

DOUTORAMENTO
CIÊNCIAS MÉDICAS

Peritoneal protein loss – the clinical impact in peritoneal dialysis patients

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D

2023



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**PERDAS PROTEICAS PERITONEAIS – O IMPACTO CLÍNICO NOS
DOENTES EM DIÁLISE PERITONEAL**

**PERITONEAL PROTEIN LOSS – THE CLINICAL IMPACT IN
PERITONEAL DIALYSIS PATIENTS**

Tese de Candidatura ao grau de Doutor em
Ciências Médicas,
Programa Doutoral da Universidade do Porto
(Instituto de Ciências Biomédicas Abel Salazar)

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2023

De acordo com o disposto no nº2 do artigo 31º e no nº1 do artigo 34º do Decreto-Lei nº74/2006, alterado pelo artigo 2º do Decreto-Lei nº 65/2018, publicado em Diário da República nº 157/2018, Série I de 16 de Agosto de 2018, que procede as restantes alterações, constam nesta tese os artigos publicados, e que a seguir se discriminam:

- I. Malho Guedes A. Peritoneal Protein Loss, Leakage or Clearance in Peritoneal Dialysis, Where Do We Stand? *Perit Dial Int.* 2019 May-Jun; 39(3):201-209. DOI: 10.3747/pdi.2018.00138. PMID: 31088933
- II. Malho Guedes A, Calças Marques R, Domingos AT, Laranjo C, Silva AP, Rodrigues A. Protein Loss in Peritoneal Effluent: different meaning for 24-hour versus PET samples. *Blood Purif.* 2022 August; 52(2):1-8. DOI:10.1159/000525502
- III. Malho Guedes A, Marques R, Domingos AT, Silva AP, Bernardo I, Neves PL, Rodrigues A, Krediet RT. Overhydration May be the Missing Link between Peritoneal Protein Clearance and Mortality. *Nephron.* 2021; 145(5):478-480. DOI:10.1159/000516531. Epub 2021 Jun 15. PMID:34130276.
- IV. Malho Guedes A, Calças Marques R, Ribeiro B, Fernandes MT, Faísca M, Silva AP, Bragança J, Rodrigues A. Peritoneal Protein Loss, Inflammation, and Nutrition: Refuting Myths. *Front Med (Lausanne).* 2022 May 26;9:884061. DOI:10.3389/fmed.2022.884061. PMID: 35692552; PMCID: PMC9178188.
- V. Malho Guedes A, Calças Marques R, Domingos AT, Laranjo C, Silva AP, Rodrigues A, Krediet RT. Peritoneal Protein Loss with time in Peritoneal Dialysis. *Semin Dial.* 2024 May-Jun;37(3):242-248. doi:10.1111/sdi.13194. Epub 2024 Feb 29. PMID:38420712.

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Abbreviations

Å - angstrom
APD – automated peritoneal dialysis
BMC – body cell mass
BIA – bioelectrical impedance analysis
BMI – body mass index
BNP- brain natriuretic peptide
CAPD – continuous ambulatory peritoneal dialysis
CKD – chronic kidney disease
CV - cardiovascular
D/P creatinine – dialysate to plasma creatinine ratio
ECW – extracellular water
ECW/TBW – extracellular water to total body water ratio
EWexc – extracellular water excess
FWT – free water transport
GFR – glomerular filtration rate
GLUT-1 – glucose transporter 1
HD – hemodialysis
hs-CPR – high sensitivity C-reactive protein
ICW – intracellular water
IL-6 – interleukin 6
JvL – peritoneal large pore fluid flux
Kt/V urea - urea clearance index
LBM – lean body mass
LBMI – lean body mass index
MTAC – mass transfer area coefficient
NAD⁺ - nicotinamide adenine dinucleotide
NADH – reduced nicotinamide adenine dinucleotide
nPNA - normalized nitrogen appearance rate
OH – overhydration
PAI-1 – plasminogen activator inhibitor -1
PD – peritoneal dialysis

PET – peritoneal equilibration test

PPCI – peritoneal protein clearance

PPL – peritoneal protein loss

PPRC – peritoneal protein restriction coefficient

PTSR – peritoneal transport solute rate

RKF – residual kidney function

SARS-CoV-2 – severe acute respiratory syndrome coronavirus 2

TBW – total body water

TGF β - transforming growth factor beta

UF – ultrafiltration

VEGF – vascular endothelial growth factor

1. ABSTRACT

2. Introduction

3. Aims and outline of the thesis

4. Methods

5. Results

6. Discussion

7. Conclusions

8. References

9. Acknowledgments

1. Abstract

- Abstract
- Resumo

1. Abstract

Peritoneal dialysis (PD) is an established home-based renal replacement therapy, representing 11% of all patients treated worldwide with dialysis. It has several advantages over hemodialysis, but also has limitations. Macromolecule, as proteins, crosses the membrane through the large pores, mainly distributed in the venular part of the peritoneal capillaries. Peritoneal protein loss (PPL) has been estimated at 5 to 15g daily, in adult patients.

A comprehensive review of the literature stood as a pillar to evidence the gap of knowledge that induced this doctoral investigation. Several questions were raised by this paper, namely methodologic issues in the quantification of PPL, regarding 24-hour or 4-hour standardized PET collections, and their biological meaning. Also, the impact of PPL on clinical outcome was not clear, with some evidence linking PPL to global mortality, other to cardiovascular events, and even other studies claiming no association with a worse outcome. Mechanistically, most studies, addressed systemic endothelial dysfunction as the corner piece explaining PPL and prognosis. In this axis also appears inflammation, as a determinant for higher PPL, but also associated with higher mortality. Nutritional impact of PPL, depending on the nutritional marker chosen, disclosed different effects. Albumin stands as an undesired marker, due to be influenced by both inflammatory and nutritional status and urinary or peritoneal losses.

To investigate the clinic impact of PPL in PD patients, specific tasks were developed aiming to test relevant hypotheses: a) different methodologies might unveil different physiologic meanings; b) overhydration could be an important associated factor with PPL; c) previously reported clinical associations with PPL (like inflammation or malnutrition) should be adjusted for overhydration to establish its prognostic value; d) ascertain longitudinal PPL changes.

Concerning methodology, PPL appraisal showed that PET and 24-hour measurements should not be considered equivalent, disclosed by the weak Pearson correlations between g/24h and g/240 minutes and between ml/24h and g/240 min. There was a consistent uni- and multivariable relationship with D/P creatinine and creatinine clearance within the 4-hour collections, sustaining PET standardized collection is applicable for membrane characterization. Independent factors

associated with 24-hour collections peritoneal loss were higher D/P creatinine and extracellular water excess (EWexc), sustaining that the overall clinical status is the target of analysis on the 24-hour determinations.

The stated association with overhydration (OH) was investigated and confirmed in a second paper, with significant associations between peritoneal protein clearance (PPCI) and bioelectric impedance analysis (BIA) parameters of OH. Multiple linear model identified EWexc and higher D/P creatinine as predictors of higher PPCI, independent of PD duration, type of PD, age, gender, albumin, cardiovascular disease, C reactive protein or lean body mass index (LBMI), supporting the importance of hydrostatic pressure-induced convection for PPCI.

To ascertain the importance of OH over classical correlated factors with PPL, inflammation, hydration and nutritional status were assessed in a cohort of PD patients. PPL correlated positively with dialysate appearance rate of IL-6, but not with serum IL-6. However, linear multivariable analysis confirmed only positive association between PPL and EWexc, LBMI and performing CAPD, excluding inflammation as a determinant factor. As for survival analysis, multivariable Cox regression showed that Charlson Comorbidity Index, OH and lower PPL were predictors for mortality. PPL were not associated with malnutrition, inflammation or higher mortality, emphasizing the importance of volume overload control.

Evaluation of PPL throughout long term PD, showed significant decrease in PPL over time, in a 4-year follow-up cohort. Natural evolution with a decrease of total Kt/V urea, creatinine clearance, PET ultrafiltration, residual kidney function and urinary volume, was confirmed. Still, parameters of systemic inflammation, nutrition, serum total protein, albumin, D/P creatinine, free water transport (FWT) and volume overload did not change significantly within the time of follow-up. No relationship was found between overall or cardiovascular mortality and PPL. PPL may act as a precocious biomarker of acquired peritoneal venous vasculopathy, even if small pore fluid transport rates and FWT are preserved.

As for conclusions, through this work, it was established that the use of PET protein loss quantification should be regarded as a marker of peritoneal membrane intrinsic permeability, and in this case, characterization of large pore transport. Measurements of 24-hour samples might be closer to patients' clinical status and prognosis, signaling opportunities of therapy intervention. In this way, this work

standardizes, for the first time, different methodology for different investigational questions.

The association of higher PPL with fluid overload, discloses the importance of increased venous pressure, what is the same as hydrostatic pressure-induced convection for PPL. As for longitudinal evaluation of PPL, it showed a reduction in the protein leakage with time on PD, which is probably due to a decreased hydrostatic filtration pressure resulting from peritoneal vasculopathy. This possibly reveals to be the most precocious sign of membrane dysfunction, previous to local fibrosis. Altogether, hydrostatic pressure and local vasculopathy were identified as major determinants for PPL.

PPL association with malnutrition was not confirmed, and even after long duration of PD treatment, the continuous loss of proteins was not associated with negative nutritional evolution. Likewise, systemic or local inflammation were not consistent with higher PPL. The controversial association of PPL with mortality, was not confirmed, if adjustment for OH was done.

Future longitudinal studies are needed to address peritoneal loss of albumin, metabolomic and proteomic analysis, clinical impact of specific proteins loss and usefulness of peritoneal protein clearance as a membrane monitoring tool.

Noteworthy, after 4 years on PD, small pore fluid transport rates and FWT were preserved, supporting the safety of PD, namely with the use of biocompatible solutions and adjusted PD prescription. It is important to control volume overload, amenable by updated dialysis regimens (eg. icodextrin, APD), diuretic and dietetic counseling. So, although PPL has been recognized as a feature of peritoneal dialysis since its inception, it shouldn't be considered deleterious, but as window to monitor peritoneal membrane health.

Keywords: bioelectric impedance analysis; convection; free water transport; hydrostatic pressure; inflammation; large pore transport; lean body mass index; malnutrition; mortality; nutritional assessment; overhydration; peritoneal dialysis; peritoneal equilibration test; peritoneal membrane; peritoneal protein clearance; peritoneal protein loss.

1. Resumo

A diálise peritoneal (DP) é uma técnica de substituição renal domiciliária estabelecida, representando 11% dos pacientes tratados com diálise em todo o mundo. Apresenta diversas vantagens em relação à hemodiálise, mas também apresenta limitações. As macromoléculas, assim como as proteínas, atravessam a membrana através dos grandes poros, distribuídos principalmente na parte venular dos capilares peritoneais. As perdas proteicas peritoneais (PPL) foram estimadas em 5 a 15g por dia, em doentes adultos.

Uma revisão abrangente da literatura constituiu o pilar que evidencia a lacuna de conhecimento e que induziu esta investigação doutoral. Várias questões foram levantadas por este artigo, nomeadamente questões metodológicas na quantificação de PPL, relativamente às colheitas de 24-horas ou 4-horas durante o teste de equilíbrio peritoneal (PET), e o seu significado biológico. Além disso, o impacto das PPL no prognóstico clínico não é claro, com evidências que associam PLL com maior mortalidade global; outros autores associam a eventos cardiovasculares, enquanto outros estudos alegam não haver associação das PPL com um pior prognóstico. A maioria dos estudos defende que a disfunção endotelial sistémica é explicação entre PPL e outcome. Neste eixo aparece ainda a inflamação, como determinante para maiores PPL, e que também se associa a maior mortalidade. O impacto nutricional das PPL, dependendo do marcador nutricional escolhido, mostra também efeitos inconsistentes. A albumina destaca-se como um marcador indesejado, por ser influenciada por fatores inflamatórios, nutricionais e perdas urinárias ou peritoneais.

Para investigar o impacto clínico das PPL em doentes em DP, os objetivos específicos visaram testar hipóteses relevantes: a) diferentes metodologias poderão revelar diferentes significados fisiológicos; b) a hipervolemia pode ser um importante fator associado com as PPL; c) importantes fatores previamente associados às PPL (como inflamação e estado nutricional) deverão ser ajustados ` volémia para poder estabelecer seu valor prognóstico; d) estudar evolução longitudinal das PPL.

Quanto à metodologia, a avaliação das PPL mostrou que as determinações de 24 horas e durante o PET não devem ser consideradas equivalentes, dada a fraca

correlação de Pearson entre g/24h e g/240 min e entre ml/24h e g/240 min. Constatou-se uma relação uni e multivariada consistente com o D/P creatinina e a depuração de creatinina nas colheitas de 4 horas, ou seja, o doseamento padronizado no PET caracteriza a membrana. Na colheita das 24h, D/P creatinina e excesso de água extracelular (EWexc) foram os fatores independentes associados a maiores PPL, enquadrando o estado clínico global como alvo de análise nas determinações de 24 horas.

A associação encontrada com EWexc foi investigada e confirmada num segundo artigo, com associações significativas entre a clearance de proteínas peritoneais (PPCI) e hipervolemia, determinada por impedância bioelétrica (BIA). O modelo linear múltiplo identificou EWexc e maior D/P creatinina como preditores de PPCI, independente da duração da DP, tipo de DP, idade, sexo, albumina, doença cardiovascular, proteína C reativa ou índice de massa corporal magra (LBMI). Fatos que apoiam a relevância da convecção, induzida pela pressão hidrostática, para aumentar a PPCI.

Para verificar a importância da hipervolemia sobre os fatores clássicos relacionados com as PPL, a inflamação, a hidratação e o estado nutricional foram avaliados numa coorte de pacientes em DP. As PPL correlacionaram-se com a taxa de aparecimento de IL-6 no dialisado, mas não com a IL-6 sérica. No entanto, a análise multivariável linear confirmou apenas associação positiva entre PPL e EWexc, LBMI e realização de CAPD, excluindo a inflamação como fator determinante. Quanto à análise de sobrevida, a regressão multivariada de Cox mostrou que o Índice de Comorbidade de Charlson, EWexc e menor PPL foram preditores de mortalidade. As PPL não foram associadas à desnutrição, inflamação ou mortalidade, enfatizando a importância do controle da sobrecarga de volume.

Na avaliação das PPL a longo prazo em DP, verificou-se diminuição longitudinal significativa das PPL, numa coorte com seguimento de 4 anos. Foi confirmada a evolução natural em DP, com diminuição do Kt/V ureia, da depuração de creatinina, da ultrafiltração no PET, da função residual renal e do volume urinário. Ainda assim, os parâmetros de inflamação sistêmica, nutrição, proteína sérica total, albumina, D/P creatinina, transporte livre de água e sobrecarga de volume não se alteraram significativamente no tempo de seguimento. Nenhuma relação foi encontrada entre mortalidade geral ou cardiovascular e PPL. As PPL

podem ser um biomarcador precoce da vasculopatia venosa peritoneal adquirida, mesmo quando o transporte de água livre e de fluido pelos pequenos poros ainda estejam preservados.

Quanto às conclusões, foi estabelecido que o uso da quantificação da perda de proteína durante o PET deve ser considerado como um marcador de permeabilidade intrínseca da membrana peritoneal e, neste caso, caracterização do transporte de grandes poros. As medições de amostras de 24 horas podem estar mais próximas do estado clínico e do prognóstico dos pacientes, sinalizando oportunidades de intervenção terapêutica. Desta forma, este trabalho padroniza, pela primeira vez, diferentes metodologias para diferentes questões investigacionais.

A associação de maior PPL com hipervolemia revela a importância do aumento da pressão venosa, o que equivale dizer, da convecção induzida por pressão hidrostática para aumentar PPL. Já a avaliação longitudinal da PPL mostrou redução da perda de proteínas com o tempo em DP, o que provavelmente se deve à diminuição da pressão de filtração hidrostática decorrente da vasculopatia peritoneal. Este possivelmente será o sinal mais precoce de disfunção de membrana, prévio à fibrose local. Sumariando, a pressão hidrostática e a vasculopatia local foram identificadas como os principais determinantes das PPL.

A associação das PPL com a desnutrição não foi confirmada e, mesmo após longa duração do tratamento em DP, a perda contínua de proteínas não foi associada a evolução nutricional negativa. Da mesma forma, a inflamação sistêmica ou local não se relacionou com PPL mais elevadas. A controversa associação de PPL com mortalidade também não foi confirmada, caso fosse feito ajuste para a EWexc.

Futuros estudos longitudinais são necessários para abordar a perda peritoneal de albumina, análise metabólica e proteômica, impacto clínico da perda de proteínas específicas e utilidade da depuração de proteínas peritoneais como ferramenta de monitorização da membrana.

De salientar que após 4 anos em DP, o transporte de fluidos por pequenos poros e transporte de água livre encontram-se preservados, apoiando a segurança da DP, nomeadamente devido à utilização de soluções mais biocompatíveis e evolução na prescrição de DP. É importante o controlo da sobrecarga volêmica, através de prescrição ajustada de diálise (ex. Icodextrina, DP automatizada),

diuréticos e restrição da ingestão de sal e água. Assim, parece que embora as PPL tenham sido reconhecidas como uma característica da diálise peritoneal desde o seu início, este transporte não deve ser considerado deletério, mas sim como uma janela para monitorizar a saúde da membrana peritoneal.

Palavras-chave: análise de impedância bioelétrica; avaliação nutricional; convecção; depuração de proteínas peritoneais; desnutrição; diálise peritoneal; hipervolemia; índice de massa corporal magra; inflamação; membrana peritoneal; mortalidade; perda de proteína peritoneal; pressão hidrostática; teste de equilíbrio peritoneal; transporte de água livre; transporte de grandes poros.

1. Abstract

2. INTRODUCTION

3. Aims and outline of the thesis

4. Methods

5. Results

6. Discussion

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8. References

9. Acknowledgments

2. Introduction

- Peritoneal dialysis – principles, advantages and concerns
- Peritoneal membrane and transport – theories and enigmas
- Peritoneal Protein Loss, Leakage or Clearance in Peritoneal Dialysis, where do we stand?
- Critical appraisal and review of the state of the art

2. Introduction

2.1 Peritoneal dialysis – principles, advantages and concerns

Peritoneal dialysis (PD) was introduced in the 1960s, for the treatment of end-stage renal failure. Nowadays it has evolved to an established therapy that is complementary to hemodialysis (HD), representing 11% of all patients treated worldwide with dialysis (1). Current evidence shows that survival outcomes are now globally, at least, equivalent for both dialysis techniques (2-5), as PD results have improved during the past decade (3, 4).

PD encompasses the exchange of solutes and water between blood in the peritoneal capillaries and the instilled solution in the peritoneal cavity (dialysate) through a catheter, using the peritoneal membrane as the dialysing surface (6). Solutes diffuse from blood in the capillaries into the dialysate and are discarded.

PD is a home-based treatment, which relies on self-care ability of the patient to perform dialysis or on the assistance of a caregiver. It has numerous potential advantages over HD, by including several dimensions of quality: it is a simpler technique, with greater feasibility of use in remote communities, generally lower cost, lesser need for trained staff, fewer management challenges during natural disasters, possibly better survival in the first few years, fewer dietary restrictions, greater ability to travel, better preservation of residual kidney function, better quality of life, greater treatment satisfaction, better outcomes following subsequent kidney transplantation, delayed need for vascular access (especially in small children but also in adults with expected longer chronic kidney disease (CKD) career migrating among renal replacement therapies), reduced need for erythropoiesis-stimulating agents, and lower risk of SARS-CoV-2 infection and of blood-borne virus infections (7-9).

On the other hand, as much as PD is associated with more independent schedules, it also obliges to a greater responsibility and compliance to avoid access related infectious complications as well as maintain euvolemia. Other concerns arise with time on PD, as weight gain, metabolic complications or hernias (6). Also,

structural changes in the peritoneal membrane can occur over time, which may compromise the effectiveness of the method.

The use of an in-built membrane, as the peritoneal membrane, with little possibility for external maneuver, impedes external control of its performance. This has brought several controversies, on supposed deleterious effects of such a “blind” filter. Several substances cross this membrane, ions, products of catabolism and proteins, just to name a few. It is the long-known loss of proteins to the dialysate, higher than in hemodialysis, that has become polemic recently, by being associated with malnutrition, and worse outcome.

2.2 Peritoneal membrane and transport – theories and enigmas

The peritoneum is a serous membrane with a surface of approximately 1 to 2 m² in adults. It is constituted by two leaflets, the parietal peritoneum (about 10% of total), which covers the inner surface of the abdominal and pelvic walls including the diaphragm; and the visceral peritoneum (about 90% of total), which covers the visceral organs and forms the omentum and the visceral mesentery. The effective peritoneal surface area, which means the functional area available for exchanging between blood and the dialysate, is determined by the number of perfused capillaries (6).

From the perspective of PD, important components of the peritoneal membrane include the mesothelial cells, an underlying basement membrane, the interstitium, the microcirculation, and the visceral lymphatics (10,11).

Mesothelium: a simple squamous epithelium, is a continuous monolayer of cells ultrastructurally similar to type II pneumocytes found in the pulmonary alveoli. These cells secrete surfactant-like lubrication for the peritoneum and are active in modulating host defense (10).

Basement Membrane: underlies the mesothelium, is composed of type IV collagen, proteoglycans, and glycoproteins, ranging between 25 and 40 nm thick.

Interstitium: is the supporting structure of the peritoneum and is composed primarily of a mucopolysaccharide matrix. This extracellular matrix consists of glycosaminoglycans, mainly proteoglycans and non-protein-bound hyaluronan (12). It contains bundles of collagen fibers, the blood vessels, the lymphatics, occasional macrophages, and fibroblasts.

Blood vessels: blood flow to peritoneum is estimated to occur at a rate of 50-100 ml/min. Capillaries represent the largest barrier to the transport of solutes and water (6). The capillary walls are believed to contain 3 distinct pore sizes, with the larger pores located primarily at the venular end and the smaller pores at the arteriolar end. The third type is “water-only” aquaporin-1 pores (13).

Peritoneal lymphatics: aid in removal of fluid and solutes from the interstitium. Intraperitoneal hydrostatic pressure, body posture and pharmacological agents alter the rate of lymphatic uptake (10).

Transport across the membrane:

Among the layers of resistance to the passage of fluids and solutes between blood and intraperitoneal fluid, the principal barrier is the vascular endothelium of capillaries and post-capillary venules of the peritoneum (14). The endothelium basement membrane, the interstitial matrix, cells of the interstitium, the mesothelium and its basement membrane offer virtually no obstacle to the transport of small molecules (15).

According to the pores model developed by Rippe *et al.*, three pore sizes would be present in the membrane, regulating the passage of molecules of different radii and molecular mass: ultra-pores, small pores and large pores (16).

The large pores of 250 Å are sparsely distributed, representing less than 0.01% of the pores, and allow the passage of molecules with a higher molecular mass, like proteins (13). The identity of these large pores has not been established, even if some structures have been postulated, including plasmalemma vesicles, vesicular-vacuolar organelles, large interendothelial clefts or venular interendothelial gaps (17).

Small pores are intercellular slits of 45 Å that allow the passage of 99.5% of small solutes. The endothelial cells of peritoneal capillaries are permeable to water through ultra-pores (3 Å radius). These transcellular pores are represented by endothelial cell membrane proteins called aquaporins that allow the passage of water molecules only (6,18).

Peritoneal transport involves diffusion, convection, ultrafiltration and fluid absorption that occur simultaneously.

Fluid transport: transcapillary ultrafiltration occurs in both directions, directed to the cavity and towards the capillary lumen. The flow of fluids from the capillaries to the peritoneal cavity occurs across small interendothelial pores and across aquaporin-1 channels, through the osmotic gradient created by the addition of osmotic agents to the dialysis solution. Approximately 40% of ultrafiltration is believed to occur via solute-free transcellular pathways (aquaporins) (19).

The peritoneal cavity fluid absorption occurs mostly by the subdiaphragmatic area lymph vessels. This flow varies according to hydrostatic, osmotic and oncotic pressure gradients.

In clinical practice it is recommended to study the transport of fluids and small solutes by using the Twardowski's peritoneal equilibration test (PET) (20), but to more accurately test ultrafiltration capacity and water removal pathways, the instillation of 3.86%/4.25% glucose solution (modified test) is advocated.

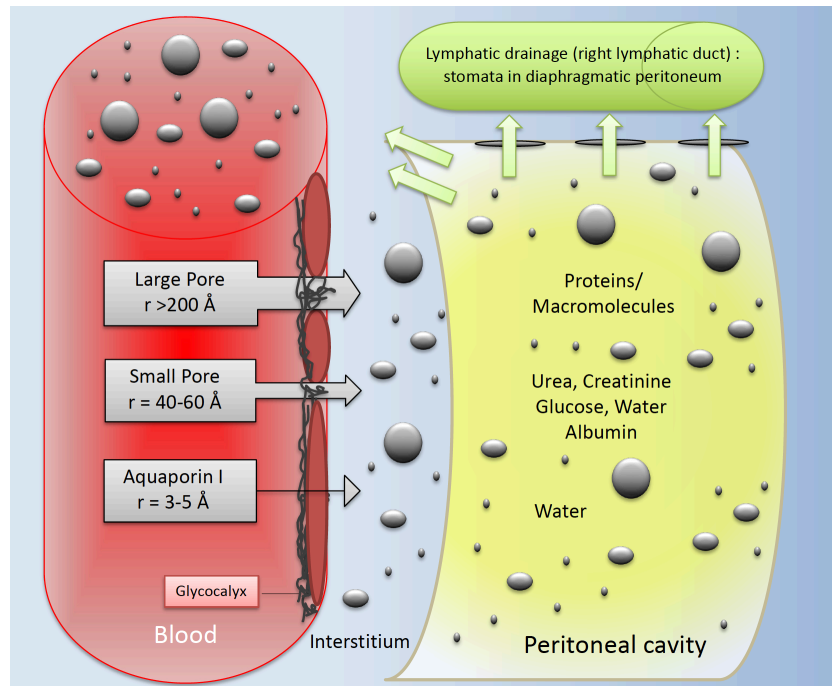


Figure 1 – schematic representation of the transport across the peritoneal membrane; image adapted from Malho Guedes, A (21)

Solute transport: occurs as a result of both diffusional and convective transport, and through both small and large pores.

Solute diffusion primarily occurs by a concentration gradient, with solutes such as urea, creatinine and potassium moving from the plasma toward the dialysate, while other solutes, such as bicarbonate, usually move in the opposite direction. According to Fick's principles, peritoneal solute transport rate is primarily determined by the number of perfused peritoneal capillaries in contact with the dialysis solution (the effective peritoneal surface area), the intrinsic permeability of the membrane and the concentration gradient between blood and dialysate (17).

The permeability of the peritoneum is attained by the ratio between the free diffusion coefficient of the solute and the distance to be traveled beyond the surface area available for exchange. The product of the permeability by the surface area is also known as mass transfer area coefficient (MTAC), a clinically used parameter to define the rate of transport of small solutes of an individual. However, Twardowski's traditional or modified PET is the most widely used test for this purpose, for being a simple and inexpensive test, with good clinical correlation (15,20).

Solute convective transport, or drag, happens together with the transport of water. It is determined by water flow, the average solute concentration in the membrane and the reflection coefficient (15).

Macromolecule transport: the transperitoneal transport of macromolecules occurs mainly by the large pores. Size and shape are the main determinants of transperitoneal transport (21), as the transport depends largely on both the effective vascular surface area (the number of pores) and the intrinsic size-selective permeability (the diameter of the large pores) (22,23). It has been demonstrated charge selectivity is absent (24).

There are controversies as to the primary mechanism of macromolecule transport, whether it is convection induced by hydrostatic force or size-restricted diffusion (15). For instances, macromolecules such as serum proteins are transported from the circulation to the peritoneal cavity at a rate lower than that of low molecular mass solutes. Thus, their concentrations are generally low in the dialysate, without reaching equilibrium with the serum ones, arguing against size-restricted diffusion only. However convective transport rate should equal ultrafiltration rate, which also does not occur (26).

On top of these riddles, marked differences related with peritoneal transport are present between individual patients, namely regarding protein leakage through the peritoneal membrane. All these enigmas prompted a review of the state of the art on peritoneal protein loss.

2.3 Peritoneal Protein Loss, Leakage or Clearance in Peritoneal Dialysis, where do we stand?

Malho Guedes A.

Perit Dial Int. 2019 May-Jun; 39(3):201-209. DOI: 10.3747/pdi.2018.00138. PMID: 31088933

Short Summary

Ever since peritoneal dialysis (PD) was used in the treatment of chronic kidney disease (CKD), high peritoneal protein loss (PPL) has been observed in each PD exchange. This loss has been estimated at 5 to 15g daily, in adult patients. The consequences of PPL have been a matter of study, namely which type of proteins are leaking and its clinical impact. This comprehensive review, was done in 2018, and stands as a pillar to evidence the gap of knowledge that induced this doctoral investigation. The paper structures what was known about the pathophysiology and predictors of PPL, quantification methodology, impact of PPL on clinical outcomes, namely survival and technique-failure analysis, cardiovascular disease and nutritional consequences. It ends pointing eventual therapeutic interventions to reduce PPL and future directions for investigation.

The peritoneal dialysate is likely to contain many, if not all, of the proteins present in serum. The amount of protein lost will depend on dialysis time, protein size and its serum concentration, and other factors including patient's clinical status. Concerning outcomes, controversial data rose from the literature. Little was evidenced concerning the mechanisms of PPL, and thereby its impact or role as a therapeutic target.

The clinical menaces of PPL, namely its association with cardiovascular conditions and nutritional outcomes, need to be addressed by prospective studies. The role of PPL as a marker or a confounding factor should be enlightened.

Nonetheless, peritoneal dialysis stands as a valuable therapy for treatment of CKD patients and an important field for research, specifically on these peritoneal effluent components.

REVIEW

PERITONEAL PROTEIN LOSS, LEAKAGE OR CLEARANCE IN PERITONEAL DIALYSIS, WHERE DO WE STAND?

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Peritoneal protein loss (PPL) through peritoneal effluent has been a well-recognized detrimental result of peritoneal dialysis (PD) treatment since its inception. Investigation has focused mainly on PPL quantitative and qualitative determinations and evaluation of its prognostic value.

A comprehensive review of the pathophysiology of PPL (3-pore model revisited), methods of quantification, dialysate protein composition, and impact on clinical outcomes is presented herein. The author summarizes a brief analysis of associated cardiovascular disease and nutritional consequences, exploring the controversial cause-effect on mortality and technique failure.

Therapeutic modalities aiming to reduce PPL (angiotensin-converting enzyme inhibitors [ACEI]s and vitamin D therapies) were explored, although it is unclear whether PPL represents a valid therapeutic target or, on the other hand, is solely a manifestation of endothelial dysfunction.

Perit Dial Int 2019; 39(3):201–209
<https://doi.org/10.3747/pdi.2018.00138>

KEY WORDS: Cardiovascular mortality, peritoneal protein excretion.

Ever since peritoneal dialysis (PD) was used in the treatment of chronic kidney disease (CKD), high peritoneal protein loss (PPL) has been observed in each PD exchange (1). In adult patients, the loss has been estimated at 5 to 15 g daily (2–4), including 2–4 g a day of amino acids (5). This loss of proteins (significantly greater in PD than in hemodialysis) accompanies the removal of both low-molecular-weight uremic metabolites and the so-called middle molecules. The removal of the latter is considered to be one of the beneficial

features of PD (2). Peritoneal protein loss requires peritoneal large-pore transport, which is correlated with systemic or local inflammatory status and probably linked to endothelial dysfunction (6).

The terms protein loss, leakage, and clearance have been used interchangeably in the literature (7–9). However, the distinction between these terms is important when comparing study results.

The consequences of PPL have been a matter of study, namely which type of proteins are leaking and its clinical impact. Interventions aimed at reducing PPL have been explored. The aim of this paper is to do a comprehensive review of the current literature.

PATHOPHYSIOLOGY AND PREDICTORS OF PROTEIN LOSS IN PERITONEAL EFFLUENT

Peritoneal transport, explained by the 3-pore model, refers to the passage of different molecules through the peritoneum via different mechanisms. According to this model, peritoneal capillaries can be described as a membrane with frequent “small” pores of approximately 45-Å radius that are permeable to small solutes and water, and a very low number of “large” pores of 250-Å radius that are protein-permeable, constituting only 0.01% of the total number of pores (10,11). “Water-only” aquaporin-1 pores contribute to osmotic ultrafiltration induced by low-molecular-weight osmotic agents (10,11). Overall protein loss is dependent on the effective peritoneal surface area (mainly determined by the number of small pores) and intrinsic permeability (average radius of large pore system) (12). Changes in dialysate-to-plasma (D/P) ratios for different proteins are correlated with molecular size, and this ratio decreases with increasing molecular radius (13). Whilst shape and size are the main determinants of transperitoneal transport, other features should be highlighted when considering protein or albumin leakage.

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Received 4 July 2018; accepted 28 December 2018.





Charge selectivity has been a matter of controversy. Studies have suggested that fixed negative charges restrict transcapillary passage of negatively charged macromolecules (14,15). Studies focused on acute tissue uptake have demonstrated a higher efflux of neutral or cationic probes, which could reflect the presence of negatively charged groups in the interstitium. A neutral or cationic macromolecule showed a larger volume of distribution in the interstitium than a polyanionic macromolecule. This unequal distribution would increase initial (non-steady-state) capillary efflux of polycationic molecules as compared with negatively charged molecules (11). Asgeirsson *et al.* found similarity of permeability across the rat peritoneal membrane for neutral (charge-modified) albumin, neutral Ficoll_{36k}, and pullulan_{36k} compared with native (negatively charged) albumin (16). Thus, overall, with regard to steady-state transport of macromolecules, the combined peripheral capillary–interstitial barrier apparently does not show any (marked) charge selectivity (11).

Furthermore, the fiber-matrix hypothesis suggests that a matrix of fibrous macromolecules (conceivably the endothelial glycocalyx) may, in addition, offer a “pre-filter” at the luminal surface of the endothelium, further complicating the small-pore pathway (17).

Despite the high selectivity of peritoneal capillaries and their long and tortuous diffusion path for small solutes across the peritoneal membrane (reflecting both tortuosity of interendothelial clefts and distribution of peritoneal capillaries within the interstitium), peritoneal albumin clearance is approximately 0.1 mL/min. According to heteroporous transport theory, “small” proteins like albumin can permeate capillary walls through small pores but also filter through large pores. Small proteins and polypeptides are sensitive to changes in net fluid transport driven by glucose osmosis. Under conditions of intense osmosis, as during the first 0 – 60 minutes of a hypertonic (4%) glucose dwell, the sieving coefficient to albumin across the human peritoneum is approximately 0.015, but it then rapidly increases as osmotic ultrafiltration falls with dwell time (11). The transfer of excess fluid into the peritoneum decreases osmotic pressure and reduces protein loss. By contrast, larger macromolecules, such as IgG and IgM, are confined to the large-pore system for transperitoneal passage, which occurs by (unidirectional) convection from blood to the peritoneal cavity (18). Nonetheless, higher molecular weight macromolecules undergo convective leak from the peritoneal cavity caused by lymphatic drainage and by transmesothelial transport to the peritoneal interstitial tissue, induced by abdominal pressure (19), diminishing total PPL (Figure 1).

The dialysate-to-plasma ratio for creatinine (D/P Cr), used to evaluate transportation of small molecules, is an indicator of peritoneal transport status. There is a correlation between PPL and D/P Cr (6). The amount of PPL is correlated with molecular weight: the lower the molecular weight of the protein, the higher its concentration in dialysate compared with plasma (as a percentage of circulating or extracellular stores) (4,20). Thus, the proportional increase in D/P Cr and

PPL may concern leakage of albumin, the predominant protein in plasma and dialysate. Several studies have suggested that the role of large pores may be affected by endothelial disorders and inflammatory states (18,21). Peritoneal albumin and microalbuminuria have been compared to determine their relevance as markers of endothelial dysfunction (22). The responsible mechanism comprises microvascular damage affecting permeability of peritoneal or glomerular membranes. Diabetic patients have been reported to have higher PPL due to a higher proportion of fast transport status, which may be explained by diabetic vascular lesions (23). Increased systemic inflammatory reaction is also correlated with peritoneal microvascular damage, and studies have confirmed this association, relating PPL with serum C-reactive protein, independently of D/P Cr (6,23). Similarly PPL is increased in peritonitis. Therefore the PPL ratio is highly variable, both within and between patients (4,7).

Total protein in dialysate originates not only from plasma but also from local production of proteins and peptides by peritoneal cells, namely vascular endothelial growth factor and interleukin (IL)-6 (12). During inflammatory processes, polymorphonuclear leukocytes and activated macrophages produce an array of proteases, acid hydrolases, and functionally active complement components. Cellular components resulting from pathological changes in the peritoneum could also contribute to dialysate protein (3).

QUANTIFICATION OF PROTEIN LOSS IN PERITONEAL EFFLUENT

The terms loss, leakage, excretion, and clearance seem to have been used indistinctly, and studies report protein determinations in grams, grams/L, grams/day, or mL/day. Although protein excretion and protein clearance have a tight correlation ($r = 0.94$) in peritoneal equilibration test (PET) samples (9), there are some issues with the protein clearance concept. The estimation equation of this clearance is given by: total protein loss in 24-h effluent/serum total protein, expressed per milliliter of plasma per day (7,9). An estimation using serum albumin as the reference value has been proposed by Haraldsson (8), and is based on albumin as the main protein implicated in peritoneal transport. However, the applied correction factor is generic, which does not add precision to the estimation of PPL (9). Preferably, clearance should be used for single proteins and not for total protein, as protein distribution of plasma and dialysate are different. Thus albumin clearance may be a good alternative (21). Moreover, the prognostic significance of albumin clearance or total protein clearance may not be the same, and the former needs a specific assessment (24). Accordingly, peritoneal protein loss or excretion, over protein clearance, is advocated, as it is simple, easy to interpret, and slightly more predictive (9). The report of PPL should be related with a timely collection (24-hour measurement or 4-hour PET assessment) or concentration. A standardized and reproducible estimation of peritoneal protein transport is obtained during PET. Although the 24-hour measurements estimate the real

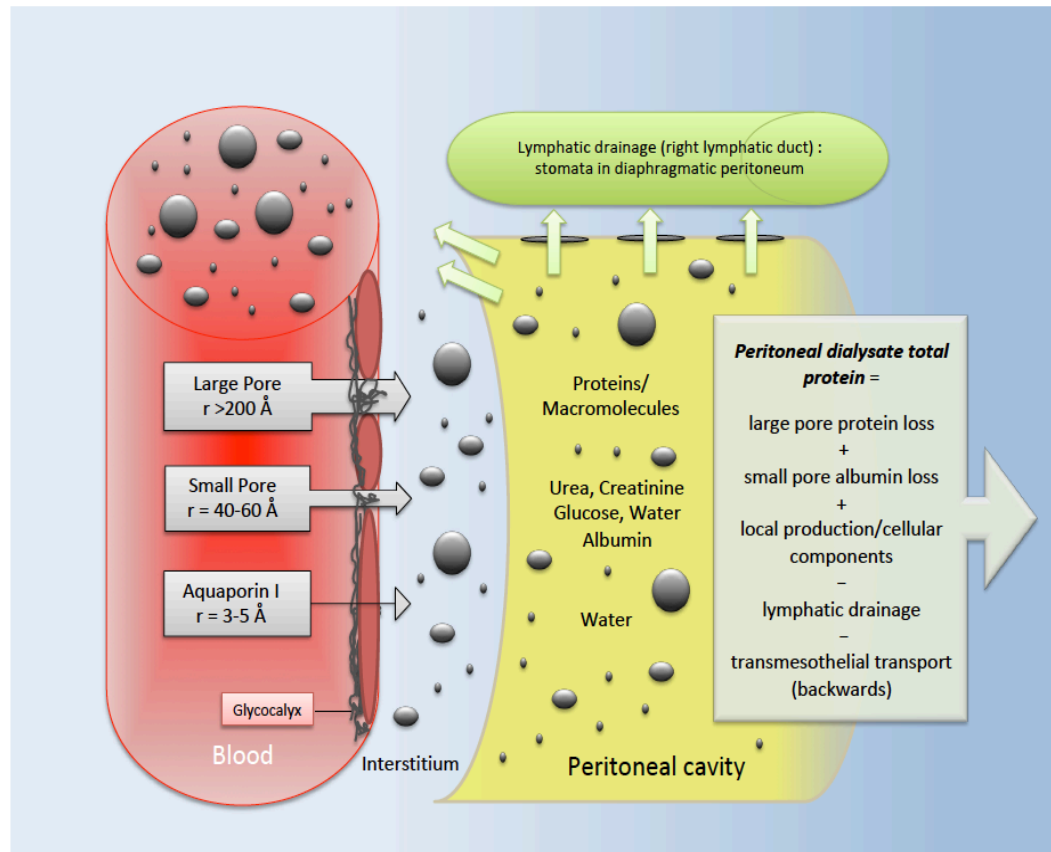


Figure 1 — Total protein in dialysate originates not only from plasma but also from local production of proteins by peritoneal cells and cellular components. Lymphatic drainage and transmesothelial transport to peritoneal interstitial tissue, induced by abdominal pressure, diminish total plasma protein loss (PPL).

amount of PPL, it may be affected by other factors unrelated to the prognosis of PD patients, such as inconsistencies in collection and management of the samples. For instance, patients treated with automated PD may be particularly prone to incorrect sampling. Overall, PET protein loss quantification may be a more specific marker of peritoneal large-pore dysfunction and is seemingly more convenient than 24-hour measurements (9,25).

Methods used for PPL quantification have been established for serum and urine. Reagent manufacturers do not usually claim satisfactory performance in other body fluids, and analyte concentration may be outside the dynamic range of the assay. The choice of an appropriate quality-control sample is difficult (26). Measurement of PPL is challenging, due to low protein concentration (0.10 – 1.40 g/L), large sample-to-sample variation, and the presence of non-protein interfering substances, such as a high content of inorganic ions. Peritoneal protein loss has been measured by chemical (e.g., biuret and Folin-Lowry reactions) (27), turbidimetric (28), and dye-binding or colorimetric (pyrogallol red molybdate

[29]) methods. Biuret test detection limit is enhanced with Folin-Ciocalteu reagent (Folin-Lowry method), resulting in a range of 0.01 – 1.0 mg/mL; the pyrogallol red molybdate method has a range of 0.01 – 2 mg/mL; turbidimetry presents detection limits of 0.002 – 0.01 mg/mL (30,31).

Specific proteins can be estimated by more discriminating methods, such as electrophoresis, specific binding techniques, immunochemical methods, and proteomics. A considerable variety of proteins, representing several functional classes, have been long quantified in peritoneal dialysate, such as albumin, immunoglobulins, complement factors, transferrin, parathyroid hormone (PTH), and vitamin D-binding protein (4). Dulaney and Hatch, back in 1984, did a detailed enumeration of protein identified in peritoneal dialysate to that date, and these are summarized in Table 1.

Proteomic analysis, which studies protein products expressed by the genome, has provided new evidence, with 2-dimensional gel electrophoresis (2D-GE) and mass spectrometry analysis becoming state-of-the-art, but few studies have focused on peritoneal dialysate (32–34). Following

2D-GE analysis, a number of these proteins were confirmed and many other were detected: haptoglobin, α -2 macroglobulin, complement component C4A, immunoglobulin κ ,

TABLE 1
Protein Found in the Analysis of Peritoneal Dialysate,
Until 1984, Adapted from Dulaney and Hatch (4)

Protein functional class/type	Protein
Carrier proteins	Ceruloplasmin
	Sex hormone-binding protein
	Transcortin
	Transferrin
Hormones	Vitamin D-binding protein
	Follicle-stimulating hormone
	Gastrin
	Luteinizing hormone
Coagulative and fibrinolytic system	Prolactin
	Parathyroid hormone
	Antithrombin III
	Factor I (fibrinogen)
	Factor II (prothrombin)
	Factor V
	Factor VIII
	Factor VIII-related antigen
Immune system	Plasminogen
	C3
	C4
	IgA
	IgA paraprotein (Bence-Jones)
	IgG
	IgG paraprotein (lambda type)
Enzymes	IgG paraprotein (kappa type), IgM
	Acid phosphatase
	Adenosine deaminase
	Alanine aminotransferase
	Alkaline phosphatase
	Aspartate aminotransferase
	γ -glutamyl transferase
	Guanase
	β -hydroxybutyrate dehydrogenase
	Lactic dehydrogenase
Lipoproteins	HDL
	LDL
	β -lipoprotein
	VLDL
	Apoprotein A
	Apoprotein B
Miscellaneous	Apoprotein C
	Albumin
	Collagen
	Globulins
	α_1 -glycoprotein
	α_2 -glycoprotein
	Pre-albumin

Ig = immunoglobulin; HDL = high-density lipoprotein; LDL = low-density lipoprotein; VLDL = very low-density lipoprotein

gelsolin, omentin, heat shock 70 kDa protein 1A/1B, Apo A-I, α -1-antitrypsin (32,34,35). Cuccurullo *et al.* published a comprehensive list of 151 proteins found in the peritoneal effluent of 15 PD patients (36), which included cystatin-C, fetuin-B, transthyretin and insulin like growth factor, amongst others. This portrays one of the most exciting aspects of proteomics technology: it allows the discovery of previously unknown proteins in peritoneal dialysate. As a note of caution, the huge potential of biomedical and biomarker research is limited by the considerable challenge of identifying the lowest abundant proteins, which may be biologically relevant, and the clinical usefulness of biomarkers. There are over 10 orders of magnitude of difference in abundance between the most and the least abundant proteins (33).

IMPACT OF PERITONEAL PROTEIN LOSSES ON CLINICAL OUTCOMES

The clinical effects of long-term PPL have been a concern for over 40 years. How well are serum levels of macromolecules preserved during PD? The impact of high PPL on patient outcome is controversial (Table 2).

SURVIVAL ANALYSIS

Baseline peritoneal transport status was found to be an independent predictor of mortality and technique failure for continuous ambulatory PD (CAPD) patients (34), but proper therapeutic adjustments (e.g., icodextrin use, automated PD) seemed to offset this negative effect on both outcomes (34,35). Perle *et al.* demonstrated that increased protein clearance on PD initiation, but not peritoneal transport status, was a predictor of death, and this was independent of serum albumin, age, transport status, and comorbidity (27). In addition, Heaf *et al.* found high peritoneal large pore flux (J_{VL}), resulting in increased PPL, was related to hypoalbuminemia and mortality after PD initiation (21). Pérez-Fontán *et al.* also reported baseline PPL as a strong independent marker of survival of PD patients (9). In contrast to the aforementioned studies, no association between PPL and an increase in mortality was found in other studies (6,12,37). Sanchez-Villanueva *et al.* showed significant association between the presence of peripheral artery disease and high PPL but this was not associated with increased mortality (6). In addition, Balafa *et al.* reported protein clearance was not an independent predictor of mortality (12). Chang *et al.*, in the largest study published, demonstrated that higher PPL is a risk factor for the occurrence of cardiovascular events, but not mortality, in PD patients (37).

TECHNIQUE-FAILURE ANALYSIS

Worse technique survival has been weakly associated with higher albumin loss, but without significance after adjustment for mass transfer area coefficient (12). This could, in part, be explained by changes in the transport status of the membrane,

as inflammation results in a fast transport status independent of PPL alone. The dialysate-to-plasma creatinine calculation is based on a standard PET that does not differentiate causes for fast transport or membrane status. Van Biesen *et al.* suggested the use of a personal capacity test to single out inflammation as the cause of this transport status. This discrimination has important prognostic and therapeutic consequences: in patients with large surface area in the absence of inflammation, the outcome can be improved by increasing fill volume or using icodextrin, whereas in patients with inflammation, the underlying cause of inflammation should be identified and

treated or PD, potentially, paused (38). Managing these different situations could translate into a loss of impact of PPL on overall technique survival, as recent studies have demonstrated (34,35). Another cause for technique failure comes with the association of baseline PPL with a higher risk of peritonitis (7,9).

CARDIOVASCULAR DISEASE

Peritoneal protein loss may be a manifestation of endothelial dysfunction, as with another type of capillary protein leakage, microalbuminuria, a recognized endothelial dysfunction

TABLE 2
Studies Concerning Peritoneal Protein Loss: Characteristics and Main Findings

Year, Author	Study population	Study follow-up duration	Measure of protein removal	Findings
2004, Szeto (40)	44 incident PD patients	Cross-sectional study	24-h peritoneal protein losses (g/24 h) and PET peritoneal total protein and albumin losses (mg/L) with adjustment for dialysate creatinine concentration (mg/mol creatinine)	Patients with cardiovascular disease prior to initiation of dialysis have higher levels of dialysate albumin and total protein excretion, indicating that dialysate protein loss is a marker of underlying cardiovascular disease.
2005, Heaf (21)	155 incident PD patients	4 years	J_v (mL/min/1.73 m ²) 24-h peritoneal albumin losses (μM/day) and 24-h peritoneal albumin clearance (no units or equation stated)	High peritoneal large pore fluid flux (J_v) results in increased peritoneal loss of albumin, mortality was increased significantly with raised J_v .
2005, Szeto (22)	43 incident PD patients	26.5 months	24-h peritoneal protein losses (g/24 h) and PET peritoneal total protein and albumin losses (mg/L) with adjustment for dialysate creatinine concentration (mg/mol creatinine)	Peritoneal albumin excretion was associated with the extent of calcified and ulcerated carotid atherosclerotic plaques by Doppler ultrasound, indirectly supporting the relation with endothelial dysfunction. Authors conclude that peritoneal albumin excretion may be an important predictor of cardiovascular disease, as for every 100 mg/L increase in peritoneal albumin excretion, the adjusted hazard ratio of all-cause mortality was 1.49 (95% CI 0.91–2.73, $p = 0.09$), which fell short of statistical significance.
2009, Perl (27)	192 incident PD patients	38.8 months	24-h peritoneal protein losses (g/24 h) and 24-h peritoneal protein clearance ^a (mL/day)	Increased protein clearance at the start of PD therapy is a predictor of death, independent of baseline small solute transport status, when measured separately, as well as serum albumin, age, and detailed comorbidity.
2009, Sanchez-Villanueva (6)	133 incident PD patients	38.1 months	24-h peritoneal protein losses (g/L and g/24 h) and 24-h peritoneal protein clearance ^a (mL/day)	Protein clearance is significantly and independently related to the presence of peripheral artery disease, but failed to demonstrate the association of protein clearance with mortality or subsequent cardiovascular events.
2009, Elsurer (24)	66 PD patients	2 years	24-h peritoneal albumin losses (g/day)	24-h peritoneal albumin leakage is not a predictor of 2-year prospective cardiovascular outcome and patient survival.

TABLE 2 (cont'd)

Year, Author	Study population	Study follow-up duration	Measure of protein removal	Findings
2010, Pérez-Fontán (9)	269 incident PD patients	29 months	PET and 24-h total protein losses (mg/day) PET and 24-h peritoneal protein clearance ^a (mL/day)	Baseline peritoneal protein losses represent a strong independent marker of survival of PD patients. Estimation of PPE during PET is more accurate than 24-hour PPE for this purpose, sustains a definite independent association with cardiovascular and infectious mortality, and shows a significant correlation with the risk of peritonitis.
2011, Balafa (12)	257 incident PD patients	51 months	Albumin loss (g/L per 4-h standard peritoneal analysis) calculated by multiplying the clearance of albumin ^b with serum albumin concentration (mL/min)	Peritoneal albumin clearances and albumin loss were not associated with death in crude and adjusted analysis. Patients with lower albumin loss had better technique survival than those with higher albumin loss (log rank $p = 0.029$). However, in the multivariate Cox model, albumin loss tested as a categorical variable and adjusted for age, serum albumin, MTAC creatinine, and Davies index lost its impact on technique survival.
2013, Chang (37)	540 incident PD patients	50 months	24-h peritoneal protein losses (g/24 h) and 24-h peritoneal protein clearance ^a (mL/day)	Baseline peritoneal protein clearance is a risk factor for occurrence of cardiovascular event, but not mortality in patients on PD.
2013, Dong (7)	305 incident PD patients	48.8 months	24-h peritoneal total protein losses. Protein clearance (total protein losses in 24-h effluent/serum total protein, in mL/day)	Baseline values of peritoneal protein leakage are significantly independent predictors of peritonitis even with adjustment for systemic inflammation.
2015, Rajakaruna (29)	300 incident PD patients	62 months	24-h peritoneal protein clearance (mL/day) (no equation stated)	Higher peritoneal protein clearance was associated with increased risk of death (hazard ratio: 1.81; 95% CI 1.11–2.95), after adjustments for age and comorbidity, and even after patients underwent transplantation or transferred to hemodialysis.

PD = peritoneal dialysis; PET = peritoneal equilibration test; CI = confidence interval; PPE = peritoneal protein excretion; MTAC = mass transfer area coefficients.

^a Equation used for peritoneal protein clearance was: 24-h dialysate protein/(serum albumin/0.4783) expressed in mL/day

^b Protein clearance (mL/min) = $(Pr_{Dr} + Pr_{Rv}) / (Pr_p) \times t$, where Pr_{Dr} is the dialysate protein content in the drained test bag, Pr_{Rv} is the dialysate protein content in the residual volume, and Pr_p is the plasma protein concentration in time (t)

marker (39). The link between endothelial dysfunction and cardiovascular disease has been shown in several studies. Szeto *et al.* reported incident PD patients with active cardiovascular disease had higher protein and albumin levels in the peritoneal effluent, and cardiovascular events were more frequent in patients with greater peritoneal albumin losses. These authors postulated that PPL could be an independent cardiovascular risk factor (40). Sanchez-Villanueva *et al.* showed PPL is independently related to the presence of peripheral artery disease, but failed to demonstrate any association with subsequent cardiovascular events (6). Chang *et al.* revealed higher PPL is a risk factor for the occurrence of cardiovascular events in PD patients (37). A small single-center study stated that a single

measure of dialysate albumin > 300 mg/L was associated with increased cardiovascular events and a tendency to more atherosclerotic disease on carotid Doppler (22). Early in treatment, PPL may serve as a marker of the severity of systemic vascular disease and injury (27).

NUTRITIONAL ISSUES

Nutrients are also lost into dialysate. Conflicting studies have been published concerning nutritional status. Peritoneal protein loss is compensated by an increase in albumin synthesis in PD patients (41). The continuous loss of proteins and amino acid can be compensated by adequate dietary intake, but a



poor appetite limits protein intake in many patients (42), and in cases of coexisting inflammation and previous malnutrition, dietary correction can be insufficient. In addition, the type of peritoneal membrane transport may influence the amount of protein loss. Peritoneal protein loss is much greater in high transporters (43). Some studies have stated that a fast peritoneal solute transport rate is independently associated with poor nutritional status (44) while others found no such association (45–47). Peritoneal protein losses contribute, in part, to the lower serum levels of albumin and higher daily protein requirements observed in patients on PD compared with those treated with hemodialysis. However, protein losses are not large enough to make a substantial contribution to protein energy wasting. The target for dietary protein intake is generally regarded as being 1.2 g/kg/day; it should not be below 0.8 g/kg/day in any patient. On average, the achieved normalized protein nitrogen appearance (nPNA) needs to be 1 g/kg/day, adjusted for the patient's age and physical activity (48).

Serum levels of a certain protein are determined by a complex equilibrium between its rate of production, rate of clearance and individual clinical status. Different studies reported different outcomes, as serum albumin and total protein levels, the most frequent comparators, "remained stable" (2,49), "in the low-normal range" (4,20,50), or evolved with "progressive hypoproteinemia and hypoalbuminemia" (51).

Individual proteins have been addressed: a 4% decrease in serum transferrin (3) and maintained vitamin D-binding protein serum levels (higher than matched hemodialysis patients) have been described (52). Serum C3 and C4 concentrations were higher than found in normal subjects or in patients on hemodialysis (53). Although thyroid binding protein excretion is high, serum levels remain in the normal range; T4 and T3 losses are relatively modest, remaining below their daily production rate. Nonetheless, 15.6%–27.5% of PD patients have subclinical hypothyroidism, a higher proportion compared with the general population (4%–10%). Clinical hypothyroidism is rare, but long-term monitoring of thyroid function is warranted (54,55). In contrast, growth hormone and growth hormone binding protein activity are relatively normal in patients on CAPD, and these patients exhibit fewer abnormalities than hemodialysis patients (56).

Peritoneal protein loss can also comprise abnormal proteins, but a real therapeutic value is difficult to establish. It may well be that nonfunctional protein, protein subunits, or protein complexes are removed by PD, which otherwise would persist in circulation longer than normal, interfering with normal homeostatic mechanisms. This could be particularly useful in the event of impaired liver or reticuloendothelial function (4). Furthermore, the benefits conferred by PD appear to offset the risks, at least to some degree. For instances, the more satisfactory removal of immunosuppressive middle molecules by PD compared with hemodialysis is suggested to be responsible for the immune system improvement of uremic patients on CAPD (4).

So far, no catastrophic or pathologic loss of any of the serum proteins assayed seems to have occurred among PD patients. It appears that on the whole (and in the absence of serious

impairment of liver function and secondary decrease in protein synthesis), malnourishment due to PPL can be compensated by adequate intake of nitrogen (2,50,51). These findings do not exclude the possibility of net losses of certain serum proteins in individual cases.

Despite conflicting studies, PPL may correlate with systemic or local vascular damage and endothelial dysfunction in PD patients.

INTERVENTIONS TO REDUCE PERITONEAL PROTEIN LOSSES AND FUTURE DIRECTIONS

Adjustments on PD prescription aiming to reduce PPL are not straightforward. Continuous ambulatory PD and automated PD (APD) present with equal dialysate protein content (g/24 h) (43), and PPLs were similar, with 1.36% and 3.86% dextrose fluids, suggesting that initial effects of hypertonicity are diminished or reversed by dilution and absorption of dextrose (57). Icodextrin did not alter peritoneal function transport parameters or PPL when compared with glucose solutions (58). For amino acid solutions, the gain of protein provided exceeds the usual 24-hour protein and amino acid losses through PD (3), but it was associated with increased PPL (59). Tjong *et al.* showed amino acid solutions did not induce a significant increase of PPL despite stimulating the release of several pro-inflammatory cytokines (3). For PPL, these amino acid-based solutions do not appear to be advantageous.

Long-term PD causes morphological changes in the membrane, including interstitial fibrosis, loss of mesothelial cell layer, and vasculopathy. One potential mechanism originates on a local renin-angiotensin-aldosterone system (RAAS), by which injured peritoneal mesothelial cell-derived angiotensin-II causes activation of vascular endothelial growth factor and transforming growth factor beta, inducing peritoneal fibrosis and angiogenesis (60). This can be a therapeutic target when reducing PPL, as RAAS blockade ameliorates peritoneal injury induced by hypertonic PD solution (61). In albino Wistar rats enalapril preserved peritoneal histology, peritoneal function, and mesothelial cell remodeling (61). Basturk *et al.* demonstrated that treatment with angiotensin-converting-enzyme inhibitors (ACEIs) significantly decreased PPL in a cohort of PD patients (1). Angiotensin-converting-enzyme inhibitor therapy acted on dual fronts in reducing protein losses: urinary and peritoneal.

Vitamin D receptor (VDR) activators also reduce urinary protein losses, although more discretely, through an inhibitory action on RAAS and expression of VDR in peritoneal mesothelial cells (62). Sánchez-Álvarez *et al.* showed no change in PPL when using paricalcitol supplementation, but there was no control group enrolled in this study (63). Other studies, with larger study populations and controls, demonstrated a reduction of PPL with paricalcitol supplementation (23,28).

Which other modalities/drugs could lower protein loss? Cinacalcet was associated with reduced PPL in a small study (64). Intraperitoneal heparin reduced albumin permeability, possibly due to its anti-inflammatory properties (65). Currently,

there is anecdotal evidence of improvement of outcome by modulating protein levels in peritoneal effluent. Animal model studies and randomized controlled trials are warranted to steer the clinical management and reduction of PPL in PD.

CONCLUSIONS

Peritoneal dialysis patients lose significant quantities of protein and albumin during the dialysis procedure. It appears that peritoneal dialysate is likely to contain many, if not all, of the proteins present in serum. The amount of protein lost will depend on dialysis time, protein size and its serum concentration, and other factors including patients' clinical status.

Proteomic analysis of patients' individual clearance and dialysis efficiency could further aid to identify and monitor specific protein culprits that may result in negative effects on patient quality of life and predict outcome.

It should be noted that, thus far, claims have not been made that loss of a specific protein (or class of proteins) via PD has resulted in a specific metabolic or other deficiency syndrome. However, it is clear that the long-term success of PD will depend heavily on proper nutrition (4).

The results of investigations showed that the key to diminish PPL is not related to dialysis prescription, as once thought (3,43,58,59), and possible alternatives may include ACEIs and vitamin D therapies (1,28). The study of new therapeutic options is warranted. Nonetheless, the effort to reduce PPL should be considered only if it diminishes cardiovascular morbidity or mortality, as it appears PPL is a marker of endothelial dysfunction, and has no pathologic concerns [opinion]. Randomized interventional studies are encouraged to address the pathological concern of PPL in the future, namely its effects on cardiovascular conditions or its role as marker or confounding marker.

Peritoneal dialysis stands as a valuable therapy for treatment of CKD patients and an important field for research, including on these peritoneal effluent concerns.

DISCLOSURES

The author has no financial conflicts of interest to declare.

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2.4 Critical appraisal and review of the state of the art

The review published in *Peritoneal Dialysis International*, in 2019 (21), raises more questions than provides answers to several issues related with PPL.

It is not feasible to compare and ascertain the meaning of peritoneal protein loss when different studies use different quantifications methodologies. Equally important to standardize measurements, it is important to understand if the different determinations have different clinical significance. It is possible to extract from this review, that estimation of protein loss can be made in grams, grams/L, grams/day or mL/day. The report of PPL should be related with a timely collection, still regarding 24-hour or 4-hour standardized PET collections, do they have the same biological meaning? Or is there a better correlate, a better methodological approach, for a specific study question?

As for the impact of PPL on clinical outcome, many papers claim certain associations with worse prognosis, but there are many others denying such impact. Controversy is far from ending, for instances, global mortality was associated with higher PPL by Perl *et al* (27), Heaf *et al* (28), Pérez-Fontán *et al* (29), contrasting with cohorts by Sanchez-Villanueva *et al* (30), Balafa *et al* (31) and Chang *et al* (32). Unequivocally, how can the existence of an association with global mortality be determined? Are the major confounders for higher mortality in PD patients being controlled in such populations? It seems the main hypothesis is centered in the presence of endothelial dysfunction as the source of cardiovascular disease (but not necessarily mortality) and higher PPL, with a general consensus among the studies by Szeto *et al* (33), Chang *et al* (32), but not Sanchez-Villanueva *et al* (30), for instances.

In this hypothesis axis of PPL related mortality, inflammation appears as a driving force for protein leakage, either systemic or local, and it is independently associated with mortality (34). Studies approaching PPL related mortality should always proceed to adjustment for this confounder. Other associated factor, either causative or consequence, has been nutritional status. Depending on the nutritional marker used, it has also been associated with higher mortality, namely when hypoalbuminemia is the chosen indicator. Again, the influence of inflammation on this equation must be taken into account since it might have a

causative role in hypoalbuminemia. If complete evaluation of nutritional status is performed, namely muscle mass, strength or sarcopenia, as long-term markers of malnutrition, PPL has not been indisputably related (35). In a conceptual thought, lower serum protein levels should prompt lower diffusive loss of proteins to the dialysate. So, shouldn't it be the other way around, better nutritional status correlated with higher PPL?

As for the transport of these macromolecules, proteins, it seems little doubts subsist on the role of large pores. Nonetheless the transport mechanism associated and how the aging of the membrane can influence the leakage in the venular side of the circulation is not known. Does this evolve in a technique limiting manner?

With the newer biocompatible solutions, it is possible to perceive, in the clinical practice, that PD patients stay longer performing the technique without membrane associated deleterious misfunctions. So, after this review, can the PPL be considered a detrimental consequence of peritoneal dialysis? Definitive conclusions were scarce.

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3. Aims and outline of the thesis

3. Aims and Outline of the Thesis

The main aim of this thesis was to investigate the clinical impact of peritoneal protein loss in peritoneal dialysis patients, namely associated factors and consequences on overall mortality in PD patients.

In order to do that, a step back was taken, after the critical review of the state of the art to standardize methods and question concepts.

Methodology details are presented in chapter 4.

The investigation specific tasks aimed to test relevant hypotheses:

In **chapter 5.1**, different PPL quantification methodology (24 h versus 4h peritoneal equilibration test collections; sampled and calculated determinations) were evaluated, and clinical meaning ascertained. The hypothesis that different methodologies relate with different physiologic meanings (membrane evaluation versus clinical status) was tested.

In **chapter 5.2**, to explore possible mechanisms underlying PPL, namely convection, associated with venous congestion. Multifrequency bioelectrical impedance was used to determine extracellular water excess. The role of overhydration was evaluated as an associated factor for PPL, in this study.

In **chapter 5.3**, previous associated factors with PPL, either clinical (demographics, nutritional status, peritoneum characteristics) or inflammatory biomarkers (serum and effluent) and overhydration were assessed. Also, their consequences on overall mortality in PD patients were explored. This study aimed to test the validity of overhydration over reported causes of PPL and establish its prognostic value.

In **chapter 5.4**, PPL evolution according to vintage on PD was evaluated in a repeated measures cohort. The hypothesis that PPL was dependent on the intrinsic changes of the membrane was investigated.

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4. Methods

A comprehensive review of the pathophysiology of PPL, methods of quantification, dialysate protein composition and impact on clinical outcomes was done to identify gaps of knowledge in the literature. This provided the outline of this thesis and identified accurate aims, as described previously.

Specific methodological issues in this investigation are highlighted here, in a sequential approach, other details such as studies design, population, demographics, clinical features registered, follow-up, exclusion and inclusion criteria are described in detail in each article. Laboratory analysis and other procedures were standardized throughout the investigation as follows:

1. Routine biochemical analyses were performed by an automatic chemistry analyzer (Architect ci8200 Abbot®). Creatinine was measured with an enzymatic method to prevent glucose interference. Serum high sensitivity C reactive protein (CRP) was determined by immunoturbidimetry at the time of the peritoneal equilibration test (PET).
2. For IL-6 analysis: serum and peritoneal fluid samples, stored at -80°C until analysis, were used. All samples had one or two freeze-thaw cycles before quantification. For the development of sandwich enzyme linked immunosorbent assays (ELISA), the DuoSet® ELISA Development System was used to measure Interleukin 6 (IL-6; DY206-05; R&D Systems Inc., Minneapolis, MN, USA), following the manufacturer's protocols and using all the recommended additional reagents. The ELISA was specific for human IL-6 and did not cross-react with human Recombinant human CNTF, G-CSF, gp130, IL-6 R, IL-11, IL-12, LIF, LIF R, and OSM. Duplicate readings were assayed using a TECAN Infinite®200 multimode reader (Mannedorf, Switzerland) for each standard, control, and sample and the average zero standard optical density (O.D.) was subtracted. A standard curve with the value of absorbance vs the concentration was generated by reducing the data using the Quest Graph™ Four Parameter Logistic (4PL) Curve Calculator (36). The sample concentrations were then calculated from the determined absorbance values through the four-parameter logistic (4PL) standard curve, using the same software.

3. Functional evaluation of the membrane was performed in all investigations using the Two-in-One PET (37). Dialysis adequacy was performed simultaneously, with collection of 24h-urine and spent dialysate. Residual kidney function (RKF) was assessed as the mean of urea and creatinine clearance from the 24-hour urine collection. Urea clearance index (Kt/V urea) was derived from the 24-hour urine and PD effluent collection. Normalized nitrogen appearance rate (nPNA) was estimated using the relevant Bergstrom equation (38).
4. Peritoneal dialysate protein loss was measured in the peritoneal dialysate effluent using the biuret reaction method. The 4-hour PET and 24-hour results were presented in grams (derived from the concentration measured times the total volume drained during the PET and 24-hour respectively). Peritoneal protein clearance (PPCl) was calculated from the total protein loss in 24-hour effluent/serum total protein, expressed as milliliter per 24-hour.
5. Body composition analysis was performed after the completion of the peritoneal equilibration test (PET), with empty abdomen, using multifrequency bioelectrical impedance assessment (InBody S10 Body Composition Analysis; Biospace, Seoul, South Korea). Lean body mass index (LBMI) was calculated by LBM correcting for body height square (kg/m^2). Extracellular water excess (ECWexc) was calculated by $\text{ECW measured} - 0.613 \times \text{intracellular water (ICW) measured}$ (39).

Statistical analysis is described, in detail, in each article. The study protocols were conducted in compliance with the ethical principles of the World Medical Association Declaration of Helsinki, and approved by the Ethics Committee of the Centro Hospitalar Universitário do Algarve (UAIF 192/2019). Written informed consent was obtained, accordingly.

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5. Results

- Methodological evaluation of peritoneal protein loss
- Peritoneal protein loss: new insights
- Old known causes vs new insights
- Valuation of peritoneal protein loss associated with dialysis duration

5. Results

5.1 Methodological evaluation of peritoneal protein loss

Protein Loss in Peritoneal Effluent: different meaning for 24-hour versus PET samples

Malho Guedes A, Calças Marques R, Domingos AT, Laranjo C, Silva AP, Rodrigues A. Blood Purif. 2022 August; 52(2):1-8. DOI:10.1159/000525502

Short Summary

Throughout different studies, different methods for protein peritoneal leakage quantification are found. One can only compare results from similar methodology. The aim of this study was to compare the results of both sampling and calculated methods with 24h and 4h collections, in order to ascertain specific clinically relevant associations and biologic meaning. PPL was measured (4-hour PET and 24-hour results) and peritoneal protein clearance (PPCI) was calculated from 24-hour collections.

When evaluating 24-hour sampling, in contrast to 4-hour determinations, it is noticeable the influence of body composition parameters, age and type of PD prescription on PPL in the univariable analysis. Independent factors associated with 24-hour collections peritoneal loss were higher D/P creatinine and extracellular water excess. Higher lean body mass and lean body mass index were associated with higher peritoneal protein clearance, indicating that a better nutritional status is the predominant feature linked with higher PPL in PD and refuting the assumption that protein loss causes malnutrition.

The assessment of peritoneal protein leak showed that PET and 24-hour measurements should not be considered equivalent, disclosed by the weak Pearson correlations between g/24h and g/240 minutes and between ml/24h and g/240 min. PET protein loss quantification should be regarded as a marker of peritoneal membrane intrinsic permeability. Measurements of a 24-hour sample might be closer to patients' clinical status and prognosis, signaling opportunities of therapy intervention.

Protein Loss in Peritoneal Effluent: Different Meaning for 24-h versus PET Samples

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Keywords

Peritoneal equilibration test · Peritoneal dialysis · Peritoneal protein clearance · Peritoneal protein loss

Abstract

Introduction: Quantification of peritoneal protein loss (PPL) may be expressed according to a timely collection (24-h measurement or 4-h PET assessment) and as a concentration. The aim of this study was to compare the quantification methods of 24-h and 4-h collections. **Methods:** This study included 81 prevalent peritoneal dialysis patients. Demographics and clinical and bioelectrical impedance features were registered. PPL was measured (4-h PET and 24-h results) and peritoneal protein clearance was calculated. A linear regression model was performed. **Results:** Age and continuous ambulatory peritoneal dialysis (compared to cycler) were positively associated with greater PPL on 24-h collections. Neither cardiovascular disease, hypertension, diabetes nor the comorbidity Charlson Index was significantly associated with PPL. There was a consistent univariable relationship with D/P creatinine, whichever sampling method was used. Only 24-h measurements of PPL correlated with body composition variables. In multiple linear regression analysis,

D/P creatinine association with PPL stands out. On the other hand, 24-h determinations (in grams or clearance) were associated with overhydration. PET protein quantification was associated with peritoneal creatinine clearance. **Discussion/Conclusion:** Different methods sign different pathophysiological pathways. PET protein loss quantification should be regarded as a marker of peritoneal membrane intrinsic permeability. Measurements of a 24-h sample might be closer to patients' clinical status and prognosis, signaling opportunities for therapy intervention.

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Introduction

Peritoneal protein loss (PPL) is a described feature of peritoneal dialysis (PD) since its inception. It has been estimated in 5–15 g daily. Over time, many studies have tried to evaluate the impact of this protein leak on patients' outcomes, namely mortality, cardiovascular events, or technique failure [1, 2]. Conclusions remain elusive possibly because of the different methodologies used to measure such protein leak.

The quantification of peritoneal protein loss may be expressed according to a timely collection (either as a 24-h measurement or a 4-h peritoneal equilibration test [PET] assessment) and as a concentration. Standardized and reproducible estimation of peritoneal protein transport is obtained during PET [3]. The 24-h measurements estimate the real amount of PPL but may be affected by other factors unrelated to the prognosis of PD patients, such as inconsistencies in collection and management of the samples. Overall, PET protein loss quantification may be a more specific marker of peritoneal large-pore dysfunction [4, 5].

There are also studies reporting the protein loss in terms of protein clearance, with added variability in the chosen denominator: instead of total protein, some prefer to use albumin level multiplied by a correction factor as an attempt to eliminate the bias of hypoproteinemia [3]. With such diversity of methods to report protein loss, how to uniformize results and conclusions? The aim of this study was to compare the results of both sampling and calculated methods with 24-h and 4-h collections in order to ascertain specific clinically relevant associations.

Materials and Methods

Study Design and Patient Population

This single-centre cross-sectional study included prevalent PD patients from January 2015 until censoring of the data in December 2020. All patients included were assessed with a modified PET, using 3.86/4.25% glucose solutions. Simultaneously, a 24-h collection of urine and spent dialysate were obtained. Patients who had active infection or malignancy, experienced acute hospital admissions, or a peritonitis episode during the preceding 3 months were excluded.

Clinical Information, Biochemical Data, PD Adequacy

Demographics and clinical features registered included age, gender, weight, height, comorbid conditions, medication, cause of kidney failure, dialysis duration, residual renal urine volume, and solute clearance. All patients were treated with reduced glucose degradation products and a normal pH dialysis solution (Baxter® and Fresenius®). The maximum glucose concentration was 2.27%/2.3%. The Charlson Comorbidity Index was calculated at the time of study inclusion [6]. Routine biochemical analyses were performed by an automatic chemistry analyser (Architect ci8200 Abbot®). Creatinine was measured with an enzymatic method to prevent glucose interference. Serum high-sensitivity C-reactive protein (CRP) was determined by immunoturbidimetry at the time of the PET. Residual renal function (RRF) was assessed as the mean of urea and creatinine clearance from the 24-h urine collection. The urea clearance index (Kt/V urea) was derived from the 24-h urine and PD effluent collection. The normalized nitrogen appearance rate (nPNA) was estimated using the relevant Bergstrom equation [7].

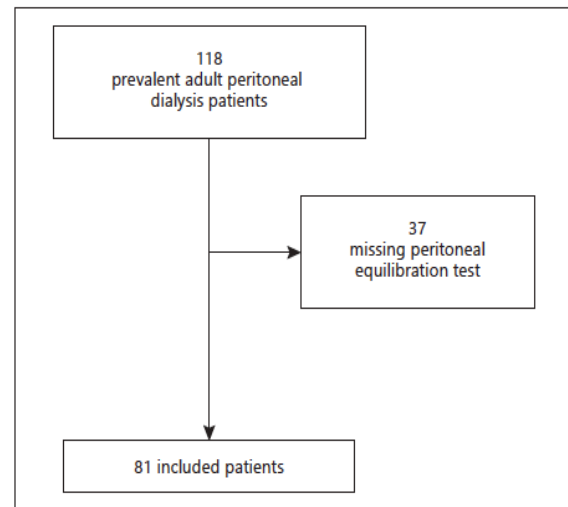


Fig. 1. Strobe diagram showing the total number of prevalent PD patients assessed for inclusion and included in the study.

Peritoneal Protein Clearance and Peritoneal Protein Loss

Peritoneal dialysate protein loss was measured in the peritoneal dialysate effluent using the biuret reaction method. The 4-h PET and 24-h results are presented in grams (derived from the concentration measured times the total volume drained during the PET and 24-h, respectively). Peritoneal protein clearance (PPCI) was calculated from the total protein loss in 24-h effluent/serum total protein, expressed as millilitre per 24-h.

Body Composition Analysis

Simultaneous multifrequency bioelectrical impedance assessment (InBody S10 Body Composition Analysis; Biospace, Seoul, South Korea) was performed with an empty abdomen immediately after completion of the PET. Lean body mass index (LBMI) was calculated by LBM, correcting for body height square (kg/m^2). Extracellular water excess (ECWexc) was calculated by ECW measured – $0.613 \times$ intracellular water measured [8].

Statistical Analysis

Data were checked for normality using the Shapiro-Wilk test. Results were expressed as frequencies and percentages for categorical variables, mean $\pm$ standard deviation (SD) for continuous variables, and median (interquartile range) for skewed distributions. For comparing groups, an unpaired *T*-test was performed. Pearson correlation test was used to relate the different PPL measurements. Univariable analysis was performed with a linear regression model. All variables with a statistical association of $p < 0.1$ and those thought to be of major clinical significance (age, gender, time on PD, high-sensitivity CRP, serum albumin, and D/P creatinine) were used to create a multiple linear regression model to determine associations with PPL. Preliminary analyses were performed to ensure there was no violation of normality and linearity. Of the large number of BIA parameters describing water distribution, ECWexc was the one used due to its biologic meaning and being an absolute value, independent of body structure. Exploratory survival analy-

Table 1. Characterization of the patient population (demographics, comorbidities, dialysis, biochemical, and BIA results)

Demographics and comorbidities		Dialysis	
Age, years	57 [40–69]	Time on PD (months)	2 [1–10]
Gender, male, %	62%	Dialysis modality	
Charlson Comorbidity Index	4 [2–6]	CAPD (vs. cycler)	31%
Diabetes mellitus	28%	Cycler	69%
Cardiovascular disease	27%	APD (no daytime dialysis)	56%
Hypertension	84%	CCPD (with daytime exchange)	14%
Systolic blood pressure, mm Hg	145±27	Icodextrin use	33%
Diastolic blood pressure, mm Hg	89±20	Peritoneal equilibration test and adequacy	
Pulse pressure, mm Hg	50 [40–68]	D/P creatinine	0.64±0.12
Patients with antihypertensives	89%	Net UF, mL/4 h	852±255
Agents, <i>n</i>	2 [1–2]	RRF, mL/min/1.73 m ²	6.0 [3.9–9.0]
β blockers	43%	Kt/V _{urea} /week	2.28 [1.94–3.03]
Calcium channel blockers	63%	Creatinine clearance, L/1.73/week	85.9 [66.1–117.2]
ACE inhibitors/All receptor blockers	63%	PNA, g/kg/24 h	0.9±0.2
		Protein loss, g/4 h	1.3 [0.9–1.8]
		24-h dialysate collection	
		Protein loss, g/24 h	5.2 [3.8–6.7]
		Peritoneal protein clearance, mL/24 h	73.5 [54.4–98.8]
Biochemical evaluation		Bioelectrical impedance assessment	
Haemoglobin, g/L	118±18	Lean body mass, kg	48.0±10.8
Blood urea nitrogen, mmol/L	20.5±6.8	Soft lean mass/height ² , kg/m ² *	17.1±2.3
Creatinine, μmol/L	548 [407–743]	Extracellular water, L	14.9±3.4
Glucose, mmol/L	7.0 [5.9–11.0]	Intracellular water, L	22.0 [18.6–25.6]
Total protein, g/L	68.4±6.7	Extracellular/total body water, %	39.6±1.5
Albumin, g/L	38.5±5.8	Extracellular water excess, L	1.01±0.99
Prealbumin, mg/dL	42.1±9.6		
High-sensitivity C-reactive protein, g/L	3.9 [1.8–10.2]		
24-h urine collection			
Volume, mL	1,467±723		
Protein loss, g/24 h	0.85 [0.41–2.44]		

Values are presented as mean±SD, or as median [75% interval]. APD, automatic peritoneal dialysis; CCPD, continuous cyclic peritoneal dialysis. * LBMI reference values: 16.7 kg/m² in males and 13.8 kg/m² in females; for the diagnosis of sarcopenia in PD patients [9].

sis was performed using Cox Regression. The statistical analysis was performed using SPSS version 22.0. Statistical significance was considered at or below the 5% level.

Results

A peritoneal equilibration test with 4-h and 24-h collection for determination of PPL was obtained in 81 out of 118 prevalent patients (Fig. 1). Simultaneous body composition analysis was possible in 75 out of those 81 patients. Population characterization is displayed in Table 1 and significant associations with peritoneal loss as measured during a 4-h PET test, in a 24-h sample, and as PPCI are shown in Table 2.

The most frequent cause of kidney failure was diabetic nephropathy (23%), followed by glomerular disease in 18%, tubulo-interstitial disease in 17%, vascular disease in 12%, or unknown in 28% of the patients. Antihypertensives were prescribed in 89% of the patients, and 68% were on loop diuretics. Anuria was present in only 3 patients.

Clinically relevant associations with each of the different procedures for quantification of peritoneal protein loss were scrutinized. The Pearson correlations between g/24-h and g/240 min (ρ : 0.482, p < 0.001) and between mL/24-h and g/240 min (ρ : 0.437, p < 0.001) were weak.

Table 2. Significant associations of the univariable linear regression with PPL in 4-h, in 24-h, and PPCI

	Protein loss 4-h determination (g/4 h)			Protein loss 24-h determination (g/24 h)			Peritoneal protein clearance (mL/24 h)					
	R ²	β ₁	p value	95% CI	R ²	β ₁	p value	95% CI	R ²	β ₁	p value	95% CI
Demographics and comorbidities												
Age (years)			ns		0.092	0.055	0.006	[0.016; 0.094]	0.090	0.965	0.007	[0.273; 1.658]
Gender, male (%)	0.087	0.512	0.008	[0.135; 0.888]	0.106	2.090	0.003	[0.722; 3.457]	0.081	32.473	0.011	[7.753; 57.193]
Charlson Comorbidity Index			ns				ns				ns	
Diabetes mellitus			ns				ns				ns	
Cardiovascular disease			ns				ns				ns	
Systolic blood pressure (mm Hg)			ns				ns				ns	
Diastolic blood pressure (mm Hg)			ns				ns				ns	
Dialysis												
Time on PD (months)			ns				ns				ns	
CAPD (vs. cycler)			ns		0.118	2.315	0.002	[0.887; 3.743]	0.119	41.541	0.002	[16.113; 66.969]
Icodextrin use			ns				ns				ns	
Peritoneal equilibration test and adequacy												
D/P creatinine	0.211	3.089	<0.001	[1.732; 4.447]	0.137	9.385	0.01	[4.080; 14.689]	0.151	175.330	<0.001	[81.504; 269.155]
Net UF (mL/4 h)			ns				ns				ns	
RRF (mL/min/1.73 m ²)	0.110	0.071	0.003	[0.025; 0.117]			ns				ns	
Kt/V _{urea} (/week)			ns				ns				ns	
Creatinine clearance (L/1.73/week)	0.147	0.008	<0.001	[0.004; 0.013]			ns				ns	
PNA (g/kg/24 h)			ns		0.232	1.800	<0.001	[1.052; 2.547]	0.191	29.081	<0.001	[15.403; 42.758]
Protein loss (g/4 h)			–				–				–	
24-h dialysate collection			<0.001	[0.075; 0.183]			–				–	
Protein loss (g/24 h)	0.232	0.129	<0.001	[0.075; 0.183]			<0.001	[0.053; 0.057]	0.967	17.528	<0.001	[16.793; 18.262]
Peritoneal protein clearance (mL/24 h)	0.191	0.007	<0.001	[0.003; 0.010]	0.967	0.055	<0.001	[0.053; 0.057]	–	–	–	
Biochemical evaluation												
Haemoglobin (g/L)			ns				ns				ns	
Blood urea nitrogen (mmol/L)			ns				ns				ns	
Creatinine (μmol/L)			ns				ns				ns	
Glucose (mmol/L)			ns				ns				ns	
Total protein (g/L)			ns		0.144	–1.814	0.001	[–2.812; –0.816]	0.256	–43.165	<0.001	[–59.746; –26.583]
Albumin (g/L)			ns		0.076	–1.536	0.013	[–2.739; –0.332]	0.116	–33.723	0.002	[–54.712; –12.735]
High-sensitivity C-reactive protein (g/L)			0.006				ns				ns	
24-h urine protein loss (g/24 h)	0.095	0.014	ns	[0.004; 0.024]			ns				ns	
Bioelectrical impedance assessment												
Lean body mass (kg)			ns		0.157	0.116	<0.001	[0.053; 0.179]	0.145	1.992	0.001	[0.853; 3.131]
Soft lean mass/height ² (kg/m ²)			ns		0.111	0.462	0.004	[0.156; 0.769]	0.112	8.288	0.004	[2.804; 13.773]
Extracellular water (L)			ns		0.221	0.443	<0.001	[0.247; 0.639]	0.209	7.713	<0.001	[4.191; 11.235]
Intracellular water (L)			ns		0.127	0.218	0.002	[0.084; 0.353]	0.115	3.713	0.003	[1.296; 6.130]
Extracellular/total body water (%)			ns		0.087	61.035	0.01	[15.004; 107.067]	0.103	1,179.176	0.005	[367.830; 1,990.552]
Extracellular water excess (L)			ns		0.199	1.415	<0.001	[0.747; 2.083]	0.212	26.137	<0.001	[14.300; 37.975]
ns, nonsignificant.												

Comorbidity and PPL

Neither cardiovascular disease, hypertension, diabetes nor comorbidity Charlson Index was significantly associated with PPL, regardless of the methodology considered. Age was positively associated with greater PPL, considering 24-h collections.

Modality of PD and PPL

This population showed a high predominance ofycler use (69%), mostly dependent on patient's preference. Continuous ambulatory peritoneal dialysis (CAPD) was done by 31%, automatic peritoneal dialysis without a long exchange (dry day) by 56%, and continuous cyclic peritoneal dialysis including a long-exchange by 14% of the patients.

Vast majority of CAPD patients were performing 4 exchanges daily of 1.5–2 L per cycle, representing a mean daily volume of 6.2 ± 1.3 L and contrasting with a mean volume of 8.9 ± 2.5 L per in automatic peritoneal dialysis patients. Higher losses were found in CAPD patients, compared to those on cycler, in every 24-h sampling method: either as total grams per day (7.4 g/24 h vs. 5.1 g/24 h, $p = 0.002$), or as peritoneal protein clearance (116.5 mL/24 h vs. 74.9 mL/24 h, $p = 0.002$). In this population, cycler patients with and without long day dwell had similar 24-h protein loss and PPCL. Differences according to modality of PD prescription were not documented in timed 4-h PET protein loss measurements.

Small Solute Transport Rate and PPL

There was a consistent and statistically significant univariable relationship with D/P creatinine, whichever the sampling method.

Nutrition, Inflammation, and PPL

Protein nitrogen appearance (PNA) averaged 0.9 g/kg/24 h and median levels of high-sensitivity CRP were within the normal range. None of these factors was associated with the protein loss, whichever the method used. PPL was associated with lower serum albumin and lower total protein levels.

Body Composition and PPL

The results of the BIA assessment are summarized in Table 1. Quantification of hydration status was performed, retrieving mean ECW/total body water of 39.6% and mean ECWexc of 1 L. Only 24-h measurements of PPL correlated with body composition variables.

Table 3. Multiple linear regression model to determine independent associations with different quantification methods for peritoneal protein loss

Variable	4-h determination (g/4 h)				24-h determination (g/24 h)				Peritoneal protein clearance (mL/24 h)			
	B	SE B	β	95% CI	B	SE B	β	95% CI	B	SE B	β	95% CI
Age	-0.003	0.005	-0.053	[-0.013; 0.008]	0.013	0.023	0.069	[-0.034; 0.060]	0.204	0.413	0.062	[-0.621; 1.029]
Gender (male gender)	0.321	0.170	0.185	[-0.018; 0.659]	1.002	0.802	0.155	[-0.600; 2.604]	13.441	14.135	0.117	[-14.797; 41.680]
Albumin	-0.087	0.148	-0.060	[-0.382; 0.207]	-0.182	0.733	-0.029	[-1.645; 1.282]	-11.510	12.915	-0.104	[-37.312; 14.291]
C-reactive protein	0.007	0.004	0.157	[-0.001; 0.016]	-0.002	0.017	-0.012	[-0.037; 0.033]	-0.210	0.307	-0.071	[-0.824; 0.403]
D/P creatinine	2.570	0.641	0.382**	[1.291; 3.849]	6.383	2.760	0.251*	[0.869; 11.897]	128.921	48.650	0.284*	[31.732; 226.110]
Time on peritoneal dialysis	0.003	0.005	0.054	[-0.007; 0.013]	-0.002	0.021	-0.012	[-0.045; 0.040]	-0.094	0.377	-0.027	[-0.848; 0.660]
Residual renal function ^a	-	-	-	-	-	-	-	-	-	-	-	-
Creatinine clearance	0.008	0.002	0.359**	[0.004; 0.012]	1.063	0.397	0.335*	[0.270; 1.855]	18.246	6.992	0.321*	[4.278; 32.213]
Extracellular water excess	-	-	-	-	0.146	0.174	0.105	[-0.202; 0.493]	2.687	3.063	0.108	[-3.433; 8.008]
LBMI	-	-	-	-	0.560	0.829	0.082	[-1.096; 2.217]	8.334	14.615	0.068	[-20.863; 37.531]
CAPD (vs. cycler)	-	-	-	-	-	-	-	-	-	-	-	-
R ²	-	-	0.430	-	-	-	0.383	-	-	-	0.401	-
F	-	-	6.613	-	-	-	4.423	-	-	-	4.755	-
Durbin Watson	-	-	2.097	-	-	-	1.800	-	-	-	1.793	-

* $p < 0.05$. ** $p < 0.001$. ^aVariable excluded due to collinearity with creatinine clearance; tolerance over 0.598, VIF under 1.672 in all analysis.

Outcome and PPL

Fourteen patients were submitted for a successful renal transplant, and there were 11 deaths (6 were related to cardiovascular events). There was no association of the 4-h PET, 24-h PPL, or PPCI with all-cause death, cardiovascular death, or events, in the median follow-up period of 30.8 months.

Independent Associations of Clinical Variables with PPL

For multiple linear regression analysis, a set of variables was considered to be clinically relevant and thus introduced in the models to explore the association with different quantification methodologies, regardless of the significant univariable linear regression: age, gender, time on PD, high-sensitivity C-reactive protein, serum albumin, and D/P of creatinine.

The multiple linear regression analysis of protein loss determination on the 4 h sample of a PET study included such a set of clinically relevant variables, plus RRF and creatinine clearance (as significant variables in the univariable linear regression). For the multiple linear regression analysis of both 24-h protein determination and peritoneal protein clearance, in addition to that set of variables, modality of PD, LBMI, and extracellular water excess were included (Table 3).

D/P creatinine stands out as an associated factor with protein loss, regardless of the setting, but with a really high confidence interval in the 24-h measurements. On the other hand, 24-h determinations (in grams or clearance) were associated with overhydration. The PET protein quantification was associated with peritoneal creatinine clearance.

Discussion

This study highlights methodological issues concerning peritoneal protein loss measurement that may have been overlooked but stand determinant to efface some controversy about its clinical associations. Peritoneal protein loss has long been presumed to be a detrimental consequence of peritoneal dialysis, possibly due to a link with inflammation, malnutrition, or CAPD modality [1, 2]; however, the literature is inconclusive.

Some studies rely on a standardized methodology to approach this theme by using the 4-h peritoneal equilibration test sample [3, 10] to quantify the amount of protein loss. Other authors consider 24-h effluent determinations as the right parameter to ascertain associations, ei-

ther as total g/24 h [11] or as peritoneal protein clearance (despite the diverse calculation methods or adjustments) [4, 12]. The effluent protein concentration (mg/dL) is not a reliable measure of protein loss [13], so it was not evaluated in this study. The key point is that different methods sign different pathophysiological pathways, as evidenced by the present investigation.

Higher protein leakage is dependent on the effective peritoneal surface area and intrinsic permeability (average radius of large-pore system) [2, 10]. Determinations during the peritoneal equilibration test ascertain peritoneal membrane transport characteristics in a controlled and reproducible assessment [14]. Therefore, variables related to small solute transport such as D/P creatinine and peritoneal creatinine clearance [15] shown to be independently associated with peritoneal protein loss. D/P creatinine stands out as an associated factor with protein loss and although this relationship is preserved regardless of the setting, there is a high confidence interval in the 24-h measurements, in agreement that this determination has more to do with other factors than just membrane solute transport; for instances, daily PD prescription or intrinsic patient characteristics, such as nutritional status, or comorbidities, which wouldn't express themselves in such standardized conditions when comparing to 24-h timed collections.

Notably, regardless of the quantification method, the present study found no association between peritoneal protein loss and the Charlson Comorbidity Index, cardiovascular disease, hypertension, diabetes mellitus, or chronic kidney disease aetiology (namely diabetic nephropathy, data not shown).

We cannot exclude that endothelial dysfunction might be an added explanation for the protein leak in some diabetic patients, as well as in some hypoalbuminaemic inflamed/atherosclerotic patients [16, 17], but in our study, no association with diabetes status, C-reactive protein, or hypoalbuminemia was documented.

The studied population was on PD for a short period of time (interquartile interval of 1–10 months), also limiting the association with vintage. Furthermore, mortality was not associated with higher protein loss. This can be explained by the early-stage DP inherent high solute transference, which is associated with high protein leakage and is expected to decrease with time [18]. This feature also explains male gender association with every measure of protein loss, as already described, independently of body surface area [19, 20].

When evaluating 24-h collections, it is noticeable the influence of body composition parameters, age, and PD type

of prescription on peritoneal protein in the univariable analysis. Independent factors associated with 24-h collection peritoneal loss were higher D/P creatinine and extracellular water excess, determined by multiple linear regression analysis. These results show no major difference between the absolute quantification of grams per day versus protein clearance calculation in mL/day, replicating the results from Davenport et al. [15], namely the association of peritoneal protein clearance with ECW expansion. Therefore, both increased effective capillary surface and increased hydrostatic pressure are drivers of peritoneal protein leak, as also stated by other investigators [21, 22].

In both timed collections, higher transporters are associated with higher peritoneal protein losses; nonetheless, the latter was not associated with malnutrition as measured by prealbumin or protein nitrogen appearance. Higher lean body mass and LBMI were associated with higher peritoneal protein clearance in univariable analysis, indicating that a better nutritional status is the predominant feature linked with higher protein losses in PD and refuting the assumption that protein loss causes malnutrition.

This study has limitations related to the limited sample size, early-stage population, and lack of effluent inflammation biomarker analysis or differentiation of specific protein clearances (for instances: β 2-macroglobulin, α 2-macroglobulin). However, by using a real-life clinical protocol, it brought novelty to the assessment of peritoneal protein leak, showing that PET and 24-h measurements should not be considered equivalent, as disclosed by the weak Pearson correlations between g/24 h and g/240 min and between mL/24 h and g/240 min.

Conclusions

PET protein loss quantification should be regarded as a marker of peritoneal membrane intrinsic permeability. Measurements of a 24-h sample might be closer to pa-

tients' clinical status and prognosis, signaling opportunities for therapy intervention.

Statement of Ethics

The study protocol was conducted in compliance with the ethical principles of the World Medical Association Declaration of Helsinki and approved by the Ethics Committee of the Centro Hospitalar Universitário do Algarve (UAIF 192/2019). The protocol of this observational study was reviewed and the need for written and informed consent was waived by the Ethics Committee of the Centro Hospitalar Universitário do Algarve.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

None.

Author Contributions

Anabela Malho Guedes – responsible for the investigational project, elaboration of the manuscript, and statistical analyses. Roberto Calças Marques and Ana Teresa Domingos – responsible for clinical care and data collection. Céu Laranjo – responsible for biological samples care. Ana Paula Silva and Anabela Rodrigues – responsible for the investigational project and for reviewing the manuscript.

Data Availability Statement

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

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5.2 Peritoneal protein loss: new insights

Overhydration May be the Missing Link between Peritoneal Protein Clearance and Mortality

Malho Guedes A, Marques R, Domingos AT, Silva AP, Bernardo I, Neves PL, Rodrigues A, Krediet RT

Nephron. 2021; 145(5):478-480. DOI:10.1159/000516531. Epub 2021 Jun 15.

PMID:34130276.

Short Summary

As stated in the previous study, hydrostatic convection is a major drive for microvascular protein transport, however most studies in peritoneal dialysis patients overlooked this mechanism. It has been shown recently that both serum NT-proBNP and right atrial area are independently and positively associated with peritoneal protein clearance (PPCI). The next study aimed to explore the importance of fluid overload in PPCI in PD patients.

A cohort of 67 prevalent PD patients was assessed by multifrequency bioelectrical impedance (BIA) and 24-hour peritoneal protein loss and PPCI were simultaneously obtained.

Significant associations between PPCI and BIA parameters of overhydration were found in both univariable and multivariable analysis. A multiple linear model identified extracellular water excess and higher D/P creatinine as predictors of higher PPCI, independent of PD duration, type of PD, age, gender, albumin, cardiovascular disease, C reactive protein or lean mass index.

The uni- and multivariable strong associations between fluid overload and PPCI support the importance of hydrostatic pressure-induced convection for PPCI. Also, peritoneal small solute transport was associated with PPCI. Both are amenable by adjusted dialysis prescription, especially focused on fluid status and avoidance of overhydration.

Overhydration May Be the Missing Link between Peritoneal Protein Clearance and Mortality

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Keywords

Peritoneal protein clearance · Bioelectrical impedance · Peritoneal dialysis · Overhydration · D/P creatinine

Abstract

Introduction: Peritoneal protein loss (PPL) has been associated with mortality. Inflammation was assumed a putative cause with malnutrition as a consequence. Hydrostatic convection is a major drive for microvascular protein transport, but most studies in peritoneal dialysis (PD) patients overlooked this mechanism. An association between peritoneal protein clearance (PPCI) and venous congestion has been reported recently. The aim of this study was to explore the importance of fluid overload in PPCI in PD. **Methods:** Sixty-seven prevalent PD patients were assessed with peritoneal equilibration test and multifrequency bioelectrical impedance assessment (BIA). PPL and PPCI were calculated from simultaneously obtained 24-h peritoneal effluent. **Results:**

PPL averaged 5.2 g/24 h. It was higher in patients on continuous treatment than in those without a long dwell. Significant associations between PPCI and BIA parameters of overhydration were found in both univariable and multivariable analyses. Lean mass index, partly dependent on hydration status, was associated with PPCI in univariable but not in multivariable analysis. A multiple linear model identified extracellular water excess and higher D/P creatinine as predictors of higher PPCI, independent of PD duration, type of PD, age, gender, albumin, cardiovascular disease, C-reactive protein, or lean mass index. **Conclusions:** The uni- and multivariable strong associations between fluid overload and PPCI support the importance of hydrostatic pressure-induced convection for PPCI. Also, peritoneal small solute transport was associated with PPCI. Both are amenable by adjusted dialysis prescription, especially focused on fluid status and avoidance of overhydration. The assumption of an association with inflammation and malnutrition was not confirmed.

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Introduction

Peritoneal protein loss (PPL) in peritoneal effluent is a well-recognized consequence of peritoneal dialysis (PD) treatment since its introduction. In adult patients, the loss averages 5–15 g daily [1, 2], and notwithstanding a number of locally produced proteins and peptides, the quantitatively most important are serum proteins, especially albumin [1].

Peritoneal protein clearance (PPCl) is size dependent and determined by both the effective vascular peritoneal surface area (the number of interendothelial pores) and the intrinsic permeability (large pore size) [3, 4]. The concentration of all serum proteins in the peritoneal effluent and their peritoneal clearances increase, often by 2-fold or more, irrespective of their molecular weight, during episodes of bacterial peritonitis, returning to baseline values after resolution of the infection [5, 6]. In contrast to small solutes, peritoneal total protein and albumin clearances show no increase with PD duration [7, 8].

The clinical significance of peritoneal protein leakage has been a matter of controversy. Several studies have associated PPL with mortality [9–13], whereas other studies have failed to show such association [14–17], but instead encountered an important relationship with cardiovascular events [16] and peripheral arterial disease [17]. These results suggested that an increased peritoneal leak of serum proteins might reflect systemic endothelial barrier dysfunction and hence be a biomarker of vascular comorbidity and worse survival [18]. Hydrostatic convection is a major drive but largely overlooked mechanism for peritoneal protein transport. In fact, hydrostatic convection is the only mechanism for the escape of albumin from the general microcirculation to interstitial tissues. It has been shown recently that both serum NT-proBNP and right atrial area are independently and positively associated with PPCl. These findings mean that venous congestion is likely to be important in PPL [19]. Fluid volume overload can lead to venous congestion and is a recognized independent predictor of cardiovascular events and mortality in PD patients [20–22]. This potential confounder, often disregarded in most survival analysis studies on PPL, can be the missing link between PPL and mortality. The aim of this study was to explore the importance of overhydration in the magnitude of PPCl in prevalent PD patients.

Materials and Methods

Study Design and Patient Population

This single-center cross-sectional study included prevalent PD patients from January 2014 until censoring of the data in December 2019. All patients included were assessed with a modified peritoneal equilibration test (PET), using 3.86/4.25% glucose and multifrequency bioelectrical impedance assessment (BIA), performed simultaneously. At the same time, all participants performed a 24-h collection of urine and spent dialysate. Patients who had malignancy or active infection, experienced a prior peritonitis episode, or acute hospital admissions in the previous 3 months were excluded. Patients with implantable cardiac devices or other conditions contraindicating BIA were also excluded. The study protocol was conducted in compliance with the ethical principles of the Helsinki Declaration and approved by the Ethics Committee of the Centro Hospitalar Universitário do Algarve.

Clinical Information, Biochemical Data, and PD Adequacy

Demographics and clinical features including gender, age, height, weight, cause of renal failure, comorbid conditions, medication, dialysis duration, residual renal function (RRF), and solute clearance data were registered. Cardiovascular disease was defined by a past history of angina pectoris with electrocardiographic changes related to myocardial ischemia, myocardial infarction, arrhythmia documented by electrocardiography, transient ischemic attacks, thromboembolic or hemorrhagic stroke, peripheral vascular disease, and cardiovascular congestion (all defined according to conventional clinical criteria). These diagnostics were determined by the attending physicians, who had no knowledge of the undergoing study data. This information was retrieved from the computerized renal registry database that keeps detailed records of all hospitalization episodes. All patients were treated with dialysis solutions that had a reduced content of glucose degradation products and a normal pH (Baxter® and Fresenius®). The maximum glucose concentration was 2.27/2.3%. Icodextrin 7.5% was used for the long dwell in one-third of the patients. Charlson Comorbidity Index was calculated at the time of study inclusion [23]. Residual renal function was assessed as the mean of urea and creatinine clearance from the 24-h urine collection. Urea clearance index (Kt/V_{urea}) was derived from the collection of the 24-h PD effluent and 24-h urine. Normalized protein nitrogen appearance (PNA) was estimated using the relevant Bergstrom equation [24]. Routine biochemical analyses were performed by an automatic chemistry analyzer (Architect ci8200 Abbott). Creatinine was measured with an enzymatic method to prevent glucose interference. Serum high-sensitivity C-reactive protein (CRP) was determined by immunoturbidimetry at the time of the PET. Prealbumin was determined by nephelometry.

PPCl and Body Composition Analysis

Peritoneal dialysate protein loss was measured in 24-h peritoneal dialysate effluent using the biuret reaction method. PPCl was calculated from the total protein loss in 24-h effluent/serum total protein, expressed as milliliter per 24 h. Simultaneous multifrequency BIA (InBody S10 Body Composition Analysis; Biospace, Seoul, South Korea) was done with empty abdomen after completion of the PET. Lean BMI (LBMI) was calculated by lean body mass (LBM) correcting for body height square (kg/m^2). Extracellular water excess was calculated by ECW measured $-0.613 \times \text{ICW}$ measured [25].

Statistical Analysis

Data were checked for normality using the Shapiro-Wilk test. Results were expressed as frequencies and percentages for categorical variables, mean $\pm$ standard deviation (SD) for continuous variables, and median (interquartile range) for skewed distributions. An unpaired *T* test was performed for comparing groups. Univariable analysis was performed with linear regression model. All variables with a statistical association of $p < 0.1$ and those thought to be of major clinical significance (CRP and PD duration) were used to create a multiple linear regression model to determine associations with PPCL. Preliminary analyses were performed to ensure there was no violation of normality and linearity. Of the large number of BIA parameters describing water distribution, extracellular water excess was the one used due to its biologic meaning and being an absolute value, independent of body structure. The statistical analysis was performed using SPSS version 22.0. Statistical significance was considered at or below 5% level.

Results

A PET with 24-h collection for determination of PPCL and simultaneous BIA was obtained in 67 out of 93 prevalent patients (Fig. 1). Population characterization and results of the investigations are given in Table 1. Diabetic nephropathy was the most frequent cause of renal failure (28%). Other etiologies were glomerular disease in 19%, tubulointerstitial disease in 14%, vascular disease in 10%, or unknown in 29% of the patients. Anuria was present in only 2 patients. Antihypertensives were prescribed in 90% of the patients, and 67% were on loop diuretics. Comorbidity was not significantly associated with PPCL.

Dialysis characterization showed a high predominance of the use of aycler (69%), mostly dependent on patient's preference. Continuous ambulatory peritoneal dialysis (CAPD) was done in 31%, continuous cyclic peritoneal dialysis including a long exchange in 12%, and automatic peritoneal dialysis (APD) without a long exchange (dry day) in 57% of the patients. One-third of the patients used icodextrin for the long dwell (14 patients on CAPD and 7 patients on continuous cyclic peritoneal dialysis).

PPL averaged 5.2 g/24 h and peritoneal albumin loss averaged 3.5 g/24 h. Higher losses were found in CAPD patients, compared to those on APD without a long dwell exchange: 7.1 g/day versus 5.2 g/24 h ($p = 0.036$). Patients with diabetic nephropathy or patients with glomerular disease had no higher PD effluent protein loss or PPCL, compared to the other ones. Median levels of high-sensitivity CRP were in the normal range.

The results of bioelectrical multifrequency impedance assessment are also summarized in Table 1. It provided

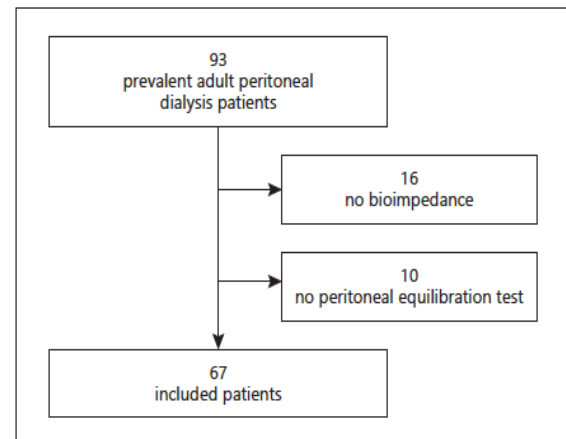


Fig. 1. Strobe diagram showing the total number of prevalent PD patients assessed for inclusion and included in the study. PD, peritoneal dialysis.

nutritional parameters that allowed computation of mean soft lean mass index. BIA also quantified hydration status, namely retrieving a median extracellular water/total body water of 39.6% and a mean extracellular water excess of 1 L.

Univariable analysis of the association of the clinical variables with PPCL is presented in Table 1. Negative relationships were found for serum total protein and albumin. The following parameters were not related to PPCL in the univariable analysis: comorbidity, blood pressure parameters, PD duration, and CRP.

The multiple linear regression model adjusted for the significant variables in the univariate analysis and the clinically relevant ones, that is, CRP, cardiovascular disease, type of PD (CAPD vs.ycler), and PD duration, identified extracellular water excess and D/P creatinine as predictors of a higher PPCL, as shown in Table 2. Each liter of overhydration was associated with a rise in PPCL of 18 mL/24 h.

Discussion

The current cross-sectional study in prevalent PD patients showed a strong association between the peritoneal clearance of total protein and parameters of overhydration, as assessed by BIA. This association was found in both univariable and multivariable analyses. The results are in close agreement with those of a previously published study in incident PD patients from the UK [19]. The presence of venous congestion, assessed by an in-

Table 1. Characterization of the patient population (demographics, comorbidities, dialysis, and biochemical and BIA results) [26]

Demographics and comorbidities		Univariable linear regression (R^2 , F , β_1 , p value)			
Age, years	54±7	0.078	5.491	0.923	0.022
Gender, male (%)	61	0.091	6.509	34.977	0.013
Charlson Comorbidity Index	4 [2–7]				ns
Diabetes mellitus, %	31				ns
Cardiovascular disease, %	25	0.057	3.918	31.157	0.052
Hypertension, %	85				ns
Systolic blood pressure, mm Hg	145±28				ns
Diastolic blood pressure, mm Hg	89±20				ns
Pulse pressure, mm Hg	52 [40–72]				ns
Patients with antihypertensives, %	90				
Agents, n	2 [1–2]				
β -Blockers, %	42				ns
Calcium channel blockers, %	64				ns
ACE inhibitors/ATII receptor blockers, %	67				ns
<i>Dialysis</i>					
Time on PD, months	2 [1–9]				ns
Dialysis modality					
CAPD (vs. cycler), %	31	0.067	5.738	34.912	0.019
Cycler, %	69				
APD (no daytime dialysis), %	57				
CCPD (with daytime exchange), %	12				
Icodextrin use, %	31				ns
PET and adequacy					
D/P creatinine	0.63±0.12	0.111	8.136	161.807	0.006
Net UF, mL/4 h	857±267				ns
RRF, mL/min/1.73 m ²	6.1 [3.8–9.8]				ns
Kt/V _{urea} , /week	2.32 [2.00–3.09]				ns
Creatinine clearance, L/1.73/week	86.4 [68.9–112.9]				ns
PNA, g/kg/24 h	0.9 [0.8–1.1]				ns
24-h dialysate collection					
Protein loss, g/24 h	5.2 [3.8–6.7]	0.968	1,978.422	17.650	<0.001
PPCl, mL/24 h	73.4 [54.8–97.2]		Mathematical coupling		
<i>Biochemical evaluation</i>					
Hemoglobin, g/L	118±19				ns
BUN, mmol/L	20.3±6.8				ns
Creatinine, μ mol/L	548 [407–716]				ns
Glucose, mmol/L	7.1 [5.9–11.6]				ns
Total protein, g/L	68.2±6.1	0.251	21.787	–46.799	<0.001
Albumin, g/L	37.6±4.8	0.094	7.831	–39.089	0.007
Prealbumin, mg/dL	42.1±9.5				ns
CRP, g/L	3.9 [1.7–10.3]				ns
24-h urine collection					
Volume, mL	1,485±712				ns
Protein loss, g/24 h	0.91 [0.42–2.79]				ns
<i>Bioelectrical impedance assessment</i>					
LBM, kg	47.8±11.0	0.127	9.282	1.828	0.003
Soft lean mass/height ² , kg/m ² *	32.3±7.6	0.091	7.521	7.940	0.008
Extracellular water, L	14.8±3.4	0.190	15.057	7.196	<0.001
Intracellular water, L	21.9 [18.5–25.6]	0.099	7.009	3.371	0.010
Extracellular/total body water, %	39.6±1.5	0.097	8.080	1,191.66	0.006
Extracellular water excess, L	1.01±1.02	0.224	18.456	26.263	<0.001

Values are presented as mean ± SD or as median [75% confidence interval]. Statistically significant associations with PPCl are shown in the right column. BIA, bioelectrical impedance assessment; ns, nonsignificant; PD, peritoneal dialysis; CAPD, continuous ambulatory peritoneal dialysis; APD, automatic peritoneal dialysis; CCPD, continuous cyclic peritoneal dialysis; PET, peritoneal equilibration test; RRF, residual renal function; PNA, normalized protein nitrogen appearance; PPCl, peritoneal protein clearance; CRP, C-reactive protein; LBM, lean body mass; LBMI, lean BMI. * LBMI reference values: 16.7 kg/m² in males and 13.8 kg/m² in females for the diagnosis of sarcopenia in PD patients [26].

Table 2. Multiple linear regression model to determine associations with PPCL

Variable	Coefficient	SE	t	p value
Age	0.185	0.426	0.435	ns
Gender (male gender)	14.782	15.022	0.984	ns
Cardiovascular disease	10.718	15.396	0.696	ns
Albumin	-12.203	14.897	-0.819	ns
CRP	-0.140	0.318	-0.441	ns
D/P creatinine	124.999	55.853	2.238	0.029
LBMI	3.052	3.171	0.963	ns
Extracellular water excess	17.954	7.668	2.341	0.023
CAPD (vs. cycler)	5.814	15.340	0.379	ns

Model R^2 0.397, constant -33.926; tolerance over 0.564, VIF under 1.773; Durbin-Watson: 2.349. PPCL, peritoneal protein clearance; ns, nonsignificant; CRP, C-reactive protein; LBMI, lean BMI; CAPD, continuous ambulatory peritoneal dialysis.

creased right atrial diameter and elevated serum NT-proBNP, was independently associated with PPCL in that analysis. Although no echocardiography was done in the present study, nor was serum NT-proBNP determined, BIA was performed in all patients and in both studies and showed similar relationships between overhydration and PPCL. Another cross-sectional analysis reported relationships between fluid overload and 4-h dialysate/serum total protein ratio, but no 24-h collections were obtained [27]. BIA results were reported in only one of the published epidemiological studies on relationships between PPCL and death [12]. Also, in that study, an increased ECW/TBW was associated with both increased protein clearance and mortality. None of the other studies on PPCL and mortality reported BIA results, but a low serum albumin was identified as a predictor of death in some [10, 15]. Volume overload is the most important cause of a low serum albumin concentration in PD patients [28]. However, the interpretation of serum albumin in studies on PPCL is hampered by mathematical coupling, because serum albumin or total protein is in the denominator of the clearance equation [29]. Furthermore, a low serum albumin concentration itself does not cause fluid retention. This has been established for patients with the nephrotic syndrome [30] and also for those with hypoalbuminemia due to kwashiorkor [31]. The explanation is the absence of a decrease in the colloid osmotic pressure gradient between vascular luminal and interstitial fluid [30]. Overhydration can manifest itself as hypertension and/or venous congestion, or both. Parameters of blood pressure were not significantly related to PPCL. One explanation

may be related to the use of antihypertensives in 90% of the patients but more likely due to autoregulation and the marked drop in the arterial pressure in the microcirculation to only 4% of that in the left atrium [32]. Proteins escape from the circulation in the venules, as has been shown in experiments with topical application of histamine in rats and cats [33] and after intraperitoneal administration of the venodilator nitroprusside in PD patients [34]. Large pores, probably represented by large interendothelial gaps in the vascular wall [33], probably constitute the route through which the venous hydrostatic pressure gradient operates [35]. Fluid overload is an important cause of an increased venous pressure. Convective transperitoneal transport of albumin and larger serum proteins does not occur through the small pore system during PD, because it is not influenced by the crystalloid pressure gradient [36] nor by the colloid osmotic one [37]. The current study, although being a cross-sectional analysis, therefore confirms a pathophysiologic mechanism: protein escapes from the microcirculation in its venular segment due to venular hydrostatic pressure. Fluid overload increases this pressure and thereby augments protein escape, the magnitude of which can be assessed in PD patients by PPCL.

Transperitoneal transport is dependent on the effective peritoneal surface area, that is, the number of perfused peritoneal microvessels, so the number of small and large pores [4]. D/P creatinine is generally considered to represent this parameter. In the univariable and multivariable analyses, D/P creatinine was a significant predictor of PPL. This relationship has been reported to disappear with PD duration, probably to the development of fibrotic interstitial peritoneal alterations [7], but the vast majority of our patients was treated for <1 year. This may also explain why PD duration showed no relationship with protein clearance.

Endothelial dysfunction and inflammation have been suggested to influence PPCL by increasing large pore permeability [18]. The absence of relationships with Charlson Comorbidity Index and serum CRP in the present study is not in support of this assumption. But, the presence of peritoneal microinflammation cannot be excluded with certainty, because no effluent IL-6 concentrations were determined in the present study. Investigations on relationships between effluent IL-6 and peritoneal small solute transport have given variable results [38, 39] and are hampered by mathematical coupling, because IL-6 is not only determined by local production but also partly by peritoneal transport [29]. Furthermore, a recent analysis showed no upregulation of

the IL-6 gene in PD patients treated for <2 years, nor in those treated for a longer time [40].

Male gender was associated with a higher PPCL than female, which has been described previously [41], and is likely due to a larger body surface area [42]. In the present population, higher PPCL was associated with higher LBM and LBMI, higher extracellular body mass/body cell mass ratio, and higher skeletal muscle mass index, challenging the conventional viewpoint that higher PPCL may cause malnutrition [1]. These are in agreement with results from a study in Chinese patients, which also showed a relationship between LBMI and PPCL in univariable analysis [43]. However, most of these nutritional parameters are also influenced by fluid status. For instance, LBMI is calculated as the difference between body weight and body fat, the former also being dependent on fluid status. In the univariable analysis, LBMI was associated with PPCL in the present study and was therefore introduced in the multivariable analysis but was excluded as an explaining mechanism. Therefore, these results argue against a role of nutritional status in the magnitude of PPCL. This is also supported by the absence of a univariable relationship between this parameter and serum pre-albumin.

The multivariable model, including relevant clinical variables, retrieved effective peritoneal surface area represented by the 4-h D/P creatinine and overhydration as major determinants of PPCL in PD patients. Both these variables have been associated with higher mortality and both have been successfully addressed by dialysis adjustments, which include APD and icodextrin. Strategic focus on better volume management probably mitigates PPL.

We acknowledge a number of limitations of this study, like being a single-center analysis with a limited number of participants, mostly in early stage of PD exposure. However, this can also be considered a strength, because it enables insight into normal peritoneal physiology. A long PD duration can cause peritoneal abnormalities that can obscure normal physiology. Strengths of this study rely on the use of measured total protein for 24-h PPCL calculation in a population with reduced exposure to glucose and glucose degradation products, because all patients were prescribed compatible solutions and none had higher than 2.27/2.3% dialysate. Especially, the use of bioimpedance to evaluate the link between peritoneal leak and body composition was an important discriminative tool.

To conclude, peritoneal effluent protein leak was significantly associated with peritoneal small solute transport and with overhydration, both amenable by adjust-

ed dialysis prescription. The importance of fluid overload explains the association between PPCL and mortality reported in some epidemiologic studies and refutes assumptions on a possible role of endothelial dysfunction or inflammation. An association with malnutrition, as measured by body cell mass or LBM, was also not found. Future longitudinal studies addressing current PD prescription and bioimpedance analysis should be focused to enlighten the clinical impact and usefulness of PPCL.

Statement of Ethics

The study protocol was conducted in compliance with the ethical principles of the Helsinki Declaration and approved by the Ethics Committee of the Centro Hospitalar Universitário do Algarve.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

The authors did not receive any funding.

Author Contributions

A.M.G. – responsible for the investigational project, elaboration of the manuscript, and statistical analyses. R.M. and A.T.D. – responsible for data collection. A.P.S., I.B., and P.L.N. – responsible for clinical care and registry. A.R. and R.T.K. – responsible for the investigational project and for reviewing the manuscript.

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5.3 Previously known associations vs new insights

Peritoneal Protein Loss, Inflammation, and Nutrition: Refuting Myths

Malho Guedes A, Calças Marques R, Ribeiro B, Fernandes MT, Faísca M, Silva AP, Bragança J, Rodrigues A.

Front Med (Lausanne). 2022 May 26;9:884061. DOI:10.3389/fmed.2022.884061.

PMID: 35692552; PMCID: PMC9178188.

Short Summary

Peritoneal protein loss (PPL) has been classically correlated with mortality, malnutrition and inflammation. In the previous studies overhydration was brought to the equation. This study aimed to review classic and recent factors associated with PPL.

Inflammation, hydration and nutritional status were assessed in a cohort of 78 PD patients. Positive associations between PPL and small solute transport, body composition (lean body mass index (LBMI) and extracellular water excess (EWexc)), comorbidities and performing CAPD (vs cycler) were disclosed. PPL correlated positively with dialysate appearance rate of IL-6, but not with serum IL-6. Linear multivariable analysis confirmed positive association between PPL and EWexc, LBMI and performing CAPD.

As for survival analysis, no relationship was found between mortality and PPL. Multivariable Cox regression showed that Charlson Comorbidity Index, overhydration and lower PPL were predictors for mortality.

Again, overhydration, was a strong predictor of PPL, overpowering variables previously reported as determinants of PPL, namely clinical correlates of endothelial dysfunction or local inflammation. Also, survival analysis demonstrates the importance of overhydration as a strong prognostic factor. PPL were not associated with malnutrition or higher mortality, emphasizing the importance of volume overload control, amenable by adjusted dialysis prescription, diuretic and water intake restriction.



Peritoneal Protein Loss, Inflammation, and Nutrition: Refuting Myths

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
Nephrology,
a section of the journal
Frontiers in Medicine

Received: 25 February 2022

Accepted: 11 April 2022

Published: 26 May 2022

Citation:

Malho Guedes A, Calças Marques R,
Ribeiro B, Fernandes MT, Faisca M,
Silva AP, Bragança J and Rodrigues A
(2022) Peritoneal Protein Loss,
Inflammation, and Nutrition: Refuting
Myths. *Front. Med.* 9:884061.
doi: 10.3389/fmed.2022.884061

Peritoneal protein loss (PPL) has been correlated with mortality, malnutrition and inflammation. More recently overhydration was brought to the equation. This study aims to review classic and recent factors associated with PPL. Prevalent and incident peritoneal dialysis (PD) patients were included. Dialysate and serum IL-6 was obtained during PET. Hydration and nutritional status were assessed by bio-impedance. Linear regression and Cox regression were performed. The 78 included patients presented median values of PPL 4.8 g/24h, serum IL-6: 5.1 pg/mL, and IL-6 appearance rate 153.5 pg/min. Mean extracellular water excess (EWexc) was 0.88 ± 0.94 L, and lean body mass index (LBMI) 17.3 ± 2.4 kg/m². After mean follow-up of 33.9 ± 29.3 months, 12 patients died. Linear univariable analysis showed positive associations between PPL and small solute transport, body composition (LBMI and EWexc), comorbidities and performing CAPD (vs. cycler). PPL correlated positively with dialysate appearance rate of IL-6, but not with serum IL-6. Linear multivariable analysis confirmed positive association between PPL and EWexc ($p = 0.012$; 95%CI: 4.162–31.854), LBMI ($p = 0.008$; 95%CI: 1.720–11.219) and performing CAPD ($p = 0.023$; 95%CI: 4.375–54.190). In survival analysis, no relationship was found between mortality and PPL. Multivariable Cox regression showed Charlson Comorbidity Index (HR: 1.896, 95%CI: 1.235–2.913), overhydration (HR: 10.034, 95%CI: 1.426–70.587) and lower PPL (HR: 0.576, 95%CI: 0.339–0.978) were predictors for mortality. Overhydration, was a strong predictor of PPL, overpowering variables previously reported as determinants of PPL, namely clinical correlates of endothelial dysfunction or local inflammation. PPL were not associated with malnutrition or higher mortality, emphasizing the importance of volume overload control in PD patients.

Keywords: peritoneal protein loss, peritoneal dialysis, inflammation, nutrition, overhydration

INTRODUCTION

The clinical significance of peritoneal protein loss (PPL) has been a matter of controversy. It has been seen as a detrimental consequence of peritoneal dialysis for many years (1, 2). Many studies correlated this protein leakage with higher mortality (3–7), malnutrition (8), and inflammation (9). Other authors found PPL to be related with cardiovascular events (10). Such conclusions are commonly explained by the concept of systemic endothelial barrier dysfunction and hence increased peritoneal leak of serum proteins could be seen a biomarker of vascular comorbidity, leading to worse survival (11). In this context, inflammation has been advocated as one of the driving forces for protein leakage. First clinical evidence arose initially from peritonitis (12), and then this debate evolved from an initial culprit systemic inflammation (9) to a consistent focus on local inflammation (11).

In spite of that, several authors have shown other survival cohorts, refuting the association with PPL with all-cause or cardiovascular mortality (10, 13–15). More recent knowledge has brought overhydration to this equation (16, 17), claiming fluid volume overload as a major culprit for PPL attributed mortality. Furthermore, other attributed consequences of PPL, such as malnutrition, have also been refuted in recent papers. Do et al. demonstrated that PPL is not associated with muscle mass, strength or sarcopenia, as long-term markers of malnutrition (18).

Controversy is far from over, and confounders should be identified. By combining critical methodological issues such as effluent and serum biomarkers, the aim of this study is to review classic and recent factors associated with peritoneal protein loss and its consequences on overall mortality in peritoneal dialysis patients.

MATERIALS AND METHODS

Study Design, Patient Population, and Variables

This single center, longitudinal study, included prevalent and incident peritoneal dialysis, from March 2015 to March 2021, with follow up until the 31st December of 2021. Inclusion criteria comprised a modified PET, using 3.86/4.25% glucose solutions and interleukin-6 determination (both in serum and 4-h peritoneal effluent). Simultaneously, a 24-h collection of spent dialysate was obtained. Effluent protein was measured using the biuret reaction method and peritoneal protein loss (PPL) is expressed as grams per 24-h. Hydration and nutritional status were assessed by simultaneous multifrequency bioelectrical impedance (InBody S10 Body Composition Analysis; Biospace, Seoul, South Korea). Extracellular water excess (ECWex) was calculated by $ECW = -0.613 \times \text{intracellular water (ICW)}$ measured (19). Exception made for overhydration control in heart failure patients (performing a daily exchange of icodextrin), all other patients were eligible for performing PET.

Baseline demographics and clinical features were registered, namely age, gender, weight, height, medication, cause of kidney failure, dialysis duration, residual renal urine volume and solute

clearance. Charlson Comorbidity Index was calculated at the time of study inclusion¹. Routine biochemical analyses were performed by an automatic chemistry analyzer (Architect ci8200 Abbot®). Creatinine was measured with an enzymatic method to prevent glucose interference. Serum high sensitivity C reactive protein (CRP) was determined by immunoturbidimetry at the time of the PET. Residual renal function (RRF) was assessed as the mean of urea and creatinine clearance from the 24-h urine collection. Urea clearance index (Kt/V urea) was derived from the 24-h urine and PD effluent collection.

All patients were treated with reduced glucose degradation products content and a normal pH dialysis solution (Baxter® and Fresenius®). The maximum glucose concentration was 2.27%/2.3%. Patients who had active infection or malignancy, experienced acute hospital admissions or a peritonitis episode during the preceding 3 months, were excluded.

Sample IL-6 Analysis

Collected serum and peritoneal fluid samples were immediately stored at -80°C , until analysis. All samples had one or two freeze-thaw cycles before quantification. For the development of sandwich enzyme linked immunosorbent assays (ELISA), we used the DuoSet® ELISA Development System to measure Interleukin 6 (IL-6; DY206-05; R&D Systems Inc., Minneapolis, MN, USA), following the manufacturer's protocols and using all the recommended additional reagents. The ELISA was specific for human IL-6 and did not cross-react with human Recombinant human CNTF, G-CSF, gp130, IL-6 R, IL-11, IL-12, LIF, LIF R, and OSM. Duplicate readings were assayed using a TECAN Infinite®200 multimode reader (Mannedorf, Switzerland) for each standard, control, and sample and the average zero standard optical density (O.D.) was subtracted. A standard curve with the value of absorbance vs. the concentration was generated by reducing the data using the Quest Graph™ Four Parameter Logistic (4PL) Curve Calculator (20). The sample concentrations were then calculated from the determined absorbance values through the four-parameter logistic (4PL) standard curve, using the same software.

Statistical Analysis

Shapiro-Wilk test was used to check normality of the data. Results were expressed as frequencies and percentages for categorical variables, mean $\pm$ standard deviation (SD) for continuous variables, and median (interquartile range) for skewed distributions. For description of the predictors of peritoneal protein loss uni- and multivariable linear regression were performed. Preliminary analyses were performed to ensure there was no violation of normality and linearity. All variables with a statistical association of $p < 0.2$ were used to create a multiple linear regression model to determine associations with PPL. The backward method was used to choose the best model, based on the highest adjusted R^2 .

Survival analysis, using Cox regression, was performed firstly as univariable analysis. The conditional backward method was used in the multivariate analysis, due to the low number of events.

¹<https://www.mdcalc.com/charlson-comorbidity-index-cci> (accessed during the study time period).

The statistical analysis was performed using SPSS version 22.0. Statistical significance was considered at or below a 5% level.

RESULTS

Patients' Characteristics

A total of 78 patients were included (54 incident, 24 prevalent) out of 118 (Figure 1). Table 1 displays the main baseline characteristics. Diabetes-associated renal disease was the most frequent etiology of renal failure (23%), followed by glomerular disease in 19%, tubulo-interstitial disease in 17%, vascular disease in 12%, and unknown in 19% of the patients. Anuria was present in three patients. The baseline evaluation was performed on the first month of technique (interquartile interval 1–8 months) and these patients had a mean follow-up of 33.9 ± 29.3 months.

Univariable Correlation With PPL and Best Multivariable Model

Linear univariable analysis showed positive associations between PPL and (1) small solute transport, as measured by D/P creatinine, (2) body composition, as measured by lean body mass index and overhydration, (3) comorbidities, namely presence of cardiovascular disease or measured by Charlson Comorbidity Index, pulse pressure, older age or male gender (Table 1, right column), (4) performing CAPD vs. cyclical. The peritoneal protein loss also correlated positively with dialysate appearance rate of IL-6, but not with serum IL-6. A strong negative correlation was seen between PPL serum albumin and total protein. No significant difference in PPL was found according to icodextrin use or time on PD.

To avoid collinearity, variables with the same biologic meaning were excluded from the multivariable analysis, specifically extracellular water excess was included (excluding extracellular/total body water), and also albumin was entered

in the model (ignoring total protein). All the other variables with p -value < 0.2 shown in Table 1 were included. By linear multivariable analysis, using the backward method, the model with the best adjusted R^2 -value showed a significant positive association between PPL and extracellular water excess (95% CI: 4.162–31.854; $p = 0.012$), lean body mass index (95% CI: 1.720–11.219; $p = 0.008$) and performing CAPD (95% CI: 4.375–54.190; $p = 0.023$) were validated. In the best model, cardiovascular disease was considered without attaining statistical significance (Table 2).

Survival Analysis

Overall, 12 patients died (5 deaths due to cardiovascular events, 2 in the context of catastrophic gastrointestinal hemorrhage, 1 death due to Covid-associated pneumonia, 1 death after aspiration pneumonia, other due to vasculitis recurrence and 2 deaths attributed to cachexia). During follow-up, the annual mortality rate of our Unit averaged 6.3%.

In the exploratory survival analysis, no relationship was found between mortality and PPL (HR: 1.020, 95% CI: 0.777–1.339, $p = 0.886$). A univariable positive association was shown with age (HR: 1.077, 95% CI: 1.014–1.144, $p = 0.016$), serum IL-6 concentration (HR: 1.024, 95% CI: 1.006–1.044, $p = 0.011$), pulse pressure (HR: 1.038, 95% CI: 1.002–1.075, $p = 0.039$), overhydration (HR: 2.771, 95% CI: 1.267–6.058, $p = 0.011$) and Charlson Comorbidity Index (HR: 1.331, 95% CI: 1.094–1.620, $p = 0.004$). The presence of cardiovascular disease at baseline assessment showed a trend for worse outcome (HR: 3.101, 95% CI: 0.976–9.854, $p = 0.055$). Higher serum albumin levels were found to be protective (HR: 0.339, 95% CI: 0.117–0.981, $p = 0.046$).

In this early-stage PD population, with globally preserved residual kidney function and lean body mass, an effect of these variables on mortality was not evident. Also, no association with gender, diabetes, dialysate appearance rate of IL-6, CAPD/APD technique or D/P creatinine was found.

Cox regression, conditional backward method, variables included are depicted in Table 3, showed Charlson Comorbidity Index (HR: 1.896, 95% CI: 1.235–2.913, $p = 0.003$), overhydration (HR: 10.034, 95% CI: 1.426–70.587, $p = 0.021$) and lower peritoneal protein loss (HR: 0.576, 95% CI: 0.339–0.978, $p = 0.041$) were predictors for mortality.

DISCUSSION

The purpose of this study was to analyze peritoneal protein loss determinants and to explore prognostic consequences. Most commonly established pathways for higher peritoneal protein leak have been inflammation, in turn associated with peritoneal solute transport rate, and endothelial dysfunction.

In the analyzed cohort, the univariable analysis showed a consistent association of PPL with small solute transport and dialysate appearance rate of IL-6. Davies et al., established that peritoneal protein clearance was a function of local inflammation (as reflected by the product of effective membrane area and local

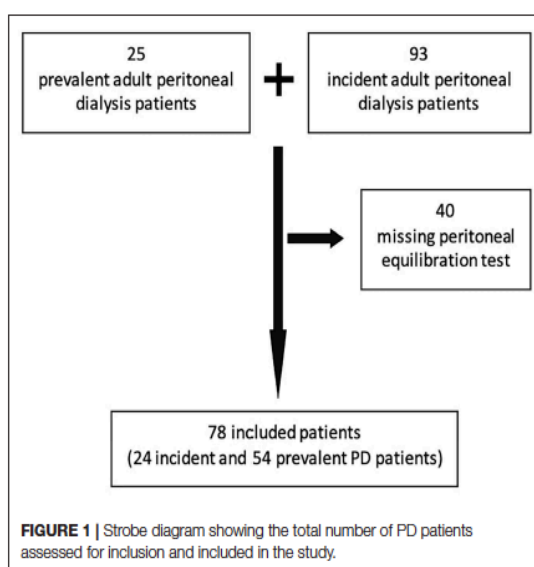


TABLE 1 | Characterization of the patient population ($n = 78$ patients) and linear univariable regression with peritoneal protein loss.

Demographics and comorbidities		Linear regression with PPL		
		<i>B</i>	95% CI	<i>p</i>
Age (years)	56 (41–70)	0.048	0.012, 0.085	0.010
Gender, male (%)	65%	1.585	0.187, 2.984	0.027
Follow-up (months)	22 (13–53)			
Charlson comorbidity index	4 (2–6)	0.341	0.062, 0.619	0.017
Diabetes mellitus	26%			n.s.
Cardiovascular disease	27%	1.888	0.401, 3.374	0.014
Systolic blood pressure (mmHg)	138 (122–160)			n.s.
Diastolic blood pressure (mmHg)	87 ± 21			n.s.
Pulse pressure (mmHg)	48 (40–67)	0.037	0.000, 0.074	0.047
Biochemical evaluation				
Hemoglobin (g/L)	118 ± 15			n.s.
Blood urea nitrogen (mmol/L)	56.2 ± 17.6			n.s.
Creatinine (μmol/L)	566 (389–796)			n.s.
Glucose (mmol/L)	7.0 (5.9–10.5)			n.s.
Total protein (g/L)	69.3 ± 8.1	−1.183	−2.001, −0.365	<0.001
Albumin (g/L)	39.7 ± 7.2			0.032
High sensitivity C-reactive protein (g/L)	3.7 (1.6–8.1)			n.s.
Interleukin-6 (pg/mL)	5.1 (3.9–9.9)	−1.036	−1.893, −0.089	n.s.
Dialysis				
CAPD (vs. cycler)	31%	2.749	1.401, 4.097	<0.001
Icodextrin use	33%			n.s.
Peritoneal equilibration test and adequacy				
Time on PD (months)	1 (1–8)			n.s.
D/P creatinine	0.65 ± 0.12	6.595	0.803, 12.387	0.026
Net UF (mL/4 h)	871 ± 259			n.s.
RRF (mL/min/1.73 m ²)	6.0 (4.1–9.1)			n.s.
Kt/V _{urea} (V/week)	2.26 (1.97–2.76)			n.s.
Creatinine clearance (L/1.73/week)	86.5 (65.8–118.6)			n.s.
Peritoneal protein loss (g/24 h)	4.8 (3.8–6.8)			
IL-6 appearance rate (pg/min)*	153.5 (80.7–347.7)	0.002	0.000, 0.004	0.020
Bioelectrical impedance assessment				
Soft lean mass/height ² (kg/m ²)**	17.3 ± 2.4	0.455	0.160, 0.750	0.003
Extracellular/total body water (%)	39.4 ± 1.5	53.277	3.981, 102.574	0.035
Extracellular water excess (L)	0.88 ± 0.94	1.365	0.646, 2.083	<0.001

n.s., non-significant (*p*-values are shown when < 0.2).

*IL-6 appearance rate = peritoneal IL-6 effluent volume/dwell time in minutes.

**LBMI reference values: 16.7 kg/m² in males and 13.8 kg/m² in females; for the diagnosis of sarcopenia in PD patients (21).

dialysate IL-6 appearance rate) and not systemic inflammation in patients commencing PD (11). Supporting this notion, in our study systemic inflammation, as assessed by serum IL-6, did not predict higher PPL.

The association of PPL and comorbidities, such as measured by Charlson Comorbidity Index, the presence of cardiovascular disease, higher pulse pressure or older age, have been described in several other cohorts, reinforcing the endothelial dysfunction as the origin for such associations (10, 13, 15, 17).

However, multivariable analysis does not support such inferences. The best explicative model enhances overhydration, better nutrition and performing CAPD as best predictors for higher PPL.

As for overhydration, previous studies have established a pathophysiologic mechanism: fluid overload as an important cause for increased venous pressure, causing protein escape from the microcirculation in its venular segment due to venular hydrostatic pressure (16, 17). The magnitude of this increase can be assessed by patients' peritoneal protein loss. Regarding nutritional status, lean body mass index (LBMI) measured by BIA and corrected for body height square has been used as a useful marker (22). Our cohort shows that higher LBMI was independently associated with higher PPL, contradicting the previous viewpoint that higher PPL may cause hypoalbuminemia and malnutrition (1). Previous cohort studies have found similar results, defending that PPL can

TABLE 2 | Risk factors associated with peritoneal protein loss.

Variable	PPL				
	B	β	95% CI	Tolerance	VIF
CAPD (vs. APD)	1.769	0.265*	(0.284, 3.254)	0.841	1.190
Extracellular water excess	0.789	0.242*	(0.01, 1.567)	0.729	1.371
Cardiovascular disease	1.110	0.150	(-0.559, 2.779)	0.816	1.225
Lean body mass index	0.373	0.293	(0.106, 0.641)	0.950	1.053

Multivariable linear regression model (backward method), variables included in the model: age, gender, Charlson comorbidity index, cardiovascular disease, pulse pressure, serum IL-6 and albumin levels, CAPD (vs. APD), D/P creatinine, residual kidney function, IL-6 appearance rate, extracellular water excess, peritoneal protein loss and lean body mass. *mean $p < 0.005$.

Adjusted R^2 : 0.324, F: 8.773, Durbin Watson: 2.059.

TABLE 3 | Risk factors predictive of mortality.

Variable	All-cause mortality		
	HR	95% CI	p
Charlson Comorbidity Index	1.896	(1.235, 2.913)	0.003
Extracellular water excess	10.034	(1.426, 70.587)	0.021
Peritoneal protein loss	0.576	(0.339, 0.978)	0.041

Multivariable Cox regression model (conditional backward method), variables included in the model: age, gender, Charlson comorbidity index, cardiovascular disease, pulse pressure, serum IL-6 and albumin levels, residual kidney function, extracellular water excess, peritoneal protein loss and lean body mass.

be compensated by an adequate dietary intake (2, 18) and those patients who have better nutritional state may have more sufficient protein reserves, as well as more active protein metabolism in the peritoneal cavity and lead to more protein loss (18, 23). As for the relation of peritoneal protein leak with PD technique, this issue has also revealed to be a controversial topic. Kathuria et al., back in 1997, found no difference in nocturnal intermittent PD vs. CAPD, in terms of peritoneal protein leakage (24). In our cohort CAPD was associated with higher PPL. This comes in accordance to an interventional study, done by Cueto-Manzano et al., demonstrating lower PPL with short dwell-time periods and extended dry periods (25). Despite the controversy, APD could be a more feasible option in nephrotic patients, in order to decrease PPL, which could aggravate the clinical features in presence of the high proteinuria (opinion).

In the exploratory survival analysis, higher mortality was higher in overhydrated patients, with higher Charlson Comorbidity Index, but not peritoneal protein losses. The importance of fluid overload explains the association between peritoneal protein clearance and mortality reported in some epidemiologic studies and refutes assumptions on a possible role of endothelial dysfunction or inflammation (17, 26).

The present study presents with a number of limitations. It was a single-center cross study with a limited number of participants, mostly in early stage of PD exposure. The low number of deaths and the lengthiness of the study could be a limitation for the survival analysis. Strengths of this study rely on the use of measured total protein for 24 h peritoneal protein loss (instead of calculation) and second, the early stage of PD exposure can also be seen as a strength, as it enables insight in normal peritoneal physiology. Third, the use of bioimpedance to evaluate the link between PPL and body composition as an important evaluation tool, allowing the adjustment for multiple confounding covariates. It is recommendable to confirm these results in larger series.

This study illustrates the importance of overhydration, as a strong predictor of PPL, overpowering variables previously reported as determinants of PPL, namely clinical correlates of endothelial dysfunction or local inflammation. Also, survival analysis demonstrates the importance of overhydration as a strong prognostic factor. Peritoneal protein losses were not associated with malnutrition or higher mortality, emphasizing the importance of volume overload control, amenable by adjusted dialysis prescription, diuretic and water intake restriction.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Centro Hospitalar Universitário do Algarve (UAIF 192/2019). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AM: responsible for the investigational project, elaboration of the manuscript, and statistical analyses. RC and BR: responsible for clinical care and data collection. BR, MTF, ME, and JB: responsible for biological samples care and analysis. AS and AR: responsible for the investigational project and for reviewing the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the grant numbers UIDB/00215/2020, UIDP/00215/2020, and LA/P/0064/2020 respectively.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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5.4 Valuation of peritoneal protein loss associated with dialysis duration

Peritoneal Protein Loss with time in Peritoneal Dialysis

Malho Guedes, Calças Marques R, Domingos AT, Laranjo C, Silva AP, Rodrigues A, Krediet RT.

Semin Dial. 2024 May-Jun;37(3):242-248. doi:10.1111/sdi.13194. Epub 2024 Feb 29. PMID:38420712.

Short Summary

Despite previous investigation, knowledge concerning the changes in peritoneal protein loss over time were still not disclosed. The “classic” associations of PD duration in membrane damage due to interstitial fibrosis and vasculopathy might have a role. The aim of this study was to identify longitudinal changes of PPL in conjunction with its associated relevant clinical conditions.

For this longitudinal study, 52 PD patients gathered the inclusion criteria: simultaneous peritoneal equilibrium test and multifrequency bioelectrical impedance, on two different circumstances with a minimum of 6 months interval. Repeated measures analysis and Cox regression, were performed.

PPL did not change significantly after a median time of 15.5 months interval. A subgroup, with more than four years of interval between evaluations, was analysed showing significant decrease in PPL over time. This cohort presented a decrease of total Kt/V urea, creatinine clearance, PET ultrafiltration, residual kidney function and urinary volume but parameters of systemic inflammation, nutrition, serum total protein, albumin, D/P creatinine, free water transport and volume overload did not change significantly within follow-up under PD. No relationship was found between overall or cardiovascular mortality and PPL.

The decrease of peritoneal protein transport with PD duration can be attributed to a decreased hydrostatic filtration pressure resulting from vasculopathy. After controlling for confounders, PPL may act as a precocious biomarker of acquired venous vasculopathy, even if small pore fluid transport rates and free water transport are preserved.

ORIGINAL ARTICLE

Peritoneal Protein Loss With Time in Peritoneal Dialysis

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Received: 15 August 2023 | **Revised:** 22 October 2023 | **Accepted:** 28 January 2024

Funding: The authors received no specific funding for this work.

Keywords: peritoneal dialysis | peritoneal dialysis duration | peritoneal membrane | peritoneal protein loss

ABSTRACT

Longitudinal evolution of peritoneal protein loss (PPL), a reflection of hydrostatic pressure-driven leak of plasma proteins through the large-pore pathway, is not clear. Time on PD causes loss of mesothelial cells, vasculopathy, and increased thickness of the submesothelial fibrous layer. Are these structural changes associated with progressive increase of PPL, in a parallel with the rise in the D/P creatinine? The aim of the present study was to identify longitudinal changes of PPL over time. This single-center, longitudinal study included 52 peritoneal dialysis (PD) patients with a median follow-up of 26.5 months, evaluated at two different time points with a minimum interval of 6 months. Repeated measures analysis was performed using paired sample *t*-test or the nonparametric Wilcoxon signed-rank test, depending on the distribution. After a median interval of 15.5 months, lower levels of residual renal function and urine volume, lower Kt/V, and creatinine clearance were found. D/P creatinine and PPL were stable, but a decrease in ultrafiltration was present. Systemic inflammation, nutrition, and volume overload showed no significant change with time on PD. Analysis of a subpopulation with over 48 months between initial and subsequential assessment ($n = 11$) showed again no difference in inflammation, nutritional and hydration parameters from baseline, but importantly PPL decreased after more than 4 years on PD (mean difference 1.2 g/24, $p = 0.033$). D/P creatinine and dip of sodium remained unchanged. The absence of deleterious effects of time on PD is reassuring, pointing to the benefit of updated PD prescription, including the standard use of more biocompatible solutions towards membrane preservation and adjusted prescription avoiding overhydration and inflammation while maintaining nutritional status. After controlling for confounders, PPL may act as a biomarker of acquired venous vasculopathy, even if small pore fluid transport rates and free water transport are preserved.

1 | Introduction

Peritoneal dialysis (PD) protein loss has been presumed to be linked with detrimental effects, such as malnutrition, inflammation, and mortality [1, 2]. Studies' results were often not consistent, but recently overhydration appeared to be the major drive for higher losses, and therefore a confounder for the worse outcome associated [3, 4]. Longitudinal changes in protein clearance, predominantly a reflection of hydrostatic pressure-driven

leak of plasma proteins through the large-pore pathway, are less clear [5].

Mechanistically, serum protein can pass through interendothelial pores in the microcirculation, via interstitial tissue and the mesothelial layer to the dialysate-filled peritoneal cavity [6]. Then time on PD causes loss of mesothelial cells, vasculopathy, and increased thickness of the submesothelial fibrous layer [7]. These structural changes are associated

with progressive increase of the D/P creatinine, indicating an increase in the number of perfused peritoneal microvessels. However, in contrast to small solutes, peritoneal total protein and albumin clearance show no increase with PD duration [5, 8].

If, on one hand, peritoneal protein loss (PPL) is related to D/P creatinine in incident patients and cross-sectionally, there are several other factors interfering in such equation: (1) inflammation, (2) nutritional status, and (3) fibrosis. If inflammation arises with time on PD, its influence on PPL due to vascular hyperpermeability could be relevant. On the other hand, evolution of nutritional status could also be of importance; considering a long-standing PD patient who develops hypoalbuminemia, then serum protein pool presumably diminishes, and PPL will also decrease. The development of fibrosis and vasculopathy is associated with ultrafiltration failure. This may cause a decrease of the convective force to drive the protein clearance, and PPL would diminish.

The result of all of these factors on PPL is unknown, and its prognostic meaning uncertain. The aim of the present study was to identify longitudinal changes of peritoneal protein losses over time in conjunction with its associated relevant clinical conditions.

2 | Material and Methods

2.1 | Study Design, Patient Population, and Variables

This single-center, longitudinal study was performed in a cohort of 52 peritoneal dialysis patients that were treated with this mode of renal replacement therapy from April 2016 to April 2022. Their median follow-up was 26.5 months [IQI: 18.0–59.0]. Each patient served as his or her own control to assess the effect of PD duration on PPL. All included patients were investigated twice with a modified PET, as described by Bernardo et al [9], using 3.86/4.25% glucose solutions with 24-h collection of spent dialysate, and simultaneously a multifrequency bioelectrical impedance, on two different time points with a minimum of 6-month interval. Exclusion criteria encompassed patients with active infection or malignancy or who had experienced acute hospital admissions or a peritonitis episode during the preceding 3 months.

Baseline demographics and clinical features were registered. These included age, gender, weight, height, medication, cause of kidney failure, dialysis schedule, duration, residual renal urine volume, and solute clearance. All patients were treated with dialysis solutions containing low concentrations of glucose degradation products and a neutral pH (N-pH/L-GDP) (Baxter® and Fresenius®). The maximum glucose concentration prescribed was 2.27%/2.3%. Charlson Comorbidity Index was calculated at the time of study inclusion. Cardiovascular-related death was defined by diagnosed myocardial infarction, arrhythmia documented by electrocardiography or thromboembolic or hemorrhagic stroke. All were defined according to conventional clinical criteria or sudden death at home with a past story of cardiovascular disease.

2.2 | Sample Analysis

Effluent protein was measured using the biuret reaction method. Peritoneal protein clearance (PPCI) was calculated from the total protein loss in 24-h effluent/serum total protein, expressed as milliliter per 24 h. Hydration and nutritional status were assessed by multifrequency bioelectrical impedance (InBody S10 Body Composition Analysis; Biospace, Seoul, South Korea). Extracellular water excess (ECW_{ex}) was calculated as the difference between measured ECW and 0.613 × measured intracellular water (ICW) measured. Free water transport was calculated accordingly [9].

Routine biochemical analyses were performed by an automatic chemistry analyzer (Architect ci8200 Abbott®). Creatinine was measured with an enzymatic method to prevent glucose interference. Serum high-sensitivity C-reactive protein (CRP) was determined by immunoturbidimetry at the time of the PET. Residual renal function (RRF) was assessed as the mean of urea and creatinine clearance from the 24-h urine collection. Urea clearance index (Kt/V_{urea}) was derived from the 24-h urine and PD effluent collection.

2.3 | Statistical Analysis

Shapiro–Wilk test was used to check normality of the data. Results were expressed as frequencies and percentages for categorical variables, mean ± standard deviation (SD) for continuous variables, and median (interquartile range) for skewed distributions.

Repeated measures analysis was performed using paired sample *t*-test, for variables with a normal distribution. Skewed variables repeated measures were evaluated with the nonparametric Wilcoxon signed-rank test. Due to the low number in the subgroup analysis, only the nonparametric test was used.

Survival analysis using Cox regression was performed for technique failure and cardiovascular and overall mortality. For this analysis, PPL expressed as grams per 24 h at the first assessment was used [10].

The statistical analysis was performed using SPSS Version 22.0. Statistical significance was considered at or below 5% level.

3 | Results

3.1 | Patients' Characteristics

A total of 52 patients were included. Table 1 displays the main baseline characteristics. The most frequent etiology of renal failure was glomerular disease in 27%, followed by diabetes-associated disease (23%). Etiology was not determined in 21% of the patients.

Baseline assessment was performed early after the start of dialysis: the interquartile interval (IQI) was 1–8 months. The second evaluation, with a minimum interval of 6 months, was performed after a median gap of 15.5 months (IQI: 8–34 months). The median follow-up of the included patients was 26.5 months: IQI: 18.0–59.0.

TABLE 1 | Characterization of the patient population at baseline (demographics and comorbidities).

Demographics and comorbidities	
Number of patients	52
Age (years)	55 ± 2
Gender, male (%)	58%
Charlson Comorbidity Index	4 [2–6]
Diabetes mellitus	33%
Cardiovascular disease	35%
Hypertension	86%
Systolic blood pressure (mmHg)	140 [126–159]
Diastolic blood pressure (mmHg)	90 ± 15
Pulse pressure (mmHg)	49 [41–64]
Dialysis	
Time on PD (follow-up)	26.5 [18.0–59.0]
Assisted PD	11%
PD first	79%
Dialysis modality	
CAPD (vs. cycler)	31%
Cycler	69%
APD (no daytime dialysis)	59%
CCPD (with daytime exchange)	10%
Icodextrin use	33%

3.2 | Repeated Measures Analysis

The results of first and second evaluations are presented in Table 2. As expected, the population had lower levels of residual renal function and urine volume, leading to reduced values of Kt/V urea, creatinine clearance, and an increase in plasma urea nitrogen and creatinine. Parameters of peritoneal function showed no effect on small solute transport and on peritoneal protein loss, but a decrease in ultrafiltration was present. Parameters of systemic inflammation, nutrition (protein nitrogen appearance rate [PNA] and soft lean mass index), and volume overload showed no significant change with time on PD.

The length of the interval between evaluations could have been insufficient to demonstrate major changes, so in order to explore a possible detrimental effect of long-term PD, a subpopulation, presenting over 48 months between the initial and subsequent evaluation ($n=11$), was analyzed. Again inflammation, nutritional, and hydration parameters were not different from baseline (Table 3), but importantly, in this population, peritoneal protein loss decreased after more than 4 years PD. In contrast, D/P creatinine, a major driver of PPL, was unchanged during long-term follow-up. The dip of sodium (11 mEq/L [5–17] vs. 12 mEq/L [9–14], $p=0.563$) or the percentage of free water transport did not change significantly (0.42 [0.31–0.53] vs. 0.53 [0.49–0.56], $p=0.424$).

3.3 | Survival Analysis (Cardiovascular and Overall Mortality)

Nine patients died during follow-up: two deaths due to cardiovascular events, one in the context of catastrophic gastrointestinal hemorrhage, one death due to Covid-associated pneumonia, two deaths due to pneumonia, other due to epileptic encephalopathy, and two deaths attributed to cachexia. In the exploratory survival analysis of the cohort, no relationship was found between overall mortality and PPL (HR: 0.936, 95% CI: 0.698–1.253, $p=0.656$) nor between cardiovascular mortality and PPL (HR: 0.598, 95% CI: 0.233–1.537, $p=0.286$).

4 | Discussion

Peritoneal protein loss did not change significantly after a median time of 15.5 months in the present cohort. However, when considering only patients with more than 4 years' interval between evaluations, PPL showed a decrease. This finding together with the stability of the associated confounders suggests changes in the pathophysiology of large pore transport over time.

With regard to various variables previously mentioned, inflammation, it is well known that the large pore flux (and thus PPL) increases dramatically during peritonitis, that is, in the presence of severe inflammation that includes an influx of neutrophils to the peritoneal cavity [11, 12]. Subsequently, Lambie et al demonstrated independent effects of systemic and peritoneal inflammation [13], and later on, Yu et al argued that peritoneal protein clearance was a function of local (and not systemic) inflammation in incident patients on PD [11]. In peritonitis episode, local IL-6 production increases PPL not just by increased effective area (peritoneal solute transport rate) but also through an increase in the density of large pores, leading to a more leaky membrane. Nonetheless, this has been less clear in the context of a stable, noninfected peritoneal cavity [11]. Lindholm et al stated that albumin, the predominant plasma and dialysate protein, is able to pass through small pores (predominantly by convection) as well as large pores [14]. As for longitudinal analysis, previous studies have stated that inflammation arises with time on PD [15, 16]. Although, in this cohort, both systemic (measured by high-sensitivity PCR) and local (measured by intraperitoneal IL-6) inflammation were similar to baseline, PPL diminished during follow-up. A small subset of patients of the present study had intraperitoneal IL-6 measurement, and there was no difference in the group with wider follow-up (data not shown). Nonetheless, local inflammation is not expected to diminish with PD duration, so it would not explain the observed decrease of PPL with longer PD duration. It is possible that this time frame is capturing a moment previous to the expected inflammatory changes. If so, the absence of change in inflammatory markers, together with the diminution of PPL, reiterates the previous paper conclusions [3]: PPL do not seem related with either systemic or local inflammation in stable noninfected patients and in incident and prevalent PD patients.

Nutritional status, the second variable related to PPL in literature, has always been mentioned either as a cause or as a consequence. Several studies have refuted the causal relationship of

TABLE 2 | Characterization of the evolution of dialysis, biochemical and BIA parameters.

n = 52	First evaluation	Second evaluation	Repeated measures analysis				
			Mean difference	t	95% CI	z	p
Dialysis							
Peritoneal equilibration test + adequacy							
D/P creatinine	0.67 ± 0.13	0.70 ± 0.13	0.03	—	—	—	n.s.
Net UF (mL/4h)	900 [700–1,040]	793 ± 216	–141	—	—	–2,905	0.004
RRF (mL/min/1.73 m ²)	6.9 ± 3.7	4.2 [1.1–6.4]	–2.8	–5232	–3.813;–1.698	—	<0.001
Kt/V _{urea} (/week)	2.23 [1.99–2.76]	1.88 ± 0.55	–0.5	—	—	–3,916	<0.001
Creatinine clearance (L/1.73 m ² /week)	86.2 [63.3–105.0]	63.6 [38.6–82.9]	–25	—	—	–4,162	<0.001
PNA (g/kg/24h)	0.95 ± 0.24	0.89 ± 0.22	–0.06	–1789	–0.133;0.008	—	n.s.
Corrected percentage FWT	0.47 [0.40–0.58]	0.53 [0.47–0.63]	0.05	—	—	—	n.s.
24-h dialysate collection							
Protein loss (g/24h)	5.1 [3.8–6.7]	4.9 [3.7–6.7]	–0.1	—	—	—	n.s.
Peritoneal protein clearance (mL/24h)	73.9 [50.0–98.0]	66.9 [54.0–97.7]	–0.6	—	—	—	n.s.
Biochemical evaluation							
Hemoglobin (g/L)	117 ± 19	121 ± 15	0.4	1.579	–0.121;1.013	—	0.034
Blood urea nitrogen (mmol/L)	21.6 ± 5.5	25.2 [20.2–27.2]	3.4	—	—	–2,848	0.004
Creatinine (μmol/L)	566 [433–707]	787 ± 318	186	—	—	–4,208	<0.001
Glucose (mmol/L)	7.7 [6.0–12.8]	8.3 [6.7–11.6]	0.6	—	—	—	n.s.
Total protein (g/L)	70.0 ± 7.1	70.0 ± 8.0	0.1	—	—	—	n.s.
Albumin (g/L)	39.5 [35.6–43.2]	40.3 ± 5.8	0.3	—	—	—	n.s.
High-sensitivity C-reactive protein (g/L)	3.3 [1.6–8.5]	5.0 [1.3–10.9]	0.6	—	—	—	n.s.
24-h urine collection							
Volume (mL)	1706 ± 753	1136 ± 728	–548	–4,435	–796.2; 299.8	—	0.047
Protein loss (g/24h)	0.98 [0.51–2.95]	0.71 [0.35–1.62]	–0.53	—	—	–2,359	0.018
Bioelectrical impedance assessment							
Lean body mass (kg)	48.7 ± 10.8	48.3 ± 10.9	–0.4	—	—	—	n.s.
Soft lean mass/height ² (kg/m ²)	17.1 ± 2.3	17.0 ± 2.5	–0.1	—	—	—	n.s.
Extracellular/total body water (%)	39.7 ± 1.4	39.7 ± 1.6	0.003	—	—	—	n.s.
Extracellular water excess (L)	1.0 ± 0.9	0.8 [0.4–1.6]	0	—	—	—	n.s.

Abbreviation: n.s., nonsignificant.

TABLE 3 | Comparison of dialysis, biochemical and BIA parameters after more than 4 years on PD ($n=11$).

Dialysis	Wilcoxon signed-rank test		
	Mean difference	z	p
Peritoneal equilibration test + adequacy			
D/P creatinine	0.01	—	n.s.
Net UF (mL/4h)	−360	—	n.s.
RRF (mL/min/1.73 m ²)	−5.5	−2.936	0.003
Kt/V _{urea} (/week)	−1.1	−3.916	0.003
Creatinine clearance (L/1.73 m ² /week)	−49.6	−4.162	0.004
PNA (g/kg/24 h)	−0.1	—	n.s.
Corrected percentage FWT	0.06	—	n.s.
24-h dialysate collection			
Protein loss (g/24 h)	−1.2	−2.134	0.033
Peritoneal protein clearance (mL/24 h)	−17.6	−1.867	0.062
Biochemical evaluation			
Hemoglobin (g/L)	−0.3	—	n.s.
Blood urea nitrogen (mmol/L)	13.8	−2.041	0.041
Creatinine (μmol/L)	3.4	−2.490	0.013
Glucose (mmol/L)	−22.6	—	n.s.
Total protein (g/L)	−0.07	—	n.s.
Albumin (g/L)	0.09	—	n.s.
High-sensitivity C-reactive protein (g/L)	1.5	—	n.s.
24-h urine collection			
Volume (mL)	−1301	−2.845	0.004
Protein loss (g/24 h)	−169	—	n.s.

Note: Bioelectrical Impedance assessment parameters showed no statistically significant difference.
Abbreviation: n.s., nonsignificant.

PPL with malnutrition [3, 17–19]. Better nutritional status have conversely been associated with higher PPL [3]. In the present cohort, no change with PD duration of the analyzed nutrition markers, like PNA and lean body mass index, was present. So, sustained protein leakage throughout time does not compromise nutritional status significantly.

Venous congestion has been considered a culprit of convective transport for serum proteins more recently [4, 18]. In patients with 4 or more years on PD, reduced residual renal function and lower urine output can be expected. The fact that there was no change in hydration parameters demonstrates that an adjusted PD prescription with preserved free water transport capacity is feasible. Nonetheless, sustained hydration parameters do not explain the observed changes in PPL in the studied cohort.

Small solute transport, measured by the dialysate-to-plasma for creatinine (D/P creatinine), has been correlated with PPL [20]. The amount of PPL is also related to molecular weight: the lower the molecular weight of the protein, the higher its concentration in dialysate compared with plasma [2, 7, 21]. So, a proportional increase in D/P creatinine and PPL, mainly concerning albumin, over time would be expected. However, a progressive uncoupling between small solute transport rate and peritoneal total protein clearance occurs with PD duration, resulting in

absence of an increase of the latter with time [5]. D/P creatinine did not increase over time, but PPL decreased in the present cohort.

The relative decrease of peritoneal protein transport with PD duration can either be due to a decreased hydrostatic filtration pressure resulting from vasculopathy or to interstitial changes. Interstitial fibrosis is associated with a smaller distribution volume of a protein in the interstitium implying the possibility of an increase protein transport in long-term PD patients [22]. However, the observed relative decrease argues against peritoneal fibrosis as the explanation of the absence a time-related rise in PPL.

Vasculopathy is likely associated with PPL. It is attributed to deposition of advanced glycosylation end products [23]. This process induces subendothelial hyalinosis leading to various minor luminal stenoses that result in a reduction of the hydrostatic pressure gradient. This reduced hydrostatic pressure gradient explains the decrease in small pore fluid transport with PD duration [24]. Importantly, this cohort had sustained free water transport during the time of observation. So, it can be hypothesized that in addition to peritoneal fibrosis, vasculopathy is another sign of membrane dysfunction causing not

only ultrafiltration failure but also leading to a decrease in PPL, caused by the lower filtration pressure [7, 25].

Mortality was not associated with higher PPL in incident PD patients in the present study, and the long-term evolution showed a reduction in protein leakage. So, PPL should be regarded as an individual characteristic, integrated in the congestive and nutritional state of the patient plus membrane transport characteristics [3], that evolves with the acquired damage to the membrane, toward reduction, without affecting global or cardiovascular mortality.

There are several limitations to this study. Mainly, the small population size, and even smaller sample when analyzing wider intervals of time elapsed, can present as a bias. Sub-analysis of albumin loss was not performed. Technique failure was not considered as an end-point, which could give different insights in the relevance of the type of protein leaking. However, the inclusion of patient as their own controls allows to mitigate the effect of uncontrolled variables as well as the risk of bias. Our results suggest the uncoupling of small solute transport and PPL and support the contention that PPL in the long term may be a surrogate of vasculopathy.

The absence of deleterious effects of PD duration is reassuring, pointing to the benefit of updated PD prescription, including the standard use of more biocompatible solution, toward membrane preservation, and adjusted prescription avoiding overhydration and inflammation while maintaining nutritional status. After controlling for confounders, PPL may act as a biomarker of acquired venous vasculopathy, even if small pore fluid transport rates and free water transport are preserved.

Acknowledgments

The authors have nothing to report.

Ethics Statement

The study protocol was conducted in compliance with the ethical principles of the World Medical Association Declaration of Helsinki and approved by the Ethics Committee of the Centro Hospitalar Universitário do Algarve (UAIF 192/2019). Written informed consent was obtained.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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1. Abstract

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6. Discussion

6.1 Knowledge gap

Peritoneal protein loss has long been presumed to be a peritoneal dialysis detrimental consequence. Many studies were performed to evaluate the putative association with mortality, cardiovascular events or nutritional consequences. For instances, studies by Perl et al, Heaf et al, Pérez-Fontain et al, Rajakaruna et al, agreed that PPL was a strong independent marker for higher mortality in PD patients (27-29, 40). Chang et al, with a massive cohort of PD patients (n=540), showed PPL was a risk factor for cardiovascular events, but not mortality in these patients (32). Sanchez-Villanueva et, Balafa et al, Elsurer et al, did not find PPL to be a predictor of cardiovascular events and/or mortality (30,31,41). How come different studies are so contradictory? Could these differences between studies be explained by a physiology-based characteristic of the peritoneal transport?

Mechanistically, the peritoneal transport for macromolecules, such as proteins, occurs through large pores. Overall protein loss is dependent on the effective vascular peritoneal surface area (representing the number of pores) and the intrinsic size-selective permeability (the diameter of large pores). Charge selectivity is nonexistent (21-24). Consequently, changes in dialysate-to-plasma ratios for different proteins are correlated with molecular size (42). This dependency of transport on their molecular weight and diffusion coefficient has led to the development of the peritoneal protein restriction coefficient (PPRC). Hence, the higher the molecular size, higher the PPRC, and more restriction to transport (43).

Still, the definition of the large pores is a controversial issue. It has been defended to be represented either by vesicular vacuolar organelles, clusters of vesicles and cytoplasmatic vacuoles in clusters interconnected by fenestrae with opening and closing regulated by diaphragms, or intercellular gaps, 3-4 times larger than regular intercellular clefts (44).

The proposed mechanisms for the PPL association with worse outcome have been inflammation, malnutrition or endothelial dysfunction. These factors would enhance, in the peritoneum, the large pore transport, and worsen vital prognosis

by the systemic effect. But again, why so dissimilar results between studies? If the proposed mechanisms were unequivocal culprits for both results, higher PPL and worse outcome, shouldn't it become an unequivocal effect when present? Other hypothesis could rely on bias of the studies, namely by not adjusting for the same confounders, or by not including known factors that are responsible for a worse outcome.

This work tried to recap the existing information, uniformize the terminology and methodology for peritoneal protein leak determinations, and adjust for known confounders that could enlighten the PPL clinical significance.

6.2 Testing hypothesis

6.2.1 Different quantification methods, different meanings, different results

The scientific method assumes that for studies results' comparison, similar methodologies should be executed, clearly stated in the methods section and they must be reproducible. Regarding peritoneal protein quantification, several factors have been identified to interfere in such standardization (21).

First, the availability of different quantitative ways to measure PPL, such as turbidimetric (45), chemical (e.g. biuret and Folin-Lowry reactions) (27), and dye-binding or colorimetric (pyrogallol red molybdate) methods (40), all presenting with different detection limits, and questionable performance in other fluids than serum or urine (46).

Second, definitions used are not unequivocal. Peritoneal protein clearance, as a measurement for PPL, should be defined. Estimation equation of this clearance is given by: total protein loss in 24-h effluent/serum total protein, expressed per milliliter of plasma per day (29,47). Applying a correction factor, for extrapolation of the value of total protein from serum albumin, does not add precision to the characterization of PPL (29). Nonetheless it should be stated that peritoneal clearance, in rigor, should be better used for single proteins, as plasma and dialysate protein distribution are different. Moreover, the prognostic significance of albumin clearance or other single protein and total protein clearance may not be the same, and this needs specific assessment (41).

Third, determinations should be performed in a standardized, timely collection and reproducible manner (21). The effluent protein concentration (mg/dL) is not a reliable measure of protein loss (42). In peritoneal dialysis there are, at least 2 major ways of reporting such timely determinations, in 4-hour peritoneal equilibration test and 24-hour samples. Both collections have disadvantages in attaining scientific rigor. For instances, in the 4-hour PET samples, the different solutions used in different cohorts might add a bias in absolute comparisons between cohorts, as 2.27% or 3.86% dextrose fluids, drive different osmotic response in the same period of observation (48). As for the 24-hour collection, there might be inconsistencies in the collection of the samples. APD

patients may be particularly prone to incorrect sampling (21). In a quick analysis, PET protein loss quantification is seemingly more convenient than 24-hour measurements (29,48).

Furthermore, beyond the concern of standardization between trials methodology, do these measurements have the same meaning? Do they signal the same pathways?

In all the papers presented, the comparisons were done within the PD population of a single center, obviating the discrepancy related to the use of a different quantitative method. Also, peritoneal protein clearance was performed with the real measurement of serum total protein, avoiding bias related to correction factors. As for the timely preferred method, evidence was lacking to determine the most adequate measurement. So, investigation was conducted to understand correlations between 4-hour (g/240 minutes), peritoneal protein clearance (mL/24-hour) and 24-hour (g/24-hour) and clinical outcomes. The aim was to unveil distinctions between the timely-sampling techniques.

The investigation presented previously (**chapter 5.1**) showed that the 4-hour and the 24-hour determinations didn't have a strong correlation, as one might expect (49). Pearson correlations between g/24-hour and g/240 minutes (ρ : 0.482, $p < 0.001$) and between ml/24-hour and g/240 minutes (ρ : 0.437, $p < 0.001$). On the other hand, the different 24-hour collections, had a quite strong Pearson correlation between each other (for ml/24-hour and g/24-hour: ρ : 0.983, $p < 0.001$), signalling a more similar biological value. So, what is the meaning behind these findings?

This contrast between the 4-hour and the 24-hour methods was evaluated in the clinical context. In the cohort studied, there was a consistent uni- and multivariable relationship with D/P creatinine and creatinine clearance within the 4-hour collections. Supporting that PET protein loss quantification should be regarded as a marker of peritoneal membrane intrinsic permeability (49). Also, previous reports have stated that this type measurement may be a more specific marker of peritoneal large-pore dysfunction (29, 48). In contrast, relying on PET protein loss quantification to establish prognostic conclusions might be misleading. For instances, a previous study reported that estimation of PPL during PET is more accurate than 24-hour PPL, as independent marker of survival of PD

patients (29), this assumption probably reflects the membrane characteristics, namely a high transporter status, and doesn't reflect the overall clinical state of the patients or PD treatment and its adjustments to such characteristics. A previous line of thought, associated high transport status with mortality (50, 51), which was refuted by other studies (52,53), and more recent findings have demonstrated that if the high transport status is addressed with related dialysis adjustments (like icodextrin and APD), mortality or transition to hemodialysis aren't higher in these patients (54-56).

If 4-hour PET peritoneal protein determinations are a reflection of the membrane intrinsic characteristics, and this is not equivalent to 24-hour PPL quantification, then what does the latter stands for? In the presented cohort, age and CAPD (compared to cycler), body composition variables (such as water in excess and lean body mass index) and D/P creatinine were positively associated with greater PPL on 24-hour collections, in univariable analysis. So, these findings probably are more in line with dialysis prescription and clinical status, rather than a pathophysiologic pathway, as these variables imply more than just the membrane intrinsic permeability. After multivariable analysis, overhydration and D/P creatinine stood out as predictors of 24-hour PPL (49). Davenport et al, had already stated that hydration status did not influence peritoneal equilibration test ultrafiltration volumes (57). Importantly, hydration status is known to be associated with survival (58,59).

In conclusion, measurements of a 24-hour sample might be closer to patients' clinical status and prognosis, signaling opportunities of therapy intervention (49).

6.2.2 Overhydration identified, coincidence or causative?

Proteins escape the circulation in the venules, as it has been shown in previous experiments (55,60), through the large pores, probably represented by large interendothelial gaps in the vascular wall (60). As stated previously, this transport is dependent on the number and diameter of large pores and molecular size (21-24, 42). But which other forces determine its transport?

Regarding fluid passage, the Starling Principle states that fluid movements between blood and tissues are determined by differences in hydrostatic and colloid osmotic (oncotic) pressures between plasma inside microvessels and fluid outside them (61). Aberrant changes in the pressures (hydrostatic and oncotic) acting across the microvascular walls, alterations in the molecular structures in the endothelial wall (manifested as changes in hydraulic conductivity and the osmotic reflection coefficient for plasma proteins), or alterations in the lymphatic outflow system, can augment this flow of fluid from the vessel to the interstitial tissue (62).

Although macromolecules cross microvascular walls by a separate route, other than the main pathway for water and small solutes described, in theory, the same mechanisms could affect macromolecule transport. Following this line of thought, for instances, higher PPL would be related with higher hydrostatic pressure or derangements in venular endothelium of the peritoneal microvessels. Moreover, it is known that hydrostatic pressure on the venous side of the microcirculation raises when blood volume is expanded or there is obstruction at the venous end (63), and progressive subendothelial hyalinization, with luminal narrowing or obliteration is a described feature with time on PD, reported with bioincompatible solutions (64).

Following this rational, if fluid overload is an important cause of an increased venous pressure, hydrostatic convection will be a major mechanism for peritoneal protein transport. Krediet et al, recently shown that both serum NT-proBNP and right atrial area are independently and positively associated with peritoneal protein clearance. These findings confirm that venous congestion is likely to be important in PPL (65).

To confirm these findings, a study was conducted to determine the importance of overhydration on the magnitude of peritoneal protein clearance in prevalent PD patients was evaluated. In the presented cohort in **chapter 5.2**, peritoneal protein clearance was positively associated, with age, male gender, presence of cardiovascular disease (trend, $p=0.052$), CAPD (compared to cycler), D/P creatinine, lean body mass index and extracellular water excess. Importantly, the association with total serum protein and albumin was inverse. The multiple linear regression model, adjusted for the significant variables, identified extracellular water excess and D/P creatinine as predictors of a higher peritoneal

protein clearance. Each litre of overhydration was associated with a rise of peritoneal protein clearance of 18 mL/24h (66).

Thereby, fluid overload increases venous pressure and augments protein escape, the magnitude of which can be assessed in PD patients by peritoneal protein clearance. In conclusion, both increased effective capillary surface and increased hydrostatic pressure are drivers of peritoneal protein leak (65-67).

6.2.3 Are nutrition and inflammation still important?

Nutritional status is an indissociable thought when addressing PPL, commonly described as a consequence. The rationale relies on the idea that continuous loss of proteins and amino acids will diminish protein reservoir. Nonetheless, studies have not been consistent demonstrating a clear association. This could also be difficulted by the lack of standardization in the clinical indicator used in studies to ascertain and compare nutritional status. In the past many studies used to rely on albumin serum levels, or even total protein, and reported different outcomes on PD, with these levels remaining stable (67,68), in the low-normal range (34,69,70) or evolving with “progressive hypoproteinemia and hypoalbuminemia (71). However, albumin serum levels (and subsequently total protein) can be influenced by many factors, like rate of production, rate of clearance, inflammation and individual clinical status; and it has been inconsistently associated with nutritional status in PD patients (21,72,73).

Protein nitrogen appearance (PNA) has been used and validated in many studies as a nutritional marker in peritoneal dialysis patients (74, 75). Also, lean body mass index (LBMI), evaluated by bioelectrical impedance analysis has proven to be valuable in these patients (76,77), and a baseline lower LBMI was associated with higher mortality (78).

To understand the effect of PPL on nutritional status, studies including albumin, PNA, BIA determinations and peritoneal protein loss were conducted (**chapter 5.2** and **chapter 5.3**). In both cohorts, univariable analysis showed a negative correlation with serum albumin levels, but a positive association between PPL and LBMI. PNA had no significant association with PPL. Exploring the second

study (**chapter 5.3**), with a wider cohort (n=78), the best model on multivariable analysis (variables included in the model: age, gender, Charlson comorbidity index, cardiovascular disease, pulse pressure, serum IL-6 and albumin levels, CAPD (vs. APD), D/P creatinine, residual kidney function, IL-6 appearance rate, extracellular water excess, PPL and lean body mass) showed a significant association between PPL and extracellular water excess, lean body mass index and performing CAPD (in this best model, cardiovascular disease was considered without attaining statistical significance). Interestingly, when reporting such association between PPL and nutritional status, evaluated by BIA, these results contradicted previous reports and showed a significant positive association amongst PPL and LBMI (95% CI: 1.720–11.219; p=0.008). Hence, the assumption of higher PPL being associated with worse nutritional parameters, like lean mass body index and PNA was refuted (49,79). On the contrary, those patients with better BIA scores had higher PPL.

Reviewing recent publications, also, Fan et al, stated higher LBMI was independently associated with higher peritoneal protein clearance, indicating that better nutritional status dominates peritoneal protein metabolism in PD patients (80). Do et al. demonstrated that PPL is not associated with loss of muscle mass, strength or sarcopenia, as long-term markers of malnutrition (35).

Additionally, in the hereby presented cohort no change with PD duration of the analyzed nutrition markers (PNA and lean body mass index) was present. So sustained protein leakage throughout time does not compromise nutritional status significantly (79). These findings confirm that the continuous loss of proteins and amino acid can be compensated by adequate dietary intake. The target for dietary protein intake is generally regarded as being 1.2 g/kg/day; it should not be below 0.8 g/kg/day in any patient (72). On average, the achieved normalized protein nitrogen appearance (nPNA) needs to be 1 g/kg/day, adjusted for the patient's age and physical activity (81).

Another factor often strongly associated with PPL is inflammation. It is well known that the large pore flux (and thus PPL) increases dramatically during peritonitis, i.e., in the presence of severe inflammation that includes an influx of neutrophils to the peritoneal cavity (82,83). Subsequently, Lambie et al, demonstrated independent effects of systemic and peritoneal inflammation (84),

and later on, Davies et al., argued that peritoneal protein clearance was a function of local (and not systemic) inflammation in incident patients on PD (82). In peritonitis episode, local IL-6 production increases PPL not just by increased effective area (peritoneal solute transport rate), but also through an increase in the density of large pores leading to a more leaky membrane. Nonetheless, this has been less clear in the context of a stable, non-infected peritoneal cavity (82). Lindholm et al, stated that albumin, the predominant plasma and dialysate protein, is able to pass through small pores (predominantly by convection) as well as large pores (85).

A study was conducted to perceive, in stable, non-infected patients, the relationship between systemic and local inflammation (by quantifying the rate of appearance of IL-6 in the dialysate and serum IL-6) with PPL. In the analyzed cohort, the univariable analysis showed a consistent association of PPL with small solute transport and dialysate appearance rate of IL-6, but the multivariable analysis neglected the hypothesis of local inflammation as a driver for PPL when overhydration was considered (79). Again, overhydration seems to overpower every other known associated factor with protein leakage to the peritoneal effluent. Possibly, the rationale in these findings, rely on the convection being the previous explanation for the former encountered association of local inflammation with higher PPL. This convective force is not present in the peritonitis episodes, due to the loss of ultrafiltration. In the presence of local inflammation, with the influx of neutrophils and local production by inflammatory cells, it becomes the major drive for the rise in PPL. In the steady state, again, after adjusting for such confounders, higher hydrostatic pressure, not local or systemic inflammation, explains PPL.

Consequently, the assumptions of an association of PPL with inflammation and malnutrition were not confirmed (66,79).

6.2.4 Variation with PD technique

As for the relation of PPL with the type of PD technique, this issue has also revealed to be a controversial topic. Kathuria et al., back in 1997, found no difference in nocturnal intermittent PD vs. CAPD, in terms of peritoneal protein

leakage (86). Cueto-Manzano et al., demonstrated that in patients with high peritoneal permeability, PPL are significantly reduced on short dwell-time periods and extended dry periods (87).

In the studies performed (**chapter 5.2** and **chapter 5.3**), population characterization included the technique performed by each patient, and the relationship with PPL was analyzed (49, 79). In both cohorts, an association between CAPD and higher PPL was found (49, 79). Again, of importance, this association is not identifiable when considering 4-hour PET protein loss measurements, as PET standardized determinations expose membrane characteristics and not the impact of prescription or clinical milieu of the patient (49). In the 24-hour samples, the differences found were of 2.3g/day (7.4 g/24h vs 5.1 g/24h, $p=0.002$).

Despite the controversy, APD could be a more feasible option in nephrotic patients, in order to decrease PPL, which could exacerbate the clinical features of hypoalbuminemia concurrent with the high proteinuria (79).

6.2.5 Evolution throughout time

During peritoneal dialysis, peritoneal cells are repeatedly exposed to a non-physiological hypertonic environment with high glucose content and low pH (88,89). Longitudinal changes in the peritoneal membrane comprehend the loss of mesothelial cells with epithelial-to-mesenchymal transition of mesothelial cells, vasculopathy and increased thickness of the submesothelial fibrous layer (12,43,88,90). This results in peritoneal dysfunction, driven by the dissipation of the osmotic driving force through increased area and solute transport that accompanied neoangiogenesis of the submesothelial microvasculature. The increase in the number of perfused peritoneal microvessels is associated with progressive increase of the D/P creatinine (88).

Assuming that serum proteins pass through interendothelial pores in the microcirculation, via interstitial tissue and the mesothelial layer, to the dialysate-filled peritoneal cavity (89), mechanistically what should we expect regarding the PPL evolution with time on PD? Are longitudinal changes in protein clearance,

predominantly a reflection of hydrostatic pressure-driven leak of plasma proteins through the large-pore pathway? Literature is scarce but has described that, in contrast to small solutes, peritoneal total protein and albumin clearance show no increase with PD duration (91,92).

To address the evolution of PPL throughout time, a longitudinal study was conducted, with repeated measures time (**chapter 5.4**). The cohort studied showed a decrease in PPL over 4 years of follow-up, with a mean difference of 1.2 g/24, $p=0.033$, without any significant differences in inflammation, nutritional and hydration parameters, D/P creatinine and free water transport from baseline (93). This is the first study that, with the stability of all major confounders throughout time, shows what is happening with large pores transport.

Exploring each one, now in a longitudinal view:

- Inflammation: previous studies state that inflammation arises with time on PD (88,94). Although in this cohort, both systemic (measured by high-sensitivity PCR) and local (measured by intraperitoneal IL-6) inflammation were similar to baseline, PPL diminished during follow-up (93). It is possible that this time frame is capturing a moment previous to the expected inflammatory changes. If so, the absence of change in inflammatory markers, together with the diminution of PPL, reiterates the previous paper conclusions (79): PPL don't seem related with either systemic or local inflammation in stable non-infected patients, in incident and prevalent PD patients.
- Nutrition: peritoneal loss of proteins was considered to cause malnutrition in prevalent PD patients, due to its continuous nature. This relationship was previously refuted (35,66,79,80), and also the cohort presented in **chapter 5.4** (93) disclosed that nutritional parameters remained stable, despite the duration of PD. Not only higher PPL were not found to be related with lower LBMI in the incident population, but also there was no evolutionary relationship with vintage. The study presented (93) showed the decrease of PPL with time in PD with stable nutritional parameters (nPNA and lean body mass index).
- Peritoneal transport solute rate, measured by D/P creatinine: in incident PD patients, D/P creatinine has been positively correlated with PPL (30).

The presented cohorts (49, 66) are consistent with such findings (**chapter 5.1** and **chapter 5.2**). However, a progressive uncoupling between small solute transport rate and peritoneal total protein clearance occurs with PD duration. This unmatched evolution is related to an increase in small-pore area, increasing D/P creatinine, due to an increase in the anatomic membrane in contact with dialysate, an increase in the density of perfused capillaries or an increase in capillary perfusion rate (or any combination of these) (91,95). This dissociation, has been proposed to be only due to increasing PTSR, with stability over time of the number of large pores (95,96). Opposing to such theory, in the studied cohort, in a time frame yet without the increase of D/P creatinine, there was a longitudinal decrease in PPL (93). So, this uncoupling must occur based on the diminution of the number of large pores, or other local alteration is the culprit to prevent protein to escape circulation.

- Interestingly, free water transport also remained unaltered in the time period analyzed. The reduction of FWT is one of the most precocious signs of membrane dysfunction, developing in about 21% of prevalent patients after 2 years of DP (97), and with a subsequent decrease of 67% of the initial value by 3 years-time (98,99). Interstitial fibrosis has been the major culprit for the reduction of FWT (98).
- Overhydration, and presumably hydrostatic pressure, thoroughly studied previously, also could not explain the diminishing of peritoneal protein loss, as the excess of extracellular water remained unchanged through time.

In conclusion, with all the confounders naturally “adjusted”, PPL diminished with PD vintage, meaning that the large pores transport is decreasing. Why is that occurring?

The documented development of fibrosis and vasculopathy with time on PD are associated with ultrafiltration failure. This could be the explanation of a decrease of the convective force to drive the protein clearance, and PPL would diminish. Against such argument is the absence of fibrosis (clinically demonstrated by the FWT maintenance). On the other hand, interstitial fibrosis is associated with a smaller distribution volume of a protein in the interstitium implying the

possibility of an increase protein transport in long-term PD patients (100). In summary, the observed relative decrease argues against peritoneal fibrosis as the explanation of the absence a time-related rise in PPL (92,93).

As for vasculopathy, the hypothesis that PPL reflected generalized endothelial dysfunction seems attractive, however there are several studies denying the association between PPL and cardiovascular morbidity, events or mortality (82,91,93). It seems illogical to endorse systemic endothelial injury as the cause of PPL, when PPL decreases with PD duration, despite the fact that comorbidity (and thus underlying systemic endothelial disease) tends to accumulate with time passing by and aging.

So, the relative decrease of PPL with PD duration can be hypothesized to be due to a decreased hydrostatic filtration pressure resulting from peritoneal vasculopathy. It is attributed to deposition of advanced glycosylation end products (101). This process induces subendothelial hyalinosis leading to various minor luminal stenoses that result in a reduction of the hydrostatic pressure gradient. This reduced hydrostatic pressure gradient also explains the decrease in small pore fluid transport with PD duration (102).

Additionally, in the view of present study (**chapter 5.4**), a timeline of injury could be drawn, with peritoneal vasculopathy being the more precocious sign of membrane dysfunction. This leads to a decrease in PPL, due to a lower filtration pressure, which, in addition to local fibrosis, evolves with FWT reduction causing ultrafiltration failure (43,93,103).

6.2.6 Mortality

One of the major controversies around peritoneal protein loss involves its direct impact on survival. If a relation of cause-effect was to be proven, this could mean the possibility to identify a therapeutic target to improve survival on PD.

Several studies confirm association with worse outcome, as stated previously (27-29,40,104,105) and updated in table 1.

Table 1 – analysis of studies associating PPL with mortality

<i>Author, year</i>	<i>Nr. of patients</i>	<i>Mortality predictors</i>	<i>OH evaluation</i>	<i>Comments</i>
Heaf et al, 2005 (28)	155	Large pore fluid flux (J _{vL})	Clinical judgement	“This study does not therefore have the power to differentiate whether J _{vL} is just a marker of increased mortality or a direct cause secondary to increased protein loss”
Perl et al, 2009 (27)	192	Age, comorbidity grade, PPCI	no	PPCI calculated with correction factor
Pérez-Fontán et al, 2010 (29)	269	Age, comorbidity grade, albumin, CRP, GFR, PET-PPL	no	Inferences made with incident PET PPL determinations
Rajakaruna et al, 2015 (40)	300	Age, comorbidity grade, %ECW/TBW, PPCI (higher quartile)	yes	Dichotomized non continuous PPL; dichotomized ECW/TBW; other significant variables were not included in the model: D/P creatinine, gender, CAPD
Lu et al, 2018 (105)	711	Comorbidity grade, overhydration, PPCI	yes	PPCI calculated with correction factor; significant variable not included in the model: D/P creatinine

However, other authors refute PPL association with higher mortality in PD patients, as shown in table 2. It is interesting to notice that several studies have come across the association of PPL with overhydration.

Fluid volume overload is a well-recognized independent predictor of cardiovascular events and mortality in these patients (106,107). So, it was hypothesized that overhydration could be a potential confounder, disregarded in many studies on peritoneal protein loss, and could be the missing link between peritoneal protein loss and mortality.

Proper PD prescription adjustments have proved to be capable of hinder high PTSR or overhydration, namely by the introduction of icodextrin or use of APD (55,56). Euvolemia is a goal to achieve in order to provide the best care for these patients, either by preserving RKF or by raising UF.

Table 2 – analysis of studies refuting the association of PPL with mortality

<i>Author, year</i>	<i>Nr. of patients</i>	<i>PPL associations</i>	<i>OH evaluation</i>	<i>Comments</i>
Szeto et al, 2005 (33)	43	Diabetes, preexisting CVD, D/P creat, body weight, proteinuria	no	Peritoneal albumin loss was a risk factor for CV events, but not higher mortality
Sanchez-Villanueva, 2009 (30)	133	MTAC creat, albumin (inv)	no	PPL was a risk factor for peripheral artery disease, but not higher mortality
Elsurer et al, 2009 (41)	66	Not studied	no	Peritoneal albumin loss was not a risk factor for CV events or mortality
Dong et al, 2010 (104)	216	Inflammation, overhydration	yes	In the model with OH, PPCI is not statistically significant for mortality
Balafa et al, 2011 (31)	257	MTAC creat	no	Peritoneal albumin loss was not a risk factor for CV events or mortality
Chang et al, 2013 (32)	540	Pulse pressure, diabetes, CRP, D/P creatinine, kt/v, albumin (inv)	no	PPL was a risk factor for CV events, but not higher mortality
Krediet et al, 2019 (65)	316	venous congestion	yes	Mortality wasn't evaluated, hypothesised to be a consequence of venous congestion
Yu et al, 2019 (82)	257	Local membrane inflammation and membrane area	no	PPL was not a risk factor for higher mortality
Malho Guedes et al, 2021 (66)	67	Overhydration, D/P creatinine	yes	Mortality wasn't evaluated, hypothesised to be a consequence of venous congestion
Niu et al, 2022 (108)	318	Albumin (inv), BMI, MTAC creat	no	PPL was not a risk factor for CV events, but not higher mortality
Malho Guedes et al, 2022 (79)	78	CAPD (vs. APD), overhydration	yes	Higher mortality in overhydrated patients with higher comorbidity grade. NOT with higher PPL

The path made, through review of previous investigations, defining the more adjusted methodology, and exploring independent associations of clinical variables with the outcomes, provided the identification of overhydration as a driver for PPL. In the study presented in **chapter 5.2**, the interference of overhydration, through raising hydrostatic pressure, in the amount of protein loss through the membrane was confirmed. The hypothesis raised by Krediet, and by our group, that mortality was a consequence of overhydration and venous congestion, instead of PPL, was addressed in **chapter 5.3**. After multivariable analysis, overhydration stood as the strongest risk factor for unfavorable outcome, with PPL in the model (79). And this is, in fact, amenable of intervention and prognostic changing.

Another interesting point of view, in terms of mortality analysis, would be the fact that PPL decreases over time. Wouldn't it be expected to raise, if it was one of the culprits for worse vital outcome?

6.2.7 Clinical Insights

Chapter 5.4 gives a reassuring perspective of how the peritoneum evolves during the course of peritoneal dialysis. Interestingly, the reduction of PPL over time, in a cohort which small pore fluid transport rates and free water transport are preserved, is supporting the benefit of updated PD prescription, including the standard use of more biocompatible solutions, towards membrane preservation, and adjusted prescription avoiding overhydration and inflammation while maintaining nutritional status (93).

Despite PPL being called “the detrimental effect”, it seems PPL should be regarded as an individual characteristic, resulting of the intrinsic membrane permeability and large pores distribution, combined with the congestive and nutritional status of the patient (68), that evolves with the acquired damage to the membrane, towards reduction, without affecting global or cardiovascular mortality (93).

6.3 Future directions

All investigations open new tracks, further questions.

6.3.1 Peritoneal Albumin Loss

Albumin loss, due to its lower molecular weight, and to its association with systemic inflammation, should be studied and addressed distinctly. The passage through small pores, namely when peritonitis ensues (47,92), and the uncoupling between higher transport evolution and albumin loss with PD duration (82,89), reveals incongruous mechanisms present.

Peritoneal albumin excretion was associated with the extent of calcified and ulcerated carotid atherosclerotic plaques by Doppler ultrasound indirectly supporting the relation with endothelial dysfunction (33). This connection, raises the hypothesis of a more complex relationship of peritoneal albumin loss, hypoalbuminemia and inflammation with systemic endothelial barrier (109, 110).

This relationship with atherosclerosis and cardiovascular disease, resembles microalbuminuria related studies. Persistent microalbuminuria was consistently associated with increased systemic transvascular albumin leakage, in a level similar to that seen among individuals with severe clinical atherosclerosis (111-113). Despite that, diabetes, an albuminuria-driven disease, has not been associated with higher peritoneal protein loss in previous studies (114) and in the cohorts studied during the course of this thesis (data not shown).

Study and analysis of peritoneal albumin loss should be focused outside the scope of total PPL.

6.3.2 Metabolomic and proteomic analysis

Characterization of the type of proteins leaking or even the final products of local metabolism, in the peritoneal effluent, could enlighten in a more specific way

the biologic value of such losses. Metabolomic and proteomic analysis of peritoneal dialysate may provide clues to the biological and pathological processes involved in peritoneal damage (115-117). Preliminary findings point out that metabolome correlates with PET findings (118) and proteome even show alterations much earlier than PET monitoring (119).

Systematic global identification and quantification of proteins or metabolites can, not only improve biomedical understanding of a particular system in individuals, but also be used for protein biomarker discovery (60). These are extremely relevant areas of study to broaden the findings regarding what is happening within the peritoneal membrane.

6.3.3 Loss of immunoglobulins and risk of infection

It has been proposed that there is a predictive role of PPL in peritonitis. Preliminary proteomic studies detected that some types of protein that leak into the peritoneal cavity can modulate the action of leucocytes and favor the onset of local infection (117). Clinical studies revealed that baseline peritoneal protein leakage was able to independently predict risk for peritonitis, which was not explained by systemic inflammation (29,47,120). It has also been postulated that local inflammation in the peritoneal cavity, possibly related to vascular endothelial growth factor and leptin released by the local adipose tissue, leading to vasodilatation and inter-cellular fenestra, may contribute to the increased risk for peritonitis with high PPL (109). It is conceivable that the immune competence of patients with higher PPL may be compromised by the continuous leak of immunoactive proteins. On the other hand, persistent overhydration has been associated with the risk of peritonitis (121). Again, is it the overhydration augmenting PPL and the loss of immunoglobulin? Does this loss of immunoactive proteins carry a higher infectious burden to these patients, resulting in more systemic or localized infections? Cohorts comparing HD and PD patients, haven't stated such differences (122-124), rather they have shown less septicemia incidence in PD patients (125).

In few and older studies, losses of other specific proteins were associated with a series of disorders observed in PD patients, including dyslipidemia,

prothrombotic and hormonal disturbances (126-128). In the light of new biocompatible solutions and PD evolution, these findings should be confirmed and its clinical impact should be ascertained.

6.3.4 Pseudohypoxia and other membrane specificities

The high content of glucose in PD solutions, led to the development of the pseudohypoxia theory by Professor Krediet, twenty years ago (129). This is based on the NADH/NAD⁺ reaction that occurs in the setting of severe hyperglycemia. In this situation the formed NADH is due to the metabolization of glucose in the sorbitol pathway and cannot be oxidized to NAD⁺; resulting in a high cytosolic NADH/NAD⁺ ratio that is very similar to the situation in hypoxia or ischemia and is therefore known as pseudohypoxia (89).

Several papers have been published in accordance to such theory, based on the increment of pro-fibrotic proteins levels in the dialysate with the duration of dialysis. Several factors have been identified throughout the years, namely GLUT-1, VEGF, TGF β , PAI-1, among others (130-132). The study of such derivatives, and news to be identified, might be a window to the peritoneal membrane, and the aging process with the exposure to PD solutions. Also, as PPL diminish with time, the evaluation of the changes on the pattern of proteins and metabolites that are lost with time, should be assessed.

6.3.5 Value as a risk stratification tool

The correlation found between PPL and overhydration could favor PPL as an indirect sign of unadjusted PD prescription. Peritoneal dialysis maintenance without increasing volume status, nor major deleterious corporal composition trends, is feasible under careful therapy strategies (133), namely with water and salt restriction, adjusting diuretics, introducing icodextrin or APD to attain normovolemia. Longitudinal application of BIA may be a useful clinical tool to evaluate adequacy, but availability might not be universal.

Future longitudinal studies addressing current peritoneal dialysis prescription and bioimpedance analysis should be focused to enlighten the clinical impact and usefulness of monitoring related peritoneal protein clearance or loss.

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7. Conclusions

This work results after a thorough investigation of the state of the art and critical appraisal of the gap of knowledge. Addressing different dimensions of the peritoneal loss, starting with methodology, exploring mechanisms and longitudinal evolution, and ending in associated prognosis, this thesis enlightens the possible reasons behind controversial findings when considering peritoneal protein loss consequences.

7.1 Improvement of methodology

Starting from the very beginning: the choice of the right methodology to measure and report peritoneal protein leakage is necessary. The scientific method requires a reproducible methodology, with adequate determinations to the purpose of research. **Chapter 5.1** testes different methodologies used in the quantification of peritoneal protein leakage.

Through this study, it was concluded that the use of PET standardized collection is applicable for membrane characterization, and in this case, characterization of large pores transport. PET protein loss quantification should be regarded as a marker of peritoneal membrane intrinsic permeability (49).

As for 24-hour determinations, the overall clinical status is the target of analysis. Measurements of a 24-hour sample might be closer to patients' clinical status and prognosis, signaling opportunities of therapy intervention (49).

In this way, this work standardizes, for the first time, different methodology for different investigational questions.

7.2 Enlightening of PPL mechanisms

Previous publications characterized PPL associated factors, like low albumin serum levels (30,32,108), high D/P creatinine (32,33) or MTAC (30,31,108) and inflammation (32,82,104), amongst others. Also, an association with malnutrition has long been stated, despite unstandardized definitions and methodologies to describe nutritional status.

These associations were tested in the PD population of the outpatient clinic of CHUA. Inflammation, systemic and local, was assessed by dosage of serum and dialysate IL-6, respectively. Bioimpedance analysis, which provides a reliable evaluation of body composition in peritoneal dialysis patients, was routinely performed. Results were contradictory with previous findings.

Scrutinizing the data available, it was possible to recognize there was no association of PPL with malnutrition, as measured by body cell mass or lean body mass index (66). Likewise, systemic or local inflammation were not consistent with higher PPL. On the other hand, characterization of the hydration state showed a direct relationship between PPL and extracellular water excess. Interestingly from the former known factors, in multivariable analysis, only peritoneal small solute transport was predictive of PPL, in conjunction with overhydration.

Fluid overload is an important cause of increased venous pressure, so these findings support the importance of hydrostatic pressure-induced convection for PPL (65,66). As for longitudinal evaluation of PPL, it showed a reduction in the protein leakage with time on PD. In the cohort presented, the reduction of PPL, is probably due to a decreased hydrostatic filtration pressure resulting from peritoneal vasculopathy, which seems to be the most precocious sign of membrane dysfunction, previous to local fibrosis.

Hydrostatic pressure and local vasculopathy were identified as major determinants for PPL (65,66,93).

7.3 Reassurance of PD technique safety

PPL does not stand as a detrimental effect of PD therapy.

The association of PPL with malnutrition was not confirmed, and even after long duration of PD treatment, the continuous loss of proteins was not associated with negative nutritional evolution or sarcopenia.

The controversial association of PPL with mortality, was not confirmed, if adjustment for extracellular water was done. Hypervolemia has long been associated with worse outcome in this population and the magnitude of fluid overload explained the association between peritoneal protein clearance and mortality reported in some epidemiologic studies, overpowering a possible role of endothelial dysfunction or inflammation. This emphasizes the importance of volume overload control, amenable by adjusted dialysis prescription, diuretic and water intake restriction.

Noteworthy, after 4 years on PD, small pore fluid transport rates and free water transport were preserved, supporting the safety of PD, namely with the use of biocompatible solutions and adjusted prescription.

In conclusion, despite previous reports, peritoneal protein loss was not associated with worse outcome, namely mortality, cardiovascular morbidity or nutritional complications. So, it seems although PPL has been recognized as a feature of peritoneal dialysis since its inception, it shouldn't be considered deleterious, but as window to monitor peritoneal membrane health.

Peritoneal dialysis stands as a valuable therapy for treatment of chronic kidney disease patients, with proper therapeutic adjustments (eg. Icodextrin, automated PD).

7.4 Limitations and strengths

This work has several limitations, namely related with the limited sample size, a single centre-study and observational nature. The lack of quantification of more effluent inflammation biomarkers analysis or differentiation of specific protein clearances (for instances: β 2-macroglobulin, α 2-macroglobulin), when studying the local peritoneal membrane inflammation or membrane transport characteristics, limiting the discussion of the findings. Sub-analysis of albumin loss was not performed. Transition to hemodialysis was not considered as an end-point, which could give different insights in the relevance of the type of protein leaking. Proteomic study was not available, for that matter.

Nonetheless, the studies presented here, came in line to confirm clinical associations explored previously. The investigation evolved to explain their meaning, like the relationship of overhydration with higher PPL and the posterior link described between fluid overload and mortality. It reassures the safety of long-term PD treatment, and relegates PPL to an associated feature. PPL was not confirmed as a risk factor for worse outcome, so it is also not a candidate for intervention, on the contrary, it stresses the need to achieve normovolemia in PD patients to better treat these patients.

This work also raises several questions to be studied in a near future.

Future longitudinal studies addressing peritoneal loss of albumin, metabolomic and proteomic analysis, clinical impact of specific proteins loss and usefulness of peritoneal protein clearance as a membrane monitoring tool (134).

Peritoneal dialysis stands as a valuable therapy for treatment of CKD patients and an important field for research, namely on these peritoneal effluent specificities.

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1. Abstract

2. Introduction

3. Aims and outline of the thesis

4. Methods

5. Results

6. Discussion

7. Conclusions

8. References

9. ACKNOWLEDGMENTS

9. Acknowledgments

9. Acknowledgments

The quest for knowledge, the quest for critical thinking, the quest for constant defiance of my comfort zone, the quest for improvement...that defines me, my life and my doctorate journey. In this last adventure I had the companionship, the support and the challenge of many important people in my life, and I am thankful.

First, I must thank Professor Anabela Rodrigues, for the person she is, for her trust and friendship, for all her challenges and dares, she has been an inspiration, a hands-on helper and a leader.

Along the way, Professor Raymond Krediet brought critical insights, improvement and growth to my academic journey. I had the privilege to learn with one of the biggest names in Peritoneal Dialysis... A living legend, thank you!

Acknowledge Dr. Ana Paula Silva, who through constant resilience example, accompanied most of my professional challenges.

I would like to recognize my team... Nurse Céu Laranjo, Dr. Idalécio Bernardo, Dr. Ana Domingos, Dr. Roberto Calças Marques, I must realize you are the heroes in this journey and it has been a pleasure to share this PD adventure with you. Simultaneously recognizing the Nephrology Department: Doctors, Nurses, Assistants, all have had a part in taking care of these patients, and helping my journey. Thank you all for your support. Moreover, I must thank some very special companions: Idália Carvalho, Nurse Joana Fontes, Dr. Joana Vidinha, for your precious words and encouragement. Professor Pedro Leão Neves, thank you for your constant challenges.

I must also thank Dr. Marília Faísca, Dr. Brigitte Ribeiro, Professor Mónica Fernandes and Professor José Fernandes, thank you for the technical help and the hours spent in the lab.

Acknowledge Dr. Patrícia Branco, for her inspiration and friendship, and for all our joint ventures and challenges, our phone calls. I thank you all the guidance!

As for the invisible forces that kept my family safe during some troubling times, Patrícia Gil, Patrícia Mendes e Joana Martins, thank you for sheltering my children every time I needed, you are their aunts, my angels, their protectors. Thank you, Carlos Alvarez, for your endearment, patience and support.

This work was inspired by my small world: Mariana, Pedro, Carolina and Tiago! You are my joy, my strength and my fiercest motivation. You have seen the ups and downs, and we did this together.

Last, but definitively the first, MOM... I am fortunate. You are the very best in the whole world.

For all of this... I am grateful!

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

