. * $P < 0.05$.

Six *Candida* isolates from the oral cavity were analysed for antifungal susceptibility profile: 4 isolates from controls (3 *C. albicans*, and 1 *C. glabrata*) and 2 isolates from PD patients (1 *C. albicans*, and 1 *C. carpophila*). All the isolates were resistant to itraconazole ($\text{MIC} > 1 \mu\text{g mL}^{-1}$); presented a non-wild type phenotype regarding posaconazole (NWT, $\text{MIC} > 2 \mu\text{g mL}^{-1}$); and were susceptible to anidulafungin ($\text{MIC} < 0.125 \mu\text{g mL}^{-1}$), voriconazole ($\text{MIC} < 0.125 \mu\text{g mL}^{-1}$),

TABLE 4: Smoking habits, oral hygiene, and saliva biochemistry of peritoneal dialysis (PD) patients and controls.

	PD patients	Controls	P value
Smoking habits			
Past (%)	58.3%	40.9%	0.331
Present (%)	16.7%	22.7%	>0.999
Visual plaque index (%)	56 (16, 100)	69 (14, 100)	0.489
Saliva biochemistry			
Flow rate (mL min ⁻¹)	0.41 (0.05, 1.06)	0.26 (0.10, 1.04)	0.432
pH	8.0 (6.5, 8.0)	6.8 (6.2, 8.0)	<0.001*
Urea (mg dL ⁻¹)	110.41 ± 36.64	47.85 ± 23.88	<0.001*
Phosphorus (mg dL ⁻¹)	22.76 ± 8.19	18.58 ± 16.86	0.486
IgA (mg dL ⁻¹)	143.0 (126.0, 178.0)	136.5 (30.0, 167.0)	0.361
Amylase (U L ⁻¹)	309.45 (127.40, 757.50)	562.6 (312.90, 571.10)	0.310

Results are prevalence (%), median (min, max) or mean ± SD; PD, peritoneal dialysis. * $P < 0.05$.

and fluconazole (MIC < 4 µg mL⁻¹). A similar susceptibility profile was obtained for all isolates regarding amphotericin B (MIC = 1 µg mL⁻¹) and flucytosine (MIC = 0.125 µg mL⁻¹). *Candida glabrata* isolated from the control group was the only *Candida* isolate resistant to micafungin (MIC = 0.5 µg mL⁻¹). A variable susceptibility to caspofungin (MIC ranging from 0.25 to 1 µg mL⁻¹) was observed for the *Candida* isolates. The susceptibility epidemiological cutoffs for antifungals are still not defined for *C. carpophila*; nevertheless, the susceptibility values were 0.125 µg mL⁻¹ for flucytosine, 0.25 µg mL⁻¹ for voriconazole, 0.5 µg mL⁻¹ for amphotericin B, 1 µg mL⁻¹ for caspofungin and micafungin, 2 µg mL⁻¹ for posaconazole, and 4 µg mL⁻¹ for fluconazole, itraconazole, and anidulafungin.

Table 4 depicts the oral factors that can play a role on yeast growth in the oral cavity. Saliva pH and urea levels were higher in PD patients when compared to the control group.

Regarding PD-related fungal infections, clinical records of this group of PD patients were analysed. In the period previous to sample collection, no peritonitis of fungal origin was recorded, and only one exit-site infection episode was recorded from fungal origin, namely, due to *C. parapsilosis*. This patient, however, did not present yeast oral colonization at the time of the study (approximately one year after the infection).

Moreover, concerning the longitudinal evaluation, PD-related infections of fungal origin were recorded during the 4 years following sample collection. During this period, 4 exit-site fungal infection episodes were recorded, 2 of them in the same patient. These 2 episodes occurred with a time difference of more than 5 months and were caused by *Candida parapsilosis*, although in one of the episodes bacterial agents were also isolated. Other two patients presented infections either by *Candida parapsilosis* or

Candida zeylanoides. No peritonitis of fungal origin was recorded for these patients within this time frame.

A comparison between the *Candida* species identified in saliva of PD patients and fungal infectious agents responsible for the exit-site infections did not reveal the existence of common species (Figure 1).

In addition, none of the patients that presented oral yeast colonization developed PD-related fungal infections. Also, no relationship was found between PD-related fungal infectious agents and oral colonization of family controls.

4. Discussion

PD-related infections from fungal origin are important complications in PD patients, and the opportunistic fungi inhabiting distant body sites may represent a major source of infection. According to our results, oral yeast colonization constitutes a limited risk for fungal infections in PD patients, due to the lack of relationship between fungal oral colonizers and PD infectious agents. Additionally, PD patients presented a lower prevalence of oral yeasts, in particular *C. albicans*, in comparison to a healthy population.

Interestingly, non-*Candida albicans* species were also found to be normal colonizers of saliva, namely, *C. carpophila* in PD patients and *C. glabrata* in controls. To our knowledge, this is the first study to detect the yeast *C. carpophila* in human saliva. It is possible that the modified oral environment of PD patients results in a shift of yeast prevalence compared with the healthy population resulting in the emergence of rare yeasts, such as *C. carpophila*.

Several factors may contribute to the altered prevalence of yeasts in the oral cavity of PD patients, in particular regarding *C. albicans*. *C. albicans* is the most prevalent fungal species in the oral cavity, being described as more sensitive than other *Candida* species to potential combined environmental factors present in the oral cavity [15]. Thus, the lower oral *C. albicans* colonization of PD patients could be justified not only by a higher exposure to antifungal therapy, recommended during an antibiotic course [16, 17], but also by alterations of the oral environment secondary to systemic ESRD effects, PD therapy, and medication. Regarding the antifungal therapy, the protocol followed in our department consists in the prescription of fluconazole (100 mg day⁻¹) whenever a patient starts antibiotic therapy after the first use without success. However, since none of our patients had an infectious episode in the month previous to sample collection, this may not be the reason for a lower level of *Candida albicans* colonization. Regarding the medication of these patients, several molecules are reported to be associated with altered susceptibility to fungal infections and can influence yeast colonization in this population. 47.6% of the PD patients were on calcium channel blockers therapy, described to have an inhibitory effect on oxidative stress response of *Candida albicans* [11]; 57.1% were prescribed with statins, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors, used to lower patients' cholesterol but that also affects ergosterol levels exhibiting antifungal properties [12]; 71.4% were supplemented with vitamin D known to affect fungal growth in

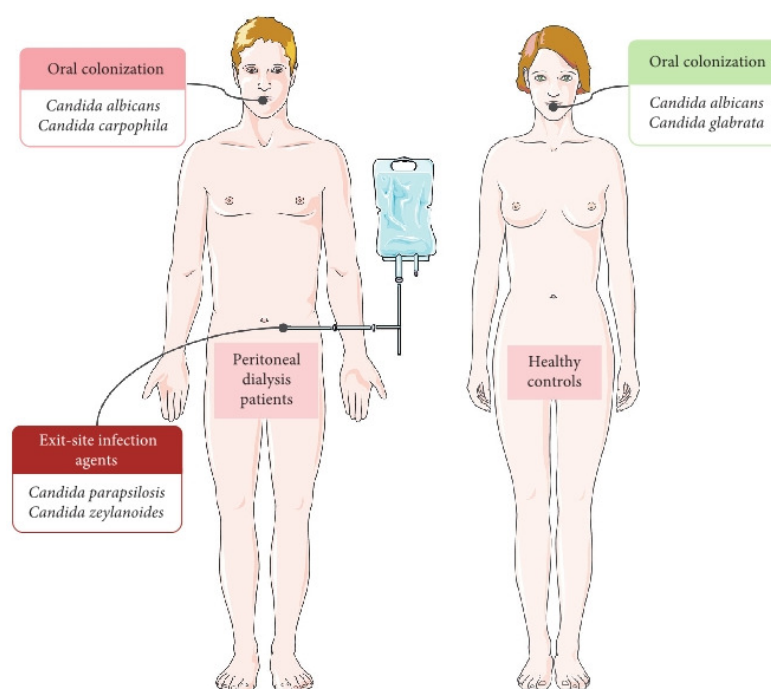


FIGURE 1: Oral *Candida* colonization of PD patients and healthy controls and *Candida* agents responsible for PD-related infections. Figure was produced using Servier Medical Art, <http://www.servier.com/Powerpoint-image-bank>.

a dose-dependent manner [13]; and 90.5% had iron supplementation, an essential element for microbial growth and known to influence host susceptibility to *C. albicans* infections [14].

In order to investigate other possible causes for the reduced oral *C. albicans* colonization of PD patients, we further evaluated specific oral parameters such as oral hygiene, smoking habits, and saliva biochemistry, given that previous studies found relevant changes in the oral status of chronic kidney disease patients undergoing PD [18]. In accordance to previous studies, saliva pH and urea levels were significantly altered in PD patients in comparison with controls [8, 19]. The high urea levels may contribute in part to the higher salivary pH, due to the ammonia production as a result of urea hydrolysis [8, 19]. This altered oral pH may have an impact on *Candida* growth, given that oral *Candida* isolates have been shown to be more adapted to acidic conditions [20]. It is known that neutral to alkaline pH can cause severe stress to *C. albicans* including impaired nutrient acquisition, as a consequence of a disrupted proton gradient, and malfunctioning of pH-sensitive proteins [21]. In addition, changes in the oral pH may be the major ecological factor that alters the oral commensal microbiome, leading to shifts in its natural diversity [22]. Recent advances on bacterial–fungal interkingdom communication have shown a negative correlation between the *Candida* load and the diversity of the salivary microbiome [22]. This suggests that, globally, the oral microbiome of PD patients may be altered; the impact of these changes on PD-related infections deserves to be further clarified.

Despite the limited number of isolates tested for antifungal susceptibility profile, we verified that all were resistant to itraconazole and presented a non-wild type phenotype regarding posaconazole. Also, the MIC values of posaconazole for *C. albicans* and *C. glabrata* isolates are higher than the previously reported values for wild-type strains [23]. This resistance profile may not be associated to the frequent prophylactic antifungal therapy prescription during an antibiotic course [16, 17], given that oral nystatin and fluconazole are the common choices [1]. However, itraconazole and posaconazole susceptibility profiles in oral *Candida* isolates are a matter of concern since both these drugs are second-line agents for the treatment of oropharyngeal candidiasis [24]. Also, we observed variable susceptibility profiles of *Candida* isolates to caspofungin. Taking into account that itraconazole, posaconazole, and caspofungin are prescribed for the treatment of systemic fungal infections [25] and that previous studies report the existence of antifungal resistance to itraconazole and caspofungin [26], we consider it relevant to determine the susceptibility to these antifungals in all clinical isolates.

This study presents some limitations, particularly the limited number of patients analysed and the methodology for yeast isolation (direct spread plate technique), which is associated with limited sensitivity [27]. However, it is important to highlight that the percentage of *Candida* that we obtained is similar to other studies that used the same methodology [28]. On the other hand, it is important to highlight that 10 elements of the control group were family members of the PD patients. This is a relevant aspect since

the oral microbial colonization is strongly correlated to the diet, oral hygiene, and familial predisposition.

In conclusion, oral yeast colonization may represent a limited risk for fungal infections in PD patients, given that in this pilot study there is an absence of relationship between patients with oral yeast colonization and the development of PD-related fungal infections; the *Candida* species found in oral cavity are different from the ones identified as PD-related fungal infectious agents; and also, PD patients present a low prevalence of oral yeast colonization, namely, *C. albicans*. Despite the low number of oral *Candida* isolates, the antifungal susceptibility profile revealed a possible resistance to some second-line drugs, suggesting the need for the assessment of antifungal susceptibilities in clinical practice. Further studies are still necessary to fully characterize the oral yeast colonization in this population.

Ethical Approval

All procedures performed in the study involving human participants were in accordance with the ethical standards of the Ethics Committee for Health and Institutional Review Board of São João Hospital Center and with the 1964 Helsinki declaration and its later amendments.

Consent

Informed consent was obtained from all individual participants included in the study.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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“Influence of dialysis therapies on oral health - a pilot study”

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L. Simões-Silva participated in the experimental design, conducted the experiments of microbiological analysis, collected the clinical history of patients, participated in the analysis and interpretation of the data and writing of the manuscript.

Influence of dialysis therapies on oral health - a pilot study

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Running title: Oral health status in HD and PD patients

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Abstract

Objectives: Chronic kidney disease (CKD) is a public health problem worldwide and it is not clear if observed differences regarding oral health status between haemodialysis (HD) and peritoneal dialysis (PD) patients are due to dialysis modality or to dialysis vintage. Therefore, the aim of this study was the evaluation of the periodontal disease, dental caries and *Candida* colonization among patients on HD, PD and PD with previous history of HD (HD/PD).

Method and Materials: The clinical history, smoking and oral hygiene habits were recorded. Decayed, Missing and Filled Teeth index, visual plaque index (VPI), clinical attachment level (CAL), bleeding on probing, saliva flow rate, pH, and yeast colonization were assessed.

Results: HD/PD patients were submitted to longer periods of dialysis therapy than the other groups. The number of decayed and filled teeth did not differ between groups, but HD patients presented a higher number of teeth. Among the three groups, HD patients presented higher VPI, CAL and *Candida* colonization, independently of the time on dialysis. The isolated species of *Candida* were *C. albicans* (HD and PD), *C. krusei* (HD), and *C. carphophila* (PD).

Conclusion: HD has a more adverse impact on oral health than PD, in particular periodontal disease and *Candida* colonization, but this impact on oral health appears to be reduced or ameliorated when patients change from HD to PD therapy.

Keywords: Renal Replacement Therapy; Renal Insufficiency; Periodontal disease; Oral health; Saliva; *Candida*.

Introduction

Chronic kidney disease (CKD) is considered a public health problem worldwide.¹ Across the world there are wide variations in the choice of the renal replacement therapy. For example, peritoneal dialysis (PD) prevalence in Mexico attains 85% whereas in Bulgaria reaches only 3.7%.² In Portugal, approximately 60% of the patients in end-stage renal disease (ESRD) are on haemodialysis (HD), 36% are transplanted and only 4% are on PD.³ Both dialysis modalities (HD and PD) aim to clear blood toxins by using a semipermeable membrane. On HD the membrane is synthetic whereas on

PD it is the patient's own peritoneal membrane.⁴ HD does not prevent many complications of the disease itself as chronic renal anaemia, phosphocalcium metabolism disorders, hormonal disorders, and others. Also, oral complications persist or are worsen in HD patients in comparison to the general population. Accumulation of dental calculus, changes in periodontal tissue, xerostomia, opportunistic infections by microorganisms⁵, as well as alterations in salivary secretion rate, pH, and composition are common in HD patients.⁶ Patients undergoing PD have a medium-term survival comparable to patients undergoing HD.⁷ PD potentially offers a number of important clinical advantages over

HD, including better preservation of residual renal function and vascular access sites, reduced erythropoietic stimulatory agent administration and blood transfusion requirements, decreased risk of blood-borne infections (such as hepatitis B and C), improved quality of life, enhanced treatment satisfaction, and improved subsequent renal allograft function.⁸ PD advantages are jeopardized by the risk of PD-related infections, namely peritonitis and catheter exit-site infections.⁹ In the literature, it is not clear if the observed differences regarding oral health status between HD and PD patients are due to dialysis modality or to dialysis vintage. Oral health may be evaluated by the two major oral pathologies, dental caries and periodontal disease, but also by the microbial load, in particular, the oral *Candida* colonization. Although *Candida* spp. are part of the normal microbiota of the oropharyngeal and gastrointestinal tract, they represent one of the most frequent opportunistic microorganisms of the human body.^{10,11} Therefore, this study aims to evaluate the dental caries, periodontal disease, and oral *Candida* colonization in an ESRD population on HD, PD and PD with previous history of HD (HD/PD) to determine the influence of the type of Renal Replacement Therapy on oral health.

Material and methods

Study design and patient selection

A cross-sectional clinical study was performed at the outpatient clinic of the Nephrology

Department of a Portuguese Hospital. According to previous studies¹², patients with active systemic infection and with less than 8 remaining teeth were excluded. A total of 63 patients were included in the present study, 17 undergoing HD, 35 undergoing PD, and 11 undergoing PD with previous history of HD (HD/PD group). The study sample included 8 patients with diabetes (12.7%), belonging to PD and HD/PD groups ($\approx 17\%$ each). The Ethics Committee of this hospital approved the study. All participants were recruited voluntarily after receiving detailed information on the study protocol. Written informed consent was obtained in all cases and the study was performed in accordance with the Declaration of Helsinki. Demographic variables including age, gender and level of education as well as medical chart information such as dialysis modality, time on dialysis, CKD aetiology, smoking habits, serum_creatinine, blood urea nitrogen and antibiotics prescription in the previous 3 months to the oral evaluation were recorded for all subjects. Data were collected from February 2009 to February 2012.

Assays

Routine laboratory testing of blood and 24-hour urinary samples were performed by the laboratory of the hospital using standard procedures. We used renal creatinine clearance (mL/min) as a measure of residual renal function (RRF).

Oral evaluation

Oral evaluation was performed in all patients undergoing HD (n=17), but only in 24 (69%) PD

patients and 7 (58%) HD/PD patients received oral assessment due to patients' availability. Oral health-related habit such as tooth brushing per day was recorded using a medical questionnaire. The intra-oral exam included the assessment of Visible Plaque Index (VPI), Clinical Attachment Level (CAL), Bleeding on Probing (BOP) and Decayed, Missing and Filled Teeth (DMFT) index. Oral hygiene was assessed using the Visible Plaque Index (VPI) in four sites of each tooth (mesio-buccal, mid-buccal,___distobuccal, mid-lingual)¹³; the percentage of the examined sites with visible plaque ranged from 0% to 100%. For each participant the DMFT index was calculated following the World Health Organization recommendations.¹⁴ In addition, a full-mouth periodontal examination was performed for all the teeth in the oral cavity, excluding the third molars, using a dental mirror, an explorer and the Florida Probe® (FP, Florida Probe® Company, Gainesville, FL, USA).¹⁵ CAL and BOP were assessed at 6 sites around each tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, disto-lingual). CAL was expressed as the distance in mm from the cemento-enamel junction to the bottom of the gingival pocket, whereas BOP was scored positive if a site bled after pocket probing within the time interval used for the buccal and lingual measurements of a quadrant. Two calibrated dentists performed the oral evaluation. Prior to the oral assessment, the dentists received a brief training; which included written instruction/information and a compact clinical practice course. For quantitative measurements, the t-test for paired samples

was used. The results reveal no statistical significant differences between the two examiners ($p>0.05$). Additionally, before the oral clinical evaluation, unstimulated whole saliva was collected in to a sterile container for 5 minutes in a quiet room, between 9:00 to 11:00 AM to minimize the circadian rhythm effects, and at least 2h after eating, tooth brushing, mouth washing or smoking. The same protocol was adopted to collect stimulated saliva, using paraffin pellets (Ivoclar vivadent, NY, USA). The volume collected over a 5-min period was registered, enabling the calculation of salivary flow rates (ml/min). The salivary pH was measured immediately after saliva collection using a pH indicator paper (5.0-8.0, Duotest, Germany) as previously reported.¹²

Microbiological analysis

Saliva samples from all patients were serially diluted with 0.9% sterile NaCl solution until 10^{-2} and immediately plated in triplicate in a selective and differential culture medium, CHROMagar Candida™. Plates were incubated aerobically for 48h at 37°C. The lower limit of detection was 10^2 CFU/ml. Colonies were isolated and identification of Candida species was performed following manufacturer instructions according to specific colour of the colonies. Non-identified yeasts were identified by sequence analysis of 18S rRNA and internal transcribed spacer (ITS) regions. Single colonies of non-identified yeast isolates were cultured on Sabouraud agar for 24h at 30°C and a sodiumhydroxide based method was used to extract DNA from yeasts

(protocol at http://www.aspergillus.org.uk/indexhome.htm?secure/laboratory_protocols, "DNA Extraction, miniprep"). PCR amplification was performed using a group of previously described primers – EF3, EF4, fung5, ITS1-F, ITS4^{16,17} in a Thermo-Hybrid-PX2 thermal cycler. Amplification products were visualized in a polyacrylamide gel followed by silver-staining. Sequence analysis was performed in a Genetic Analyzer ABI-Prism-3100 (Applied Biosystems). Genomic data was compared with a database that comprises a large collection of yeast sequences of 18S rRNA and ITS regions obtained from GenBank.¹⁸

Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics version 22.0. The categorical variables were described through absolute and relative frequencies (%) and analysed by Pearson's chi-squared test or Monte Carlo simulation when more than 20% of the cells had expected counts less than 5. Continuous variables were described using mean $\pm$ standard deviation (SD) and analysed by one way ANOVA test when following a normal distribution, or described by median (minimum, maximum) and analysed by Kruskal-Wallis test, when there was no normality of data. Statistical significance was assumed when the p values were less than 0.05.

Results

Age, gender, dialysis vintage, creatinine and urea blood levels, residual renal function, CKD

aetiology, educational level and antibiotic therapy from the three groups are described in table 1.

The age was similar between groups, but regarding the gender balance, the HD group included a higher percentage of men. HD/PD patients presented higher average dialysis vintage than the other two groups. This group of patients had a median time on HD of 65.6 months (min: 1.2 months; max: 208.9 months), followed by a median time on PD of 7.49 months (min: 1.3; max: 28.9).

No significant differences were observed among the three groups regarding antibiotic therapy intake at the time of the study, although HD patients were less exposed to antibiotic therapy than the other two groups (Table 1).

Both HD and PD patients reduced the smoking habit through time (McNemar's test, $p < 0.001$), however HD patients included more smokers at the time of sample collection (Table 2).

Concerning oral hygiene habits, no differences were found among the three groups (Table 2). Although DMFT index was similar among the three groups, differences were observed in Missing teeth values, with HD patients presenting the lowest level of tooth loss (Table 2). Both VPI and CAL values differ among groups, with HD patients presenting the highest level. No significant differences were observed among the three groups concerning BOP, unstimulated and stimulated-saliva flow rate and pH (Table 2).

Table 1. Demographic and clinical parameters of Chronic Kidney Disease patients undergoing haemodialysis, peritoneal dialysis or peritoneal dialysis with previous history of haemodialysis.

	HD	PD	HD/PD	p
Age (years)	53.8±6.8	46.6±12.3	45.3±13.6	0.080
Gender (%)				0.048
Male	82.4%	48.6%	45.5%	
Female	17.6%	51.4%	54.5%	
Time on dialysis modality (months)	33.5 (4.0,115.0)	5.8 (1.0,84.0)	79.9 (4.2,213.7)	<0.001
Blood creatinine (mg/dL)	9.2 (4.4,35.8)	7.0 (3.0,12.4)	9.3 (3.8,15.9)	0.088
Blood urea (mg/dL)	103.0 (35.0,242.0)	128.0 (90.0,224.0)	134.5 (69.0,172.0)	0.534
Residual renal function (mL/min)	<5.0	7.4±3.9	5.1±3.9	
CKD aetiology (%)				0.198
Chronic glomerulonephritis	38.5%	20.0%	9.1%	
Polycystic kidney disease	15.4%	17.1%	0%	
Other	7.7%	34.3%	36.4%	
Undetermined	38.5%	28.6%	54.5%	
Education level (%)				0.308
Sixth grade	81.8%	60.7%	50.0%	
Higher than sixth grade	18.2%	39.3%	50.0%	
Antibiotic therapy (previous 3 months)	0%	29.0%	30.0%	0.052

Values are in mean±standard deviation, median (min,max) or in percentage. CKD - chronic kidney disease; HD – haemodialysis; PD - peritoneal dialysis; HD/PD - peritoneal dialysis with previous history of haemodialysis.

Oral *Candida* spp. prevalence was significantly different among the three groups, presenting the highest value in patients undergoing HD (Table 3).

Candida albicans was the most prevalent yeast, but other *Candida* species were also found, namely *C. krusei* in HD patients and *C. carpophila* in PD patients (Table 3).

Table 2. Smoking habits, oral hygiene habits, decayed missing filled teeth index, visual plaque index, bleeding on probing, clinical attachment level, saliva flow rate, and saliva pH in chronic kidney disease patients undergoing haemodialysis, peritoneal dialysis or peritoneal dialysis with previous history of haemodialysis.

	HD	PD	HD/PD	p
Smoking habits (%)				
Current	47.1%	9.1%	0%	0.007
Past	64.7%	66.7%	33.3%	0.340
Oral hygiene habits (%)				
Daily tooth brushing (<2times/day)	53.8%	63.6%	50.0%	0.766
DMFT index (n)	12.0±9.0	13.0±7.0	15.0±4.0	0.790
Decay teeth	3.0±3.0	3.0±3.0	4.0±3.0	0.295
Missing teeth	2.0±4.0	6.0±6.0	7.0±6.0	0.001
Filled teeth	8.0±8.0	4.0±5.0	4.0±4.0	0.163
VPI (%)	85.0±26.0	69.0±30.0	60.0±38.0	0.030
CAL (mm)	3.4±1.3	2.5±0.9	2.5±0.5	0.002
BOP (%)	13±24	32±37	40.0±49.0	0.243
Saliva flow rate (mL/min)				
Unstimulated saliva	0.3±0.2	0.4±0.4	0.4±0.4	0.751
Stimulated saliva	1.0±0.5	1.1±1.4	0.8±0.5	0.227
Saliva pH				
Unstimulated	7.3±0.7	7.6±0.6	7.4±0.5	0.069
Stimulated	7.8±0.4	7.8±0.4	7.8±0.5	0.708

Values are presented in percentage or mean±standard deviation. HD – haemodialysis; PD - peritoneal dialysis; HD/PD - peritoneal dialysis with previous history of haemodialysis; DMFT - decayed missing filled teeth index; VPI - visual plaque index; BOP - bleeding on probing; CAL - clinical attachment level.

Table 3. Prevalence of yeast colonizers in Chronic Kidney Disease patients undergoing haemodialysis, peritoneal dialysis or peritoneal dialysis with previous history of haemodialysis.

	HD	PD	HD/PD	p
<i>Candida</i> spp.	35.3%	15.2%	0%	0.044
<i>C. albicans</i>	29.4%	12.1%	0%	
<i>C. krusei</i>	5.9%	0%	0%	
<i>C. carpophila</i>	0%	3.0%	0%	

Values are in percentage. HD – haemodialysis; PD - peritoneal dialysis; HD/PD - peritoneal dialysis with previous history of haemodialysis.

Discussion

The main goal of the present study was to compare oral health status among patients on HD and PD with or without previous history of HD. Taken together our results evidence that HD patients presented poorer oral hygiene, higher rates of periodontal disease and were more colonized in the oral cavity by *Candida* spp. in comparison to PD and HD/PD patients. These facts suggest that the adverse impact of HD therapy on oral health may be reduced or ameliorated when patients change from HD to PD therapy.

The higher dental plaque accumulation observed in HD patients is consistent with previous studies, namely from Bayraktar and co-workers that justified this observation with the lower educational level of HD patients in comparison to PD.¹⁹ In this study albeit no significant differences were observed among groups regarding education level, PD and HD/PD patients presented slightly higher percentages of education level when compared to HD patients. Note that CKD patients in order to engage the PD program have to present a high comprehension level to be able to

autonomously comply with the technical requirements of this technique. Also, dental plaque correlates with poor oral hygiene that strongly and positively correlates with periodontal disease. In fact, our HD population showed higher apical migration of the epithelial attachment when compared to PD patients. This gingival weakness may be attributed to the use of repeated systemic anticoagulation therapy administered to HD patients, promoting gingival bleeding and enhancing bacterial colonization.²⁰ Although presenting longer dialysis vintage, PD patients with previous history of HD presented an oral health status more similar to PD patients, hypothesizing that the alterations induced by HD in the oral health status of CKD patients may be reversible when the patient changes to other renal replacement therapy. Nevertheless, HD patients gather more risk factors than PD patients for periodontal disease, namely poor oral hygiene, smoking habits and higher male percentage.^{21,22} It is well known that, besides tooth decay or infection, periodontal disease is one of the most common reasons for dental extraction. In our study, although HD patients presented a poorer periodontal status, a higher

number of teeth were observed, in comparison to PD and HD/PD patients. Given that HD patients have more bleeding tendencies than PD patients, and special drug dosage adjustments are necessary for all patients in dialysis therapy, one can hypothesize that dental care was more conservative in HD patients possibly to avoid surgical procedures, when compared to the other two groups.

In PD, infections are still one of major complication of this renal replacement therapy, mainly associated to contamination at the time of the PD fluid exchange in patients' home. However, HD patients are in a clinical environment several times a week, being therefore more susceptible to acquire health care associated infections than PD patients.²³ The fact that *Candida* species are frequent nosocomial fungal pathogens²⁴ may in part justify the higher oral *Candida* colonization of HD patients.

Nevertheless, *Candida* spp. are common commensal microorganisms of most individuals.²⁵ Furthermore, antibiotic therapy could not justify the differences on oral *Candida* colonization given that PD patients that were less colonized were under more antibiotic therapy than HD patients. The higher *Candida* colonization of HD patients may be linked to the more severe periodontal disease or higher smoking habits, two factors known to promote *Candida* growth.^{26,27}

Higher oral *Candida* prevalence and more severe periodontal disease are two features that may represent increased risk of systemic infection in renal transplants. Thus, to control

and minimize the risk of developing invasive infections, it is important to promote a good oral hygiene in order to reduce periodontal disease and oral *Candida* prevalence.¹¹ Educational programs focusing on the promotion of oral health are urgently needed among CKD population as recently highlighted by Ruokonen and colleagues.²⁸

Conclusion

HD has a more adverse impact on oral health than PD, in particular periodontal disease and *Candida* colonization, independently of the time on dialysis. Therefore, and despite the limited number of patients, this study highlights some relevant risk factors for HD patients and suggests that the adverse impact of HD therapy on oral health may be reduced or ameliorated when patients change from HD to PD therapy.

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- Conflict of Interest:**
- The authors declare that they have no conflict of interest.
- Ethical approval:**
- All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

CHAPTER V

“Asymptomatic effluent protozoa colonization in peritoneal dialysis patients”

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“Low prevalence of oral protozoa colonization in Portuguese healthy and end-stage renal disease patients undergoing peritoneal dialysis”

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Taking into account the low immunity of CKD patients, the high relevance of infection in PD, and the increased prevalence of parasites in CKD patients, the aim of the present study was to screen the protozoa colonization of the dialysis effluent and oral cavity of PD patients.

“Asymptomatic effluent protozoa colonization in peritoneal dialysis patients”

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L. Simões-Silva participated in the collection, analysis and interpretation of the data and writing of the manuscript.

Asymptomatic Effluent Protozoa Colonization in Peritoneal Dialysis Patients

Currently, chronic kidney disease (CKD) is a global health problem. Considering the impaired immunity of CKD patients, the relevance of infection in peritoneal dialysis (PD), and the increased prevalence of parasites in CKD patients, protozoa colonization was evaluated in PD effluent from CKD patients undergoing PD. Overnight PD effluent was obtained from 49 asymptomatic stable PD patients. Protozoa analysis was performed microscopically by searching cysts and trophozoites in direct wet mount of PD effluent and after staining smears. Protozoa were found in PD effluent of 10.2% of evaluated PD patients, namely *Blastocystis hominis*, in 2 patients, and *Entamoeba* sp., *Giardia* sp., and *Endolimax nana* in the other 3 patients, respectively. None of these patients presented clinical signs or symptoms of peritonitis at the time of protozoa screening. Our results demonstrate that PD effluent may be susceptible to asymptomatic protozoa colonization. The clinical impact of this finding should be further investigated.

Perit Dial Int 2016; 36(5):000–000 [www.PDConnect.com](http://dx.doi.org/10.3747/pdi.2015.00226)
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Supplemental material available at www.pdconnect.com

KEY WORDS: Peritoneal dialysis effluent; protozoa colonization, *Blastocystis hominis*; *Entamoeba* sp; *Giardia* sp; *Endolimax nana*.

Alterations in immune response induced by the uremic state of chronic kidney disease (CKD) patients increase the susceptibility to infection and appear to play an important role in determining the type, incidence, and outcome of infectious complications (1,2). In this setting, opportunistic infections depend upon the state of immunosuppression and the epidemiological exposure of the patients. Despite prevention strategies, peritonitis remains a leading complication of peritoneal dialysis (PD). Therefore, prevention of infection is crucial for the success of PD therapy. The factors that influence the PD-related infections occurrence are still not entirely understood. Peritoneal dialysis-related peritonitis occur more frequently due to bacterial infection, but culture-negative peritoneal infection in some PD units still accounts for 20% of peritonitis episodes (3,4). In these culture-negative cases, consideration should be given to unusual microorganisms.

More than 4,000 million people worldwide are infected with parasitic infections (5). Nowadays, with the increase in number of patients with immune deficiency, increased organ transplantation, immune-suppressing drugs, and radiation therapy, higher statistics related to parasitic infection are common in developed countries (6). In accordance with the fact that parasitic infections are more prevalent in immunocompromised patients, several reports found higher prevalence of intestinal parasites in CKD patients than in healthy individuals (2,6). Although very scarce, some reports describe parasite

infections in dialysis fluid in end-stage renal disease (ESRD) patients undergoing PD (7–9). Considering the low immunity of CKD patients, the high relevance of infection in PD, and the increased prevalence of parasites in CKD patients, the aim of the present study was to screen protozoa colonization in PD effluent from ESRD patients undergoing PD.

MATERIALS AND METHODS

A group of 49 CKD patients undergoing PD in the outpatient clinic of the nephrology department of Centro Hospitalar S. João (Porto, Portugal) participated in the present study and their written informed consent was obtained (approved by the Ethics Committee of Centro Hospitalar de S. João). Peritoneal dialysis effluent (50 mL) was collected aseptically and centrifuged at 1,500 rpm, 10 minutes, 4°C. Six smears per patient were analyzed corresponding to 50 µL of pellet. For protozoa identification by microscopic evaluation, both latency and vegetative cellular forms were searched on direct wet mount smears with Lugol's iodine solution and stained with Giemsa, Trichrome, and Modified Acid-Fast Kinyoun (10). Protozoa evaluation in saliva and feces was performed as described for PD effluent. Feces were also analyzed by the local microbiology department. Feces were collected only from patients with PD effluent protozoa colonization.

RESULTS AND DISCUSSION

The mean age of the 49 asymptomatic stable PD patients was 47.7 ± 12.9 years with a balanced proportion of each gender (55.1% male [M], 44.9% female [F]). In general, the evaluated population presented a low educational level (illiterate 4.1%, elementary school 53.0%, high school 6.1%, university 8.6%, unknown 22.4%). The most prevalent CKD etiologies were chronic glomerulonephritis (22.4%, including high prevalence of IgA nephropathy) and diabetic nephropathy (16.3%), followed by polycystic kidney disease (10.2%), others (26.5%), and undetermined (24.5%). These patients presented a mean time in a PD program of 10.8 ± 13.7 months, ranging from 1 to 72.3 months, with 89.8% on continuous ambulatory PD (CAPD) and 10.2% on automated PD (APD). From the 49 PD patients, 26.5% had 1 or more previous peritonitis episodes, bacteria and fungi being responsible for 70.6% and 5.9%, respectively, whereas culture-negative accounted for 23.5% of the total peritonitis episodes. Among 49 CKD patients undergoing PD, 5 presented PD effluent protozoa colonization without any clinical signs or symptoms of peritonitis. These 5 PD patients did not present leukocytosis or eosinophilia. In the PD effluent of 3 PD patients, cysts were observed, namely, 1 patient with cysts of *Endolimax nana* (Figure 1A) and 2 patients with cysts of *Blastocystis hominis* (Figure 1D). Also, we found trophozoites of *Entamoeba* sp. (Figure 1C) in 1 PD patient and trophozoites of *Giardia* sp. (Figure 1B) in another patient. In each positive PD patient sample, we observed less than

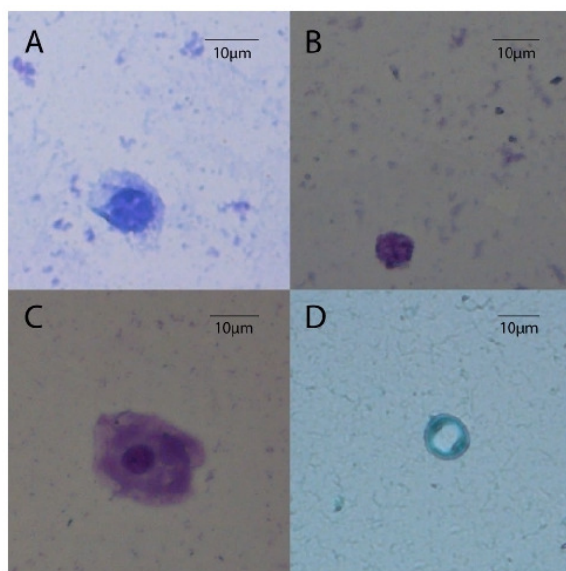


Figure 1 — Protozoa found in PD effluent from CKD patients undergoing PD: A) *Endolimax nana*; B) *Giardia* sp.; C) *Entamoeba* sp.; and D) *Blastocystis hominis*. Images obtained after Giemsa stain observed at amplification of 400x. PD = peritoneal dialysis; CKD = chronic kidney disease.

5 cellular forms (cysts or trophozoite) per smear. This may be due to the low number of protozoa found in each patient that can represent a non-infectious dose. However, even if these protozoa are considered symbionts, it should be noted that this population is immune-impaired, and thus commensals may represent relevant opportunistic microorganisms. In the few clinical reports describing the presence of parasites in dialysis effluent, a *Pseudomonas* sp. peritonitis with concomitant presence of a protozoon, *Balantidium hominis*, was described (9). The patient was treated efficiently with specific antibiotic therapy for *Pseudomonas* sp. infection, raising the question of the role of this specific protozoon: symbiont or pathogen.

Recent findings associated CKD to an impairment of the intestinal epithelial barrier structure and function that may enable translocation of microorganisms and other microbial products across the intestinal wall to systemic circulation (11,12). Peritoneal dialysis effluent protozoa colonization may represent a marker for the loss of intestinal epithelial barrier integrity in CKD patients. In the fecal samples of 3 PD patients, no intestinal protozoa were found by the research team or by the local laboratory. Since during the study, 2 protozoa-positive PD patients died, 1 with a peritonitis episode caused by *Escherichia coli* due to an intestinal perforation 5 months after sampling and the other due to sudden death, the collection of feces from these patients was not possible. The clinical history of the 5 PD patients colonized by protozoa, regarding signs and symptoms, peritonitis, and catheter exit-site (CES) infections revealed that 2 patients had an intestinal deregulation 6 months previous to effluent sampling and 1 patient

had 2 peritonitis episodes by *Streptococcus* sp. and *E. coli* with intestinal perforation 1 and 5 months after sampling, respectively. Thus, our results could represent an accidental gut colonization acquired by water or food consumption. The absence of correlation with intestinal colonization was previously described in the literature (13), but it is important to note that the stools were collected after the PD effluent analysis and in some cases the time gap was superior to 6 months.

Entamoeba protozoa are recognized oral colonizers, although with low prevalence (14). Interestingly, this protozoon also colonized the saliva of the patient presenting *Entamoeba* sp. in PD effluent, suggesting that the digestive tract may represent in this case the source of this microorganism.

In order to understand the transmission routes of these microorganisms among the PD patients, it is crucial to characterize other environmental factors. In the group of PD patients presenting effluent protozoa colonization, clinical and environmental/social characterization was performed (Supplemental Table 1). In general, these 5 patients presented a low education level, different CKD etiologies, 1 being diabetic. Moreover, 2 out of 5 of these patients combined the following features: female, had contact with and frequently ate domestic poultry, water source was a private water-well, which made them more prone to contamination given their open access to the environment.

Despite the importance of these results, this study presented some limitations, such as the limited number of patients analyzed, and the applied methodology that presents lower sensitivity and specificity than other methods, such as enzyme immunoassays, polymerase chain reaction (PCR), as well as flow cytometry (15). Thus, one can hypothesize that with a more sensitive methodology, a higher prevalence of patients with PD effluent protozoa colonization could be detected. In conclusion, PD effluent of CKD patients undergoing PD may be susceptible to asymptomatic protozoa colonization. The clinical impact of this finding should be further investigated.

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DISCLOSURES

The authors have no financial conflicts of interest to declare.

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“Low prevalence of oral protozoa colonization in Portuguese healthy and end-stage renal disease patients undergoing peritoneal dialysis”

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INTRODUCTION

Compared to other groups of microorganisms, few parasites colonize the oral cavity. The protozoan flagellate *Leishmania* can rarely, but severely, affect the human oral cavity and it does so indirectly by causing granulomatous growth disfigurements involving the mouth and nose (Bergquist, 2009). Other parasites can cause severe systemic disease after entering through oral cavity, such as *Naegleria fowleri* and *Acanthamoeba* encephalitis that may reach nasal cavity, penetrating the olfactory epithelium and, eventually, invading the brain (Cervantes-Sandoval, Serrano-Luna Jde, Garcia-Latorre, Tsutsumi, & Shibayama, 2008; Kaushal *et al.*, 2008). However, the most frequent human oral protozoa are usually non-pathogenic commensals such as *Entamoeba gingivalis*, followed by *Trichomonas tenax* (Wantland & Lauer, 1970). The oral colonization by these protozoa is associated with poor oral hygiene and with the severity of periodontal disease (Bergquist, 2009; Feki, Molet, Haag, & Kremer, 1981; Ghabanchi, Zibaei, Afkar, & Sarbazie, 2010; Kurnatowska, Dudko, & Kurnatowski, 2004; Pomes *et al.*, 2000; Wantland & Lauer, 1970).

Although literature reports are scarce, recent studies have revealed that oral protozoa colonization is more frequent than previously thought, and that its prevalence may vary significantly with the worldwide geographic distribution (Bergquist, 2009; Ghabanchi, *et al.*, 2010). In central Europe two studies from the 80s report an oral protozoa prevalence between 28% and 85% (Cambon *et al.*, 1979; Feki, *et al.*, 1981) whereas in eastern Europe two more recent studies showed an oral protozoa prevalence of 18% and 37% in Slovakia and Poland, respectively (Kurnatowska, *et al.*, 2004; Vrablic, Tomova, Catar, Randova, & Suttova, 1991). In Iraq and Iran, the prevalence varies between 4% and 46% (Ghabanchi, *et al.*, 2010; Madhi & Saeed, 1993) and, in Borneo, Indonesia, an oral protozoa prevalence of 18% was reported (Palmieri, Halverson, Sudjadi, Purnomo, & Masbar, 1984). In 1970, Wantland reported a prevalence of up to 55% in north of America (Wantland & Lauer, 1970) whereas in central and south America, studies from the 90s, described a prevalence of 21% and 62% in Guatemala and Brazil, respectively (Favoreto Junior & Machado, 1995; Pomes, *et al.*, 2000). To our knowledge no studies were performed so far in Portugal.

Parasitic disease continues to cause significant morbidity and mortality throughout the world (Stark *et al.*, 2009). Specifically protozoa are one of the most important pathogens that affect immune compromised patients (Ferreira, 2000). Individuals with impaired cell immunity such as carriers of the human immunodeficiency virus, patients with haematologic neoplasias, patients submitted to transplant of solid organs or under high-dose corticosteroid therapy are among the most infected by this pathogens group.

Immunosuppressed hosts are more susceptible to acquire infection after microorganisms exposure, and, although not commonly valorised, the oral cavity may act as a source of systemic pathogens (Bahrani-Mougeot *et al.*, 2008; Kshirsagar *et al.*, 2009; Li, Kolltveit, Tronstad, & Olsen, 2000). Once the infection is established, disease becomes more severe and disseminated, since the patients' immune response has more difficulty in eliminating the parasites, becoming chronic carriers (Stark, *et al.*, 2009). Being the immune system dysfunction a characteristic of end-stage renal disease (ESRD) patients due to uraemia (Kato *et al.*, 2008), parasite colonization can represent a risk factor in ESRD patients. In accordance, the major causes of death in ESRD patients are cardiovascular disease and infections, together accounting for up to 70% of all deaths in this patient population (Kato, *et al.*, 2008). In this population several reports demonstrate the risk of intestinal protozoa colonization (Barazesh, Fouladvand, Tahmasebi, Heydari, & Fallahi, 2015; Gil *et al.*, 2013; Visvesvara *et al.*, 2013). In Brazil, Gil and colleagues reported a prevalence of 51.6% of intestinal parasites in hemodialysis patients, but although the prevalence of colonization did not differ from control patients, ESRD patients were more easily symptomatic than controls (Gil, *et al.*, 2013). In Iran, Barazesh and colleagues reported a 28.4% of hemodialysis patients infected with one or more intestinal parasites (Barazesh, *et al.*, 2015). Blastocystidiosis, *Entamoeba coli* and *Cryptosporidium* were the most common parasitic infections in ESRD patients (Barazesh, *et al.*, 2015; Gil, *et al.*, 2013).

Peritoneal dialysis (PD) is a home-based widely used renal replacement therapy, and represents an alternative to hemodialysis (HD), with comparable survival, lower cost, and improved

quality of life (Chaudhary, Sangha, & Khanna, 2011). In PD, the patients peritoneal membrane is used as a dialysis membrane. Sterile dialysis fluid is introduced through a catheter into the abdominal cavity, drained and refreshed several times during the day and throughout the night. Despite the improvements in the last decades, infection-related morbidity is still a significant complication in PD patients, accounting for 16 to 18% of the deaths in this population, as well as catheter loss, transfer to HD or prolonged hospitalization (Bloembergen & Port, 1996; Pereira, Sayegh, & Blake, 2005; Szeto, Wong, Chow, Leung, & Li, 2003). Therefore, prevention of infection is crucial for the success of PD therapy (Barraclough, Hawley, Playford, & Johnson, 2009).

Taking into account that there were no studies evaluating oral protozoa colonization in Portugal and that in ESRD patients undergoing PD the focus of infection should be a major concern, the aim of this study was to evaluate protozoa colonization in saliva of Portuguese healthy individuals and ESRD patients undergoing PD therapy at the Hospital S. João, the main hospital from the North of Portugal.

MATERIAL AND METHODS

Study participants

A group of ESRD patients undergoing PD therapy at the outpatient clinic of the Nephrology Department of "Centro Hospitalar de S. João" (Porto, Portugal) was invited to participate in the present study. Also, non-ESRD family members living at the same house and closest in age were invited to participate as control group. The study was explained orally and a written informed consent was obtained from all participants. The written informed consent as well as the study protocols were in accordance with the Helsinki Declaration of 1975, as revised in 1983, and were approved by the Ethics Committee of "Centro Hospitalar de S. João". Exclusion criteria were applied, namely: (a) inability to give informed consent, (b) pregnancy, and (c) severe acute illness. The study sample included 39 PD patients and 18 family volunteers.

Clinical patient information was gathered, including: (a) age, (b) gender, (c) ethnicity, (d) education level, (e) aetiology of renal disease, (f) time on renal replacement therapy (RRT), (g) past

and present peritonitis episodes, and (h) microbial agents responsible for peritonitis episodes. Also, information regarding the water source consumption was obtained.

Sample collection

Saliva was collected from all participants for protozoa analysis. Patients were instructed not to eat, drink or perform the normal oral hygiene at least two hours before the procedure. Samples of stimulated saliva were collected by the spitting of whole-mouth saliva during chewing paraffin pellets for 5 minutes (Ivoclar Vivadent, NY, USA).

Protozoa evaluation

Stimulated saliva was collected from each patient and placed in eight smears (50 µl each). Microscopic evaluation was performed by direct mounts with Lugol's iodine solution and after staining with Giemsa, Trichrome and Kinyoun techniques, in duplicate. Microscopic observation and photographs were performed using a Leica DM4000B microscope (Wetzlar, Germany) connected to a camera Leica DFC320 with 400x and 1000x amplification. The images were processed using Leica Application Suite software (version 2.3.1 R1).

Data analysis

Statistical analyses were performed by using IBM® SPSS® version 21.0 (Statistical Package for Social Sciences). The categorical variables were described through relative frequencies (%) and analysed by Chi-square independence test. Continuous variables were described using mean ± standard deviation (SD) and analysed by student's t-test. A $P < 0.05$ was assumed to denote a significant difference.

RESULTS

Demographic data from PD patients and controls are shown in Table I. Control family members were slightly younger and females were more prevalent in controls than in PD patients, although no significant differences were observed regarding age and gender. In general, education level was low in both control and study groups, with no significant differences between groups.

Table I. Demographical data from ESRD patients undergoing peritoneal dialysis (PD) and healthy family members controls.

	PD patients	Controls	Pvalue
Age (years)	45.4±14.6	37.5±16.8	0.800a
Gender			0.082b
Male	51.2%	27.2%	
Female	48.8%	72.2%	
Education level			0.064b
Illiterate	4.9%	0%	
Elementary School	63.4%	38.9%	
High school	9.8%	33.3%	
University	9.8%	16.7%	

Results are shown in prevalence (%) or mean±SD. ^aStudent's t-test and ^bChi-square independence test.

In PD patients, the most prevalent aetiologies of chronic kidney disease were chronic glomerulonephritis (19.5%, with a high prevalence of IgA nephropathy) and diabetic nephropathy (14.6%). The mean time on PD program was 12.7 months. Before the study, 25.6% of the PD patients had previous peritonitis episodes and gram-positive cocci were the most prevalent isolated microorganisms. None of the patients had reported peritonitis caused by fungi or protozoa.

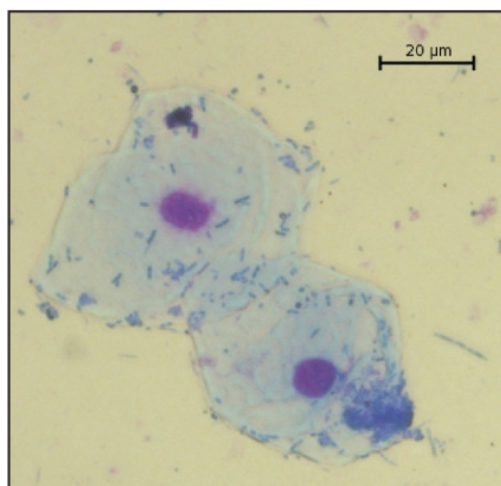
Most participants drank treated water from urban water supply network (68.3%), although 19.5% still used water from private water-well or water-borehole. We were not able to obtain information regarding the water source from 12.2% of PD patients.

In this study, oral protozoa colonization was assessed in stimulated saliva due to the low salivary rates observed in many PD patients. Concerning the microscopic observation of saliva, normal epithelial cells and oral microbiota (cocci, bacilli and, in some cases, yeasts) (Fig. 1) were observed in the wet mounts of all samples. Despite the use of specific staining methods for *E. gingivalis*, *T. tenax*, *Giardia* sp and *Cryptosporidium* sp, an amoeba was observed in only one PD patient (2.6%) (Fig. 2). The respective family member control, as well as all other participants of the study, did not present oral protozoa colonization.

DISCUSSION

This is the first study to evaluate oral protozoa colonization in a Portuguese population of

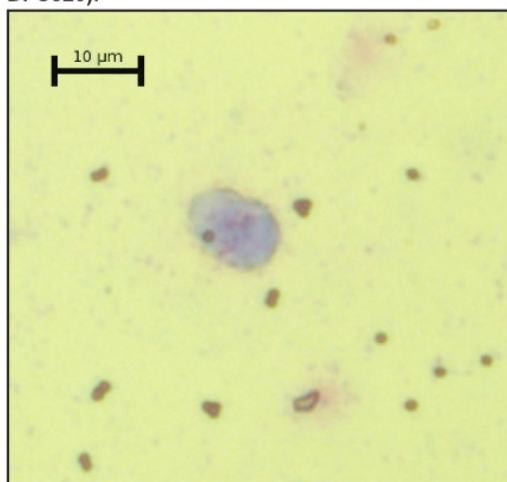
Fig. 1. Microscopic view of saliva samples after giemsa staining, showing an assortment of epithelial cells and bacteria (400x, Leica DFC320).



ESRD patients undergoing PD and respective family controls, as no other study was found in the literature.

It is well known that oral microbial colonization is strongly correlated with oral hygiene, dietary habits and familiar pre disposition; additionally, oral protozoa prevalence is associated with living environment conditions as well as alimentary habits (Almeida *et al.*, 2010; Smith, Caccio, Cook, Nichols, & Tait, 2007). Thus, for this type of studies, the recommended control group should be family members of the patients, as here employed, rather than unrelated healthy individuals (C. Arcias *et*

Fig. 2. Microscopic view of saliva sample after trichrome staining, showing a trophozoite of an amoeba with a centrally karyosome, peripheral chromatin and a granular cytoplasm (400x, Leica DFC320).



al., 2012; C. M. Areias, Sampaio-Maia, Guimaraes, Melo, & Andrade, 2011). In addition, evaluation of oral protozoa colonization in PD patients' family members is crucial since they could represent potential source of infection and act as vehicles for opportunistic microorganism transmission.

In this study only one PD patient presented oral protozoa colonization, probably by *Entamoeba gingivalis* due to the fact that is the most frequent amoeba in the oral cavity. Interestingly, protozoa colonization was not observed in his corresponding control. Despite the very low oral protozoa colonization among the studied PD population, no protozoa were observed in the control group, probably suggesting a higher susceptibility of PD patients to protozoa colonization in comparison to non-ESRD individuals.

In particular, the patient presenting an amoeba in saliva was suggested to be immune compromised, given that *Aspergillus fumigatus* and *Acinetobacter baumannii* were identified in his bronchial secretions before his death, 6 months after saliva collection for protozoa isolation. Despite the fact that no peritonitis episodes were described in this patient before the study, two peritonitis occurred one and five months after saliva collection, caused by

Streptococcus sp. and *Escherichia coli* respectively, this last in the context of an intestinal perforation that resulted in the patient's death. Future studies with a larger sample group are required to confirm this difference between health and CKD patients.

It has been previously documented that oral protozoa prevalence may vary significantly with the geographic distribution (Wantland & Lauer, 1970). Despite the fact that no data are available for the Portuguese population this study, performed at the main reference hospital of the North of Portugal, could suggest a low colonization among the northern Portuguese population. In accordance to this, a Portuguese study performed on the water basins and rivers from the north of Portugal reported the existence of a low infection risk for protozoa with surface raw and drinking water samples (Almeida, *et al.*, 2010). However, it is well established that drinking water from private wells or boreholes may represent a significant risk for infection transmission. In our study, 20% of the individuals obtained their drinking water from private wells or boreholes; so, the low rate of protozoa colonization may be explained by the generalized access of our population to water from the public network system. Despite this, the water source of the PD patient colonized by protozoa in saliva was the public network system, what may suggest that others transmission routes may have a significant impact in the oral colonization by these infectious agents.

The low rate of oral protozoa in our PD patients, namely 2.6%, and its absence in their healthy family controls may also be conditioned by the limited number of patients analysed, as well as by the methodology applied. The direct visualization of cells (cysts or trophozoites) in the samples presents lower sensitivity and specificity than other methods, such as immunofluorescence and enzyme immunoassays, culture methods, PCR as well as flow cytometry (J. Barbosa, Costa-de-Oliveira, Rodrigues, & Pina-Vaz, 2008; J. M. Barbosa *et al.*, 2008).

In conclusion, very low oral protozoa colonization was found in this study, suggesting that this may reflect the oral protozoa colonization of Portuguese population. Future studies are required to evaluate the possible higher susceptibility to oral protozoa colonization of ESRD patients undergoing PD.

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Conflict of interest

The authors declare that they have no conflict of interest.

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CHAPTER VI

**“Peritoneum microbiome in end-stage renal disease patients and
the impact of peritoneal dialysis therapy”**

Submitted for publication

Factors influencing the occurrence of PD-related infections are not fully understood. Recent studies describe the existence of specific microbiomes in body sites previously considered microbiome-free, unravelling new pathways of microbial translocation in the human body. Additionally, in CKD occurs an impairment of intestinal epithelial barrier allowing the translocation of intestinal microorganisms and microbial products to the intestinal wall, systemic circulation, and the internal milieu. These facts led us to hypothesise that the peritoneum of CKD patients may harbour a unique microbiome. In this chapter, we characterized the peritoneum microbiome in ESRD patients and the impact of PD therapy.

“Peritoneum microbiome in end-stage renal disease patients and the impact of peritoneal dialysis therapy”

Submitted for publication

L. Simões-Silva participated in the experimental design, conducted the experiments, participated in analysis and interpretation of the data and wrote the manuscript.

Peritoneum microbiome in end-stage renal disease patients and the impact of peritoneal dialysis therapy

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Running title: Peritoneum microbiome in ESRD

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Abstract

Background: Factors influencing the occurrence of peritoneal dialysis (PD) related infections, namely peritonitis and catheter exit-site infections, are still far from being fully understood. Recent studies describe the existence of specific microbiomes in body sites previously considered microbiome-free, unravelling new pathways of microbial translocation in the human body. The aim of the present study was to understand if the peritoneum of end-stage renal disease (ESRD) patients harbours a unique microbiome and if it is altered in patients on PD therapy.

Methods: We conducted a cross-sectional study where the microbiome of peritoneal tissue from ESRD patients with intact peritoneal cavity (ESRD-nonPD, n=11) and ESRD patients undergoing PD therapy (ESRD-PD, n=9) was analysed by a metagenomic approach including PCR amplification of the V3-V4 region of 16S rRNA gene using MiSeq Illumina® technology.

Results: Peritoneum tissue of both groups of ESRD patients harbours a unique low abundance microbiome dominated by Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes. Patients undergoing PD therapy presented lower species richness with a dominance by Pseudomonadaceae and Prevotellaceae families.

Conclusions: This study provides the first characterization of the peritoneum microbiome in ESRD patients bringing new insights to the human microbiome. Additionally, PD therapy may be accompanied by changes in this unique microbiome. The clinical relevance of these observations should be further explored, in particular by uncovering the role of the peritoneum microbiome as a key element in the onset or aggravation of ESRD patients' infections, especially patients undergoing PD.

Key-words: microbiome, peritoneum, chronic kidney disease, peritoneal dialysis, end-stage renal disease.

Introduction

The human microbiome influences the well-being of the host by contributing to its nutrition, metabolism, physiology and immune function, serving to maintain the balance between health and disease states.¹⁻³ Disturbances of normal gut microbiome are currently associated with the pathogenesis of several chronic diseases, including chronic kidney disease (CKD).⁴⁻¹² CKD is an emerging public health problem, with 11–13% of the world's population presenting CKD in its different stages (1 to 5).^{13, 14}

The number of patients with end-stage renal disease (ESRD) requiring renal replacement therapy (RRT) is increasing every year.¹⁵ Peritoneal dialysis (PD) is a home-based RRT for almost 200 thousand worldwide patients suffering from ESRD.¹⁶

In most parts of the world PD is still underused, even though it has comparable risks and is significantly less costly than haemodialysis.¹⁷ Major complications of patients undergoing PD are peritonitis and exit-site infections (ESI);^{18, 19} however, factors influencing the occurrence of these infections are still far from being fully understood.

The most recognized factor contributing to PD patients' peritonitis is the contamination via an exogenous route with skin pathogenic bacteria such as *Staphylococcus*.²⁰⁻²² This may occur during connection and disconnection of the dialysis transfer-set. Although microorganisms may access the peritoneum through the catheter exit-site and tunnel, the association between peritonitis and catheter

ESI was described in just less than 13% of all peritonitis episodes.²³⁻²⁵ In parallel, endogenous contamination routes of the peritoneal cavity have been recognized. These include the haematogenous route, vaginal leak, and translocation of microorganisms through the intestinal epithelial barrier.^{26, 27} Valadez and colleagues²⁸ suggested that the decrease of intestinal permeability to microorganisms could be relevant for the prevention of several complications of liver cirrhosis, namely bacterial spontaneous peritonitis. In CKD, the impairment of intestinal epithelial barrier structure and function allows the translocation of intestinal microorganisms, endotoxin, antigens and other microbial products through the intestinal wall, towards the systemic circulation and the internal milieu.²⁹⁻³⁵ A blood microbiome is presently acknowledged to exist in healthy, non-infectious states.³⁶⁻⁴⁰ Some authors postulate that the haematogenous route may be responsible for the colonization of body sites that were previously considered sterile, as is the case of the placenta,⁴¹ and breast tissue.⁴² Although the peritoneal cavity and surrounding tissues have so far been considered as being sterile, previous reports described the in vitro intracellular viability of *Staphylococcus aureus* within mesothelial cells, as well as the presence of bacterial DNA and protozoa colonization of peritoneal dialysate in PD patients.⁴³⁻⁴⁶

In this study, we postulated that the seemingly sterile peritoneum could host its own microbiome. We thus implemented a metagenomic approach to investigate whether the peritoneum of ESRD patients harboured a

unique microbiome, one that could differ in patients undergoing PD therapy.

Methods

Study design, subjects and sample collection

This cross-sectional study included adult ESRD patients followed up in the outpatient clinic of the Nephrology Department of “Centro Hospitalar de S. João”, scheduled for peritoneal catheter insertion, replacement or removal, or hernia repair. Twenty ESRD adult patients accepted to participate in the study between December of 2014 and June of 2016, and were distributed into two groups: 11 ESRD patients scheduled for peritoneal catheter insertion (ESRD-nonPD) and 9 ESRD patients undergoing PD scheduled for catheter replacement or removal, or hernia repair (ESRD-PD). Clinical evidence of infection during the preceding month was an exclusion criteria. The study protocol was approved by the Ethics Committee for Health and Institutional Review Board of “Centro Hospitalar de S. João” and with the 1964 Helsinki declaration and its later amendments; all recruited patients were asked to give their written informed consent.

Clinical information was gathered from all patients including age, gender, aetiology of CKD, residual renal function and time on PD therapy. Information about PD-related infectious episodes was gathered in the ESRD-PD group before and after peritoneal biopsy collection, and in the ESRD-nonPD group after

peritoneal tissue collection (i.e. their engagement in PD therapy). Peritoneal tissue samples (1cm² sections) were collected by trained surgeons under strict sterile conditions and placed in DNA-free sterile microtubes. Samples were immediately frozen in liquid nitrogen and stored at -80°C.

Specimen processing and microbiome analysis

Genomic DNA was isolated in a strictly controlled environment at Vaiomer SAS (Labège, France) and the methodology used was described by Lluch and colleagues.⁴⁷ Briefly, PCR amplification was performed using 16S rRNA gene universal primers targeting the V3-V4 region of the bacterial 16S rRNA gene.⁴⁷ Illumina sequencing length, by use of the 2x300 paired-end MiSeq kit V3, was designed to encompass the 476 base pair amplicon. Sample multiplexing was performed using tailor-made 6 bp unique indexes and for each sample, a sequencing library was generated. All libraries were pooled in the same quantity in order to generate equivalent number of raw reads with each library. The DNA concentration in the pool was quantified by qPCR using the 7900HT Fast Real-Time PCR System (Life Technologies) and KAPA Library Quantification Kits for Illumina Platform (Kapa Biosystems, Inc., Wilmington, MA, USA) as recommended by the manufacturer (Illumina, San Diego, CA, USA). The final pool, at a concentration after dilution between 5 and 20 nM, was used for sequencing. The sequencing effort was performed using a paired-end sequencing run in a MiSeq Illumina device.

16S rRNA Gene Sequence Analysis

The targeted metagenomic sequences were analysed using the FROGS bioinformatics pipeline established by Vaiomer SAS (Labège, France).⁴⁸ Briefly, bar code Illumina paired reads were demultiplexed, single read sequences were removed, and paired reads were assembled into longer contigs. Operational taxonomic units (OTU) were produced via single-linkage clustering, and taxonomic assignment was performed to determine community profiles. This included quality-filtering, alignment against a 16S rRNA gene reference database, and OTU clustering with the Swarm algorithm. Outputted OTUs files were uploaded and formatted for LEfSe⁴⁹ analysis using the per-sample normalization of sum values option. The linear discriminant analysis effect size was determined using default values (alpha value of 0.5 for both the factorial Kruskal-Wallis test among classes and the pairwise Wilcoxon test between subclasses, threshold of 2.0 for the logarithmic LDA score for discriminative features) and the strategy for multi-class analysis set to 'all-against-all'. LEfSe cladograms from the LDS effect size data were generated with Bacteria as the tree root. Differential features detected as biomarkers from the raw data were used to generate the cladograms, plotted as abundance histograms with class and subclass

information. Principal Coordinate Analysis (PCoA) was performed on the normalized OTU table, for comparison of sample groups/class based on four methodologies for β -diversity evaluation: 1) Bray-Curtis, 2) Jaccard, 3) Unifrac, and 4) Weighted Unifrac.

Statistics

Categorical variables were described through relative frequencies (%) and analysed by Chi-square independence test or Fisher Exact test when more than 1 cell had expected counts less than 5. PERMANOVA was used to test the significance of beta diversity. Continuous variables were described using mean $\pm$ standard deviation (SD) and analysed by student's t test. $P < 0.05$ was assumed to denote a significant difference. The presence or absence of OTUs were used to make a heat map.

Results

To investigate the existence of a microbiome in the peritoneum of ESRD patients we generated a cohort of ESRD patients undergoing PD (ESRD-PD), and ESRD patients before PD onset (ESRD-nonPD). The demographic and clinical characteristics of the two groups of patients included in this study were similar (Table 1)).

Table 1: Age, sex, education level, aetiology of chronic kidney disease (CKD), time on peritoneal dialysis of ESRD patients undergoing PD (ESRD-PD) and ESRD patients (ESRD-non PD).

	ESRD-PD (n=9)	ESRD-non PD (n=11)	P value
Age (years)	45.11±11.27	43.27±12.42	0.736
Sex (male, %)			0.070
Male (%)	77.8%	23.7%	
Female (%)	22.2%	72.7%	
Aetiology of CKD (%)			0.515
Glomerular disease	44.4%	45.5%	
Diabetic nephropathy	11.1%	18.2%	
Other GD	33.3%	27.3%	
Tubulointerstitial disease	22.2%	45.5%	
ADPKD	22.2%	18.2%	
Other TID	0%	27.3%	
Vascular disease	11.1%	9.1%	
Unknown	22.2%	0%	
Time on PD (months)	20.37±24.97	-	

Results are shown in prevalence (%) or mean ± SD. ESRD, End-stage renal disease, CKD, chronic kidney disease; PD, peritoneal dialysis; GD, glomerular disease; ADPKD, Autosomal Dominant Polycystic Kidney Disease; TID, Tubulointerstitial disease.

Both groups were subjected to bacterial metagenomic analysis of peritoneal tissue. Samples displayed a median of 39,116 reads (range: 33,085–46,171), of which 64.2±2.7% were classified into OTUs. A total of 339 OTUs were detected (median was 62 OTUs per sample), with samples presenting between 22-129 OTUs. The rarefaction curve analysis demonstrated the sufficiency of our read coverage to capture sample diversity (Supplementary figure 1). OTUs were then clustered with the Swarm algorithm, revealing the relative proportion of different taxonomic

levels in each individual sample (figure 1; Supplementary figure 2). The taxonomic profiles of the peritoneal tissue microbiome in ESRD-PD and ESRD-nonPD were similar, dominated by Phylum Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (figure 1). Taxonomic differentiation was nicely resolved at the family level, with an overrepresentation of Burkholderiales Genera incertae sedis (figure 1), whereas at the species level, an overabundance of multi-affiliation and unidentified OTUs was observed (Supplementary figure 2).

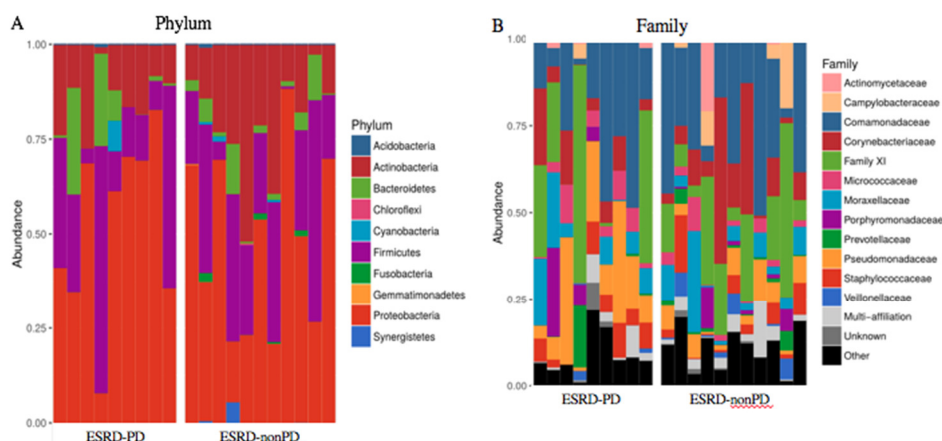


Figure 1: Relative proportion taxa for taxonomic level phylum (left) and family (right). Taxa are identified by name in the plot for abundance >1%. Taxa are merged into the “Other” category only if it exists in any sample with abundance greater than 0.01%. Taxa are merged into the “Multi-affiliation” category when they can correspond to two or more different taxa.

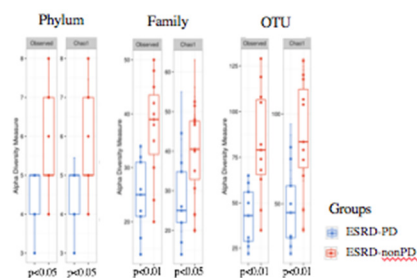


Figure 2: Alpha diversity of the peritoneum microbiome community at phylum, family and OTU taxonomic levels with observed (left panels) and Chao1 (right panels) indexes.

Alpha-diversity analysis was estimated across different indexes (figure 2; Supplementary figure 3), and presented statistical significance in Observed and Chao1 analysis. Collectively, alpha-diversity analysis revealed that ESRD patients with intact peritoneal cavity (ESRD-nonPD) presented higher bacterial species richness than ESRD patients undergoing PD (ESRD-PD). Principal Coordinate Analysis for Beta-diversity assessment did not show differences in the microbial communities in

peritoneal tissue between ESRD-PD and ESRD-nonPD groups (Supplementary figure 4), indicating that the observed differences may be mainly associated with rare and/or less abundant lineages. In fact, a higher species richness was observed in the peritoneal microbiome from ESRD-nonPD group, as can be seen in the heatmap (figure 3) where more OTUs, mostly of low abundance, are observed in the ESRD-nonPD microbiome.

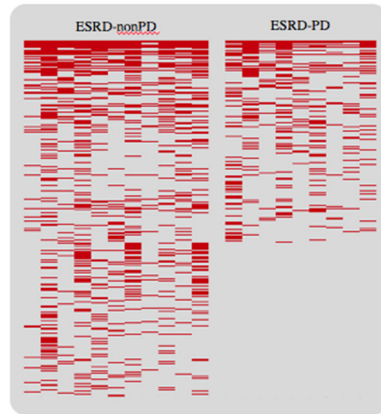


Figure 3: Heat map shows the OTU presence (in red) and absence of all the peritoneum samples from ESRD-PD and ESRD-nonPD (column: samples, row: OTUs).

The metagenomic biomarker discovery approach LEfSe (linear discriminate analysis coupled with effect size measurements) revealed that ESRD-PD patients, when compared to ESRD-nonPD patients, showed an enrichment of the microbial families Prevotellaceae, Alcanivoracaceae, Pseudomonadaceae, specifically from the genus *Prevotella*, *Alcanivorax* and *Pseudomonas*. This was associated with a decrease in the abundance of the families Fusobacteriaceae, Peptococcaceae,

Lactobacillaceae, Bifidobacteriaceae, Synergistaceae, and in the genus *Peptococcus*, *Lactobacillus*, *Bifidobacterium*, *Gardnerella* and *Corynebacterium* (figure 4A). The metagenomic derived bacterial taxa identified as differentially abundant are projected as a cladogram (figure 4B). The barplots representing the abundances of the genera and families different in ESRD-PD and ESRD-nonPD are shown in Supplementary figure 5.

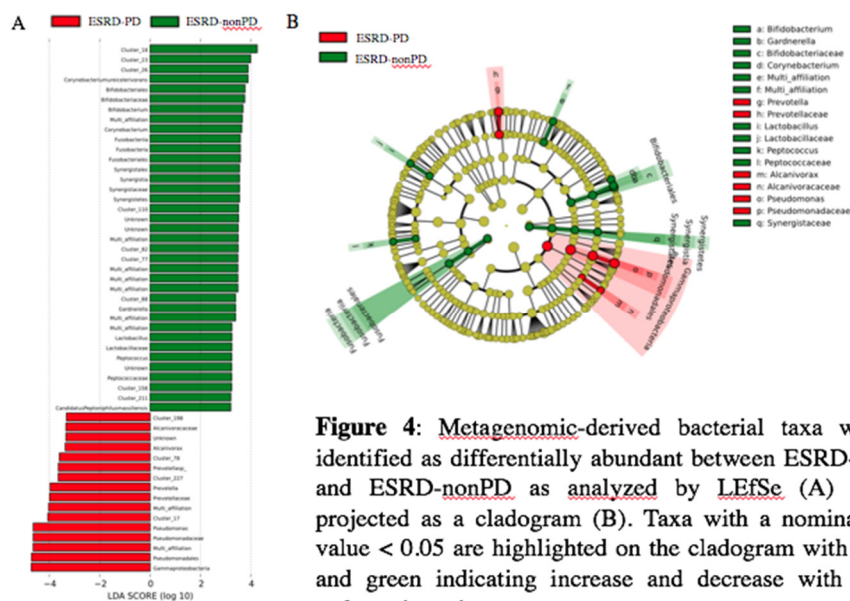


Figure 4: Metagenomic-derived bacterial taxa were identified as differentially abundant between ESRD-PD and ESRD-nonPD as analyzed by LEfSe (A) and projected as a cladogram (B). Taxa with a nominal p value < 0.05 are highlighted on the cladogram with red and green indicating increase and decrease with PD catheter insertion.

Regarding the infectious episodes of ESRD patients from the study group, the majority of PD-related infectious episodes observed were ESI, being *Corynebacterium* and *Staphylococcus* sp. the main infectious agents (Table 2). In all PD-related infections, before and after sampling, the genus of the ESI infectious agent was also present in the patient's peritoneum microbiome, with the

exception of 1 ESRD-nonPD patient that presented an ESI by *Staphylococcus*. Moreover, all infectious agents were within the 10 most prevalent known genera that composed the peritoneal microbiome of each patient, with the exception of the *Streptococcus* genus (Supplementary table 1). This seems to suggest the existence of infectious agents in the peritoneum microbiome of ESRD patients.

Table 2: Bacterial infection history of ESRD patients (ESRD-PD and ESRD-nonPD), namely peritonitis and exit-site infections before and after the peritoneum biopsy.

Infection history	ESRD-PD (n=9)		ESRD-non PD (n=11)
	Before biopsy	After biopsy	After biopsy
Peritonitis	<i>Staphylococcus epidermidis</i> (1)		<i>Staphylococcus aureus</i> (1)
	<i>Streptococcus mitis/oralis</i> (1)		<i>S. mitis/oralis</i> (1)
	No ID (1)		No ID (2)
Exit-site infections	<i>Corynebacterium</i> (2)		
	<i>Corynebacterium xerosis</i> (1)		<i>Corynebacterium</i> (2)
	<i>S. epidermidis</i> +		<i>S. epidermidis</i> +
	<i>Sphingomonas paucimobilis</i> (1)	<i>Corynebacterium</i> (1)	<i>Corynebacterium</i> (1)
	<i>Staphylococcus hominis</i> +	<i>Corynebacterium simulans</i> (1)	<i>Pseudomonas aeruginosa</i> (1)
	<i>Corynebacterium</i> (1)	No ID (1)	<i>S. aureus</i> (1)
	<i>Pseudomonas</i> (1)		No ID (3)
	No ID (3)		

Results are expressed as "Agent (number of patients)".

Discussion

To the best of our knowledge, this study is the first report of a distinctive microbiome in the

peritoneum of ESRD patients, dominated by Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (figure 5).

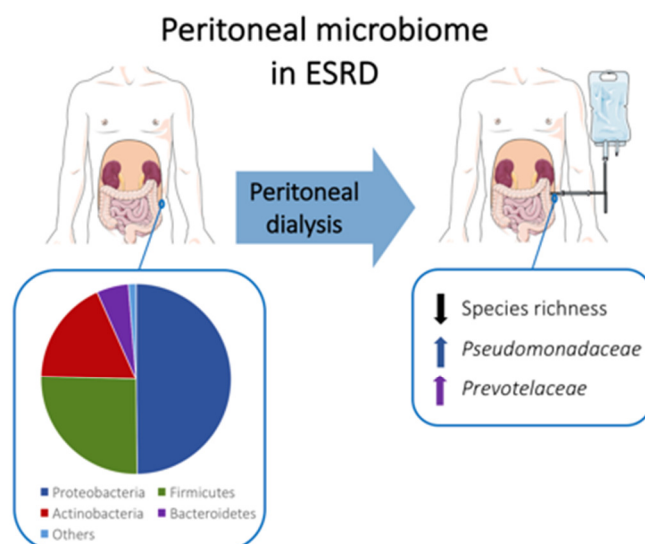


Figure 5: Schematic representation of peritoneum microbiome at phylum level in End-stage Renal Disease (ESRD) patients, and the impact of peritoneal dialysis on peritoneum microbiome. Figure was produced using Servier Medical Art, <http://www.servier.com/Powerpoint-image-bank>.

In line with our findings, previous studies have unravelled microbiomes in body sites that were previously considered microbiome-free, as is the case of the placenta⁴¹ and breast tissue.⁴² These newly identified microbiomes are unravelling new routes of microbial translocation in the human body: the breast tissue microbiome may originate via the nipple-areolar orifices, nipple-oral contact via lactation or sexual contact as well as bacterial translocation from the gut;⁴² the placenta microbiome may be established by hematogenous spread.⁴¹

Collectively, these studies uncover a scenario of mobility pathways of the human microbiome through different body habitats. This atypobiosis (microbes that appear in places other than their normal location) has long been

recognized as an important route for endogenous infections, and more recently, was implicated in the pathogenesis of a variety of inflammatory diseases.³⁶

Although there are no studies exploring the role of the blood microbiome in CKD, the existence of an impairment of intestinal epithelial barrier structure and function is recognized in this chronic disease. A previous study from our group reported the asymptomatic protozoa colonization in peritoneal dialysate of PD patients,⁴⁶ providing evidence for the intestinal translocation, not necessarily linked to an infectious state.

The fact that the peritoneal tissue of ESRD patients harbours a unique low-abundance microbiome dominated by Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes

may be linked to the increased microbial intestinal translocation observed in these patients. In fact, all these phyla dominate the intestinal microbiome of ESRD patients.⁵⁰ This is also the case of spontaneous bacterial peritonitis (SPB) in cirrhotic patients, that is well known to occur in the absence of any intra-abdominal, surgically treatable source of infection.⁵¹ Several mechanisms were proposed for SPB occurrence, namely intestinal bacterial overgrowth, alterations (structural and functional) of the intestinal mucosal barrier and deficiencies of the local immune response. Moreover, a previous study reported a microbiome dominated by Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes phyla in peritoneal tumours of Pseudomyxoma peritonei.⁵²

In this study, we demonstrate that patients on PD therapy presented a decrease in peritoneal microbiome species richness and a dominance by Pseudomonadaceae and Prevotellaceae families (figure 5). In ESRD, the PD therapy per se could represent a source of microbial translocation, either through the close contact with the skin microbiome due to the existence of a catheter connecting the skin and the peritoneal cavity, or via the presence of the PD catheter biofilm itself. However, we did not observe a direct evidence of skin microbiome translocation, since the most abundant colonizer organisms of umbilical and inguinal crease, *Staphylococcus* and *Corynebacterium* spp.,^{1, 53} were not altered or increased in ESRD-PD patients in comparison to ESRD-nonPD (Supplementary figures 5 J to O and

AA). However, the existence of biofilm in PD catheters may explain the dominance of the Pseudomonadaceae family in peritoneal microbiome of ESRD-PD patients since they are common PD catheter colonizers.^{54, 55} Regardless, other factors may alter the peritoneum environment favouring certain species in detriment of other less adapted ones. These factors include dialysis solution composition, frequent daily exchanges, and pressure variations.

In PD patients it is especially critical to understand the role of the peritoneal microbiome in the development of PD-related infections, due to patients' local immune impairment.⁵⁶ Also, infection-related morbidity is still a major complication in these patients, and all possible routes of infection should be identified in order to prevent infection more efficiently.^{57, 58} Interestingly, the majority of the genera of the PD-related infectious agents of the present study population were within the 10 most prevalent known genera of the peritoneum microbiome of each patient. This strengthens the possible association between peritoneal microbiome and PD-related infections. However, the lack of identification at the species level, implicit in the metagenomics approach used in this study, is a limitation that does not allow the establishment of a more accurate relationship. The dominance of Pseudomonadaceae family in peritoneal microbiome of PD patients could be of major clinical importance given that Gram-negative peritonitis have worse clinical outcomes,¹⁷ are more likely to require hospital admission, and

result in catheter loss and failure of PD.^{17, 59-63} *Pseudomonas* spp. is probably the most relevant agent of severe peritonitis in ESRD patients undergoing PD.¹⁷ However, within our patients' population we did not observe any *Pseudomonadaceae* infection either before or after biopsy sampling.

Overall, this study brings new insights to the characterization of the human microbiome. Considering that the peritoneum has its own microbiome, there is still a long way to go before we can understand its role in health and disease. The peritoneum microbiome in particular can contribute to the onset or aggravation of ESRD patients' infections, in particular patients undergoing PD.

In conclusion, peritoneum of ESRD patients harbours a specific microbiome and PD therapy may be associated with changes in this peritoneal microbiome, namely an increased dominance of *Pseudomonadaceae* and *Prevotellaceae* families. The clinical relevance of these facts, in particular regarding PD-related infections, should be further explored.

Authors contribution

L.S.S., M.P., I.S.S. and B.S.M. participated in the experimental design; L.S.S., I.S.S. and B.S.M. conducted the experiments; L.S.S., I.S.S., B.S.M., M.P. participated in interpretation of the studies and analysis of the data; L.S.S., I.S.S. and B.S.M. wrote the manuscript. All authors read and approved the final manuscript.

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Access to data: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Supplementary material

Peritoneum microbiome in end-stage renal disease patients and the impact of peritoneal dialysis therapy

Liliana Simões-Silva, Manuel Pestana, Isabel Soares-Silva, Benedita Sampaio-Maia

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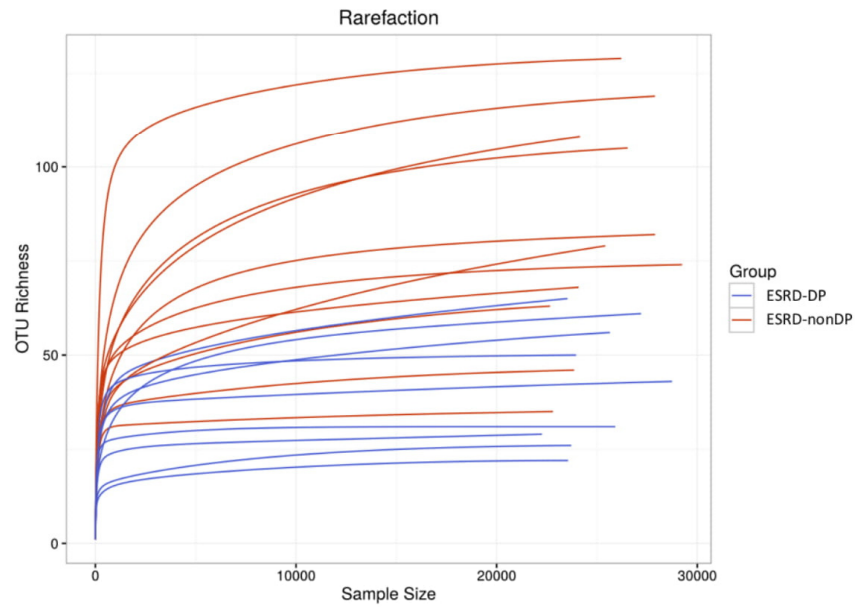
Supplementary table

Supplementary table 1: Bacterial infection history of ESRD-PD and ESRD-nonPD patients, namely peritonitis and exit-site infections before and after the peritoneum biopsy and the ranking position of the infectious agent genus within the known microbiome genera in each patient.

Infections	Groups	Infectious agent genus	Genus ranking position
Peritonitis	PD	<i>Streptococcus</i> (before)	28 th
		<i>Staphylococcus</i> (before)	3 rd
	Non-PD	<i>Streptococcus</i> (after)	18 th
		<i>Staphylococcus</i> (after)	1 st
ESI	PD	<i>Corynebacterium</i> (before and after)	8 th
		<i>Corynebacterium</i> (before)	2 nd
		<i>Corynebacterium</i> (after)	3 rd
		<i>Staphylococcus</i> (before)	3 rd
		<i>Sphingomonas</i> (before)	2 nd
		<i>Pseudomonas</i> (before)	1 st
		<i>Staphylococcus</i> (after)	3 rd
		<i>Staphylococcus</i> (after)	-
	Non-PD	<i>Corynebacterium</i> (after)	1 st
		<i>Corynebacterium</i> (after)	1 st
		<i>Corynebacterium</i> (after)	5 th
		<i>Pseudomonas</i> (after)	10 th

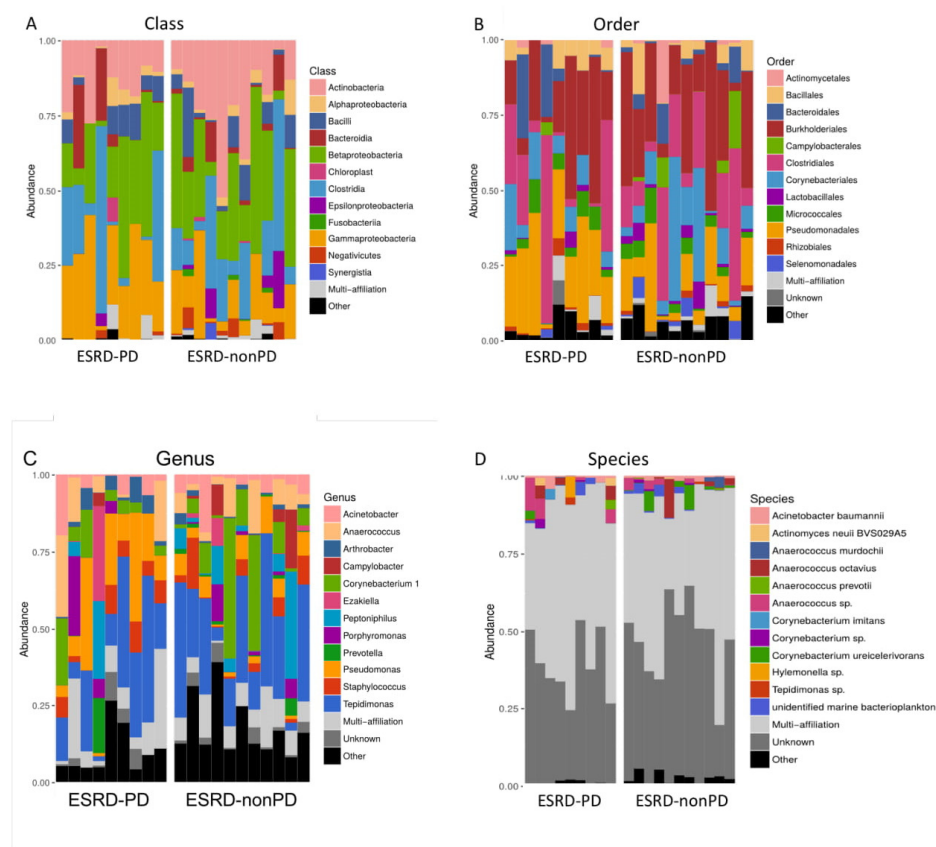
Supplementary Figures

Supplementary figure 1



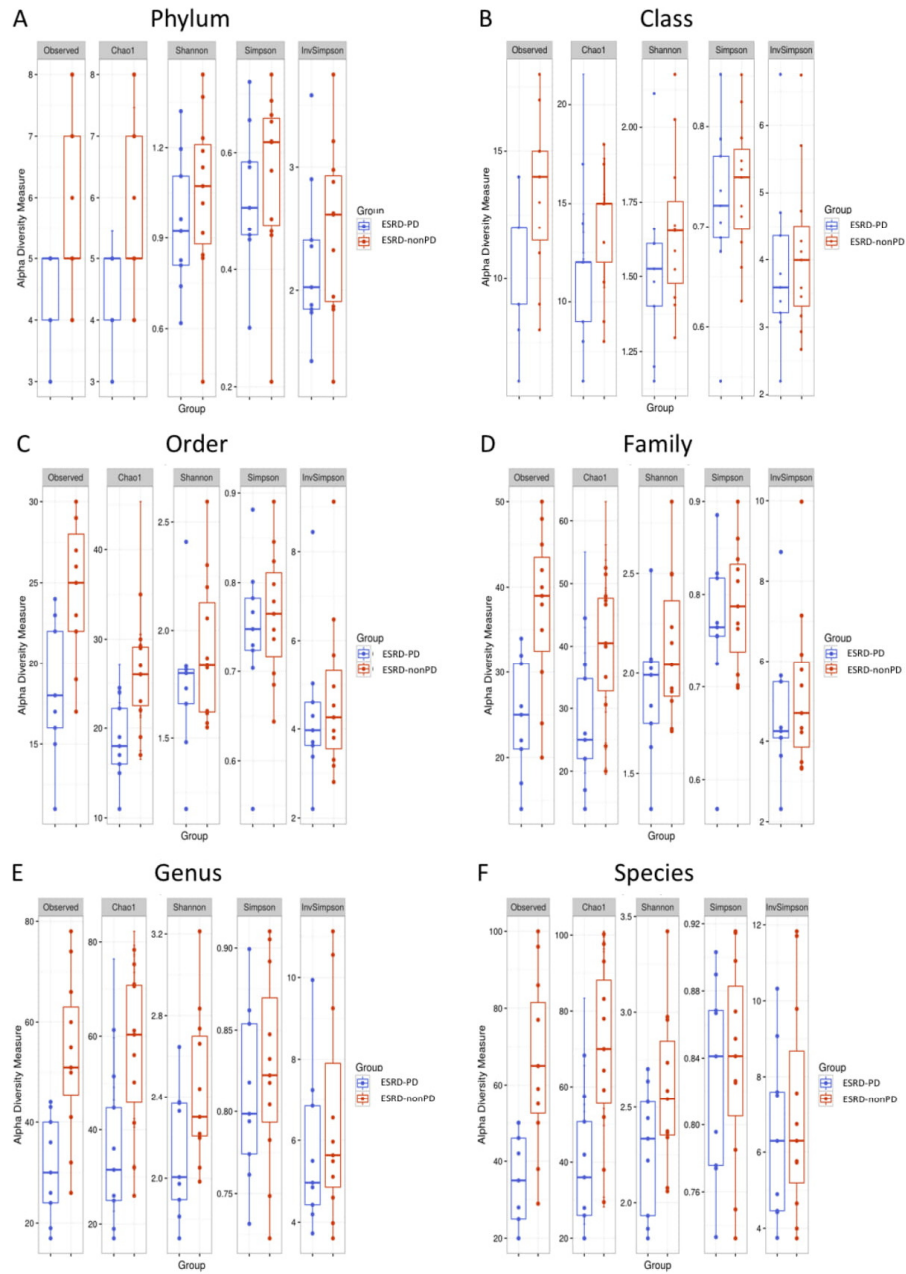
Supplementary figure 1: The rarefaction curves plotted with all groups cumulatively. The rarefaction analysis curves suggest the sample diversity was captured as expected given the average sequence pairs classified into operational taxonomic units (OTU).

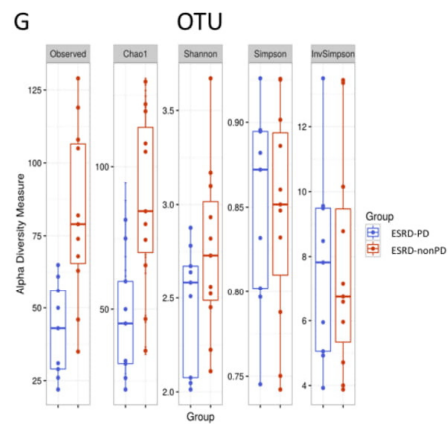
Supplementary figure 2



Supplementary figure 2: Relative proportion taxa for class (A), order (B), genus (C), and species (D). Taxa are identified by name in the plot for abundance >1%. Taxa are merged into the “Other” category only if it exists in any sample with abundance greater than 0.01%. Taxa are merged into the “Multi-affiliation” category when they can correspond to two or more different taxa. ESRD-PD – End-stage renal disease patients undergoing peritoneal dialysis; ESRD-nonPD – End-stage renal disease patients with intact peritoneal cavity.

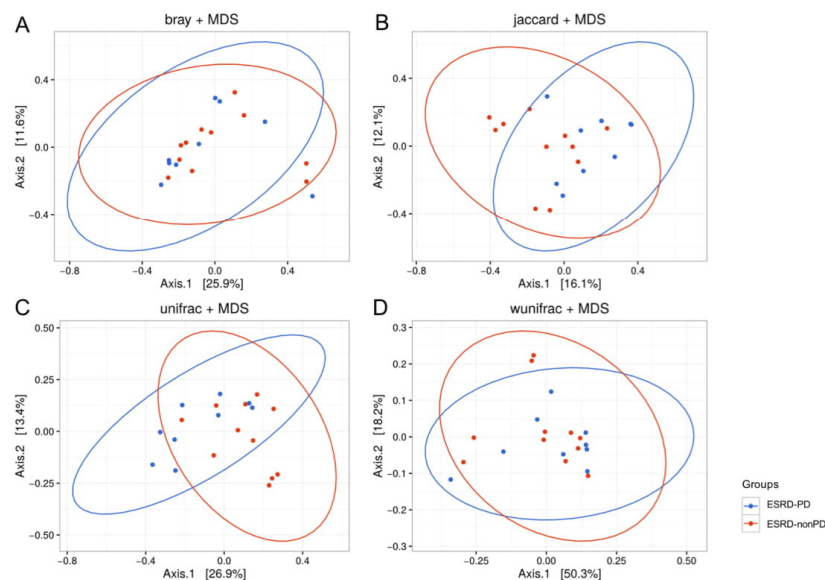
Supplementary figure 3





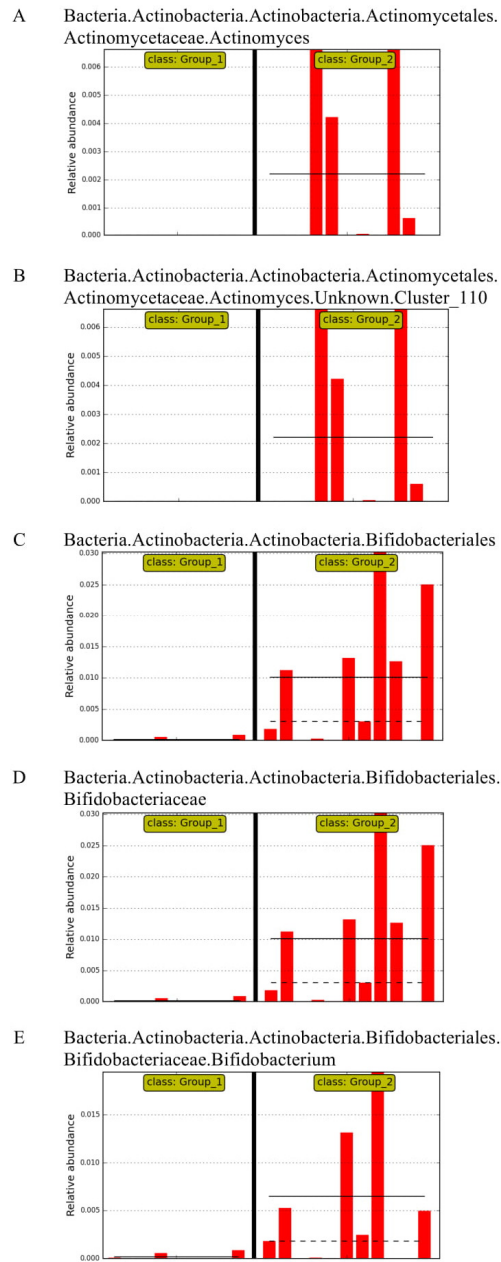
Supplementary figure 3: Alpha diversity of the peritoneum microbiome community at phylum (A), class (B), order (C), family (D), genus (E), species (F) and OUT (G) taxonomic levels calculated with Observed, Chao1, Shannon, Simpson, and Inverse Simpson indexes. ESRD-PD – End-stage renal disease patients undergoing peritoneal dialysis; ESRD-nonPD – End-stage renal disease patients with intact peritoneal cavity.

Supplementary figure 4

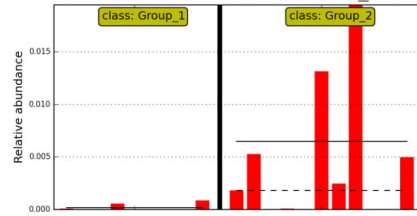


Supplementary figure 4: Beta-diversity of the peritoneum microbiome community at OTU level with Bray-Curtis (A), Jaccard (B), unweighted Unifrac (C), and Weighted Unifrac (D) distance derived from 16S rRNA gene -based sequencing data. ESRD-PD – End-stage renal disease patients undergoing peritoneal dialysis; ESRD-nonPD – End-stage renal disease patients with intact peritoneal cavity.

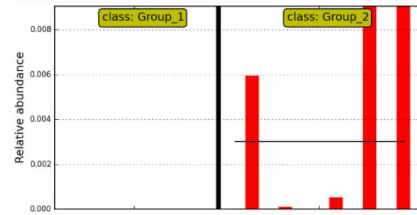
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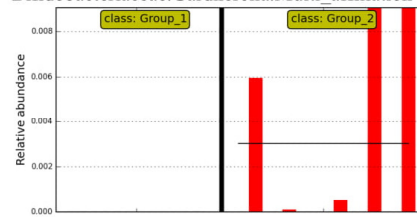
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Bifidobacteriaceae.Gardnerella



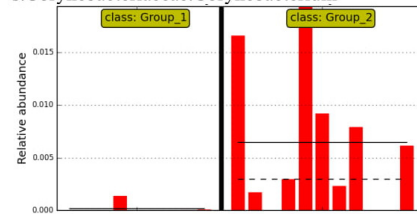
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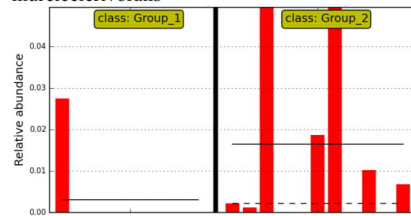
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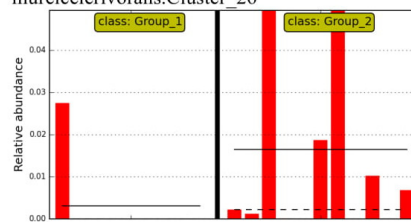
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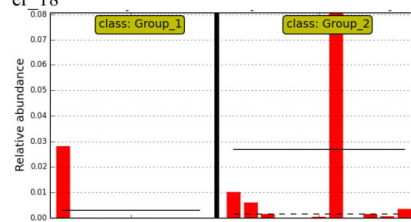
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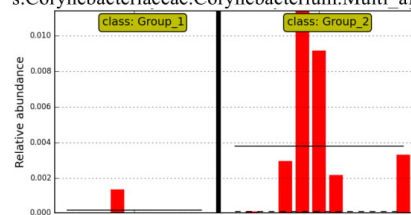
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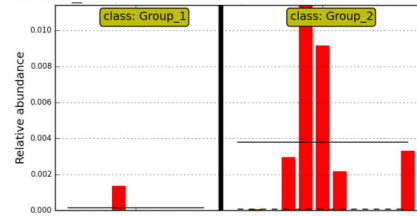
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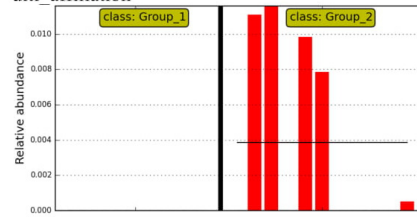
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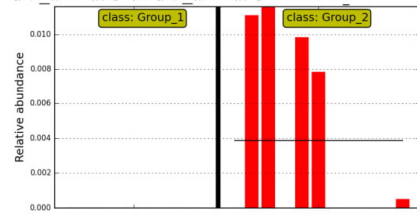
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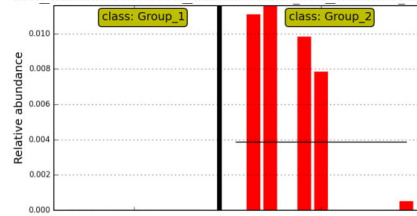
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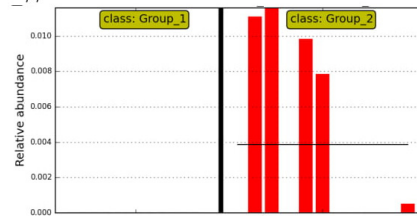
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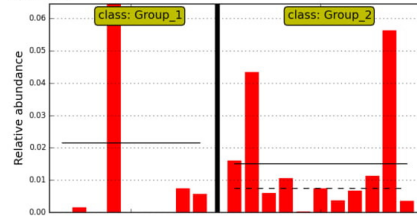
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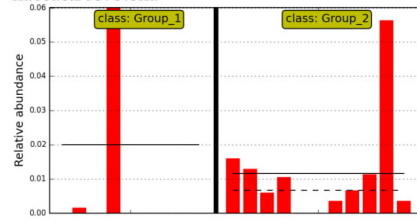
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77



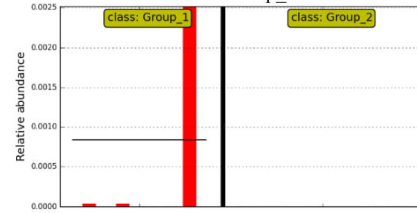
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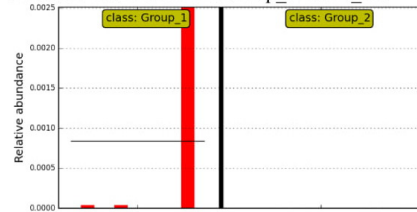
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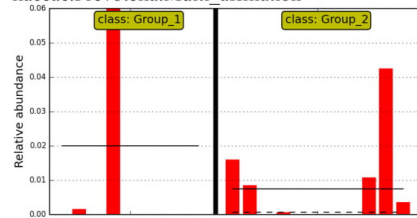
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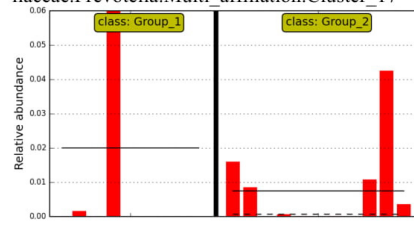
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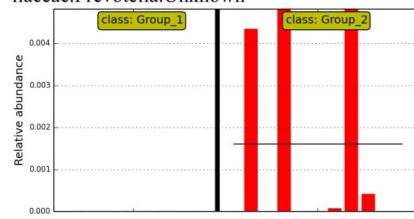
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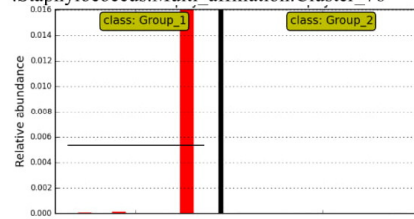
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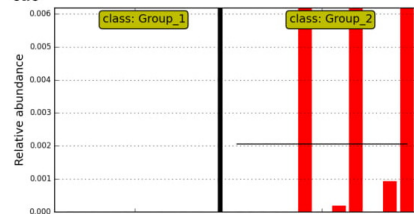
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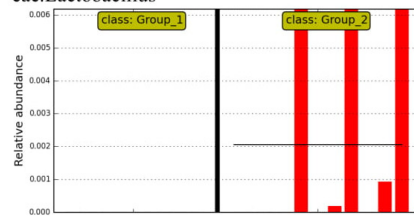
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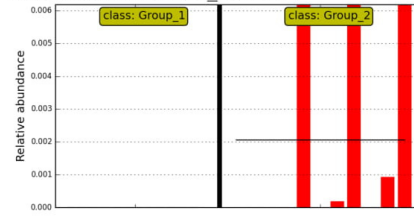
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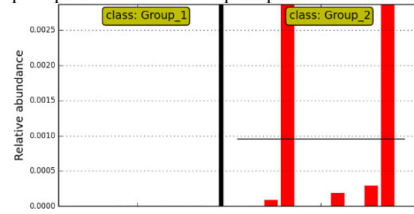
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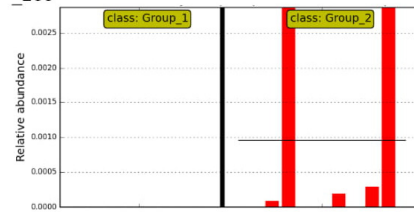
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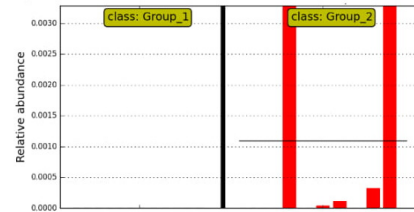
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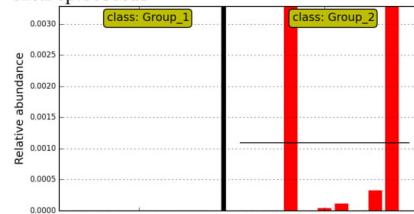
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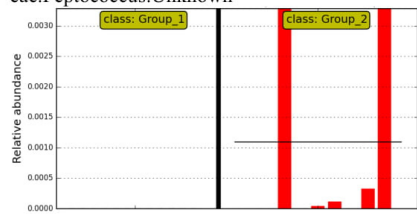
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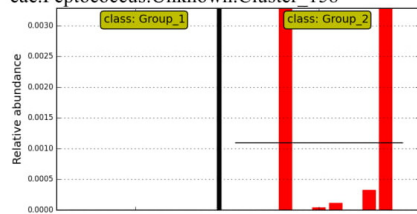
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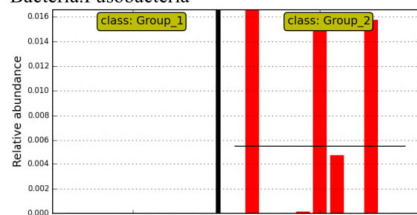
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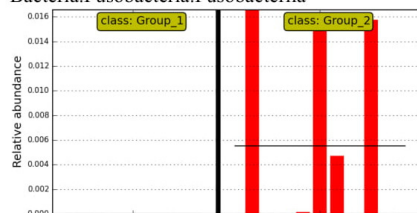
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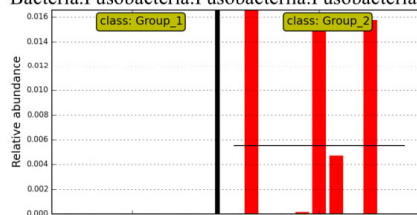
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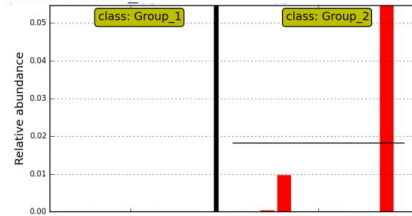
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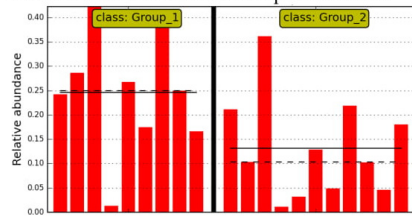
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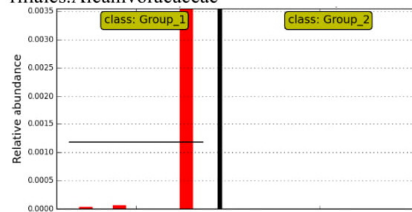
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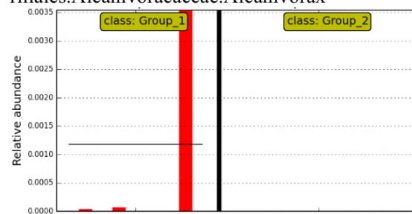
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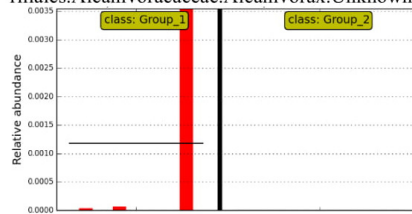
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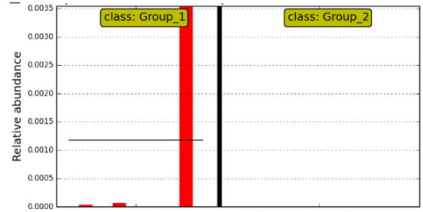
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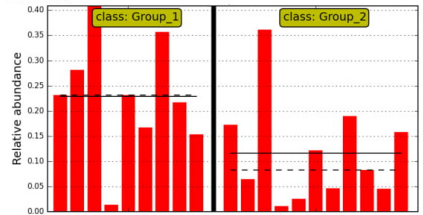
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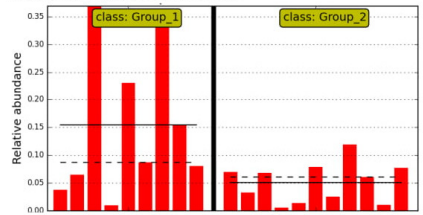
AS Bacteria.Proteobacteria.Gammaproteobacteria.Oceanospirillales.Alcanivoracaceae.Alcanivorax.Unknown.Cluster 198



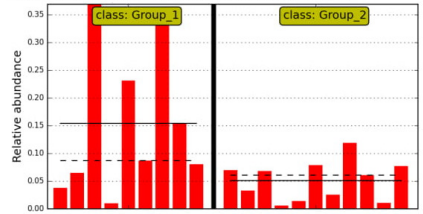
AT Bacteria.Proteobacteria.Gammaproteobacteria.Pseudomonadales



AU Bacteria.Proteobacteria.Gammaproteobacteria.Pseudomonadales.Pseudomonadaceae

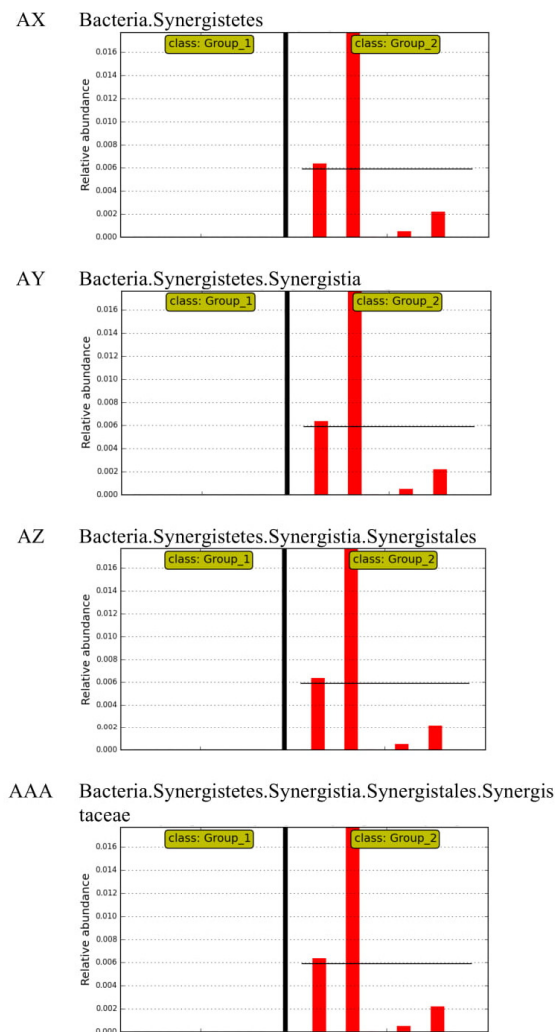


AV Bacteria.Proteobacteria.Gammaproteobacteria.Pseudomonadales.Pseudomonadaceae.Pseudomonas



AW Bacteria.Proteobacteria.Gammaproteobacteria.Pseudomonadales.Pseudomonadaceae.Pseudomonas.Multi-affiliation





Supplementary figure 5: A–AAA Barplots show the abundances of the genera and families between the ESRD-PD and ESRD-nonPD. Each bar represents a sample. Group 1 – End-stage renal disease patients undergoing peritoneal dialysis (ESRD-PD); Group 2 – End-stage renal disease patients with intact peritoneal cavity (ESRD-nonPD).

GENERAL DISCUSSION AND CONCLUSIONS

Discussion

Understanding the role of the patients' microbiome on the development of PD-related infections was the driving force of this work that emerged from the need to better understand the mechanisms underlying the occurrence of PD-related infections. The data presented in this work may contribute to the identification of possible sources of infection, which ultimately can lead to the decrease of PD-related infection occurrence.

With the development of the Human Microbiome Project a huge amount of knowledge on the microbiome was unraveled, enabling not only the characterization of the human microbiome but also the analysis of its role in human health and disease. Nowadays it is known that Humans and microbes co-evolved and that the host physiology is influenced by host-microbe interactions (Wang, B. *et al.* 2017). Curiously, only a third of microbiome constituent genes were found in the majority of healthy individuals and understanding this variability has been the main challenge in microbiome research (Lloyd-Price, J. *et al.* 2016). The focus of most microbiome studies has been the establishment of associations between the gut microbiome and several diseases, as gut microbiome is currently the most well studied microbiome.

In CKD, the gut microbiome has been associated to disease progression (Wang, F. *et al.* 2012, Vaziri, N. D. *et al.* 2013, Barrios, C. *et al.* 2015, Sampaio-Maia, B. *et al.* 2016). However, it still necessary to clarify the pathogenic role of this microbiome. Several studies described the existence of lower levels of Bifidobacteriaceae and Lactobacillaceae and higher levels of Enterobacteriaceae in the gut of CKD patients, when compared to healthy individuals. This picture is however incomplete, as it lacks characterization of bacterial dysbiosis at the species level, as well as the characterization of other communities such as archaea, fungi and virus. Only when this is achieved we will be able to fully understand gut dysbiosis in CKD and its role in disease progression. Regarding the different dialysis therapies carried out by CKD patients, HD and PD have unique characteristics that may contribute to changes in the human microbiome, not only restricted to the gut, but also affecting the blood microbiome (in HD) and the peritoneal microbiome (in PD). This may be related to specific dietary restrictions and different types of dialysis access (vascular vs. peritoneal), as well as the continuous inflow of peritoneal dialysate, containing microbial nutrients. Notwithstanding, the impact of HD or PD on the human microbiome is still not fully understood.

In PD, infections are still one of major complication of this RRT, mainly associated to contamination at the time of PD dialysate exchange. *Staphylococcus* species are common agents of PD-related infections and are frequently identified in the oral cavity of adults in health and disease (Percival, R. S. *et al.* 1991, Smith, A. J. *et al.* 2001). Nevertheless, the role of staphylococci in the oral ecology is not fully understood (Smith, A. J. *et al.* 2001). The likelihood that these microorganisms could act as opportunistic infectious agents in distant body locations led us to evaluate the oral colonization status of PD patients and healthy controls. In the Human Oral Microbiome Database (<http://www.homd.org/>) few *Staphylococcus* species are described namely *S. aureus*, *S. epidermidis*, *S. warneri*, and *Staphylococcus caprae*. In our study we detected the presence of six other *Staphylococcus* species, namely *S. saprophyticus*, *S. capitis*, *S. cohnii*, *S. pasteuri*, *S. hominis* and *S. lugdunensis*. Taking in consideration that we found a prevalence close to 100% of *Staphylococcus* in both PD patients and control groups, we suggest that this genus is a common member of the oral microbiome. In agreement, other authors also described *Staphylococcus* species as frequent oral colonizers of adults, in health and oral disease (Percival, R. S. *et al.* 1991, Smith, A. J. *et al.* 2001). Nonetheless, this was the first report of *S. pasteuri*, considered a rare CNS clinical isolate (Morfin-Otero, R. *et al.* 2012), as member of the oral microbiome.

No differences were observed in specific *Staphylococcus* species oral colonization between controls and PD patients, however PD patients presented less diversity which may be justified by more frequent antibiotic therapy of these patients, as well as by changes in the oral biochemical milieu, including higher pH and urea content observed in patients on PD therapy. Also, PD patients, when compared to controls, included more individuals that were smokers in the past and its known that tobacco use significantly reduces bacterial richness which is maintained over time (Thomas, A. M. *et al.* 2014). The fact that the oral cavity of PD patients presented lower staphylococci diversity together with the existence of common species between the oral colonization and PD infectious agents, supports the hypothesis that PD-related infections could have origin in the oral cavity. Since, prevention of infection is a critical step to the success of a PD-program we believe that the oral cavity should be considered as a possible infection route. In addition to the protocol already applied, as the use of mask, we consider that in order to prevent infections by this route, oral health care should be promoted in this population.

In the course of this work we highlight the importance of the method-of-choice for the identification of *Staphylococcus* genus. Our data support that phenotypic methods are useful in preliminary identification of bacteria, as they lead to important data concerning phenotypic or

physiological traits in the absence of more sophisticated analysis. Nevertheless, our data reinforces the notion that molecular methods are more accurate and should be preferably used, especially when dealing with atypical isolates. Even though MALDI-TOF MS methodologies, such as VITEK MS, are considered very accurate, rapid and high-throughput techniques for bacteria identification, we demonstrate that in a sub-group of isolates it did not achieve the described levels of accuracy necessary for the clinical setting, and its replacement by molecular methods such as *DnaJ* sequencing, in the case of *Staphylococcus* species, should be considered.

Fungal infections associated to the PD technique constitute a minority, however they are usually more difficult to treat and therefore are important complications in PD patients. The opportunistic fungi inhabiting distant body sites are thought to be the major source of infection. Taking into account our results, that showed lack of relationship between fungal oral colonizers and PD infectious agents, we suggest that oral yeast colonization may be not associated with PD-related fungal infections. Also, in comparison to healthy controls PD patients presented a lower prevalence of oral yeasts, namely *C. albicans*. This may be related not only with a higher exposure to antifungal therapy, recommended during an antibiotic course (Lo, W. K. *et al.* 1996, Restrepo, C. *et al.* 2010), but also with alterations of the oral environment secondary to systemic ESRD effects, PD therapy and medication. Several molecules prescribed to PD patients are reported to be associated with altered susceptibility to fungal infections that may impact on yeast colonization. Additionally, the high urea levels may contribute in part to the higher salivary pH and changes in the oral pH may be the major ecological factor that alters the oral commensal microbiome, leading to shifts in its natural diversity (Kraneveld, E. A. *et al.* 2012). This modified oral environment of PD patients could result in the emergence of rare yeasts, such as *C. carpophila*.

In this study, the antifungal susceptibility profile of the yeast isolates revealed: 1) resistance to itraconazole; 2) non-wild type phenotype for posaconazole; 3) variable susceptibility profiles to caspofungin. Taking into account that these antifungals are prescribed for the treatment of systemic fungal infections (Allen, U. 2010) and that the existence of resistant strains to itraconazole and caspofungin was previously reported (Zomorodian, K. *et al.* 2016) we consider that it is important to determine the susceptibility of clinical isolates to these antifungals.

In another work the analysis of oral yeast colonization of HD patients and PD with or without previous history of HD revealed the existence of relevant differences. In comparison to PD patients with or without previous history of HD (PD or HD/PD), HD patients present higher oral

prevalence of *Candida* species and more severe periodontal disease, which are risk factors that may contribute to systemic infection. Dental plaque accumulation was higher in HD patients, probably due to the higher educational level of PD patients, and higher gingival weakness of HD population that may be attributed to the use of repeated systemic anticoagulation therapy, promoting gingival bleeding and enhancing bacterial colonization (Borawski, J. *et al.* 2007).

Overall HD patients are more susceptible to acquire health care associated infections than PD patients, since they attend to clinical environment several times a week (Gulcan, A. *et al.* 2016), which may justify the higher oral *Candida* colonization since *Candida* species are frequent nosocomial fungal pathogens (Delaloye, J. *et al.* 2014). In comparison to PD patients, HD patients presented a more acidic saliva that may be associated to the higher oral *Candida* prevalence. As previously described *Candida* is a well-known acidogenic microorganism and is frequently associated to higher rates and severity of periodontal disease (Canabarro, A. *et al.* 2013).

In the light of recent findings that associated CKD to an impairment of the intestinal epithelial barrier structure and function that may enable translocation of microorganisms and other microbial products across the intestinal wall to systemic circulation (Vaziri, N. D. 2012, Vaziri, N. D. *et al.* 2013), we hypothesised that peritoneal dialysis effluent protozoa colonization may represent a marker for the loss of the intestinal epithelial barrier integrity in CKD patients. Five out of 49 PD patients presented PD effluent protozoa colonization without any clinical signs or symptoms of peritonitis. However, this population is immune-impaired so commensal microorganisms may represent relevant opportunistic microorganisms. Based in the clinical history of these 5 PD patients and given that all protozoa found in the peritoneal effluent may inhabit the human intestine, our results could mirror an accidental gut colonization acquired by water or food consumption that then resulted in the colonization of the peritoneal cavity due to loss of the intestinal epithelial barrier integrity.

We also evaluated the oral protozoa colonization in PD patients and healthy controls, and only one PD patient presented oral protozoa colonization by *Entamoeba* sp., possibly by *Entamoeba gingivalis*, given that this is the most frequent amoeba in the oral cavity, although with low prevalence (Ghabanchi, J. *et al.* 2010). Interestingly, this protozoon also colonized the PD effluent of the same patient, suggesting that the digestive tract may represent in this case the source of this microorganism; however it was not observed in the oral cavity of his corresponding healthy familiar control. The fact that no protozoa were observed in the control group, despite the

very low oral protozoa colonization among the studied PD population, probably suggests a higher susceptibility of PD patients to protozoa colonization in comparison to non-ESRD individuals. Our data supports that among the northern Portuguese population the protozoa colonization is low, which is in accordance to a Portuguese study that reported the existence of a low infection risk for protozoa with surface raw and drinking water samples (Almeida, A. *et al.* 2010). The water source of the PD patient colonized by protozoa in saliva was the public network system, suggesting that other transmission routes may have been involved in the oral colonization by this infectious agent.

Recently several studies described the existence of specific microbiomes in tissues considered sterile, such as placenta, breast tissue and blood (Aagaard, K. *et al.* 2014, Bhattacharyya, M. *et al.* 2017). These observations uncover a scenario of mobility pathways of the human microbiome through different body habitats. This atropobiosis has long been recognized as an important route for endogenous infections, and more recently, was implicated in the pathogenesis of a variety of inflammatory diseases (Potgieter, M. *et al.* 2015). Since there was no data regarding the existence or absence of a peritoneum microbiome, we analysed the peritoneum of ESRD patients. Our data allowed us to identify a unique microbiome of peritoneum of ESRD patients, dominated by Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes, that may be linked to the increased microbial intestinal translocation observed in these patients since all these phyla dominate the intestinal microbiome of ESRD patients (Crespo-Salgado, J. *et al.* 2016).

We also evaluated the influence of PD therapy and the results show that the peritoneum of PD patients presented a decrease in species richness, prevalence and a dominance by Pseudomonadaceae and Prevotellaceae families. PD therapy could represent a source of microbial translocation, either through the close contact with the skin microbiome due to the existence of a catheter connecting the skin and the peritoneal cavity, or via the presence of the PD catheter biofilm itself. However the most abundant colonizer organisms of umbilical and inguinal creases, *Staphylococcus* and *Corynebacterium* spp. were not altered or increased in ESRD-PD patients in comparison to ESRD-nonPD (Costello, E. K. *et al.* 2009, Grice, E. A. *et al.* 2009). The existence of PD catheters biofilm may explain the dominance of the Pseudomonaceae family in peritoneal microbiome of ESRD-PD patients since they were reported as being common PD catheter colonizers (Bayston, R. *et al.* 1999, Sampaio, J. *et al.* 2016). In addition, other factors may alter the peritoneum environment favouring certain species in detriment of other less adapted ones, such as dialysis solution composition, frequent daily exchanges, and pressure variations. The local immune impairment of PD patients justifies that it is especially critical to understand the role of the peritoneal microbiome in the development of

PD-related infections (Burkart, J. 2014). The possible association between peritoneal microbiome and PD-related infections is strengthened by the fact that the majority of the genera of the agents of PD-related infectious in our study population were within the 10 most prevalent known genera of the peritoneum microbiome of each patient.

The present characterization of the peritoneum microbiome is another piece added to the so far incomplete puzzle of the human microbiome. The peritoneum microbiome could then contribute to the onset or aggravation of PD-related infections.

The studies that compose this work had some limitations, particularly the limited number of patients analysed and in some cases the methodology used. Regarding the number of patients, we need to emphasize that the collection of human samples was not always as fast as predicted and several setbacks diffculted the task. Despite being a smaller group than initially planned, the collaboration of these PD patients and controls allowed the completion of this PhD thesis. On the other hand, the methodology used, for example for yeast isolation (direct spread plate technique) is associated with limited sensitivity (Samaranayake, L. P. *et al.* 1986). However, the percentage of *Candida* colonization obtained in controls was similar to other studies that used the same methodology (Rio, R. *et al.* 2017). In the *Staphylococcus* study, besides the traditional culture method, we used a DNA sequencing approach for species identification. Regarding protozoa colonization, one can hypothesize that with a more sensitive methodology, eg. enzyme immunoassays, polymerase chain reaction (PCR) and flow cytometry, a higher prevalence of patients with PD effluent protozoa colonization could be detected. In chapter VI the lack of identification at the species level, implicit in the metagenomics approach used in this work, is a limitation that does not allow the establishment of a more accurate relationship between peritoneal microbiome and PD-related infections.

It is important to highlight that in this study, the control groups used were constituted by family members of the PD patients. This is a relevant aspect since the oral microbial colonization is strongly correlated to the diet, oral hygiene, and familial predisposition (Lamont, RJ *et al.* 2010). In the specific case of the studies presented in chapter V, oral protozoa prevalence is associated with living environment conditions as well as alimentary habits (Smith, H. V. *et al.* 2007, Almeida, A. *et al.* 2010) being family members of the patients the ideal control group (Areias, C. M. *et al.* 2011). In addition, evaluation of oral colonization in PD patients' family members is crucial since they could represent a potential source of infection and act as vehicles for opportunistic microorganism transmission.

Conclusions

The major conclusions of the present work can be grouped as follows:

Chapter I

- Gut dysbiosis is being characterized in CKD and several studies described lower levels of Bifidobacteriaceae and Lactobacillaceae and higher levels of Enterobacteriaceae; however, the characterization up to species level will be important. Also, information on other microbial groups (archaea, fungi, and virus) in the gut of CKD patients, and its impact in disease progression may be relevant to fully understand gut dysbiosis in CKD. This will allow to understand if gut dysbiosis or uremic toxins of microbial origin can be used as CKD biomarkers as well as if the manipulation of gut microbiota can be used to prevent CV risk and CKD progression.

Chapter II

- The specific impact of RRT, namely HD and PD, on the microbiota imbalance of CKD patients is still underexplored. HD and PD have unique characteristics that may contribute to changes in the human microbiome, not only restricted to the gut, but also affecting the blood microbiome (HD) and the peritoneal microbiome (PD). Factors promoting these changes may be related to specific dietary restrictions and different types of dialysis access (vascular vs. peritoneal), as well as the continuous inflow of peritoneal dialysate, containing microbial nutrients.

Chapter III

- PD patients and controls present both high prevalence of staphylococci in the oral cavity, although PD patients presented less diversity of species. The association between oral *Staphylococcus* carriage and PD-related infections was present for *S. epidermidis*; however, in the case of *S. aureus* it doesn't seem to be a relation between oral colonization and occurrence of PD-related infection.
- The *Staphylococcus* spp. most commonly found in the saliva, nasal cavity or CES of the studied population corresponded to the most prevalent spp. associated with infections, suggesting that staphylococci PD-related infections may be strongly related to normal human microbiota. The human *Staphylococcus* population should be characterized using molecular methods, that are more accurate, given that morphological plus biochemical

approaches, including API 32 STAPH methodology and high-throughput techniques such as MALDI-TOF MS methodologies, have limitations, especially with atypical isolates.

Chapter IV

- PD patients present low prevalence of *Candida* species, especially *C. albicans*, on the oral cavity. Also, no relation was found between oral colonization by yeasts and PD-related yeast infections.
- HD has a more adverse impact on oral health than PD, in particular periodontal disease and colonization by *Candida* species, independently of the time on dialysis. Some relevant risk factors for HD patients were highlighted, and also suggested that the adverse impact of HD therapy on oral health may be reduced or ameliorated when patients change from HD to PD therapy.

Chapter V

- PD effluent may be susceptible to asymptomatic protozoa colonization, highlighting the need for a more systematic screening of protozoa colonization in this population, and the impact of these sub-clinical infections should be further explored.
- Oral protozoa colonization was found to be very low in both PD patients and controls, suggesting that this may reflect the oral protozoa colonization of Portuguese population. However, future studies are required to evaluate the possible higher susceptibility to oral protozoa colonization of ESRD patients undergoing PD.

Chapter VI

- The peritoneum of ESRD patients harbours a specific and a unique low-abundance microbiome dominated by Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes. Additionally, PD therapy may be associated with changes in this peritoneal microbiome, namely characterised by an increased dominance of Pseudomonadaceae and Prevotellaceae families. The clinical relevance of these facts, in particular regarding PD-related infections, should be further explored.

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ETHICAL COMMISSION APPROVALS



Ministério da Saúde



Hospital de S. João, E.P.E.

Exma. Sra. Dra.
Benedita Sampaio Maia
Rua Oliveira Monteiro 465
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Sua referência

Sua comunicação de

Nossa referência

Data

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ASSUNTO

Submissão de pedido para a realização de Projecto de Investigação

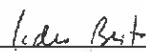
Projecto de Investigação – “Oral health in Peritoneal dialysis patients”

Junto envio em anexo cópias do parecer da Comissão de Ética para a Saúde, e decisão do Conselho de Administração, para poder iniciar os seus trabalhos.

Com os melhores cumprimentos.

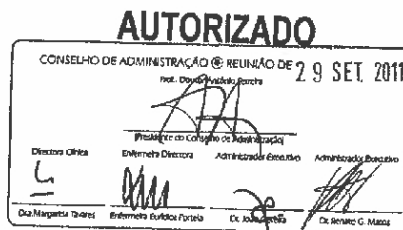
Porto, 3 de Outubro de 2011

O Secretário da Comissão de Ética para a Saúde


Dr. Pedro Brito

Na resposta indicar a «Nossa referência» - Em cada ofício tratar só de um assunto.

H.S.J. - Mod. 1-D - 80 gr. Pré-Print COD. 551011



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Admissão da Direção Clínica

Exma. Sra.
Dra. Margarida Tavares
Directora Clínica do Centro Hospitalar de São João

Assunto: Parecer da Comissão de Ética para a Saúde do Centro Hospitalar de São João

Projecto de Investigação: "Oral health in Peritoneal dialysis patients"

Investigadora Principal: Dra. Benedita Sampaio Maia

Junto envio a V. Exa. para obtenção de decisão final do Conselho de Administração o parecer elaborado pela Comissão de Ética para a Saúde relativo ao projecto em epígrafe.

Com os melhores cumprimentos.

Porto, 23 de Setembro de 2011

Dr. Pedro Brito

Secretário da Comissão de Ética para a Saúde



COMISSÃO DE ÉTICA PARA A SAÚDE

8. TERMO DE RESPONSABILIDADE

Eu, abaixo-assinado, Benedita Sampaio Maia, na qualidade de Investigador Principal, declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsínquia (com as emendas de Tóquio 1975, Veneza 1983, Hong-Kong 1989, Somerset West 1996 e Edimburgo 2000) e da Organização Mundial da Saúde, no que se refere à experimentação que envolve seres humanos.

Porto, 28 / junho / 2011

29/07/11

A Comissão de Ética para a Saúde tendo aprovado o parecer do Relator, aguarda que o Investigador/Promotor esclareça as questões nele enunciadas para que possa emitir parecer definitivo.

Benedita Sampaio Maia

O Investigador Principal

[Assinatura]

PARECER DA COMISSÃO DE ÉTICA PARA A SAÚDE DO HOSPITAL DE S. JOÃO

Em resposta a uma solicitação —
esclarecimento passado pela
investigadora,

A Comissão de Ética para a Saúde
APROVA por unanimidade o parecer do
Relator, pelo que nada tem a opor à
realização deste projecto de investigação.

[Assinatura]
Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética

emitido na reunião plenária da CES

de
23 / setembro / 2011

Comissão de Ética para a Saúde do HSJ
Parecer

Projecto de investigação: “Oral health in peritoneal dialysis patients”.

Promotor:

- Não aplicável.

Concepção e pertinência do estudo

- Trata-se de um estudo que tem como objectivo principal avaliar o estado de saúde oral em doentes inseridos no programa de diálise peritoneal, da consulta de Nefrologia do Hospital de S. João EPE. O estado de saúde oral será aferido pela avaliação dos índices de placa dentária e cárie, pela avaliação da doença periodontal e pela observação da mucosa oral.
- Tem ainda como outros objectivos a caracterização da microbiota oral e a avaliação do perfil bioquímico salivar. Serão pesquisados géneros específicos de bactérias, leveduras e parasitas que serão correlacionados com o estado de saúde oral, higiene oral e episódios de peritonite. Adicionalmente será determinado o fluxo salivar e avaliados na saliva, o pH e as concentrações de iões e proteínas, que serão correlacionados com o estadio da doença renal crónica e o perfil peritoneal.
- Este estudo incluirá cerca de 100 doentes em diálise peritoneal e um grupo controlo constituído por cerca de 100 familiares saudáveis mais próximos em idade, que co-habitem com os doentes avaliados.
- O estudo terminará em 2014 e terá o financiamento da Unidade de I&D de Nefrologia da Faculdade de Medicina do Porto e da Faculdade de Medicina Dentária da Universidade do Porto.
- Será realizada uma consulta de Medicina Dentária a todos os participantes, que terá como objectivo fazer o diagnóstico (e apenas o diagnóstico) de problemas de saúde oral (inquérito sobre hábitos de higiene oral; exame objectivo dos lábios, mucosa oral, pavimento da boca, língua, glândulas salivares, palato, orofaringe; estado da polpa dentária; Index DMFS; gengivas; fluxo e características bioquímicas da saliva). Caso sejam identificados problemas de saúde oral, os indivíduos serão orientados para uma consulta de tratamento, de acordo com a sua escolha (e a expensas próprias).
- Em todos os indivíduos seleccionados serão efectuados estudos de microbiologia oral por métodos clássicos e de genética molecular.
- O estudo é muito importante, pertinente, está muito bem fundamentado.
- Serão ainda avaliadas características sócio-demográficas, antecedentes patológicos e resultados analíticos.
- O protocolo do estudo, os critérios de inclusão e exclusão estão suficientemente detalhados.
- A Investigadora Principal, Dra Benedita Sampaio Maia, licenciada em Microbiologia e doutorada em Biologia Humana, actualmente Professora Auxiliar da Faculdade de Medicina Dentária da Universidade do Porto (tendo como elo de ligação aos doentes do hospital, a médica nefrologista Dra Carla Araújo), dispõe das competências técnicas e científicas para a realização do estudo.

- O estudo será realizado no Serviço de Nefrologia do Hospital S. João EPE que dispõem das condições necessárias para a realização do estudo. O estudo está autorizado pelo Director de Serviço Professor Manuel Pestana.

- Benefício/Risco

- Todos os procedimentos clínicos e analíticos são os que habitualmente se realizam nos doentes em diálise peritoneal e que são candidatos a incluir neste estudo. Haverá alguns procedimentos específicos do estudo mas que não causam incómodo com significado e que estão devidamente referenciados na folha de informação ao doente.
- No protocolo submetido à CES consta que será efectuada uma consulta de Medicina Dentária. Se esta consulta obrigar a uma deslocação adicional por parte do participante, as despesas com ela relacionada, deverão ser ressarcidas.

- Respeito pela liberdade e autonomia do sujeito do ensaio

- A folha de informação submetida à CES contém toda a informação relevante relacionada com o tipo de estudo proposto.
- A gratuidade do sujeito do ensaio em participar no estudo é igualmente garantida.
- Estão consignados os direitos e referenciadas as (não) consequências para o sujeito do estudo em não participar e, uma vez dado o seu consentimento, ter a possibilidade de exercer o direito de não continuar no estudo sem que daí resulte qualquer modificação dos cuidados médicos a prestar.

- Confidencialidade dos dados

A confidencialidade dos dados será garantida.

- Indemnização por danos

Não aplicável.

- Continuação do tratamento

Não aplicável.

- Propriedade dos dados

Não aplicável.

Conclusão

Em face da análise do protocolo do estudo "Oral health in peritoneal dialysis patients", proponho a sua aprovação pela CES do HSJ, depois de obtida a resposta à questão formulada em itálico e em sublinhado.

Porto, 28 de Julho de 2011

O Relator
Prof. Manuel Vaz Silva

RESPOSTA ADEQUADA
À QUESTÃO FORMULADA.

[Assinatura]
23/09/2011

DECLARAÇÃO DE CONSENTIMENTO

*Considerando a "Declaração de Helsínquia" da Associação Médica Mundial
(Helsínquia 1964; Tóquio 1975; Veneza 1983; Hong Kong 1989; Somerset West 1996 e Edimburgo 2000)*

Designação do Estudo (em português):

Saúde oral em doentes em diálise peritoneal

Eu, abaixo-assinado, (nome completo do participante) _____

_____, compreendi a explicação que me foi fornecida, por escrito e verbalmente, da investigação que se tenciona realizar, para qual é pedida a minha participação. Foi-me dada oportunidade de fazer as perguntas que julguei necessárias, e para todas obtive resposta satisfatória.

Tomei conhecimento de que, de acordo com as recomendações da Declaração de Helsínquia, a informação que me foi prestada versou os objectivos, os métodos, os benefícios previstos, os riscos potenciais e o eventual desconforto. Além disso, foi-me afirmado que tenho o direito de decidir livremente aceitar ou recusar a todo o tempo a minha participação no estudo. Sei que se recusar não haverá qualquer prejuízo na assistência que me é prestada.

Foi-me dado todo o tempo de que necessitei para reflectir sobre esta proposta de participação.

Nestas circunstâncias, decido livremente aceitar participar neste projecto de investigação, tal como me foi apresentado pelo investigador(a).

Data: ____ / ____ / 20____

Assinatura do(a) participante: _____

O(A) Investigador(a) responsável:

Nome: Benedita Sampaio Maia

INFORMAÇÃO AO PARTICIPANTE

O estudo para o qual estamos a solicitar a sua participação tem como objectivo avaliar a saúde da sua boca e pesquisar na sua saliva microrganismos (micróbios) e algumas substâncias que poderão ser responsáveis pelo grau de saúde da sua boca.

Este estudo não é possível sem a participação de doentes renais crónicos a realizar diálise peritoneal assim como um dos seus familiares que co-habite na mesma casa (grupo controlo).

Se decidir participar neste estudo, ser-lhe-á facultada toda a assistência prevista para a sua situação clínica. Depois de ser observado pelo seu médico e de lhe ter sido colhida a história da sua doença, será observado por um médico dentista.

As avaliações do médico dentista incluirão a recolha da sua saliva e um exame à sua boca onde se observarão os lábios, bochechas, gengivas, palato, pavimento da boca, glândulas salivares e dentes.

A sua participação nesta avaliação não implica quaisquer incómodos ou riscos previsíveis para além dos inerentes a uma consulta de Medicina Dentária de rotina.

Esta consulta especializada de Medicina Dentária permitirá uma avaliação completa do seu estado de saúde oral e o rastreio de qualquer doença da sua boca, permitindo o aconselhamento sobre o tratamento mais indicado para o seu caso particular. Os custos relativos aos tratamentos que decidir efetuar serão da sua responsabilidade, no entanto, terá a oportunidade de os realizar na Faculdade de Medicina Dentária da Universidade do Porto, usufruindo de uma tabela de honorários inferior à praticada na consulta privada.

O seu processo clínico só será consultado por um médico do Serviço de Nefrologia, de forma a transferir para uma ficha clínica avaliável todos os dados da sua história clínica que forem considerados relevantes. Nessa ficha clínica figurará apenas um número de código e portanto não haverá registo de qualquer elemento que o possa identificar, respeitando-se assim o seu anonimato e a confidencialidade dos seus dados.

Mesmo depois de dar o seu consentimento para participar neste estudo, é livre de retirar esse consentimento em qualquer momento, sem ter que fornecer qualquer explicação. Tal atitude não terá quaisquer implicações para si, e não afectará de modo algum a sua relação com o médico que o irá assistir ou prejudicará o tratamento de que necessita para a doença de que sofre presentemente.

O estudo iniciar-se-á só depois de obtida a respectiva autorização pela Comissão de Ética para a Saúde do Hospital de S. João.

Dada a natureza do estudo não são previsíveis quaisquer riscos ou benefícios imediatos para os doentes que nele aceitarem participar. No entanto, se forem identificadas associações entre a sua doença renal crónica e a sua saúde oral, os seus microrganismos orais e as substância salivares, essa informação poderá ser muito útil para o tratamento de doentes em diálise peritoneal.

Se porventura lhe restar alguma dúvida ou desejar algum esclarecimento adicional sobre o estudo, por favor, contacte a investigadora responsável.

Nome do investigador responsável: **Prof.ª Doutora Benedita Sampaio Maia**

Telefone do investigador: **917411727**

Correio electrónico: **bmaia@fmd.up.pt**



A Direcção Clínica 157/11

8/9/2011

MACA - m
Paulo Bettencourt
Adjunto da Direcção Clínica

AUTORIZADO

CONSELHO DE ADMINISTRAÇÃO REUNIÃO DE 22 SET. 2011			
Prof. Doutor António Pereira			
Presidente do Conselho de Administração			
Directora Clínica	Enfermeira Directora	Administrador Executivo	Administrador Burocrático
Dra. Margarida Soares	Enfermeira Buiçosa Pereira	Dr. João Oliveira	Dr. António M. Barros

Exma. Sra.

Dra. Margarida Tavares

Directora Clínica do Hospital de São João EPE

Assunto: Parecer da Comissão de Ética para a Saúde do Hospital de São João

Projecto de Investigação – “Prevenção e diagnóstico de infeções em diálise peritoneal”

Investigadora Principal: Doutora Isabel João Soares da Silva

Junto envio a V. Exa. para obtenção de decisão final do Conselho de Administração o parecer elaborado pela Comissão de Ética para a Saúde relativo ao projecto em epígrafe.

Com os melhores cumprimentos.

Porto, 7 de Setembro de 2011

O Secretário da Comissão de Ética para a Saúde

Dr. Pedro Brito

Comissão de Ética para a Saúde do HSJ
Parecer

Projecto de investigação: “Prevenção e diagnóstico de infeções em diálise peritoneal”.

Promotor:

- Não aplicável.

Concepção e pertinência do estudo

- Trata-se de um estudo que tem como objectivo principal o melhoramento do diagnóstico e prevenção das infeções na diálise peritoneal (DP), através de diferentes abordagens: 1) diagnóstico das infeções na diálise peritoneal por aplicação de abordagens genómicas e moleculares; 2) estudo das vias de infecção e origem dos agentes patogénicos.
- Um dos primeiros objectivos será a caracterização da microbiota corporal, de pacientes em DP (todos os doentes seguidos em consulta externa do Serviço de Nefrologia do Hospital de S. João EPE – cerca de 140 doentes previsivelmente) de localizações corporais consideradas fontes frequentes de infecção, tais como o nariz (zaragatoa), a saliva, o cateter (zaragatoa), assim como o fluido peritoneal, peritонеu (biósia durante a inserção do cateter para DP) e sangue periférico. Será também efectuada uma identificação precisa dos agentes causadores de peritonites e a avaliação da sua relação coma microbiota normal do paciente.
- A identificação microbiana será realizada através de abordagens clássicas, assim como técnicas moleculares (amplificação da zona 16S do rDNA por PCR seguida de sequenciação).
- O estudo terminará em 2014 e terá o financiamento da Fundação para a Ciência e Tecnologia e Unidade de I&D de Nefrologia da Faculdade de Medicina do Porto.
- O estudo é muito importante, pertinente, está muito bem fundamentado.
- Serão ainda avaliadas características sócio-demográficas, antecedentes patológicos e resultados analíticos.
- O protocolo do estudo, os critérios de inclusão e exclusão estão suficientemente detalhados.
- A Investigadora Principal, Dra Isabel João Soares da Silva, licenciada e doutorada em Biologia e, actualmente Assistente de Investigação da Unidade de I&D de Nefrologia da FMUP/Hospital de S. João EPE (tendo como elo de ligação aos doentes do hospital, a médica nefrologista Dra Carla Araújo), dispõe das competências técnicas e científicas para a realização do estudo.
- O estudo será realizado no Serviço de Nefrologia do Hospital S. João EPE que dispõem das condições necessárias para a realização do estudo. O estudo está autorizado pelo Director de Serviço Professor Manuel Pestana.

– Benefício/Risco

- Todos os procedimentos clínicos e analíticos são os que habitualmente se realizam nos doentes em diálise peritoneal e que são candidatos a incluir neste estudo. Haverá alguns procedimentos específicos do estudo mas que não causam incómodo com significado (uma colheita adicional de sangue e zaragatoas da

cavidade bucal e do cateter de DP) que estão devidamente referenciados na folha de informação ao doente.

- Não haverá deslocações adicionais dos doentes para realizarem procedimentos específicos do estudo.

– Respeito pela liberdade e autonomia do sujeito do ensaio

- A folha de informação submetida à CES contém toda a informação relevante relacionada com o tipo de estudo proposto.
- A gratuidade do sujeito do ensaio em participar no estudo é igualmente garantida.
- Estão consignados os direitos e referenciadas as (não) consequências para o sujeito do estudo em não participar e, uma vez dado o seu consentimento, ter a possibilidade de exercer o direito de não continuar no estudo sem que daí resulte qualquer modificação dos cuidados médicos a prestar.

– Confidencialidade dos dados

A confidencialidade dos dados será garantida.

– Indemnização por danos

Não aplicável.

– Continuação do tratamento

Não aplicável.

- Propriedade dos dados

Não aplicável.

Conclusão

Em face da análise do protocolo do estudo “Prevenção e diagnóstico de infeções em diálise peritoneal”, proponho a sua aprovação pela CES do HSJ.

Porto, 28 de Julho de 2011


O Relator

Prof. Manuel Vaz Silva

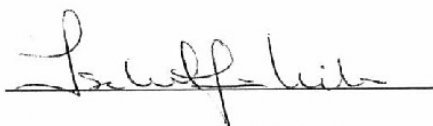
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COMISSÃO DE ÉTICA PARA A SAÚDE

8. TERMO DE RESPONSABILIDADE

Eu, abaixo-assinado, Isabel João Soares da Silva, na qualidade de Investigador Principal, declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsínquia (com as emendas de Tóquio 1975, Veneza 1983, Hong-Kong 1989, Somerset West 1996 e Edimburgo 2000) e da Organização Mundial da Saúde, no que se refere à experimentação que envolve seres humanos.

Porto, 4/Julho / 2011



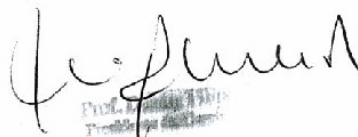
O Investigador Principal

PARECER DA COMISSÃO DE ÉTICA PARA A SAÚDE DO HOSPITAL DE S. JOÃO

emitido na reunião plenária da CES

de
28/Julho 2011

A Comissão de Ética para a Saúde
APROVA por unanimidade o parecer do
Relator, pelo que nada tem a opor à
realização deste projecto de investigação.



*Recd: o Jpr, empossado
o V. Ex. do relator
2014-02-28
L. F. F. F.*

Exmo. Senhor

Presidente da Comissão de Ética para a Saúde do
Hospital de S. João – EPE

Assunto: Adenda ao projecto de investigação “Prevenção e Diagnóstico de infeções em Diálise Peritoneal”

Nome do Investigador Principal: Isabel João Soares da Silva

Título do projecto de investigação: “Prevenção e Diagnóstico de infeções em Diálise Peritoneal”

Estando a realizar no **Serviço de Nefrologia do Hospital de S. João – EPE em colaboração com a Faculdade de Medicina da Universidade do Porto e a Faculdade de Medicina Dentária da Universidade do Porto** projecto de investigação em epígrafe aprovado pela comissão de ética para a Saúde do Hospital de S. João a 22/09/2011, solicito a V. Exa., na qualidade de Investigador, a sua apreciação da adenda ao projecto e a elaboração do respectivo parecer.

Esta adenda reporta-se à colheita de um pequeno fragmento de peritонеu. Este procedimento já estava descrito no projecto submetido para aprovação à Comissão de Ética do Hospital de S. João, no entanto não tinha sido incluído na folha de informação ao participante. Esta colheita será efectuada por um cirurgião, nas situações em que haja necessidade de colocação, remoção ou reposicionamento do cateter peritoneal do doente em Diálise Peritoneal.

Para o efeito, envio a nova informação ao participante respeitante ao projecto de investigação.

Com os melhores cumprimentos.

Porto, 20 / fevereiro / 2014.

O INVESTIGADOR/PROMOTOR

Isabel João Soares da Silva

DECLARAÇÃO DE CONSENTIMENTO

Considerando a “Declaração de Helsínquia” da Associação Médica Mundial
(Helsínquia 1964; Tóquio 1975; Veneza 1983; Hong Kong 1989; Somerset West 1996 e Edimburgo 2000)

Designação do Estudo (em português):

“Prevenção e diagnóstico de infecções em Diálise Peritoneal”

Eu, abaixo-assinado, (nome completo do participante) _____

_____, compreendi a explicação que me foi fornecida, por escrito e verbalmente, da investigação que se tenciona realizar, para qual é pedida a minha participação. Foi-me dada oportunidade de fazer as perguntas que julguei necessárias, e para todas obtive resposta satisfatória.

Tomei conhecimento de que, de acordo com as recomendações da Declaração de Helsínquia, a informação que me foi prestada versou os objectivos, os métodos, os benefícios previstos, os riscos potenciais e o eventual desconforto. Além disso, foi-me afirmado que tenho o direito de decidir livremente aceitar ou recusar a todo o tempo a minha participação no estudo. Sei que se recusar não haverá qualquer prejuízo na assistência que me é prestada.

Foi-me dado todo o tempo de que necessitei para reflectir sobre esta proposta de participação.

Nestas circunstâncias, decido livremente aceitar participar neste projecto de investigação, tal como me foi apresentado pelo investigador(a).

Data: ____ / ____ / 20____

Assinatura do(a) participante: _____

O(A) Investigador(a) responsável:

Nome: Isabel João Soares da Silva

Assinatura: _____

FOLHA DE INFORMAÇÃO PARA O DOENTE

As infecções do peritoneu designadas por peritonites são complicações que podem ocorrer nos doentes em tratamento de diálise peritoneal, cuja origem nem sempre é fácil de identificar.

O estudo para o qual estamos a solicitar a sua participação tem como objectivo detectar no seu sangue, líquido peritoneal, cateter de diálise peritoneal, nariz e no peritoneu, a presença de microrganismos possivelmente responsáveis pelo desenvolvimento de infecções designadas por peritonites.

Se concordar em participar neste estudo, ser-lhe-á facultada toda a assistência prevista para a sua situação clínica. Depois de ser observado pelo seu médico e de lhe ter sido colhida a história da sua doença, ser-lhe-ão colhidas amostras de sangue, de líquido peritoneal de 24 horas, do orifício de saída do cateter e do nariz para os objectivos do presente estudo.

Caso não aceite colaborar neste estudo, em nada ficará prejudicado na assistência que lhe é prestada.

A colheita de sangue será efectuada em repouso, 30 minutos depois de lhe ser colocado um cateter (um pequeno tubo) numa veia do braço, através do qual será retirado sangue sem ser necessário repetir a picada de outra veia. Será colhida uma amostras de sangue de 5 ml para pesquisar a presença de microrganismos.

A colheita de líquido peritoneal de 24 horas será efectuada por si, de acordo com o procedimento a que está habituado na avaliação de rotina do Sector de Diálise Peritoneal.

A colheita de amostras do nariz e do orifício de saída do cateter de DP será efectuada com uma zaragatoa de algodão.

A colheita de um pequeno fragmento de peritoneu <1cm² será efectuada por um cirurgião no momento da cirurgia indicada pela necessidade de colocação, remoção ou reposicionamento do cateter peritoneal.

O seu processo clínico só será consultado por um médico do Serviço de Nefrologia, de forma a transferir para uma ficha clínica avaliável todos os

dados da sua história clínica que forem considerados relevantes. Nessa ficha clínica figurará apenas um número de código e portanto não haverá registo e qualquer elemento que o possa identificar, respeitando-se assim o seu anonimato e a confidencialidade dos seus dados.

Mesmo depois de dar o seu consentimento para participar neste estudo, é livre de retirar esse consentimento em qualquer momento, sem ter que fornecer qualquer explicação. Tal atitude não terá quaisquer implicações para si, e não afectará de modo algum a sua relação com o médico que o irá assistir ou prejudicará o tratamento de que necessita para a doença de que sofre presentemente.

O estudo foi autorizado pela Comissão de Ética para a Saúde do Hospital de S. João.

Dada a natureza do estudo não são previsíveis quaisquer riscos ou benefícios imediatos para os doentes que nele aceitarem participar. No entanto, se forem identificadas associações entre os microrganismos presentes no doente e os microrganismos causadores de peritonites poderão ser esclarecidas possíveis fontes de contaminações e essa informação poderá ser muito útil para a prevenção de futuras infecções. Paralelamente, estes estudos poderão ainda contribuir para o desenvolvimento de novos métodos, mais rápidos e eficazes, de diagnóstico de peritonites.

Investigadora responsável:

Doutora Isabel João Soares da Silva

Unidade de I&D de Nefrologia

(contacto 965673251)