PERITONEAL DIALYSIS AND ITS LOCAL AND SYSTEMIC COMPLICATIONS: FROM THE BENCH TO THE CLINIC

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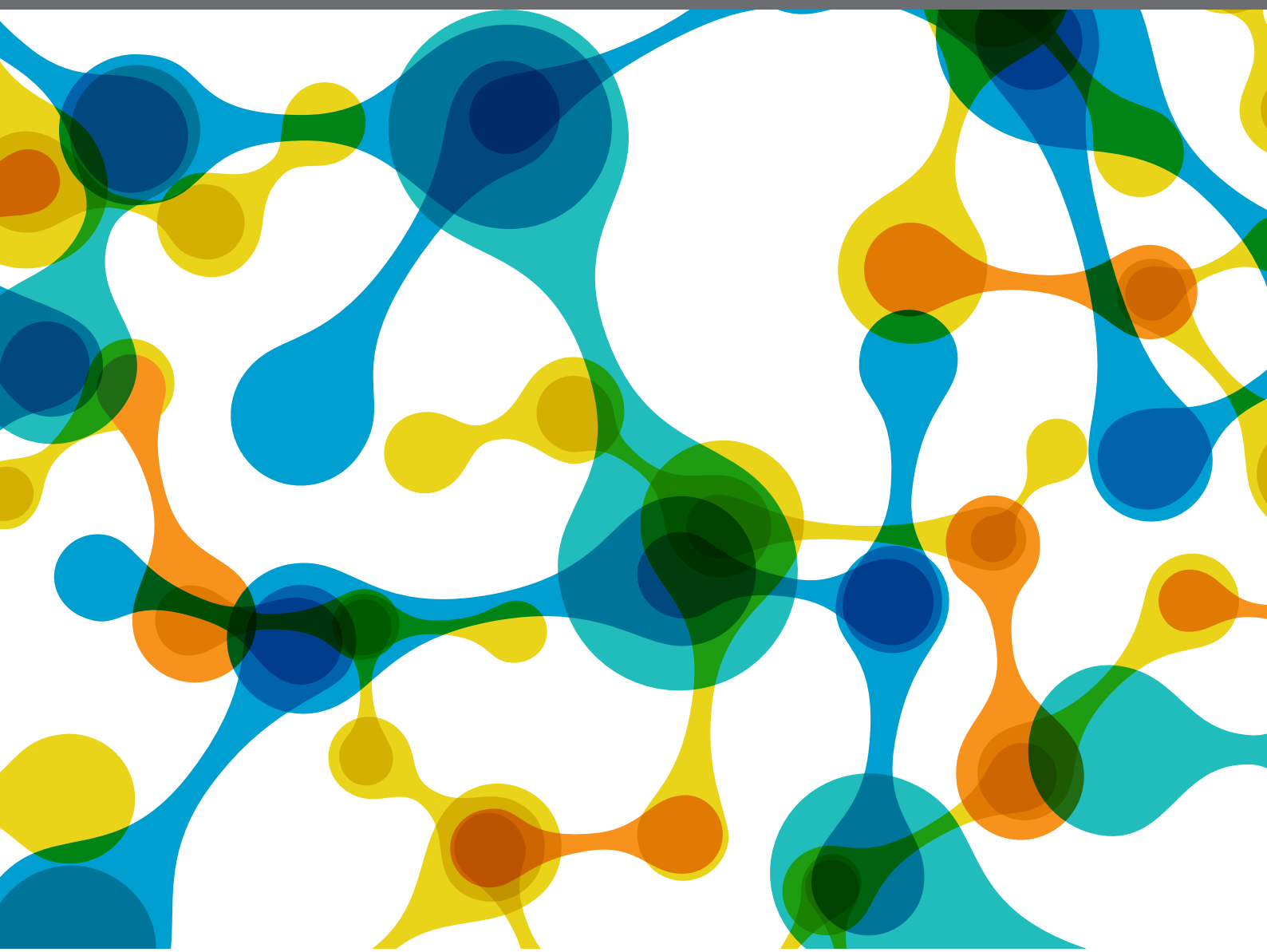

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PERITONEAL DIALYSIS AND ITS LOCAL AND SYSTEMIC COMPLICATIONS: FROM THE BENCH TO THE CLINIC

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EDITORIAL
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Peritoneal Dialysis and Its Local and Systemic Complications: From the Bench to the Clinic

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Editorial on the Research Topic

Peritoneal Dialysis and Its Local and Systemic Complications: From the Bench to the Clinic

Kidney failure is an increasingly common medical problem, posing a serious challenge to health-care systems worldwide and requiring renal replacement therapy at some point. Peritoneal dialysis (PD) is a well-established and cost-effective form of such treatment. It is particularly suitable for home-based therapy and is associated with generally good outcomes and favorable patient experience. However, compared to haemodialysis (HD)—the other dialysis modality—PD is largely underutilized. This can be partly attributed to health care policies but also to limited awareness and availability of the services. Recent experience shows that the improvements in these aspects may increase interest in PD as a treatment option (see Briggs et al., 2019 for a review).

An important problem that hampers PD proliferation is limited technique survival, with only ∼50% of patients remaining on PD after 2 years (Mehrotra et al., 2009). The reasons for technique failure can be multiple and of different nature. Among those, adverse remodeling of the peritoneum is a significant problem as it may result in the inability of the peritoneal membrane to sustain effective ultrafiltration and toxin removal. This scenario is often a consequence of peritoneal infection. Thus, peritonitis became one of the key topics and drivers of research in PD. This led to an improved understanding of peritoneal physiology and pathophysiology and produced results whose significance went beyond PD practice alone and contributed to general immunology and pathology. On the other hand, advances in other disciplines opened up new opportunities for improvements in PD.

Despite these substantial implications, PD research is often perceived as a niche that is of no particular interest to non-specialists and practicing physicians. It is therefore of importance that the results of studies on PD are widely and promptly disseminated within the medical community, as this can increase awareness about PD treatment. Thus, the initiative of Frontiers in Physiology to dedicate a special section to problems of PD should be welcomed and appreciated. Here, the reader will find a number of original and review articles devoted to the mechanisms underlying local and systemic complications of PD.

In two articles Bartosova et al. and Bartosova and Schmitt investigate the effects of new PD fluids, which are considered more biocompatible than conventional ones. Of particular interest is the fact that these were studies of peritoneal biopsies obtained from children undergoing PD, which eliminated the impact of age-related morbidities. These analyses revealed some unexpected morphological features (especially in the peritoneal vasculature), indicating that the effect of new PD fluids is more complex than previously thought. In addition, they showed that the development of peritoneal alterations (at least in a population of pediatric patients) was associated predominantly with the duration of exposure to PD rather than with a history of peritonitis.

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Nevertheless, the classic view holds that vascular changes are a hallmark of inflammation. Increasing evidence points to a role for interleukin-17 (IL-17) during inflammation associated with PD. Here, Witowski et al. present the mechanisms by which IL-17 may affect peritoneal vascularity. In turn, Raby and Labeta discuss how toll-like receptors (TLRs) expressed by peritoneal macrophages and mesothelial cells contribute to the course of peritoneal infection. They also highlight the therapeutic implications of inhibiting peritoneal TLRs during peritonitis.

In another article, Krediet a renowned expert in the field of peritoneal transport, discusses how changes in peritoneal vasculature and interstitium lead to impaired fluid transport and ultrafiltration failure during PD. On a similar note, Yu et al. present an original analysis of plasma and dialysate samples from the GLOBAL fluid study. It aimed to determine whether the peritoneal clearance of proteins depended on local or systemic inflammation (Yu et al.). The value of this study is largely that the parameters of local and systemic inflammation, and of solute and protein transport were measured simultaneously (for the first time) in the same large group of well-characterized patients. Peritoneal transport was also evaluated by Olszowska et al., who examined the kinetics of peritoneal ultrafiltration induced by icodextrin. They demonstrate that icodextrin can generate sustained net ultrafiltration even during very long dwells, which—of course—bears important clinical implications for PD patients. The use of Icodextrin-induced ultrafiltration as a treatment option for refractory heart failure was examined by Wojtaszek, Grzejszczak, Niemczyk, et al. The same group looked into the outcomes of PD used as an alternative to HD for patients requiring urgent renal replacement therapy (Wojtaszek, Grzejszczak, Grygiel, et al.).

Other studies focused on metabolic and cardiovascular aspects of PD. As PD patients are exposed to increased loads of glucose from dialysis fluids, Avila-Carrasco et al. examined concentrations of appetite-regulating peptides and eating behavior in relation to patients' body mass and insulin sensitivity. Radunz et al. analyzed more than 4,000 patients from the BRAZPD-II study to determine how glucose exposure impacts on therapy outcomes. They report that higher cumulative glucose exposure does not independently compromise patients' survival, but may be associated with an increased risk of technique failure. Borràs et al. measured the thickness of the carotid intima media in patients from the NEFRONA study assessing the progression of atherosclerosis during kidney disease. Using different models of multivariate analysis, they suggest that the intima in patients on PD may be less thickened than in patients on HD. In turn, Sánchez-González et al. compared patients on PD and HD in terms of parameters related to calcium metabolism. They showed that factors associated with PD affected the diagnostic value of different PTH fragments for metabolic bone disease.

Finally, two articles from the Klaus Kratochwill group showed exciting prospects for the application of proteomics and metabolomics in PD research. These approaches were used to assess the potential of alanyl-glutamine as a cytoprotective additive to PD fluids. This was investigated by proteomic profiling of the peritoneal surface harvested from rats subjected to experimental exposure to PD (Boehm et al.). It was also examined by analyzing the metabolome of the effluent drained from PD patients who were infused with solutions supplemented with alanyl-glutamine (Wiesenhofer et al.).

We believe that the above articles provide a representative selection of studies conducted currently in the field of PD. They should offer good reading for both physicians and basic science researchers.

AUTHOR CONTRIBUTIONS

JW and ML-C wrote the editorial.

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Guest editors of this topic would like to dedicate this work to the memory of a dear friend and colleague, Dr. Abelardo Aguilera, who sadly passed away while working on this venture.

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ORIGINAL RESEARCH
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Higher Proportion of Non-1-84 PTH Fragments in Peritoneal Dialysis Patients Compared to Hemodialysis Patients Using Solutions Containing 1.75 mmol/l Calcium

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Background: The prevalence of low- turnover bone disease (LTBD) in peritoneal dialysis (PD) patients is higher than in hemodialysis (HD) patients. LTBD patients may be at risk for vascular calcification, and cardiovascular disease. Current therapy for chronic kidney disease metabolic bone disorders (CKD-MBD) is guided by biochemical parameters, as bone biopsy is not used in routine clinical care.

Methods: We assessed intact PTH (iPTH: 1-84PTH plus non-1-84PTH), 1-84PTH, and the 1-84PTH/non-1-84PTH ratio in 129 hemodialysis and 73 PD prevalent patients dialyzed with solutions containing 1.75 mmol/L calcium.

Results: Hemodialysis and PD patients presented similar iPTH and tCa values and prevalence of putative LTBD as defined according to KDOQI iPTH cut-off levels or 1-84 PTH levels. However, iCa accounted for a higher percentage of tCa in PD (53%) than in hemodialysis (39%) $p < 0.001$, and the 1-84PTH/non-1-84PTH ratio was lower in PD than in hemodialysis patients (0.44 ± 0.12) vs. (0.60 ± 0.10), $p < 0.001$. The prevalence of putative LTBD when using the coexistence of 1-84PTH/non-1-84PTH ratio < 1.0 and iPTH < 420 pg/m, was higher in PD than in hemodialysis patients (73 vs. 16% respectively, $p < 0.001$). In a multivariate logistic regression analysis, dialysis modality was the main determinant of the 1-84PTH/non-1-84PTH ratio.

Conclusion: Solutions containing 1.75 mmol/L calciums are associated to a higher proportion of non-1-84PTH fragments in PD than in HD patients. Different analytical criteria result in widely different estimates of LTBD prevalence, thus impairing the ability of clinicians to optimize therapy for CKD-MBD.

Keywords: PTH fragments, non-1-84PTH fragments, 7-84PTH fragments, peritoneal dialysis, low calcium dialysate, low turnover bone disease, 1-84PTH/7-84PTH ratio

INTRODUCTION

Bone disorders in patients with chronic kidney disease (CKD) encompass high and low turnover bone disease (HTBD and LTBD) (Moe et al., 2006). The prevalence of LTBD appears to be higher in peritoneal dialysis (PD) than in hemodialysis (HD) patients (Rodriguez-Perez et al., 1992; Sherrard et al., 1993; Torres et al., 1995; Couttenye et al., 1997; Sánchez et al., 2000; Levy and Gal-Moscovici, 2008; de Oliveira et al., 2015). Low PTH status and LTBD might be an independent strong risk factor for vascular calcification (VC) (Hutchison et al., 1994; Guérin et al., 2000; London et al., 2004, 2015). Therefore, diagnosis and prevention of LTBD is of great clinical importance in order to identify patients that might benefit from interventions to limit the morbidity and mortality resulting from VC. In parallel, it has been established knowledge that PTH is present in uremic serum in different PTH fragments with variable half-life (Martin et al., 1979). Some of these fragments may even behave as antagonists of the PTH receptor (Langub et al., 2003; Huan et al., 2006). Thus, PTH assays may quantify different peptides with different biological actions and clinical significance (Souberbielle et al., 2006). Second-generation iPTH assays now in widespread clinical use recognize both the full-length molecule (1-84 PTH) and PTH fragments of different sizes missing N-terminal aminoacids, including a 7-84 PTH molecule. 7-84 PTH fragments are found in CKD patients and may behave as partial antagonists of 1-84 PTH, opposing its biological activity. Variants with missing N-terminal aminoacids are generated in the parathyroid glands. Intraglandular aminoterminal degradation is regulated by extracellular ionic calcium (iCa) concentration, which suppresses the release of 1-84 PTH and increases the release of 7-84 PTH fragments from parathyroid cells (Kawata et al., 2005; Friedman and Goodman, 2006). We will use the term non-1-84 PTH throughout the manuscript to refer collectively to these fragments.

There are limited data on the distribution of different PTH fragments according to dialysis modality (Gardham et al., 2010). Recently we have published the first data about such differences (González-Casaus et al., 2014). The aims of the present study were to investigate whether there are any differences in the distribution of circulating PTH fragments in PD vs. HD patients, and additionally we wanted to determine any relationship between PTH fragments and metabolic markers of bone turnover such as the serum Carboxy-terminal telopeptides of collagen type I (βCTx), a marker of bone resorption (Bonde et al., 1995); as well as the possible role of PTH fragments in bone remodeling.

PATIENTS AND METHODS

Patients

This was a cross-sectional study from a historical cohort of 202 Caucasian patients with CKD stage 5 that were dialyzed in two Nephrology centers. Data were collected from all continuous ambulatory peritoneal dialysis CAPD ($n = 73$) and HD ($n = 129$) patients dialyzed with solutions containing 1.75 mmol/L calcium (**Table 1**). We had blood samples stored at −86°C from these patients. This study was conducted according to the Declaration of Helsinki and approved by the Institutional Review Board and Ethics committee of the Jimenez Díaz Foundation (Ref. 2016/15). Participants were identified by a number and no other identifying material. All included patients gave verbal informed consent.

TABLE 1 | Baseline characteristics of the patients.

	Total group ($n = 202$)	HD group ($n = 129$)	PD group ($n = 73$)	*P*-value
Age (y)				<0.001
-Mean ± SD	60.17 ± 16.4	64 ± 14.8	52 ± 16.0	
-range	19–82	19–82	22–82	
Sex (% males)	58.4	54.8	63.9	NS
Diabetes (%)	21.4	25.2	14.5	NS
Time on dialysis (y)	2.01 ± 0.82	2.2 ± 0.2	1.63 ± 0.7	<0.001
-Mean ± SD				
Calcitirol (%)	46.7	56.3	29	<0.001

Clinical characteristics of total study population and the two dialysis subgroups. All patients were being dialyzed with solutions containing the same calcium concentration 1.75 mmol/L calcium. Data expressed as mean ± SD. y, years; HD, hemodialysis; PD, peritoneal dialysis; SD, standard deviation; NS, no significant.

Biochemical Parameters

Blood samples were drawn fasting prior to the midweek session in HD and fasting for PD patients, immediately centrifuged, aliquoted and stored at −86°C until analysis. Serum total calcium (tCa) was measured by standard methods in an automated analyzer Cobas Modular Roche. Additionally, ionized calcium (iCa) was quantified in 55 HD patients and all PD patients by ion selective electrode ISE (Rapidpoint 400; Siemens). Serum total 25-hydroxyvitamin D (D2 plus D3) levels were determined by an electrochemiluminiscent assay (ECLIA) in an automated platform/analyzer (LIAISON Vitamin D 25OH Total; DiaSorin Inc) and serum Carboxy-terminal telopeptides of collagen type I (βCTx), a marker of bone resorption (González-Casaus et al., 2014), were measured by ECLIA (CrossLaps, Roche) in an Elecsys 2010 automated system.

The following biochemical parameters were measured simultaneously using a single batch (for automated methods) to minimize analytical variability.

Plasma whole PTH (1-84 PTH, also called bioPTH, determined by a third generation assay) and intact PTH (iPTH: 1-84 plus non-1-84 determined by a second generation assay) were determined simultaneously by an immunoradiometric assay (CA-PTH duo; Scantibodies Laboratory Inc.).

Both assays use an antibody specific for the 39–42 sequence of PTH to immobilize the molecule, but they differ in the second radiolabeled antibody. In the third generation method the second antibody recognizes exclusively the first four aminoacids of the molecule (aminoacids 1–4) to avoid the interference of PTH fragments with larger N-terminal truncations, while in the second generation iPTH assay the second antibody recognizes the 1–34 sequence of PTH. Results were expressed as serum whole 1-84 PTH levels, iPTH levels (1-84 plus non-1-84 PTH) and as a 1-84 PTH/non-1-84 PTH ratio calculated as (1-84 PTH)/iPTH-(1-84 PTH).

According to PTH values, patients were stratified as below, on target, and above PTH values as recommended by KDOQI guidelines (National Kidney Foundation, 2003) and Herberth criteria (Herberth et al., 2010) because both sets of criteria were validated by bone biopsy. As the Allegro iPTH assay (Nichols) (1–84 plus non-1-84 PTH) used to establish the KDOQI reference values was not available, we used the adjustment reported by Souberbielle et al (2006) for iPTH and 1-84 PTH Scantibodies methods to obtain theoretical Allegro iPTH values. Thus, according to KDOQI recommendations, patients with serum iPTH levels < 134 pg/mL (equivalent to < 150 pg/mL Allegro iPTH) were classified as at risk of LTBD, while patients with serum iPTH values > 262 (equivalent to > 300 pg/mL Allegro iPTH) were considered as at risk of HTBD. When whole 1-84 PTH was considered, according to KDOQI recommendations, patients with serum whole 1-84 PTH < 84 pg/mL (equivalent to < 150 pg/mL Allegro iPTH) were classified as at risk of developing LTBD, while patients with serum whole 1-84 PTH > 165 pg/mL (equivalent to > 300 pg/mL Allegro iPTH) were considered as HTBD. In addition, according to Herberth et al. (2010), a 1-84 PTH/ non-1-84 PTH ratio < 1.0 combined with iPTH level < 420 pg/mL was used to diagnose LTBD and a ratio > 1.6 combined with iPTH 340-790 pg/mL for risk of HTBD.

Statistical Analysis

Standard descriptive statistical analysis was performed and distribution of data was tested using Shapiro-Wilk normality test. Results are expressed as mean or median and 95% confidence interval (CI). Group means were compared using the two-tailed non-paired Student's *t*-test. Pearson correlation coefficient was used to study the association between quantitative variables. Stepwise multiple regression analysis and partial correlation analysis were used to assess the independent contribution of several variables to bone turnover. All test were two-tailed and the level of significance was set at $p < 0.05$.

RESULTS

The total study population comprised 118 males and 84 females, with a mean age of 60 years (95% CI: 58–62 years). There were no significant differences in gender distribution or in the presence of diabetes between the two dialysis modalities. Age and time on dialysis were higher in the HD group than in the PD group (**Table 1**).

Almost half (47%) of participants were treated with active vitamin D (calcitriol), but none received calcimimetics. The percentage of patients treated with calcitriol was higher in the HD group (**Table 1**). No significant differences were found in cumulative amount of calcium element (g) based on the prescribed treatment of calcium carbonate and calcium acetate during the 12 months prior to the study.

There were no differences between dialysis modalities in serum phosphate (mg/dL) [HD: 4.59 (4.4–4.8) vs. PD: 4.73 (4.4–5.1), $p = 0.294$]; tCa (mg/dL) [HD: 9.13 (8.99–9.27) vs. PD: 9.03 (8.8–9.26), $p > 0.460$] or total 25-hydroxyvitamin D [HD: 20.15 (17.25–23.05) vs. PD: 19.46 (17.5–21.4), $p = 0.546$]. However, iCa (mg/dl) levels were lower in the HD [3.52 (3.39–3.61)] vs. PD group [4.76 (4.6–4.84), $p < 0.001$]. In this regard, differences in the distribution of serum calcium were observed; we found a high percentage of iCa in PD patients vs. HD patients (**Figure 1A**). HD and PD patients presented similar iPTH (pg/ml) values [HD: 204.8 (172–237) vs. PD: 211.2 (139–283), $p = 0.995$]. However, there were differences in the distribution of circulating PTH fragments, which were evident when serum 1-84 PTH levels were expressed as percentage of intact PTH. 1-84 PTH as a percentage of iPTH was significantly lower in PD than in HD (**Figure 1B**), and 1-84 PTH/non 1-84 PTH ratio much lower in PD as compared to HD patients [PD: 0.88 (0.7–1.1) vs. HD: 1.79 (1.6–2.0), $p < 0.001$]. That finding was corroborated after a multivariate logistic regression analysis including age, gender, diabetes, residual renal function, dialysis vintage, serum phosphate and total serum calcium leves, and vitamin D treatment. iCa showed an inverse correlation with serum 1-84 PTH and 1-84 PTH/non-1-84 PTH ratio in the total study population (**Table 2**). Serum levels of the bone turnover

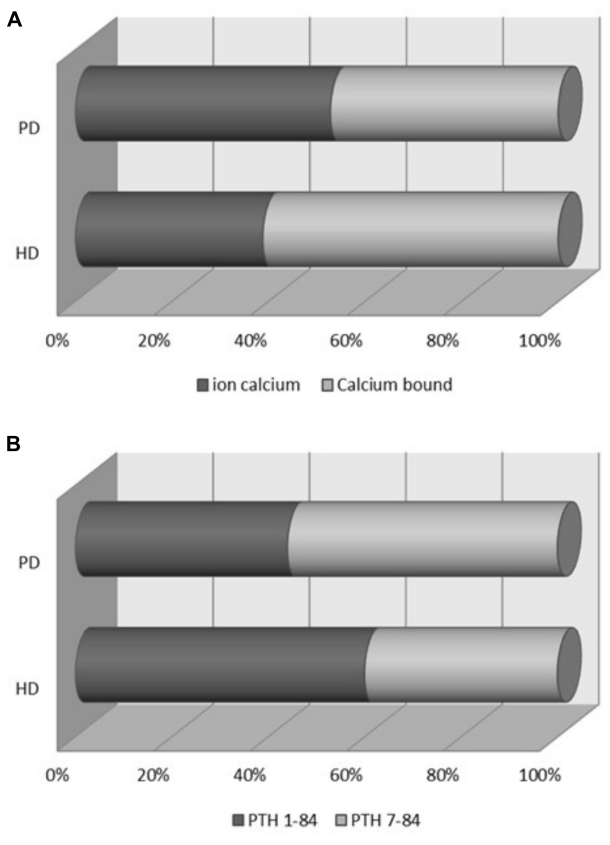

FIGURE 1 | (A) Distribution of calcium ion and calcium bound expressed as percentage of total calcium. iCa accounted for 39% of tCa in the HD group and for 53% of tCa in PD patients, $P < 0.001$. **(B)** Distribution of circulating PTH fragments (1-84 PTH and non- 1-84 PTH) expressed as percentage of intact PTH (1-84 PTH plus non-1-84 PTH). Percentage of 1-84 PTH in PD patients (44.0 ± 12.28%) vs. HD patients (60.3 ± 10.82%), $p < 0.001$. HD, hemodialysis; PD, peritoneal dialysis.

TABLE 2 | Correlations.

Total group	1-84 PTH	Ipth	1-84PTH/ non 1-84 PTH ratio
iCa	−0.240 ($P < 0.01$)	−0.087 ($P = 0.336$)	−0.636 ($P < 0.001$)
1-84 PTH	–	0.975 ($P < 0.001$)	0.423 ($P < 0.001$)
iPTH	–	–	0.242 ($P < 0.001$)
βCTx	0.441 ($P < 0.001$)	0.399 ($P < 0.001$)	0.280 ($P < 0.001$)
PD group βCTx	0.472 ($P < 0.001$)	0.491 ($P < 0.001$)	0.114 ($P = 0.411$)
HD group βCTx	0.429 ($P < 0.001$)	0.434 ($P < 0.001$)	0.106 ($P = 0.232$)

Correlations were analyzed by Pearson's correlation. iCa, ionized calcium; βCTx, carboxy-terminal telopeptides of collagen type I; HD, hemodialysis; PD, peritoneal dialysis; NS, no significant.

marker β-CTx correlated with both 1-84 PTH and iPTH in the whole population and also in PD and in HD (**Table 2**).

In a multivariate logistic regression analysis including age, dialysis vintage, 1-84 PTH/non-1-84 PTH ratio, vitamin D treatment and dialysis modality, the main determinant of the percentage of calcium present as iCa (iCA/tCa ratio) was the dialysis modality. For this analysis the mean percentage of iCA/tCa ratio (0.47) was used as a cut-off point (constant: 3.91; Estimate: 0.001, 95% CI: 0.000-0.012, $P < 0.001$). Similarly, the main determinant of LTBD (defined as the coexistence of a 1-84 PTH/non-1-84 PTH ratio < 1.0 and iPTH < 420 pg/mL), was iCa concentration (**Table 3**).

There were no significant differences in the distribution of HD or PD patients into LTBD or HTBD when bone turnover was defined according to KDOQI recommended cut-off levels for iPTH, either when iPTH was normalized to Allegro iPTH values (**Figure 2A**) or when whole 1-84 PTH was normalized to Allegro iPTH values (**Figure 2B**). However, defining LTBD or HTBD according to 1-84 PTH/ non-1-84 PTH ratio for the diagnosis of bone turnover in dialysis led to a significantly higher prevalence of LTBD in PD than in HD patients (**Figure 2C**). In accordance with these findings, serum β-CTx (pmol/L) was significantly lower in the PD group [1181 (946–1393)] vs. HD patients [2084 (1633- 2238). $p < 0.001$], suggesting also a lower bone turnover activity in PD patients.

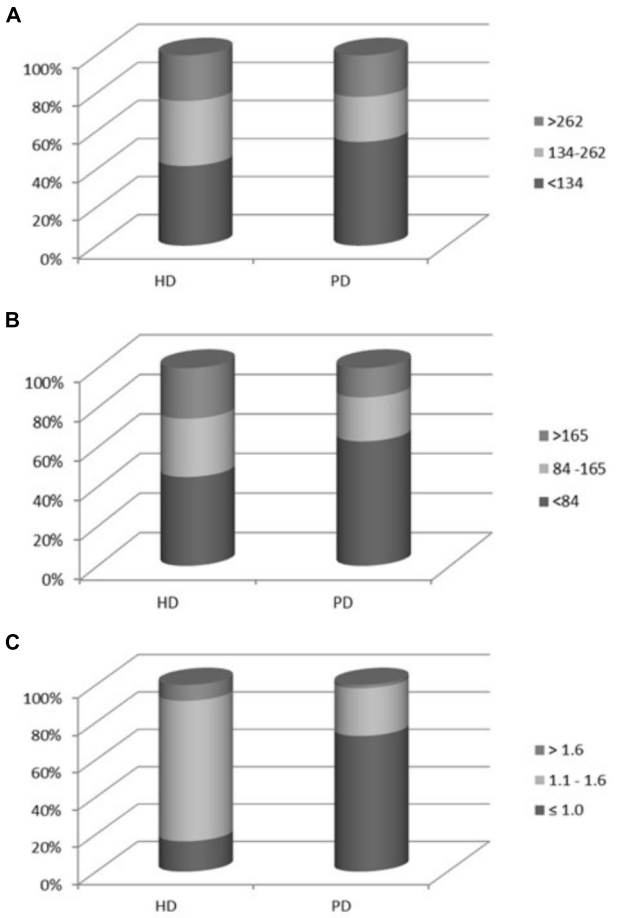

FIGURE 2 | Diagnosis of bone turnover in dialysis according to: **(A)** KDOQI recommended cut-off levels for *iPTH Scantibodies assay* normalized according to Soubervielle to obtain the equivalent Allegro iPTH values. [*HTBD*: iPTH Scantibodies > 262 pg/ml = Allegro iPTH > 300 pg/ml; *NORMAL*: iPTH Scantibodies 134–262 pg/ml = Allegro iPTH 300-150 pg/ml; *LTBD*: iPTH Scantibodies < 134 pg/ml = Allegro iPTH < 150 pg/ml]. **(B)** *Whole 1-84 PTH assay* normalized according to Soubervielle to obtain the equivalent Allegro iPTH values. [*HTBD*: whole 1-84 PTH > 165 pg/ml = Allegro iPTH > 300 pg/ml; *NORMAL*: whole 1-84 PTH: 84–165 pg/ml = Allegro iPTH 300–150 pg/ml; *LTBD:* 1-84 PTH < 84 pg/ml = Allegro iPTH < 150 pg/ml]. **(C)** *1-84 PTH/ non-1-84 PTH ratio* < 1.0 combined with iPTH level < 420 pg/mL for developing LTBD and a ratio > 1.6 combined with iPTH 340–790 pg/mL for risk of HTBD. Prevalence of LTBD in PD (72.7%) vs. HD patients (16.3%), $P < 0.0001$. HD, hemodialysis; PD, peritoneal dialysis, HTBD, high turnover bone disease; LTBD, low turnover bone disease.

TABLE 3 | Independent contributing factors to low bone turnover disease, defined by a 1-84 PTH/ non 1-84 PTH ratio ≤ 1 and iPTH < 420 pg/mL, in the multiple regression analysis.

Coefficient	iCa	iCa* (a)	iCa* (a & v)
Constant	10.67	7.19	6.93
Estimate	−9.85	−8.68	−8.75
Exp (B) (95% CI)	0.000 (0.000–0.002)	0.000 (0.000–0.006)	0.000 (0.000–0.006)
P-value	<0.001	<0.001	<0.001

iCa (a), ionized calcium corrected by age; iCa* (a & v), ionized calcium corrected by age and dialysis vintage; iCa, ionized calcium.*

DISCUSSION

This study investigated differences in serum levels of different PTH fragments between PD and HD patients using solutions containing 1.75 mmol/l calcium. 1-84 PTH, as a percentage of iPTH, and 1-84 PTH/non-1-84 PTH ratio were lower in the PD than in the HD group. The association between 1-84

PTH/non-1-84 PTH ratio and dialysis modality was corroborated by multivariate regression models. In addition, this study the concordance between different cut-off points to biochemically suspect LTBD according to guidelines or individual author's suggestions. The main finding is that there is little concordance between guideline-based cut-off points for iPTH or 1-84PTH and the proposal by Herbeth et al. based on a study of HD using a combination of iPTH and 1-84 PTH/non-1-84 PTH ratio (Herberth et al., 2010). A higher percentage of PD patients and a lower proportion of HD patients were diagnosed of suspected LTBD when 1-84 PTH/ non-1-84 PTH ratio were applied for the diagnosis of bone turnover in dialysis. This view would be further supported by the lower levels of serum β-CTx in PD patients. By contrast iPTH alone did not disclose differences in putative LTBD between HD and PD. Furthermore, these findings are consistent with the previous observation that high serum iPTH levels, assessed by second-generation assays, reflecting the sum of potentially opposing effects of 1-84 PTH and its fragments, do not correlate with histomorphometric data in patients with LTBD (Torres et al., 1995; Wang et al., 1995; Sánchez et al., 2000; Barreto et al., 2008), more frequently observed in PD patients, as well as in patients with HTBD (Herberth et al., 2009; Garrett et al., 2013). The recommendations of different guidelines on the target PTH levels are not uniform between the different guidelines an even within some guidelines; the recommended range has changed through years. In the past the aim was to maintain patients between 150 and 300 pg/ml; more recently the upper limit was increased to 6 times the upper normal (Kidney Disease, 2009). While the present study represents routine clinical practice and, thus, lacks bone biopsies to confirm LTBD, it does raise a series of issues regarding currently used cut-off points and their trustworthiness to guide therapy for secondary hyperparathyroidism. Matters are complicated by the commercial availability of different PTH assays with wide inter-method variability (Gardham et al., 2010). The individual PTH values obtained in the same sample using different assays may potentially point to opposing diagnostic and therapeutic attitudes (Souberbielle et al., 2006).

Although controversy exists, it has been proposed that assessment of the different PTH fragments may provide information on bone turnover in patients undergoing dialysis (Monier-Faugere et al., 2001; Herberth et al., 2010). According to this view, the higher percentage of PD patients with evidence of LTBD when 1-84 PTH/7-84 PTH ratio was applied for the diagnosis of bone turnover in dialysis than when iPTH criteria was applied, might represent a true higher incidence of LTBD and would point to the inaccuracy of KDOQI suggested thresholds for higher risk of LTBD. However, the lack of bone biopsy precludes confirmation of this hypothesis in the present cohort.

Renal replacement therapy is associated with net influx of Ca to the patient when dialysate calcium concentration is higher than serum calcium. Furthermore, serum iCa level in PD patients, as a consequence of its continuous nature, may be higher than in HD patients (Kurz et al., 1995) as illustrated by the present report, especially when 1.75 mmol/L calcium PD fluids are used (Hutchison et al., 1992; Weinreich et al., 1995; Sanchez et al., 2004; Haris et al., 2006; Yamamoto et al., 2008; Soroka et al., 2011; Yee-Moon Wang, 2014). Changes in serum iCa concentration are sensed by the CaSR that signals to regulate PTH secretion and regulate processing of PTH to yield different fragments (Habener et al., 1975; D'Amour et al., 1992; Brown and MacLeod, 2001; D'Amour, 2002). High serum iCa levels favor non-1-84 PTH fragments secretion over whole 1-84 PTH decreasing the 1-84 PTH/non-1-84 ratio. Thus, in our PD cohort higher serum iCa favored by the 1.75 mmol/L calcium PD fluid led to higher secretion of non–1-84 PTH fragments such as 7-84 PTH thus promoting LBTD (Slatopolsky et al., 2000; Divieti et al., 2002; Ok et al., 2016). Other factors than PTH level might regulate osteoblast function in renal osteodystrophy in PD such as high serum glucose, cytokines and local bone growth factors that decrease bone formation making LTBD.

Despite the uncertainty, given the available evidence, including the fact that the current cohort of PD patients used 1.75 mmol/L calcium PD fluid, a known risk factor for LTBD (Merle et al., 2016; Ok et al., 2016), we would concur that the combination of iPTH and 1-84 PTH/non-1-84 PTH ratio might be useful to diagnose LBTD also in PD patients and may be more sensitive that the iPTH currently used for this purpose.

Several weaknesses should be recognized. We did not perform bone biopsies in our population as they are not part of the current standard of care. The absence of this gold standard impedes to draw definitive conclusions about the true prevalence of LBTD in our PD patients and the relative accuracy of the combination of iPTH and 1-84 PTH/non-1-84 PTH ratio to non-invasively diagnose LBTD.

In conclusion, these findings cast doubt on the reliability of available cut-off points for PTH values that are in use or have been proposed to guide therapy for secondary hyperparathyroidism in dialysis patients. While the lack of bone biopsies does not allow to validate any of the cut-off vales as accurate, the higher iCa and lower β-CTx levels in the present cohort of PD patients are consistent with the hypothesis that conventional KDOQI cut-off points do underestimate the prevalence of LTBD in PD patients. Given that there is no biopsy, we can only conclude that there is increased likelihood of LTBD in PD patients based on biomarker using Herberth/KDOQI criteria.

AUTHOR CONTRIBUTIONS

CS-G, MA, VS, J-VT, and MR performed the clinical research. MG-C performed the analysis. MR wrote the manuscript. SM, AO, and EG-P corrected and edited the manuscript.

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frontiers in Physiology

REVIEW
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IL-17 in Peritoneal Dialysis-Associated Inflammation and Angiogenesis: Conclusions and Perspectives

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Long-term peritoneal dialysis (PD) is associated with peritoneal membrane remodeling. This includes changes in peritoneal vasculature, which may ultimately lead to inadequate solute and water removal and treatment failure. The potential cause of such alterations is chronic inflammation induced by repeated episodes of infectious peritonitis and/or exposure to bioincompatible PD fluids. While these factors may jeopardize the peritoneal membrane integrity, it is not clear why adverse peritoneal remodeling develops only in some PD patients. Increasing evidence points to the differences that occur between patients in response to the same invading microorganism and/or the differences in the course of inflammatory reaction triggered by different species. Such differences may be related to the involvement of different inflammatory mediators. Here, we discuss the potential role of IL-17 in these processes with emphasis on its impact on peritoneal mesothelial cells and peritoneal vascularity.

Keywords: IL-17, inflammation, peritonitis, angiogenesis, fibrosis, VEGF, peritoneal dialysis

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PERITONEAL MEMBRANE DYSFUNCTION IN PD

Although peritoneal dialysis (PD) is a well-established treatment modality and the most commonly practiced form of home dialysis, its penetration is well below the utilization rate of 25–30% considered as optimal (Lameire and Van Biesen, 2010). One of the barriers to PD proliferation is the fear that durability of the peritoneum is limited and that the membrane may become unable to sustain treatment at some point. It has been estimated that peritoneal membrane dysfunction is responsible for approximately 30% of all cases of technique failure (Davies et al., 2011). Indeed, longitudinal studies show that peritoneal ultrafiltration gradually decreases with time on PD (Davies et al., 1996). The onset of a decline in ultrafiltration capacity occurs usually 2–4 years after the initiation of PD (Smit et al., 2004) and appears to result from progressive membrane injury and (to some extent) from the loss of residual kidney function. Studies of peritoneal structure and function indicate that two major processes occur during long-term PD treatment: (i) changes in the peritoneal vasculature resulting in increased transport of small solutes, (ii) changes in the peritoneal interstitium leading to reduced osmotic conductance of the membrane

(Davies et al., 2011). These processes can be mediated through a number of intertwined pathophysiological mechanisms (**Figure 1**). By applying an extended 3-pore model of the peritoneum (Davies et al., 2011), it is possible to explain how the peritoneal membrane displays increased transport rates for small solutes and, at same time, becomes more restrictive to water flow. As the transport of small solutes down an osmotic gradient depends largely on area, formation of new blood vessels will increase the surface area available for solute diffusion. On the other hand, fibrotic thickening of the peritoneum will increase resistance to fluid flux and will ultimately decrease water flow through the interstitium. Thus, it appears that the gradual loss of peritoneal ultrafiltration with time is initially related to increased solute transport leading to proportional dissipation of the osmotic gradient. Fibrosis that develops at later stages will then uncouple the osmotic conductance from solute transport resulting in further, and disproportionately severe, reduction in ultrafiltration. In the above scenario, neovascularization plays a key role, both contributing to increased small-solute transport and fuelling fibrosis (Wynn, 2007). Indeed, it has been estimated that up to 75% of patients with ultrafiltration failure may have increased vascular area (Heimburger et al., 1990; Ho-Dac-Pannekeet et al., 1997). Moreover, peritoneal biopsies taken from PD patients show that fibrosis occurs significantly more often in the presence of vasculopathy (Williams et al., 2002), and the density of peritoneal blood vessels and submesothelial and perivascular fibrosis are significantly greater in patients with membrane failure (Mateijsen et al., 1999; Williams et al., 2002). Animal models of PD confirm the existence of inverse correlation between increased vascularization and ultrafiltration (Margetts et al., 2002). These studies also demonstrate that a decline in ultrafiltration can be partially prevented by anti-angiogenic therapy (Margetts et al., 2002).

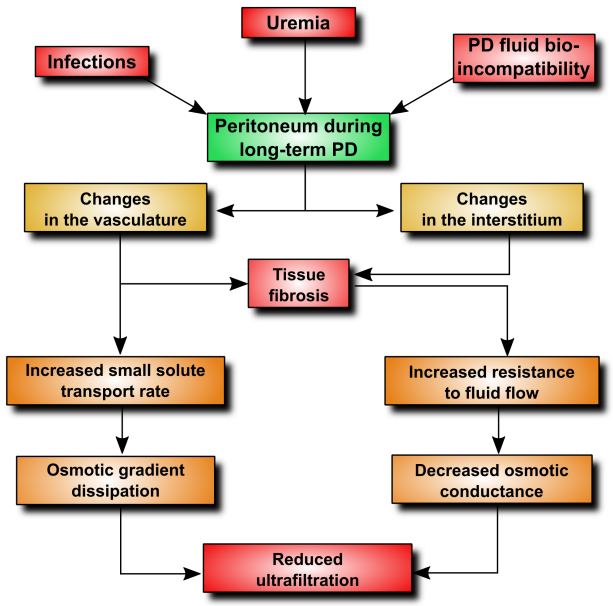

FIGURE 1 | Pathophysiological alterations contributing to ultrafiltration dysfunction during PD. An increase in peritoneal vascularity plays a key role, as it increases vascular surface area available for the transport of small-solutes, including glucose. This leads to an early loss of the glucose osmotic gradient and a decrease in fluid removal. In addition, angiogenesis and adverse vascular remodeling promotes excessive extracellular matrix deposition and tissue fibrosis.

PERITONEAL VASCULATURE DURING PD

Because the alterations in peritoneal vasculature develop in relation to the time spent on PD, their causes are likely to be therapy-related. They may include (i) exposure to PD fluid components, (ii) a progressive decline in residual renal function, and (iii) the occasional episodes of peritonitis. Although acute peritoneal inflammation may cause a profound decrease in ultrafiltration, a single and uncomplicated episode of peritonitis will usually have little long-term effect on the peritoneum (Albrektsen et al., 2004). In contrast, recurrent or clustered episodes of infection with highly pathogenic species may lead to a sustained increase in peritoneal solute transport and a permanent decrease in ultrafiltration (Davies et al., 1996; Wong et al., 2000). This effect is particularly evident in the first year of PD treatment, however, at later stages even patients who never experienced peritonitis may show a similar increase in solute transport rate (Davies et al., 2011). It appears therefore that peritonitis can exacerbate the development of membrane dysfunction over time but it is not the prime and sole determinant of the process.

The components of PD solutions that may be injurious to the peritoneum include non-physiological pH (approximately 5.2), lactate buffer, increased osmolality, and high concentrations of glucose and glucose degradation products (GDPs). A longitudinal analysis has revealed that extensive use of hypertonic PD solutions with high glucose contents precedes an increase in solute transport (Davies et al., 2001). This change in membrane function may lead to less efficient ultrafiltration, which creates a vicious circle by increasing the need for more hypertonic glucose exchanges. These requirements may be further compounded by loss of residual kidney function and decreased urine output. GDPs present in PD fluids in proportion to the concentration of glucose may also contribute to membrane dysfunction by affecting the peritoneal vasculature. It has been demonstrated that GDPs can induce capillary recruitment and vasodilation (Mortier et al., 2002), as well as angiogenesis and hyperpermeability (Hirahara et al., 2006).

The direct effect of uremia on the peritoneal membrane function is less clear. It appears that in many uremic patients some changes in the peritoneum occur even before the start of PD (Williams et al., 2002). Compared with healthy individuals, such patients often have vasculopathy and significant thickening of the submesothelial compact zone (Kihm et al., 2008). These changes are generally attributed to the build-up of uremic toxins, however, their exact nature is poorly defined. The peritoneum of rats made uremic by subtotal nephrectomy shows increased permeability, focal areas of vascular proliferation

(Combet et al., 2001), and interstitial fibrosis (De Vriese et al., 2006).

THE ROLE OF VEGF DURING PD

Vascular endothelial growth factor (VEGF) is a key mediator of pathological changes in blood vessels (Nagy et al., 2007, 2012). Its effect on peritoneal vascularity during PD can be inferred from the association between genetic polymorphisms resulting in increased VEGF production and increased transport rates for small solutes (Szeto et al., 2004). Mesothelial cells are the main source of peritoneal VEGF (Mandl-Weber et al., 2002; Gerber et al., 2006; Boulanger et al., 2007), which can be secreted in response to many stimuli. These are related to both PD fluid exposure and peritonitis (reviewed in Witowski and Jörres, 2011). Expression of the *VEGF* gene is tightly regulated at multiple levels, including transcription, mRNA stabilization, alternative splicing, translation, and subcellular localization (Arcondeguy et al., 2013). Owing to this complexity, the exact molecular mechanism controlling VEGF production during PD is only partially understood. We have previously demonstrated that different cytokines, which are present in the dialysate during peritonitis (e.g., IL-1β, TNFα, TGF-β, and IL-6) can regulate VEGF production by the mesothelium in a context dependent manner by engaging different sets of transcription factors (Catar et al., 2013, 2017).

SOURCES AND FUNCTION OF IL-17

The discovery of IL-17 in the 1990s opened a new chapter in immunology. It led to the identification of a distinct type of T helper (Th) cells and shed new light on the role of T cells in inflammation. IL-17 (also known as IL-17A) is the prototypic, the most potent and the best-characterized member of the IL-17 family of cytokines comprising IL-17A through IL-17F (see Beringer et al., 2016 for review). The source of IL-17 has been identified as a subset of $CD4^+$ effector T cells that was designated Th17 as it was clearly different from previously known Th1 and Th2 subtypes. It has transpired that naïve $CD4^+$ T cells can differentiate into various subsets of effector Th cells (Th1, Th2, and Th17) depending on the exact cytokine milieu. Each Th cell differentiation program is governed by specific transcription factors [T-bet, GATA3 and the retinoic acid-related orphan receptor-γt (RORγt), respectively] and each type of terminally differentiated Th cells produces a specific set of effector cytokines. The polarizing mediators involved at various stages of Th17 cell differentiation include TGF-β, IL-6, IL-1β, IL-21, and IL-23. These combined signals activate the transcription factor ROR-γt, which is required for the production of Th17 cell-specific effector cytokines, including IL-17, IL-22, IL-26, and CCL20 (**Figure 2**).

IL-17 can originate not only from Th17 cells but also from innate-like immune cells including $CD8^+$ T cells, invariant natural killer T cells (iNKT), lymphoid tissue inducer (LTi) cells, group 3 innate lymphoid (ILC3) cells, $CD4^-CD8^-$-double negative (DN) αβ T cells, and unconventional T cells, such as γδ T cells and mucosal-associated invariant T (MAIT) cells (**Figure 3**). And it came as a surprise when the main source of IL-17 turned out to be not the expected Th17 cells but γδ T cells, which constitute only a small fraction of lymphocytes. In contrast to naïve Th cells, γδ T cells do constitutively express IL-23R and can immediately respond to IL-23 by secreting IL-17 (Papotto et al., 2017). As IL-23 is derived mainly from sentinel cells such as dendritic cells or macrophages, IL-17-producing γδ T cells can also be viewed as belonging to the category of cells performing surveillance tasks and responding quickly to pathogens.

BIOLOGICAL ACTIVITY OF IL-17

By virtue of its ability to induce several cytokines and chemokines, IL-17 has typically been linked to inflammation. In this respect, IL-17 is critically involved both in essential protection against infections and in several disorders characterized by chronic inflammation. By blocking IL-17 signaling in murine models, it has been demonstrated that IL-17 contributes to host defense against extracellular bacterial and fungal pathogens. These include *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Candida albicans*, *Salmonella enterica*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Helicobacter pylori*, *Citrobacter rodentium*, and *Trypanosoma cruzi* (see Gu et al., 2013 for review). Here, IL-17 acts mainly as a potent inducer of neutrophil recruitment and granulopoiesis. It does so by promoting the release of chemokines that specifically attract neutrophils (e.g., CXCL1, CXCL6, and CXCL8) and stimulate granulopoiesis in the bone marrow (e.g., IL-6, G-CSF, and GM-CSF). Interestingly, IL-17-producing Th17 cells produce also CCL20 that serves to attract more Th17 cells to the site of inflammation (**Figure 3**).

Acting together with IL-22, another mediator of sentinel cells, IL-17 contributes also to the maintenance of tissue integrity by enhancing the synthesis of tight junction proteins (claudin) and a number of antimicrobial proteins such as defensins, lipocalin, lactoferrin, and regenerating (REG) and S100A proteins (Cua and Tato, 2010). Moreover, it has been shown that early innate production of IL-17 can influence the generation of antigen-specific Th17 or γδ T cells and contribute to adaptive immunity. Thus generated memory cells persist as long-lived tissue-resident cells, which generate more robust effector responses enhancing pathogen clearance (Lalor and McLoughlin, 2016).

IL-17 SIGNALING

IL-17 signaling from the cognate IL-17 receptor has been partially deciphered (reviewed in Gu et al., 2013 and Song and Qian, 2013). It involves the adaptor protein Act1 as evidenced by unresponsiveness of Act1-deficient mice to IL-17 (Qian et al., 2007). Upon IL-17 stimulation Act1 recruits tumor necrosis factor receptor associated factor-6 (TRAF6) that mediates transcription of several target genes through activation of NF-κB and AP-1 transcription factors. In addition, Act1 forms a complex with TRAF5 and TRAF2 to operate

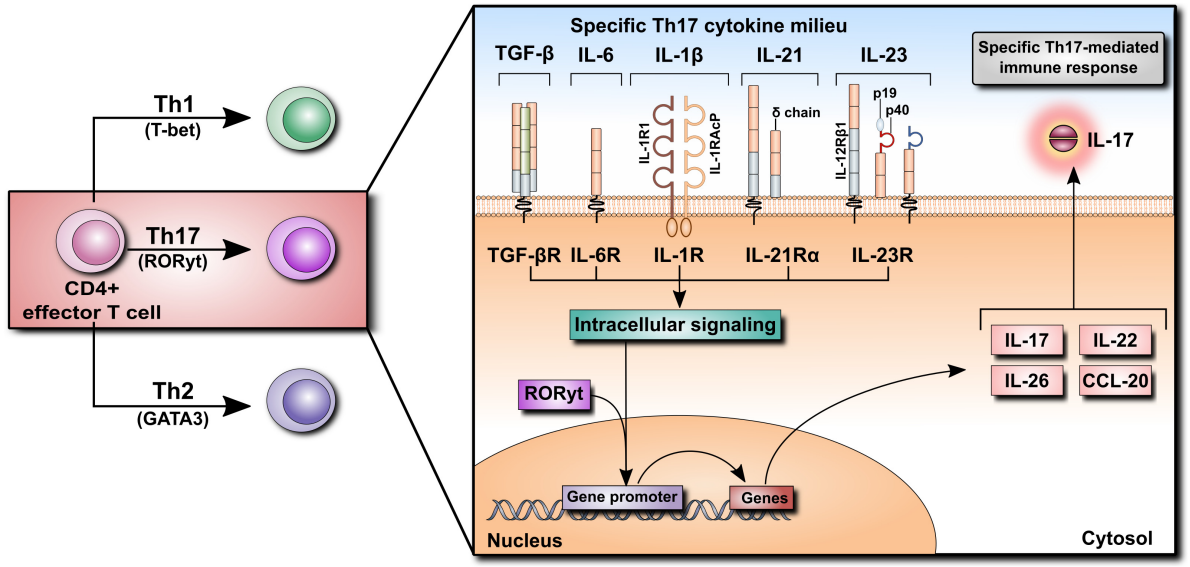

FIGURE 2 | Differentiation of $CD4^+$ T cells into Th17 cells. Naïve $CD4^+$ T cells can differentiate into Th1, Th2, or Th17 cells according to their specific cytokine milieu. For differentiation of $CD4^+$ cells into Th17 cells, TGF-β, IL-6, and IL-1β, as well as IL-21 and IL-23, are required. Cytokine receptor binding initiates an intracellular signaling cascade leading to the translocation of RORγt into the nucleus, inducing the transcription of various cytokines such as IL-17, IL-22, IL-26, and CCL20. Their secretion promotes the Th17-mediated immune response.

at the post-transcriptional level and control mRNA stability (Sun et al., 2011). This IL-17 function is aided by the RNA-binding protein HuR (Herjan et al., 2013). Although Act1 serves primarily as an adaptor protein linking the intracellular domain of the IL-17 receptor with transcription factors (typically of the NF-κB pathway), it has recently been discovered that Act1 itself may exert transcriptional activity by binding to the promoter region of IL-17-responsive genes (Velichko et al., 2016). Although these mechanisms have been found to control the expression of many IL-17-induced cytokines and neutrophil-attracting chemokines (Shen et al., 2006), it is not known whether they are also involved in the regulation of other IL-17 target genes that do not fall into these categories or are expressed in cell types not previously examined.

IL-17 IN THE PERITONEUM

IL-17 is virtually undetectable in a healthy human peritoneum, but it can be found in peritoneal biopsies from patients undergoing PD (Rodrigues-Diez et al., 2014). The cells expressing IL-17 were identified as predominantly Th17 cells and γδ T cells, and occasionally as mast cells and neutrophils. The appearance of these cells seemed to correlate with the duration of PD treatment and the extent of tissue fibrosis (Rodrigues-Diez et al., 2014). Using a model of daily PD fluid injections in mice (Gonzalez-Mateo et al., 2009), it has been demonstrated that after 30 days of exposure to PD fluids, but not to control saline, the peritoneum became markedly infiltrated by Th17 and γδ T cells, and its thickness increased in correlation with the levels of IL-17 in the peritoneal cavity (Rodrigues-Diez et al., 2014). Moreover, this increased presence of IL-17-producing cells was associated with increased activity of IL-6, TGF-β, and RORγt, all being instrumental in differentiating Th17 cells. To confirm that IL-17 did indeed contribute to PD fluid-induced alterations, the same experiments were performed in the presence of anti-IL-17 antibodies. These studies showed that the neutralization of anti-IL-17 alleviated the extent of peritoneal fibrosis. Conversely, repeated intraperitoneal administration of exogenous IL-17 led to increased expression of several fibrosis-related genes (fibronectin, TGF-β, α-smooth muscle actin, and fibroblast specific protein-1) and build-up of extracellular matrix (Rodrigues-Diez et al., 2014). Interestingly, a study assessing paricalcitol, a synthetic activator of vitamin D receptor, showed that in the same experimental setting in mice the addition of paricalcitol to PD fluids reduced the extent of peritoneal fibrosis (Gonzalez-Mateo et al., 2014). This effect was attributed partially to inhibition of IL-17-mediated responses as both the numbers of IL-17-producing T cells and the intraperitoneal IL-17 concentrations were significantly reduced (**Figure 4**).

As indicated earlier, MAIT cells can be another important source of IL-17 (Xiao and Cai, 2017). Like γδ T cells, they accumulate in the peritoneum of patients receiving PD and expand significantly during infections caused by pathogens producing appropriate ligands (Liuzzi et al., 2016). Less clear is the role of IL-17-producing DN T cells in the dialyzed peritoneum. Their contribution to intraperitoneal IL-17 can be surmised from the observation that DN T cells accumulate and secrete IL-17 in the peritoneum of mice infected with *L. monocytogenes* (Riol-Blanco et al., 2010). It will be interesting to see whether DN T cells infiltrate the peritoneum as a result of kidney failure and uremia. In this respect, it has been demonstrated in murine models that DN T cells expand in the

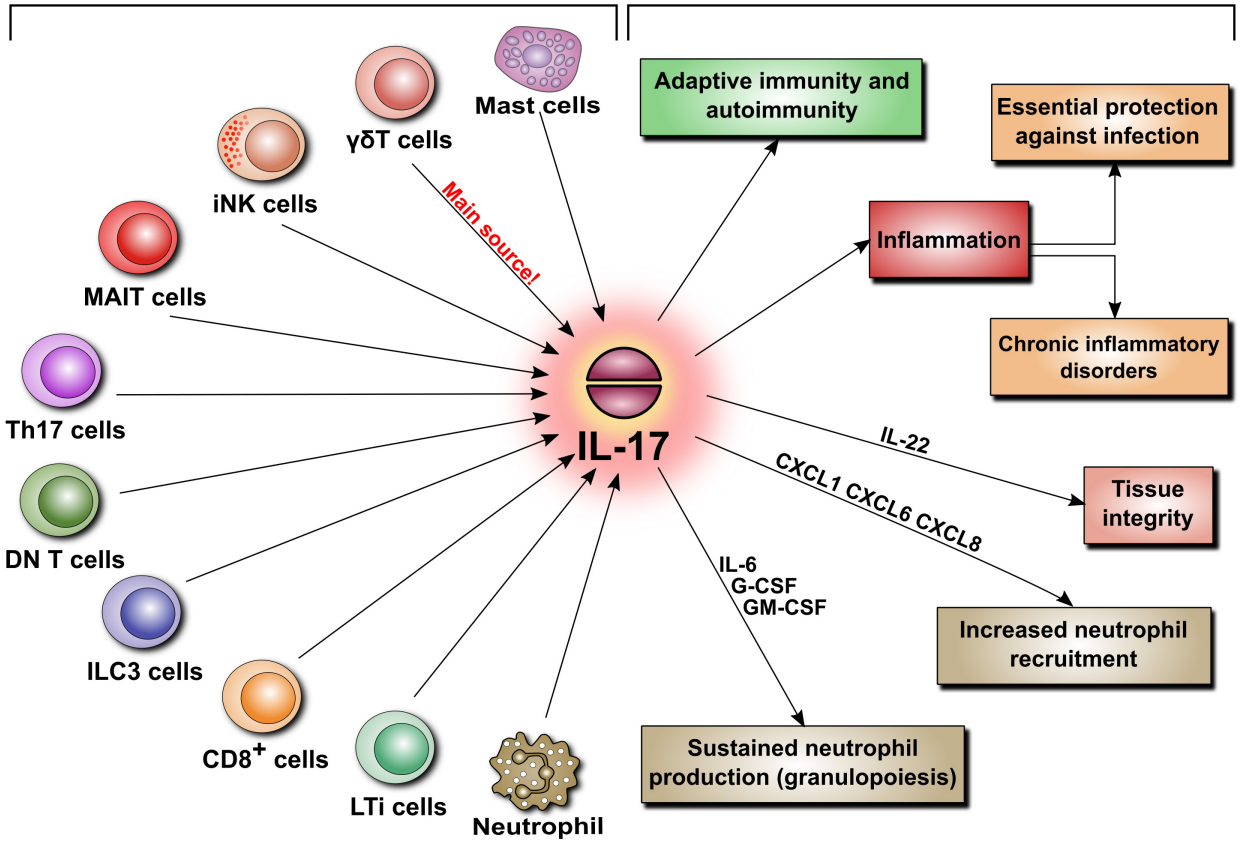

FIGURE 3 | Sources and functions of IL-17. IL-17 secretion is not limited to Th17 cells. When released, IL-17 is involved in various pathophysiological mechanisms. These include adaptive immunity, autoimmunity, and inflammation. The latter is a key element of host defense, but can also lead to chronic inflammatory disorders and tissue remodeling. Additionally, IL-17 regulates tissue integrity and is a potent inducer of neutrophil recruitment and granulopoiesis.

kidney after acute ischemia-reperfusion injury (Martina et al., 2016) and are an important subset of IL-17-producing cells in the inflamed kidney (Turner et al., 2012).

It is thought that the differentiation of Th17 cells and their activity during PD can be critically modulated by regulatory T cells (Treg) (Liappas et al., 2015). In this respect, CD69, a membrane glycoprotein induced rapidly on lymphocytes upon activation, has been implicated in promoting Treg development and limiting Th17 differentiation (Martin et al., 2010). In comparison with wild-type mice, the exposure of *cd69*$^{-/-}$ mice to PD fluids for 40 days led to an increase in Th17/Treg ratio and, consequently, to augmented Th17 cell infiltration and increased IL-17 production and peritoneal fibrosis (Liappas et al., 2016). Significantly, exacerbated fibrosis in *cd69*$^{-/-}$ mice could be alleviated by the blockade of IL-17. On the other hand, the effects seen in *cd69*$^{-/-}$ mice could be reproduced in wild-type mice by intraperitoneal administration of CD69-neutralizing antibodies. Similar results were achieved by transplantation of a mixture of bone marrow cells obtained from *Rag2*$^{-/-}{}_{\gamma}c^{-/-}$ double mutant mice and from either *cd69*$^{-/-}$ or wild-type animals. As *Rag2*$^{-/-}{}_{\gamma}c^{-/-}$ mice lack lymphocytes, these were derived only from *cd69*$^{-/-}$ or wild-type mice. This elegant strategy made it possible to demonstrate that CD69 expression in the lymphocytic rather than myeloid compartment of the bone marrow is responsible for controlling Th17 cells (Liappas et al., 2016).

Interestingly, CD69 appears to be constitutively expressed at low levels by tissue-resident memory T (T_{RM}) cells and by non-recirculating sessile innate-like lymphocyte subsets, including γδ T cells and MAIT cells (Kimura et al., 2017). The exact role of CD69 expression on these cells is not fully understood, but it appears to be important for cell retention in tissues (Kimura et al., 2017). There is a growing appreciation of the contribution of tissue-resident lymphocytes both to the maintenance of tissue homeostasis and to swift response to infection (Fan and Rudensky, 2016; Gebhardt et al., 2018). In this respect, it has been observed that γδ T cells in mice rapidly produced IL-17 in response to peritoneal infection with *E. coli*, which preceded the influx of neutrophils (Shibata et al., 2007).

It has been proposed that supplementation of PD fluids with the dipeptide alanyl-glutamine (Ala-Gln) could restore an impaired stress response in peritoneal cells and improve peritoneal host defense (Kratochwill et al., 2012, 2016). Indeed, the administration of Ala-Gln to rats and mice treated with

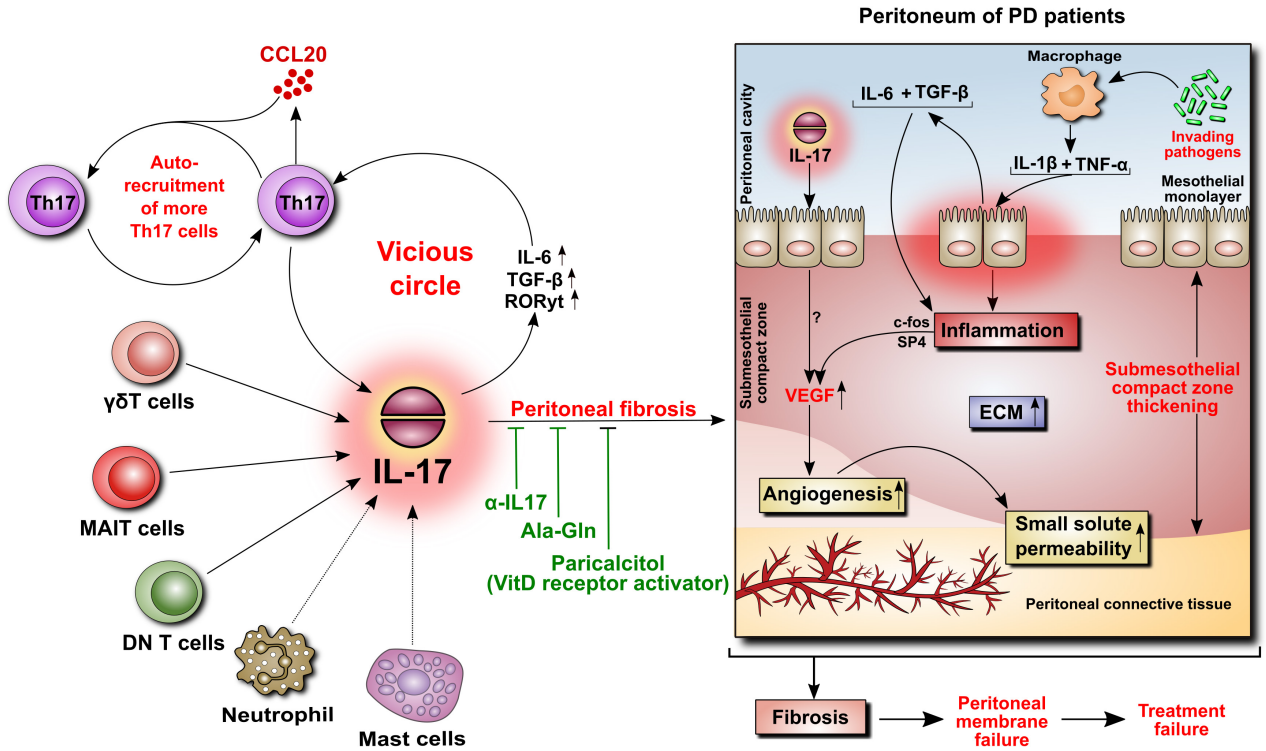

FIGURE 4 | Role of IL-17 in the peritoneum. IL-17 may be critically involved in initiating peritoneal fibrosis. IL-17 is produced primarily by γδT cells and possibly by other innate-like tissue-resident lymphocytes. The increase in IL-17 is associated with increased peritoneal levels of IL-6, TGF-β, and RORγt, leading to the formation of additional IL-17-secreting Th17 cells in a vicious circle. Moreover, Th17 cells release CCL20, a chemokine that boosts the recruitment of further Th17 cells. In the peritoneum of PD patients, IL-17 promotes thickening of the submesothelial compact zone. In addition, inflammation-induced angiogenesis leads to increased small solute transport. In this respect, we previously demonstrated that peritoneal inflammation is linked with angiogenesis through IL-6- and TGF-β-induced VEGF production involving c-Fos and SP4 transcription factors. In rodents, the attenuation of IL-17-mediated responses reduces the extent of peritoneal fibrosis.

PD-fluids markedly reduced the associated peritoneal fibrosis (Ferrantelli et al., 2016). Interestingly, this effect was paralleled by a reduction in peritoneal IL-17 expression and was thus attributed to inhibition of IL-17-driven reactions.

While the above studies clearly documented the peritoneal expansion of IL-17-producing cells in animals infused repeatedly with PD fluids, it remains to be determined, which PD fluid components are responsible for the effect. It has recently been observed that the fraction of IL-17-expressing T cells in peritoneal lavage fluid was greater in mice treated for 8 weeks with a conventional lactate-based PD solution with low pH and high GDP contents than in mice treated with a new neutral-pH low-GDP solution buffered with a mixture of lactate and bicarbonate (Vila et al., 2018). The new solution is viewed as more biocompatible and its use has also been associated with an increase in the dialysate levels of cancer antigen 125 (CA125) (Jones et al., 2001; Fusshoeller et al., 2004; Pajek et al., 2008). As CA125 is thought to reflect mesothelial cells mass (Krediet, 2001), one may hypothesize that less IL-17-mediated inflammation contributes to a better preserved mesothelium.

Clinical PD is frequented by episodes of peritonitis. It has been observed that the effluent concentrations of IL-17 in stable PD patients are very low (typically <5 pg/ml) but increase many-fold at the onset of peritonitis (Lin et al., 2013; Zhang et al., 2017). The magnitude of this increase depends clearly on the class of an invading microorganism; the highest IL-17 levels were recorded during peritonitis caused by Gram-positive bacteria other than streptococci and coagulase-negative staphylococci (e.g., by *S. aureus*) (Zhang et al., 2017). Moreover, it has been reported that patients with a delayed response to seemingly adequate antibiotic treatment had persistently low IL-17 levels (Wang et al., 2011). These observations suggest that IL-17 is an important component of peritoneal host defense. In this respect, it has recently been demonstrated that γδ T cells are the predominant source of IL-17 during *S. aureus*-induced peritonitis in mice (Murphy et al., 2014). Intriguingly, there were two waves of γδ T cell recruited with two distinct γδ T cell subsets involved. An initial rapid influx of $V\gamma1^+$ and $V\gamma2^+$ cells was followed by a more sustained infiltration by $V\gamma4^+$ cells. These $V\gamma4^+$ cells were retained in the peritoneum and responded by augmented IL-17 production during secondary infection. This led to increased phagocyte recruitment and enhanced bacterial clearance. Accordingly, transfer of *S. aureus*-primed $V\gamma4^+$ T cells to naïve hosts offered protection against *S. aureus* infection.

On the other hand, it has been observed that extensive peritoneal accumulation of IL-17-producing cells after infection or surgical injury may precede formation of peritoneal adhesions and intra-abdominal abscesses (Chung et al., 2002, 2003).

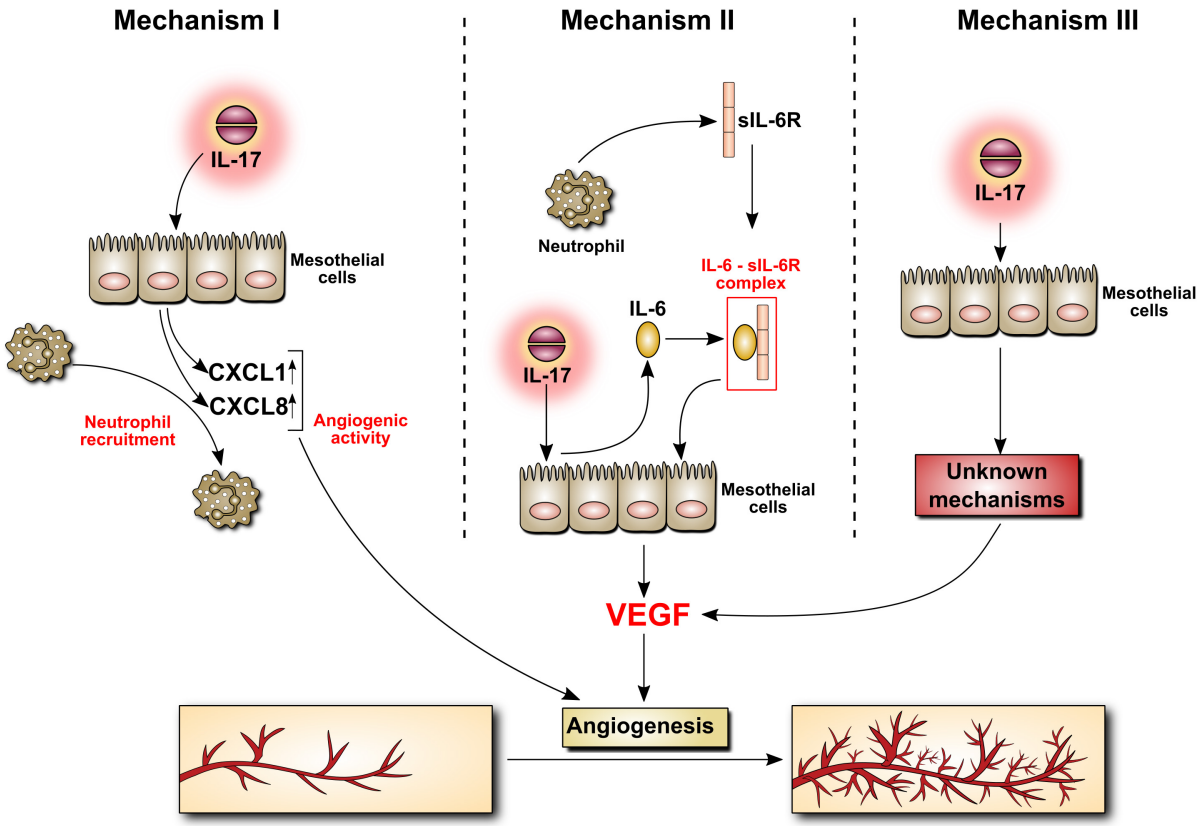

FIGURE 5 | Induction by IL-17 of mediators with angiogenic activity in mesothelial cells. The mechanism behind IL-17 induction of angiogenesis is poorly understood. It may involve the secretion of chemokines with angiogenic activity by mesothelial cells stimulated with IL-17 (Mechanism I). It may also be related to IL-17-induced mesothelial production of IL-6, which may then form a complex with sIL-6R, stimulating VEGF synthesis (Mechanism II). Other, so far unknown scenario(s), cannot be excluded (Mechanism III).

These could be prevented by neutralization of either IL-17 or IL-17-induced CXC chemokines that promote intraperitoneal neutrophil trafficking (Chung et al., 2002). In this respect, we have demonstrated that the peritoneal mesothelium is the main source of CXC chemokines released in response to IL-17 (Witowski et al., 2000). Moreover, IL-17-treated peritoneal mesothelial cells secrete G-CSF that acts to sustain neutrophil production (Witowski et al., 2007). In addition, we have previously demonstrated that mesothelial cells are the main source of intraperitoneal IL-6 (Witowski et al., 1996), which can exert some effects through so-called IL-6 *trans*-signaling (Chalaris et al., 2011). These include selective recruitment of T-cells into the peritoneal membrane (McLoughlin et al., 2005) and maintenance of their Th17 phenotype (Jones et al., 2010).

IL-17 IN ANGIOGENESIS

It has long been suspected that IL-17 may impact on the vasculature, as it can induce CXC chemokines with a characteristic ELR (glutamic acid-leucine-arginine) motif, which are potent angiogenesis promoters (Keeley et al., 2011). These chemokines, including CXCL1, CXCL5, CXCL6, and CXCL8, act via the receptor CXCR2 on endothelial cells stimulating their migration and proliferation. The angiogenic activity of ELR^+-CXC chemokines has been documented in several animal models of disease, including cancer, corneal neovascularization, and fibrosis (reviewed in Strieter et al., 2007; Keeley et al., 2011, and Santoni et al., 2014). The potential role of IL-17 in angiogenesis was further inferred from the observations that microvessel density in tumors correlated with the number of infiltrating IL-17-producing cells (Numasaki et al., 2003; Zhang et al., 2009; Wakita et al., 2010; He et al., 2011; Meng et al., 2012; Pan et al., 2015a; Huang et al., 2016). Moreover, it has been found that IL-17-transfected cancer cells formed larger and more vascularized tumors when transplanted in mice (Numasaki et al., 2005; Huang et al., 2016), and these effects could be significantly abrogated by the blockade of the CXCR2 receptor. Similarly, an increase in synovial vascularization observed in IL-17-induced arthritis in mice could be reduced by the administration of antibodies against the ELR^+ chemokine CXCL5 (Pickens et al., 2011).

Less clear is the relationship between IL-17 and VEGF. It has been reported that serum concentrations of IL-17 and VEGF correlate both with each other and with adverse prognosis in patients with colorectal (Liu et al., 2011) and non-small cell lung cancer (Pan et al., 2015a). In this respect, IL-17 has been

shown to directly induce VEGF in several malignant cell lines, including gastric (Meng et al., 2012), breast (Amara et al., 2016), and lung cancer (Pan et al., 2015b; Huang et al., 2016), as well as in tumor-associated neutrophils (Benevides et al., 2015). IL-17 can also stimulate VEGF release by normal fibroblasts from the lung, skin, and cornea (Numasaki et al., 2004, 2016; Suryawanshi et al., 2012), by synoviocytes (Honorati et al., 2006; Ryu et al., 2006), and chondrocyte-like cells from the nucleus pulposus (Hu et al., 2016). Such an effect, however, does not seem to be a general phenomenon, as VEGF secretion was not detected in IL-17-stimulated dermal microvascular endothelial cells (Takahashi et al., 2005) and in a number of cancer cell lines (Wu et al., 2016). In the latter, the absence of VEGF induction was attributed to the lack or weak expression of functional IL-17 receptor (Wu et al., 2016).

The exact mechanism of VEGF induction in cells responsive to IL-17 is poorly understood. It appears to be largely cell type-dependent. Few reports presented to date indicate that the regulation of IL-17-VEGF axis may occur via either STAT3- (Pan et al., 2015b; Hu et al., 2016; Wu et al., 2016) or STAT1-controlled (Huang et al., 2016) pathways. Interestingly, it has been suggested that IL-17-stimulated STAT3 activation in some cells required IL-6 induction (Wang et al., 2009). However, in other cells types (e.g., in corneal stromal fibroblasts) IL-17-induced VEGF production did not appear to be related to IL-6 and could not be inhibited by the IL-6 receptor blockade (Suryawanshi et al., 2012). The relationship between IL-17 and VEGF in tumor microenvironment may become even more complex during anti-VEGF therapy. It has been demonstrated that treatment with anti-VEGF drugs leads to an increase in IL-17 in the tumor micro-environment, which initiates a paracrine network that elicits an angiogenic response independently of VEGF and thus contributes to drug resistance (Chung et al., 2013).

IL-17 AND PERITONEAL VASCULATURE

IL-17 can affect peritoneal vasculature through at least three mechanisms, all involving mesothelial cells (**Figure 5**). Firstly, IL-17 can stimulate mesothelial cells to produce ELR^+ CXC chemokines such as CXCL1 (GROα) and CXCL8 (IL-8), which – in addition to being powerful neutrophil chemoattractants – do possess angiogenic activity. Secondly, IL-17 can stimulate mesothelial cells to release IL-6. During peritonitis mesothelial cell-derived IL-6 interacts with soluble IL-6 receptor shed from neutrophils and the complex activates mesothelial cells to produce VEGF. Finally, IL-17 can probably directly induce VEGF in mesothelial cells through as yet undefined mechanism.

It is not known what determines the choice of a given pathway *in vivo*. It is probably the presence of a specific combination of cytokines which drives a particular mechanism in mesothelial cells. Not only may such a cytokine cocktail promote differentiation of IL-17-producing cells, but also modulate the effector functions of IL-17. For example, TNFα can synergistically amplify IL-17-induced CXCL1 secretion through both transcriptional and post-transcriptional mechanisms involving stabilization of mRNA transcripts (Sun et al., 2011). Thus, this specific cytokine microenvironment (with IL-17 included) may arise in response to different types of infection and determine the course of inflammation and lead ultimately to changes in peritoneal vasculature.

AUTHOR CONTRIBUTIONS

EK, RC, and JW searched the literature and performed the experiments that suggested the mechanisms depicted in **Figure 5**. JK-M prepared the graphics. JW designed the paper and drafted the manuscript. JW and AJ wrote the manuscript. All authors revised, read, and approved the submitted version.

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Preventing Peritoneal Dialysis-Associated Fibrosis by Therapeutic Blunting of Peritoneal Toll-Like Receptor Activity

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Peritoneal dialysis (PD) is an essential daily life-saving treatment for end-stage renal failure. PD therapy is limited by peritoneal inflammation, which leads to peritoneal membrane failure as a result of progressive fibrosis. Peritoneal infections, with the concomitant acute inflammatory response and membrane fibrosis development, worsen PD patient outcomes. Patients who remain infection-free, however, also show evidence of inflammation-induced membrane damage and fibrosis, leading to PD cessation. In this case, uraemia, prolonged exposure to bio-incompatible PD solutions and surgical catheter insertion have been reported to induce sterile peritoneal inflammation and fibrosis as a result of cellular stress or tissue injury. Attempts to reduce inflammation (either infection-induced or sterile) and, thus, minimize fibrosis development in PD have been hampered because the immunological mechanisms underlying this PD-associated pathology remain to be fully defined. Toll-like receptors (TLRs) are central to mediating inflammatory responses by recognizing a wide variety of microorganisms and endogenous components released following cellular stress or generated as a consequence of extracellular matrix degradation during tissue injury. Given the close link between inflammation and fibrosis, recent investigations have evaluated the role that TLRs play in infection-induced and sterile peritoneal fibrosis development during PD. Here, we review the findings and discuss the potential of reducing peritoneal TLR activity by using a TLR inhibitor, soluble TLR2, as a therapeutic strategy to prevent PD-associated peritoneal fibrosis.

Keywords: peritoneal dialysis, inflammation, peritoneal fibrosis, toll-like receptors, soluble toll-like receptor 2

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INTRODUCTION

Peritoneal dialysis (PD), an essential therapy for end-stage kidney disease, depends on the integrity of the peritoneal membrane. Despite advantages over other dialysis techniques, PD failure due to peritoneal membrane damage remains the major limiting factor (Davies et al., 1999; Williams et al., 2003; Cho et al., 2014). Damage is driven by local peritoneal inflammation, which results in structural alterations of the peritoneal membrane, typically fibrosis – thickening of the sub-mesothelial compact zone – and vascular damage. This leads to altered solute transport through the membrane and dialysis failure (Lambie et al., 2013; Fielding et al., 2014).

Peritoneal infections and the concomitant inflammation resulting from the activity of pathogen-associated molecular patterns (PAMPS) derived from microbial components, are believed to be responsible for 20–40% of PD failure (Cho et al., 2014; Pajek et al., 2014). However, peritoneal inflammation and fibrosis are also observed in PD patients without defined infectious episodes (Tomino, 2012; Cho et al., 2014). In this case, uraemia, prolonged exposure to bio-incompatible PD fluids and surgical catheter insertion have all been reported to induce sterile peritoneal inflammation, fibrosis and membrane failure by promoting tissue damage and cellular stress. This leads to the release and/or generation of endogenous cellular components and matrix degradation products, acting as damage-associated molecular patterns (DAMPs). The DAMPs trigger pro-inflammatory and pro-fibrotic responses (Anders and Schaefer, 2014) that result in local angiogenesis, vasculopathy, epithelial-to-mesenchymal transition in mesothelial cells and collagen deposition in the sub-mesothelial compact zone (Flessner et al., 2007; Johnson et al., 2012; Tomino, 2012; Cho et al., 2014; Strippoli et al., 2016).

The immune mechanisms linking infection-induced or sterile inflammation with the onset, development and regulation of PD-associated peritoneal fibrosis are poorly defined and thus the focus of intense investigation (Fielding et al., 2014; Liappas et al., 2015, 2016; Raby et al., 2018). Consequently, effective therapies to prevent PD-associated fibrosis remain to be developed.

Critical to triggering pro-inflammatory responses is the activity of the Toll-like family of innate immune receptors (TLRs) (Kawai and Akira, 2010; Kawasaki and Kawai, 2014). TLRs are expressed in a variety of cell types, including peritoneal leukocytes and mesothelial cells (Colmont et al., 2011; Raby et al., 2017). They recognize a wide range of microorganisms and their PAMPs (e.g., lipopolysaccharide/endotoxin/LPS; lipopeptides) as well as DAMPs released as a consequence of cellular stress [e.g., High Mobility Group Box-1 (HMGB-1); heat shock proteins (Hsp)], or generated following extracellular matrix degradation during tissue damage (e.g., hyaluronan, fibronectin) (Chen and Nuñez, 2010; Anders and Schaefer, 2014). TLR triggering results in the production of potent pro-inflammatory and fibrotic mediators, e.g., IL-6, TGF-β, TNF-α, IL-8, IFN-γ, and IL-1β (Fielding et al., 2014; Kawasaki and Kawai, 2014).

Inappropriate TLR activation may result in serious inflammatory conditions, therefore, they are being considered as therapeutic targets for the prevention and/or treatment of a number of inflammatory pathologies (Riedemann et al., 2003; Kanzler et al., 2007; Mollnes et al., 2008; Dunne et al., 2011; Raby et al., 2013). Given the close link between inflammation and fibrosis, and the recognized involvement of TLRs in tissue fibrosis (Anders and Schaefer, 2014), we recently assessed the role that TLRs play in peritoneal fibrosis development during PD (Raby et al., 2017, 2018). Here, we review the findings and discuss the potential of reducing peritoneal TLR activity by using the soluble form of TLR2, a TLR modulator, as a therapeutic strategy to prevent PD-associated peritoneal fibrosis.

CRITICAL CONTRIBUTIONS OF TLR2 AND TLR4 TO PD-ASSOCIATED PERITONEAL MACROPHAGE AND MESOTHELIAL CELL PRO-INFLAMMATORY AND FIBROTIC RESPONSES

TLR2- and TLR4-Mediated Peritoneal Macrophage and Mesothelial Cell Responses to Infection

Recent studies have focused on TLR2 and TLR4, as these TLRs recognize the widest range of microbial components involved in PD-associated infections and are also the main TLRs involved in sterile inflammatory responses (Anders and Schaefer, 2014; Kawasaki and Kawai, 2014).

Consistent with their expression detected in PD effluent (PDE)-isolated uremic leukocytes, TLR2 and TLR4 were found to mediate pro-inflammatory (IL-6, IL-8, and TNF-α) and fibrotic (TGF-β, IL-6, IL-13, MMP1, MMP3, MMP9, and TIMP-1) responses in PDE leukocytes stimulated with the Gram-positive bacterium *Staphylococcus epidermidis*, Pam_3-Cys-Ser-$(Lys)_4$ (Pam_3Cys, a synthetic bacterial lipopeptide) – both TLR2 agonists – the Gram-negative bacterium *Escherichia coli* and the Gram-negative bacterial cell-wall component LPS – both TLR4 agonists. Macrophages were the main cell type responsible for the observed leukocyte responses, consistent with their high TLR receptor expression compared with lymphocytes (Raby et al., 2017).

Similar to peritoneal leukocytes, human peritoneal mesothelial cells (HPMC, from greater omentum) were found to respond to Pam_3Cys, *S. epidermidis* and *E. coli*, but not to LPS. HPMC's lack of response to LPS reflected the documented lack of TLR4 expression in HPMC (Colmont et al., 2011). However, HPMC responded to *E. coli*, most likely by recognizing bacterial lipopeptides through TLR2 and flagellin – the protein component of the flagellum of Gram-negative bacteria – through TLR5 expressed in these cells (Colmont et al., 2011).

In vivo studies confirmed the critical role that TLR2 and TLR4 play in infection-induced peritoneal inflammation and fibrosis (Raby et al., 2017). A mouse model of peritoneal inflammation and fibrosis induced by repeated intraperitoneal injections of *S. epidermidis* (TLR2 agonist) or *E. coli* (TLR4 agonist) was used. This model mimics the typical clinical episodes of recurrent bacterial peritonitis leading to peritoneal fibrosis observed in PD patients (Fielding et al., 2014). Repeated injection of *S. epidermidis* in wild-type (WT) mice resulted in substantial peritoneal fibrosis, whereas *S. epidermidis* injection in TLR2-deficient mice did not result in fibrosis development (**Figure 1A**). By contrast, injection of *E. coli* in TLR4-deficient mice resulted in a partial reduction in fibrosis when compared with WT mice (**Figure 1B**). This is consistent with the possibility that *E. coli*-induced pro-fibrotic responses may involve other receptors (e.g., TLR2, TLR5) in addition to TLR4. Together, these findings indicated a major role for TLR2 and to a lesser extent for TLR4 in bacteria-induced peritoneal fibrosis associated

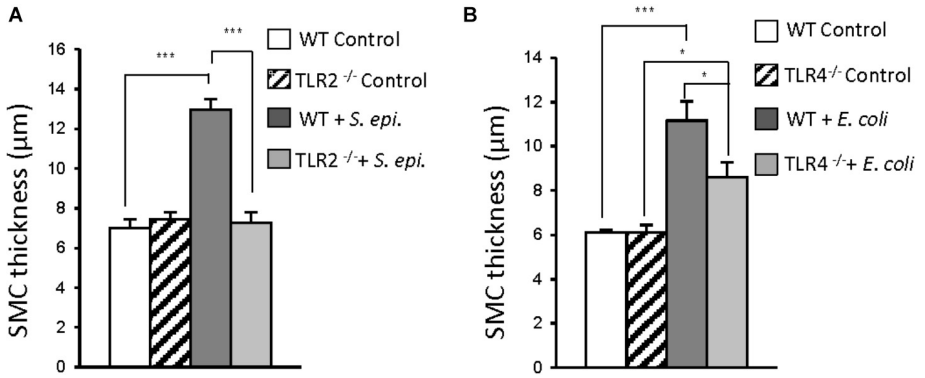

FIGURE 1 | Critical contribution of TLR2 and TLR4 to bacteria-induced peritoneal fibrosis development. **(A,B)** Wild-type (WT), TLR2 deficient (TLR2$^{-/-}$) or TLR4$^{-/-}$ mice (n = 5 per group) were inoculated intraperitoneally 4 times at weekly intervals with *S. epidermidis* (*S. epi*., 5 × 10^8 CFU/mouse) or *Escherichia coli* (*E. coli*, 2 × 10^7 CFU/mouse) or left untreated (control). Four weeks after the last injection, histological analysis of the peritoneal membrane was conducted and the thickness of the sub-mesothelial compact zone (SMC, layer between the muscle and membrane surface) was determined. Bar plots show the mean (± SEM) of SMC thickness in each experimental group. * $P < 0.05$; ***, $P < 0.005$. Adapted with permission from Raby et al. (2017).

with PD, and pointed at controlling infection-induced TLR-mediated activation as a potential therapeutic against peritoneal fibrosis.

TLR2- and TLR4-Mediated Peritoneal Macrophage and Mesothelial Cell Responses to PD Solutions

The role of TLR2 and TLR4 in sterile inflammatory and fibrotic responses of peritoneal cells resulting from exposure to PD solutions (PDS) was also evaluated (Raby et al., 2018). A number of PDS elicited pro-inflammatory and pro-fibrotic responses (CXCL-8/IL-8, IL-6, TNF-α, TGF-β, and IL-1β) from PDE-isolated uremic peritoneal leukocytes and mesothelial cells (from greater omentum), including those glucose-based (1.36 and 2.27% glucose Dianeal®, Physioneal®, Stay Safe®) or icodextrin-based (Extraneal®), having low pH (Dianeal®, Extraneal®, Stay Safe®) or physiologic pH (Physioneal®).

Interestingly, analysis of the expression of inflammatory and immunity-related genes in uremic peritoneal leukocytes and HPMC exposed from 16 h to low glucose Dianeal® (1.36% glucose), a commonly used PDS, showed substantial modulation of a number of genes. In leukocytes, 15 genes were found significantly up-regulated by Dianeal®, and only 5 were down-modulated. The transcripts up-modulated by PDS included those coding for inflammatory mediators (CXCL8/IL-8, TNF-α, IFN-γ, monocyte chemoattractant CCL2/MCP-1, the chemokine receptor CCR4, IL-1β) as well as for TLR2, TLR1, and TLR6 (TLR2 signaling partners), TLR3 and TLR signal intermediates.

In HPMC, 8 genes were found up-regulated and 6 down-regulated following exposure to Dianeal®. The transcripts for the pro-inflammatory cytokines IL-1α, IL-1β, and CXCL8/IL-8 were strongly up-modulated, whereas that for CXCL10/IL-10 – an anti-inflammatory cytokine – was found down-modulated. Fibrosis-related gene expression analysis in Dianeal®-exposed HPMC – the cell type that contributes to peritoneal fibrosis by acquiring a fibroblastic phenotype following epithelial-to-mesenchymal transdifferentiation (EMT) – showed a 3-fold increase in *VGEFA* (main isoform of VGEF) expression and a reduction in *E-cadherin*, both effects indicating EMT (Yung and Chan, 2012; Ruiz-Carpio et al., 2017).

Notably, peritoneal leukocyte TLR2 or TLR4 blocking with specific monoclonal antibodies inhibited the pro-inflammatory cytokine release induced by Dianeal®, and the extent of the inhibition depended on the PD patient tested. Simultaneous blocking of TLR2 and TLR4 resulted in a stronger inhibition of a number of pro-inflammatory and fibrotic cytokines released by the PDS-exposed uremic peritoneal leukocytes. TLR2 blockade in PDS-exposed HPMC also showed a significant reduction in pro-inflammatory mediator release. Together, these findings indicated that peritoneal TLR2 and TLR4 control inflammatory and fibrotic responses to PDS exposure.

Interestingly, it was found that the cellular stress resulting from PDS exposure induces DAMP generation which in turn triggers TLR2 and TLR4 activation, and that the PDS does not contain pre-existing components capable of TLR activation. Of note, Hsp70 and low (∼33 kDa) and medium (∼289 kDa) molecular mass hyaluronan (HA) were identified as the main PDS-induced DAMPs. They elicited inflammatory responses from peritoneal cells through TLR2/TLR4 activation, as Hsp70 and HA are ligands of both TLR2 and TLR4 and their specific inhibition reduced PDS-induced inflammation in peritoneal leukocytes.

It is worth noting that, in addition to eliciting inflammatory responses, heat-shock proteins have shown cytoprotective activity against cytotoxicity resulting from PDS exposure (Kratochwill et al., 2009). It is believed that peritoneal damage due to PD exposure may reflect an imbalance between cellular injury-induced inflammation and cytoprotective processes. The extracellular exposure to otherwise intracellular cytoprotective molecules such as Hsp70, released as a consequence of tissue damage/cell death, may trigger DAMP signals leading to pro-inflammatory responses and exacerbating peritoneal damage (Kratochwill et al., 2011).

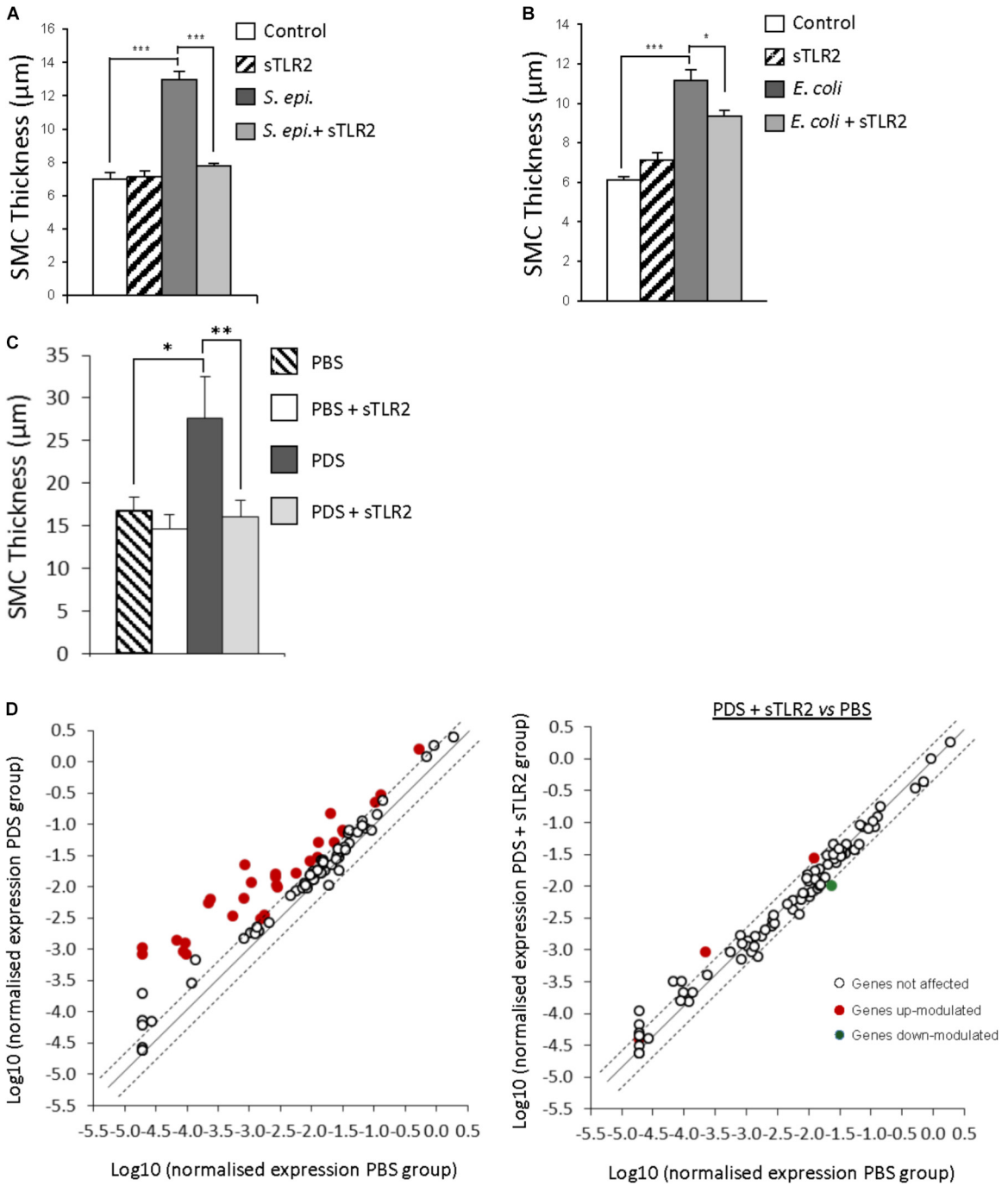

FIGURE 2 | Therapeutic potential of soluble Toll-like receptor 2 (sTLR2) against bacteria- and PD solution-induced peritoneal fibrosis development. **(A,B)** mice (n = 5 per group) were inoculated intraperitoneally 4 times at weekly intervals with *S. epidermidis* (*S. epi.*, 5×10^8 CFU/mouse) or *Escherichia coli* (*E. coli*, 2×10^7 CFU/mouse) in the presence or absence of sTLR2 (250 ng/mouse), or left untreated (control). Four weeks after the last injection, histological analysis of the peritoneal membrane was conducted and the thickness of the sub-mesothelial compact zone (SMC) was determined. Bar plots show the mean (± SEM) of SMC thickness in each experimental group. * $P < 0.05$; ***, $P < 0.005$. **(C,D)** Mice were instilled twice daily with 2 ml of PBS (n = 5) or Fresenius Standard glucose solution (PDS, n = 8) in the presence or absence of sTLR2 for 40 days before sacrifice, tissue sample collection and histological analysis of the peritoneal membrane for SMC thickness determination. Results show the mean (± SEM) for each experimental group. *$P < 0.05$; **$P < 0.01$. Scatter plots in **(D)** show the effect of PDS on the expression of fibrosis-related genes in the absence and presence of sTLR2, as assessed by quantitative RT-PCR on RNA extracted from peritoneal membrane samples. Dotted lines indicate the 0.5 and 2 fold change thresholds. Open circles outside the dotted lines correspond to genes modulated in a non-statistically significant manner. Adapted with permission from Raby et al. (2018).

These findings suggested that inhibiting DAMP-TLR associations may have therapeutic potential against peritoneal fibrosis induced by PDS exposure.

THERAPEUTIC POTENTIAL OF SOLUBLE TLR2 AGAINST INFECTION-INDUCED AND STERILE PERITONEAL INFLAMMATION AND FIBROSIS ASSOCIATED WITH PD

The therapeutic potential of inhibiting infection- or PDS-induced TLR activation to prevent peritoneal fibrosis development was evaluated by testing the ability of soluble Toll-like receptor 2 (sTLR2), a TLR inhibitor, to regulate peritoneal inflammation. It is well documented that sTLR2 reduces TLR-mediated inflammation by both acting as a decoy receptor, binding to TLR2 ligands, and by interfering with the co-receptor activity of CD14, the main co-receptor for most TLRs (Lebouder et al., 2003; Raby et al., 2009, 2013).

Inhibitory Effect of sTLR2 on PD-Associated Peritoneal Infection-Induced Inflammation and Fibrosis

When administered together with the repeated peritoneal injection of *S. epidermidis* in mice, sTLR2, was found to prevent fibrosis development (**Figure 2A**; Raby et al., 2017). This effect was accompanied by a substantial reduction of inflammatory parameters, including the peritoneal levels of a number of pro-inflammatory cytokines and chemokines, neutrophils (PMN) and monocytes at the peak time of their influx to the peritoneum as well as the prototypical pro-fibrotic cytokine TGF-β. Of note, in spite of reducing inflammation and phagocyte recruitment, the capacity of the mice to clear the infection was not found affected by the presence of sTLR2, as no difference in bacterial load (peritoneum and blood) between mice treated and non-treated with sTLR2 was observed.

Fibrosis-related gene transcripts were also markedly inhibited by sTLR2 administration. Of the 85 genes tested, 21 were found markedly up-regulated by *S. epidermidis*, and sTLR2 reduced this effect in 18 of them. The transcripts reduced by sTLR2 included *Fasl*, central to apoptosis, which impairs bacterial clearance during PD (Hohlbaum et al., 2001; Catalan et al., 2003); *STAT-1*, a critical signal intermediate for fibrosis development (Fielding et al., 2014), and *IL-6* – a major promoter of peritoneal fibrosis (Fielding et al., 2014). Notably, sTLR2 counteracted *S. epidermidis*' negative effect on matrix metalloproteinases (MMPs) Mmp-1, Mmp-3 and Mmp-9, and *S. epidermidis*' positive effect on Mmp-13 and the MMP inhibitor Timp-1 (Raby et al., 2017).

Of note, peritoneal fibrosis induced by Gram-negative bacteria was also inhibited by sTLR2, as simultaneous peritoneal inoculation of sTLR2 with the repeated injection of *E. coli* resulted in reduced peritoneal fibrosis (**Figure 2B**). This reflects the fact that, in spite of not acting as a TLR decoy receptor for most Gram-negative bacterial components, sTLR2 can still reduce TLR-mediated fibrotic signaling induced by Gram-negative bacteria by inhibiting CD14, a co-receptor for most TLRs (Raby et al., 2009), including TLR4. Thus, peritoneal fibrosis resulting from repeated peritoneal bacterial infections like those associated with PD can be inhibited by sTLR2 by acting on a variety of pro-inflammatory and fibrotic mediators, but notably, without affecting infection clearance.

Inhibitory Effect of sTLR2 on PDS-Induced Peritoneal Inflammation and Fibrosis

The therapeutic potential of sTLR2 against inflammation and fibrosis development resulting from prolonged peritoneal exposure to PDS was tested in a murine model of sterile peritoneal fibrosis consisting of daily peritoneal catheter infusions of a standard PDS (Raby et al., 2018). This mouse model mimics the changes in the peritoneal membrane (morphological and functional) observed in non-infected patients on PD (Gonzalez-Mateo et al., 2009; Loureiro et al., 2011). The peritoneal administration of sTLR2 together with the PDS twice weekly prevented the development of peritoneal fibrosis (**Figure 2C**). In agreement with this finding, sTLR2 was found to suppress the PDS-induced increased expression of inflammatory and fibrotic mediators (TNF-α, IL-1β, KC, IL-6, and IFN-γ). The suppressive effect of sTLR2 on inflammatory mediators correlated with a substantial reduction in the number of peritoneal leukocytes and the percentage of infiltrating neutrophils in particular (Raby et al., 2018). Notably, sTLR2 counteracted the negative effect of PDS on regulatory T cell (Treg) numbers, recovering their numbers to the levels observed following PBS inoculation. Tregs, an anti-inflammatory T cell subset, control T cell expansion, including that of Th17 cells, an inflammatory T cell subset involved in peritoneal damage and fibrosis development (Liappas et al., 2016). sTLR2's positive effect on Treg cells resulted in an increased in the Treg:Th17 ratio.

Analysis of fibrosis-related gene transcripts in mice peritoneal membranes carried out after the last inoculation of PDS+sTLR2 showed that sTLR2 also counteracted the positive effect of PDS on mRNA coding for several inflammatory mediators and fibrosis markers (**Figure 2D**). Of the 85 genes tested, 29 were markedly up-regulated by PDS at this time point, and sTLR2 was found to reduce this effect in 27 of them, including in the transcripts for FasL, STAT-1, IFN-γ, MMPs, TIMP1/3, TGF-β, IL-1β, and TNF-α. Thus, the development of peritoneal fibrosis by long exposure to PDS can be prevented by administering sTLR2, which inhibits pro-inflammatory and fibrotic mediator production and controls the expansion of inflammatory cells.

CONCLUSION

The results of recent investigations reviewed here revealed the critical role that peritoneal TLR2 and TLR4, main members of the Toll-like family of innate immune receptors, play in mediating inflammation and fibrosis induced either by recurrent peritoneal infections during PD or prolonged exposure to PD

solutions. Furthermore, the investigations showed the potential of a novel therapeutic strategy that targets TLRs to blunt peritoneal inflammation and thus prevent fibrosis development (either infection-induced or sterile) during PD by the use of a decoy receptor, sTLR2. This soluble receptor also inhibits the activity of CD14, the common TLR co-receptor. Thus, sTLR2 can reduce pro-inflammatory and fibrotic responses to different pathogens (e.g., Gram-positive and Gram-negative bacteria) and their PAMPs and to endogenous TLR ligands (DAMPs) activating different TLRs, not only TLR2. These findings pave the way for future clinical trials to test the clinical efficacy of sTLR2 as a therapy for patients in long-term PD.

Notably, the preclinical studies showed that peritoneal inflammation and fibrosis induced by bacteria in mice can be inhibited by sTLR2 without affecting the animal's capacity to resolve the infection. Given that PD patients are prone to infections, this ability of sTLR2 would be advantageous when comparing with complete TLR blockade-based therapies, e.g., by combination of anti-TLR2 and -TLR4 antibodies (Lima et al., 2015), as these may have a detrimental effect on infection clearance. However, preclinical studies have shown the potential of combining anti-TLR2 and TLR4 antibodies with antibiotics to reduce inflammation whilst controlling infection (Spiller et al., 2008; Lima et al., 2015). Thus, a comparative evaluation of the efficacy of both TLR-targeting therapeutic strategies in PD models of infection/fibrosis will be required. Similarly, the efficacy of sTLR2 as a treatment for established fibrosis and membrane failure remains to be evaluated, since in the reported studies sTLR2 was inoculated together with the infecting bacteria or the PD solution in an initially healthy peritoneal membrane.

The pro-fibrotic cytokine TGF-β has been a main target for therapeutic interventions. Inhibition of its synthesis or activity showed promising effects (Duman et al., 2001; Margetts et al., 2002; Kyuden et al., 2005; Loureiro et al., 2011; Tomino, 2012; Zhang et al., 2014; Nongnuch et al., 2015). However, given TGF-β pleiotropic functions, its blockade is potentially hazardous (Blobe et al., 2000; Yoshimura et al., 2010), and it is just one of several mediators of fibrosis acting down-stream of TLR activation.

Thus, the reported sTLR2-based anti-fibrotic strategy may be a valuable complement to antibiotic therapies during PD infections, to biocompatible PDS or to PDS supplemented with immunomodulatory dipeptides to mitigate the PDS' adverse effects (Ferrantelli et al., 2016). sTLR2 may also be useful in other inflammatory conditions associated with PD, for example to help reduce the increased risk of cardiovascular diseases.

AUTHOR CONTRIBUTIONS

ML proposed the subject and conceived the general structure of the review. A-CR and ML revised the existing literature and contributed to all the sections.

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Peritoneal Dialysis Is an Independent Factor Associated to Lower Intima Media Thickness in Dialysis Patients Free From Previous Cardiovascular Disease

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Carotid intima media thickness (cIMT) displays prognostic value as a marker of cardiovascular risk in dialysis patients. However, few data are available regarding the impact of dialysis modality on cIMT. The aim of this study is to determine whether the modality of dialysis influences cIMT values. We compared 237 peritoneal dialysis (PD) and 451 hemodialysis (HD) patients without previous cardiovascular disease included in NEFRONA, a prospective, observational and multicenter study. This cross sectional study included the determination of cIMT in 6 carotid territories by arterial ultrasound. cIMT was determined in territories without atheroma plaque and averaged. A second analysis was performed using all territories, giving a truncated cIMT value of 1,5 mm to territories presenting with atheroma plaque. Age and plaque presence at baseline were the clinical variables more closely associated to cIMT in dialysis patients. The evaluation of the impact of the modality of dialysis on cIMT showed that PD patients had lower cIMT than HD patients, both in territories with no plaques and when using truncated cIMT values. No differences were found between right and left sides, neither in cIMT or truncated cIMT values. Lineal multivariate analysis adjusted by several clinical variables showed a statistically significant association of PD with a lower cIMT (slope −0.036; SD 0.010). These results were also confirmed when truncated cIMT values were used. We conclude that the modality of dialysis has an impact on cITM. HD patients have greater global cIMT than PD patients, and PD is and independent factor associated with a lower cIMT.

Keywords: intima and media thickness, dialysis, cohorts study, ultrasound, atherosclerosis

INTRODUCTION

In the last 35 years, the incidence of end-stage renal disease (ESRD) has raised dramatically. ESRD patients have a 5-year survival probability of 50% (United States Renal Data System (USRDS), 2017; Kramer et al., 2018) and, compared to general population, have a crude excess mortality rate of 80 to 175.(Foster et al., 2018) Main contributors to this increased mortality rates are dialysis patients,

and among them, cardiovascular disease (CVD) accounts for 53% of the deaths.(United States Renal Data System (USRDS), 2017) In chronic kidney disease (CKD) patients, atherosclerosis hallmarks are more advanced than in the general population.(Betriu et al., 2014) This increase in atherosclerosis burden is parallel to the degree of kidney impairment, being higher in dialysis patients (Drueke and Massy, 2010). The contribution of atherosclerosis to the increase in mortality of ESRD patients has been questioned because most of the deaths are attributed to sudden death, and related to electrolyte imbalance. However, recent results have shown that atherosclerosis presence is an independent factor associated with cardiovascular events (CVE) in ESRD, and that each new arterial territory with plaque raised a 86% the hazards of having a CVE (Valdivielso et al., 2017).

Traditional risk factors are not useful to predict CVE on dialysis patients. The use of the traditional algorithms in risk stratification in CKD consistently underestimate the risk of the CKD patients of having a CVE (Coll et al., 2010). Therefore, new markers of cardiovascular risk in dialysis patients are needed. Among the candidates, vascular determination of intima media thickness of carotids (cIMT) has shown promising prognostic value in this population (Benedetto et al., 2001). Current knowledge about the impact of the type of dialysis on cIMT is based on studies in small cohorts, without adjustment for another possible confounding factors (Konings et al., 2002; Al-Hweish et al., 2010; Shi et al., 2012). These sparse data obtained in different cohorts, and with different methodology and criteria, precludes knowing whether peritoneal dialysis (PD) and hemodialysis (HD) affect cIMT differently.

There are significant differences between both modalities of dialysis. PD patients have a potentially higher atherogenic profile than their counterparts on HD, due to the recurrent peritoneal loading with glucose-based dialysis solutions, and to the continuous peritoneal leak of proteins. On the contrary, HD patients show a worse preservation of residual kidney function, which may contribute to inflammation, endothelial dysfunction and vascular calcification. Moreover, PD is associated with a stable fluid status and blood pressure pattern compared to periodic fluctuations found in HD. Therefore, the modality of dialysis itself could have a differential effect on cIMT determining factors.

With the aim of shedding some light on this clinical concern, we evaluated cIMT in a selected sub-cohort of the NEFRONA study, in order to determine whether the modality of dialysis influences cIMT.

MATERIALS AND METHODS

Study Design and Participants

The ethics committee of University Hospital Arnau de Vilanova from Lleida, Spain, approved the study. All included patients signed informed consent and the study complied with the principles of the Declaration of Helsinki.

The NEFRONA project is a prospective, multicentre, observational cohorts study from Spain aimed to assess the atherosclerotic burden in CKD patients, including patients with ESRD. The rationale and baseline description of NEFRONA cohort have been reported in detail elsewhere. (Junyent et al., 2010a,b) Briefly, 2445 CKD patients free from previous CVE, aged 18 to 75 were enrolled from 81 Spanish hospitals between October 2009 and June 2011. The exclusion criteria were previous CVE, active infections (HIV, tuberculosis), pregnancy, having received any organ transplantation or having a life expectancy of less than 1 year. This study is a cross-sectional analysis in a subcohort of the NEFRONA study, including all the dialysis patients recruited (451 on HD and 237 on PD).

Clinical and Biochemical Data

At recruitment, patients were asked to complete a questionnaire including family history regarding premature CVD, clinical history (diabetes, hypertension and dyslipidaemia), cardiovascular risk factors (such as smoking habit), and medication use. Anthropometric data and medical history were also obtained from all patients at the time of recruitment. Biochemical data were obtained from a routine blood test 3 months either before or after the vascular ultrasound. For HD patients, blood samples were retrieved just before the second session of the week. High-sensitivity C reactive protein (hsCRP), 25-hydroxy-vitamin D and 1,25-hydroxy-vitamin D were quantified in a centralized laboratory. ABI measurements were performed with a protocol previously described (Arroyo et al., 2017).

Carotid Ultrasound

Patients underwent B-mode ultrasound in both carotid arteries with the Vivid BT09 device (General Electric Instruments, Freiburg, Germany) and a 6–13 MHz broadband linear array probe. For imaging, patients were in supine position with the head turned 45° contralateral to the side of the probe. cIMT was measured in the last centimeter of the far wall of the common carotid artery, the bulb section and in the first centimeter of the internal carotid artery. Measurements were made in plaque-free arterial segments. Atheromatous plaque, following the recommendations of the ASE Consensus Statement (Stein et al., 2008) and the Mannheim cIMT Consensus report (Touboul et al., 2004), was defined by a cIMT $\geq$ 1.5 mm protruding to the lumen of the imaged sections. In order to account for the values of cIMT in territories with plaques, a truncated cIMT value was also calculated. Truncated cIMT calculations were made giving a cIMT value equal to 1.5 mm to the territories with plaque. In each patient, averaged cIMT true values or truncated values of the six territories explored were calculated.

Ultrasound explorations were carried out by the same itinerant team of five trained technicians. Images were analyzed by a single reader in a blinded fashion using the EchoPAC Dimension software (General Electric Healthcare, Harten, Norway) in the UDETMA (Unit for the Detection and Treatment of Atherothrombotic Diseases, Hospital Universitari Arnau de Vilanova, Lleida, Spain). To assess the quality of the

measurements a sample of 20 individuals was measured 3–5 times on different days, obtaining an intraclass correlation coefficient of 0.93.

Statistical Analysis

Quantitative variables are shown as means and standard deviations, and its differences between groups were compared with the Student's *t*-test. Qualitative variables are summarized as absolute and relative frequencies, and chi-squared test (Fisher test for expected frequencies <5) was used to perform comparisons between groups. Pearson′s correlations were used to determine univariate relationships between cIMT values and linear or categorical variables. Significant variables in univariate analyses and potential confounders were used to develop appropriate multivariate linear regression models. A forward step procedure was used to build the multivariate model, including the variables showing maximum contribution identifying those patients with higher cIMT, according to the likelihood ratio test (LRT).

RESULTS

Baseline Characteristics

A total of 451 HD and 237 PD patients were included. **Table 1** shows anthropometrical, clinical and biochemical data comparisons between both groups. PD patients were younger, showed increased ratios of hypertension and dyslipidemia, and had higher systolic arterial pressure, total cholesterol, HDL and LDL cholesterol, haematocrit, calcium and phosphate; they had been less time on dialysis and presented with lower levels of potassium and 25-hydroxy-vitamin D.

Variables influencing cIMT on dialysis patients.

Figure 1 shows averaged cIMT values in HD and PD patients both in territories with no plaques (IMT) and in territories in

TABLE 1 | Demographic data.

	HD (*n* = 451)	PD (*n* = 237)	*p*
Age (years)	54 (14)	51 (14)	0,004
Sex (man)	272 (60,3)	137 (57,8)	0,567
Smoker	250 (55,4)	135 (57,0)	0,381
Diabetes	107 (23,7)	47 (19,8)	0,143
Hypertension	406 (90,0)	229 (96,6)	0,001
Dyslipidemia	214 (47,5)	153 (64,6)	0,000
Plaque presence at baseline	325 (72,1)	156 (65,8)	0,054
BMI (kg/m^2)	26,3 (5,0)	26,7 (4,8)	0,341
SBP (mmHg)	136 (23)	144 (24)	0,000
Time on dialysis (months)	33 (43)	20 (19)	0,000
Total cholesterol (mg/dL)	156 (39)	180 (43)	0,000
HDL cholesterol (mg/dL)	46 (16)	49 (15)	0,016
LDL cholesterol (mg/dL)	84 (32)	104 (34)	0,000
Triglycerides (mg/dL)	141 (81)	137 (66)	0,498
Glucose (mg/dL)	103 (40)	101 (37)	0,526
Hematocrite (%)	35,7 (4,3)	36,6 (4,4)	0,013
Calcium (mg/dL)	9,0 (0,7)	9,2 (0,7)	0,005
Phosphate (mg/dL)	4,8 (1,4)	5,1 (1,2)	0,008
Uric acid (mg/dL)	6,1 (1,4)	6,0 (1,2)	0,22
iPTH (pg/mL)	309 (281)	277 (255)	0,164
Sodium (mEq/L)	139 (3)	139 (3)	0,036
Potassium (mEq/L)	5,1 (0,8)	4,5 (0,6)	0,000
usCRP (mg/L)	5,6 (10,7)	6,1 (13,9)	0,624
25OH vitamin D (ng/mL)	16,4 (8,3)	12,8 (5,5)	0,000
$1,25(OH)_2$ vitamin D (pg/mL)	8,6 (5,1)	7,7 (4,8)	0,039
Treatment with Calcium-containing P binders	186 (41,2)	119 (50,2)	0.015
Total Kt/V	1,56 (0,39)	2,52 (0,63)*	NC
Calcium content in dialysis fluid			0.001
2.5 mEq/L	182 (41,9)	72 (35.8)	
3 mEq/L	221 (50,9)	NA	
3,5 mEq/L	31 (7,1)	129 (64.2)	

*Quantitative data are shown as means (SD). Qualitative data are shown as number (%). HD, Hemodialysis; PD, Peritoneal Dialysis; BMI, Body mass index; SBP, Systolic blood pressure; iPTH, Intact parathyroid hormone; usCRP, Ultra-sensitive C reactive protein; NA, Not available. The concentration of 3 mEq/L is not available for PD dialysis fluid. NC, non comparable; *weekly Kt/V.*

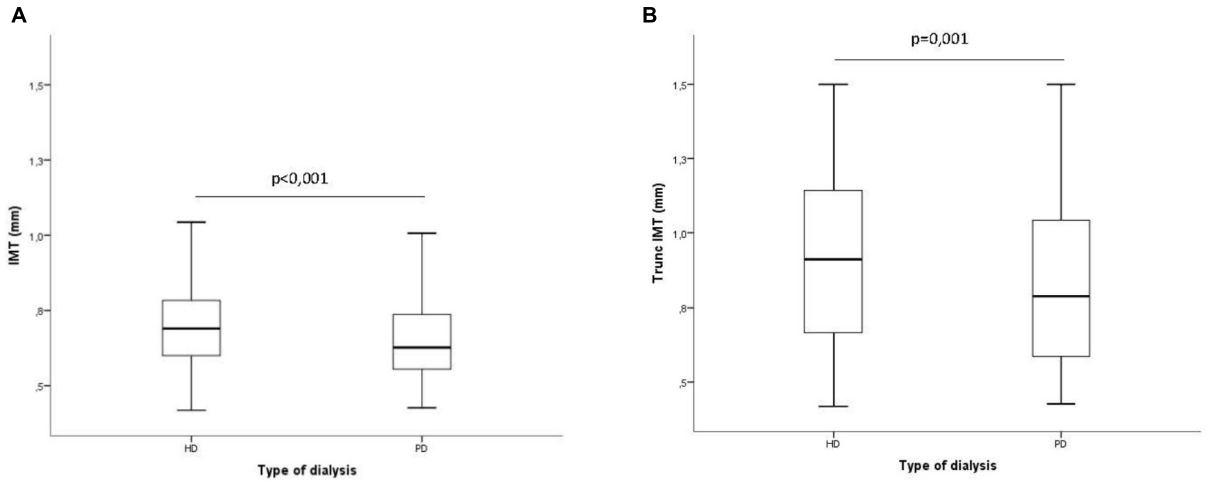

FIGURE 1 | Averaged intima media thickness in carotid arterial territories depending on the dialysis modality. **(A)** Excluding territories with atheroma plaque; **(B)** Including territories with atheroma plaque. In territories with plaque, the IMT value was truncated to 1,5 mm.

which the values of cIMT were truncated to 1.5 mm when there was a plaque present (truncated cIMT). In both cases, PD patients showed lower levels of cIMT than HD patients.

Among all clinical and biochemical variables considered, the ones more closely related to cIMT were age and plaque presence at baseline, both positively correlated (**Table 2**). The other variables with positive correlation with cIMT were body mass index (BMI), diabetes, dyslipidemia, ankle-brachial index (ABI), potassium and glucose. The only variable with negative correlation to cIMT was phosphate levels. We also found that being male and in HD were correlated with a higher cIMT. When truncated cIMT was considered, all the correlations and significances were exacerbated except the one with phosphate, which became non-significant.

When patients were stratified according to sex and age, the relationship of these variables to cIMT was clearly visible, with cIMT values increasing with age (showing a statistically significant *p*-value for the trend), and more abruptly in men (both in normal cIMT and truncated cIMT) (**Figure 2**). Furthermore, the difference related to sex was maintained in all age groups. The differences in laterality are also depicted in **Figure 3**. No differences in cIMT were observed between left and right carotids, being always higher in HD patients, regardless of the side or whether we analyzed cIMT or truncated cIMT values.

To better assess the influence of dialysis type on cIMT, we generated different models by means of lineal multivariate analysis. Univariate regression assessing the effect of dialysis type on cIMT showed a statistically significant association of PD with a lower cIMT value. (**Table 3**). After adjusting by sex and age (Model 2) the association between cIMT and type of dialysis remained significant. This association was maintained even after adjusting by several clinical variables in model 3 (Diabetes, Dyslipidemia, Hypertension, BMI, ABI, Plaque presence at baseline, Total Cholesterol, LDL Cholesterol, Glucose, Potassium, SBP, time on dialysis) confirming an association of PD with lower cIMT values. When truncated cIMT values were used as the dependent variable, the effect of dialysis type on cIMT remained. Only statistically significant variables were maintained in the final model and shown in the table.

DISCUSSION

Our results show that on dialysis patients, cIMT values correlate to gender, age, plaque presence at baseline, BMI and diabetes. In non-dialysis population, previous studies also found association of cIMT with age and sex (Dobs et al., 1999; Mutluay et al., 2012), plaque presence (Ebrahim et al., 1999), BMI (Berni et al., 2011) and diabetes (Brohall et al., 2006). Furthermore, our results show that the modality of dialysis has an impact on cIMT, even when adjusting by possible confounders. Thus, HD patients have greater global cIMT than PD patients and being in PD is independently associated with a lower cIMT.

Currently, studies about dialysis impact on ultrasound measured cIMT have shown contradictory results. Despite all studies have found higher cIMT on dialysis patients than in non-dialysis population,(Cengiz and Dolu, 2007; Mutluay et al., 2012; Shi et al., 2012) some of them reported higher cIMT in PD than in HD (Ozdemir et al., 2001; Shi et al., 2012), some other did not find significant differences on cIMT between dialysis type (Cengiz and Dolu, 2007; Yilmaz et al., 2007; Al-Hweish et al., 2010), and some others reported increased cIMT in HD patients (Mutluay et al., 2012). However, it is important to remark that, in contrast to most previous studies, the NEFRONA study excluded patients with a previous CVE. Indeed, the only similar study that also excluded patients that suffered a CVE (although with a quite lower number of patients that our study) also found that HD dialysis patient have higher cIMT (Tonbul et al., 2006).

Recently, the same NEFRONA cohort with a matched case-control design revealed that the modality of dialysis did not influence atheromatous vascular disease progression (assessed as an increase in the number of atheromatous plaques) neither

TABLE 2 | Statistically significant correlations between different parameters and cIMT values.

	IMT		IMT truncated	
	R	*p*	*r*	*p*
Sex (women)	−0.138	0.000	−0.137	0.000
Age	0.545	0.000	0.613	0.000
BMI	0.232	0.000	0.233	0.000
Diabetes	0.213	0.000	0.271	0.000
Dyslipidemia	0.077	0.049	0.129	0.001
ABI	0.082	0.037	0.137	0.000
Phosphate	−0.086	0.027		
Potassium	0.112	0.004	0.130	0.001
Glucose	0.122	0.002	0.158	0.000
Plaque presence at baseline	0.421	0.000	0.663	0.000
Type of dialysis (PD)	−0.165	0.000	−0.130	0.001

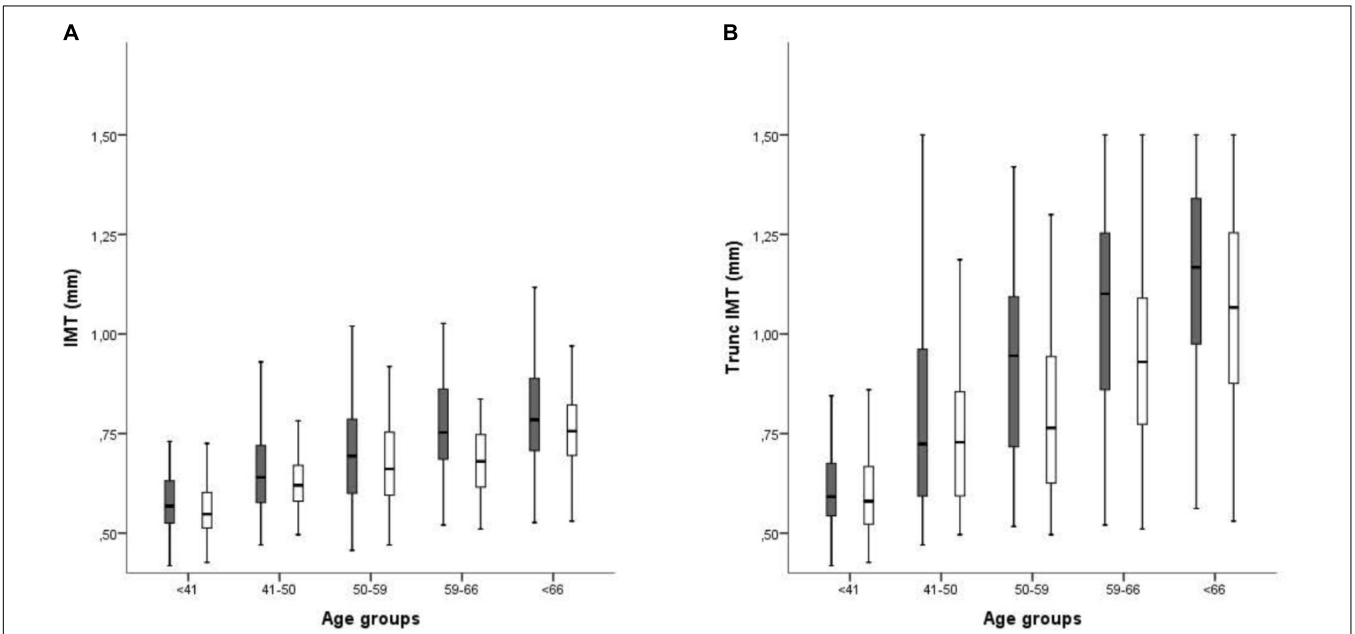

FIGURE 2 | Averaged intima media thickness in carotid arterial territories stratified by age and sex. Dark bars: Males; White bars: Females. **(A)** Excluding territories with atheroma plaque; **(B)** Including territories with atheroma plaque. In territories with plaque, the IMT value was truncated to 1,5 mm. *p* trend > 0,01.

did cardiovascular outcomes (Borràs Sans et al., 2017). These results suggest that plaque and cIMT may be distinct phenotypes rather than a manifestation of the same phenotype at different stages or phases in the progression of atherosclerosis, being in line with recent studies. Thus, the Northern Manhattan Study showed that the association between elevated baseline cIMT and the risk of new plaque formation disappeared after adjusting for demographic and vascular risk factors (Rundek et al., 2015). Similarly, Baroncini et al. (2015) found that increased cIMT was related to hypertension, but plaque presence was associated to age and dyslipidemia. Therefore, the results seems to show that despite increased cIMT and atherosclerosis are commonly found together, the risk factors influencing one or another could be different and may be genetically and biologically distinct atherosclerotic phenotypes with a heterogeneous etiology (Spence, 2006; Della-Morte et al., 2012). Therefore, plaque may not be a simple result of progressive intima-media thickening, but rather a new and different event. Indeed the hypothesis that increases in cIMT might be an adaptive event of the median layer to increased shear stress, rather than an atherosclerotic sign, is gaining adepts nowadays.

The possible causes of higher cIMT in HD patients than in PD patients are unknown. However, it is well stablished that hypertension, and particularly blood pressure variability (BPV), plays a major role in cIMT increase (Mancia et al., 2001; García-García et al., 2013). Short and long-term BPV are inherent to HD treatment because of day-to-day fluctuations of volume status. Moreover, cIMT in HD patients is more associated with long (24- and 48-h ambulatory BP measurements) than routine dialysis center BP measurements (Ekart et al., 2009). On the contrary,

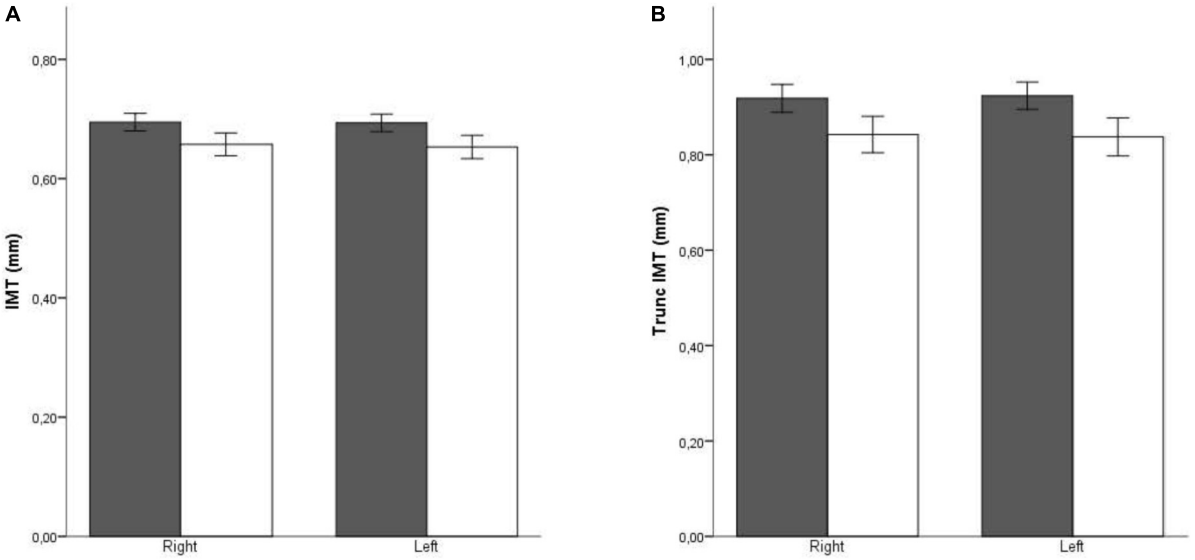

FIGURE 3 | Averaged intima media thickness in carotid arterial territories stratified by side. Dark bars: HD patients; White bars: PD patients. **(A)** Excluding territories with atheroma plaque; **(B)** Including territories with atheroma plaque. In territories with plaque, the IMT value was truncated to 1,5 mm.

TABLE 3 | Lineal multivariate analysis.

	Model 1			Model 2			Model 3		
	Slope	***SD***	***p***	**Slope**	***SD***	***p***	**Slope**	***SD***	***p***
cIMT									
Type of dialysis (DP vs. HD)	−0.051	0.012	0.000	−0.034	0.010	0.001	−0.033	0.010	0.001
Sex (women vs. men)				−0.041	0.010	0.000	−0.036	0.010	0.000
Age (year)				0.013	0.001	0.000	0.004	0.000	0.000
Hypertension							0.041	0.017	0.016
Plaque presence at baseline							0.036	0.012	0.002
Diabetes							0.033	0.012	0.006
Truncated cIMT									
Type of dialysis (DP vs. HD)	−0.084	0.025	0.001	−0.041	0.019	0.033	−0.044	0.017	0.008
Sex (women vs. men)				−0.076	0.019	0.000	−0.038	0.017	0.023
Age (year)				0.005	0.000	0.000	0.008	0.001	0.000
BMI							0.004	0.002	0.019
SBP (mmHg)							0.001	0.000	0.005
Plaque presence at baseline							0.269	0.021	0.000
Diabetes							0.063	0.020	0.002

Model 1 unadjusted. Model 2 adjusted for sex and age. Model 3 adjusted by sex, age, diabetes, dyslipidemia, hypertension, BMI, ABI, plaque presence at baseline, Total Cholesterol, LDL Cholesterol, Glucose, Potassium, SBP, time on dialysis, treatment with calcium-containing phosphate binders, calcium levels in dialysis fluid.

patients in PD have more volume status stability and, accordingly, they should present less BPV. Indeed, HD is associated with higher systolic BPs and greater systolic loads than PD in a study monitoring BP by continuous ambulatory blood pressure measurements (Rodby et al., 1994). One may hypothesize that the difference in short and long-term BPV between HD and PD patients could explain the higher cIMT in HD patients despite having lower casual BP measurements. In the same line of reasoning, Günal et al. (2006) showed a decrease in cIMT with an strict volume control in HD patients, a fact that has not been proven to be true in PD patients (Hiramatsu et al., 2007). As a last hypothesis for the increased cIMT in HD patients, there is the possibility that the previously described increased levels of pro-BNP in HD patients (Nakatani et al., 2002; Al-Hweish et al., 2010; Sanjuan et al., 2011) could favor an increased cIMT, as it has been published for CKD patients (DeFilippi et al., 2005; Sathi et al., 2014); a similar effect has been described for Troponins (Al-Hweish et al., 2010; Caliskan et al., 2012).

There is growing evidence that calcium supplements can increase atherosclerotic CVE (Bolland et al., 2008, 2010; Li et al., 2012; Mao et al., 2013), although some reports have shown no statistically significant effect (Wang et al., 2010;

Lewis et al., 2011). Therefore, there is a concern that calcium supplements can increase atherosclerosis. Dialysis patients can have a significant calcium intake, mainly due to the calcium content in the dialysis fluid and in phosphate binders administered to control hyperphosphatemia. In our study, there was a statistically significant difference in calcium supplements, as a higher percentage of PD patients received calcium-containing phosphate binders and were treated with dialysis fluid containing higher concentrations of calcium. This can be the cause of the small but significant increase in serum calcium levels. However, and although an inverse relationship between calcium load and IMT is suggested in the bivariate analysis, the difference did not reach statistical significance after adjustment for potential confounders.

Another risk factor for atherosclerosis development is hypertension (Hurtubise et al., 2016). Again, the percentage of patients diagnosed with hypertension and the SBP levels are higher in patients in PD, precisely those with lower IMT values. The multivariate analysis demonstrated that the statistical significance for both parameters was lost after adjustment. Therefore, either the effect of HD on IMT is so strong that can overcome the effect of common risk factors like hypertension, or the increases in IMT are influenced by different risk factors than those affecting atheroma plaque formation. Interestingly, PD patients were more dyslipidemic and presented with higher serum LDL-cholesterol levels than those in HD. This could be considered not only as one more piece of evidence supporting the hypothesis of increases in cIMT beyond an atherosclerotic sign or a consequence of some protective effect of PD versus HD, but also as another example of the controversial significance of LDL-cholesterol levels in dialysis patients.

Our results also show that there are no differences between left and right sides in cIMT neither, in PD nor HD patients. Several reports have shown a significantly higher proportion of cerebral ischemic events diagnosed in the left hemisphere than in the right (Hedna et al., 2013). An explanation for the higher incidence of events in the left hemisphere may be related to a higher prevalence, severity, or vulnerability of atherosclerotic disease in the left carotid artery. This is primarily attributable to differences in flow velocity in the left carotid artery, resulting in higher stress and intimal damage (Rodríguez Hernández et al., 2003). However, recent reports show that the higher incidence in left hemisphere strokes might be due to an increase in the vulnerability of the plaques, rather than in plaque formation (Adams et al., 2002; Selwaness et al., 2014). Thus, in our study, no differences in cIMT were found between right and left sides, suggesting that a systemic factor is involved in the increased cIMT found in HD patients.

Strengths of our study are the relatively high number of patients being, as far as we know, the biggest cohort used to determine the effects of dialysis on cIMT. Therefore, we have been able to adjust the models for many potential confounders. Furthermore, and although the study is multicentric, the execution and the analysis of the ultrasounds have been made by a single team, avoiding the high variability associated to the cIMT measurements. On the other hand, we have to mention some limitations. First, the exclusion of patients that presented CVE on the recruitment phase avoid extrapolating our results to the entire ESRD population. Second, we only have a single BP measurement and no fluid status data that could support the hypothesis that higher BPV in HD patients may be related to thicker cIMT. Third, the lack of determinations of cardiovascular biomarkers that could affect cIMT like pro-BNP or troponins, preclude us to determine whether the effect of dialysis modality is related to these biomarkers or to some other factor.

In summary, we have shown that cIMT is higher in HD than in PD and that being in PD is an independent risk factor associated with lower cIMT in dialysis patients.

AUTHOR CONTRIBUTIONS

MeB, EF, ÀB, and JV designed the study. SC, MP-F, and JV analyzed the data. MC-M, MiB, and MB-L performed the experiments. JV wrote the manuscript.

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REVIEW
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Ultrafiltration Failure Is a Reflection of Peritoneal Alterations in Patients Treated With Peritoneal Dialysis

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Ultrafiltration (UF) failure is a common and important complication of peritoneal dialysis (PD), especially in long-term patients without residual urine production, because it often causes overhydration, which is an important cause of death in this population. The current review provides an overview of the pathways of peritoneal fluid transport, followed by the mechanisms and causes of UF failure. The egression of circulating fluid to the tissue compartment and its subsequent re-uptake by the colloid osmotic pressure are markedly influenced by PD, because the dialysis solutions contain glucose as a low molecular weight agent causing removal of fluid from the circulation by crystalloid osmosis. Pores involved in transcapillary UF consist of inter-endothelial small pores and the intra-endothelial water channel aquaporin-1. The former allows transport of plasma fluid with dissolved low molecular weight solutes and accounts for 60% of the filtered volume, the latter transports 40% as pure water. This free water transport (FWT) is driven by the crystalloid pressure gradient, while small pore fluid transport (SPFT) is dependent on both hydrostatic and crystalloid osmotic pressure. The number of perfused peritoneal microvessels as assessed by small solute transport parameters, is differently associated with UF: a positive relationship is present with SPFT, but a negative one with FWT, because the effect of more vessels is counteracted by a faster disappearance rate of glucose. Ultrafiltration failure can be present shortly after the start of PD, for instance due to mesothelial-to-mesenchymal transition. Late UF failure develops in 21% of long-term patients. Both FWT and SPFT can be affected. Patients with encapsulating peritoneal sclerosis have severely impaired FWT, probably due to interference of interstitial collagen-1 with the crystalloid osmotic gradient. This mechanism may also apply to other patients with reduced FWT. Those with mainly impaired SPFT likely have a reduced hydrostatic filtration pressure due to vasculopathy. Deposition of advanced glycosylation end products is probably important in the development of this vasculopathy. It can be concluded that long-term UF failure may affect both SPFT and FWT. Vasculopathy is important in the former, interstitial fibrosis in the latter. Measurements of peritoneal transport function should include separate assessments of small pore-and FWT.

Keywords: ultrafiltration failure, fluid transport, vasculopathy, AGE's, peritoneal dialysis, fibrosis, AQP-1

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INTRODUCTION

Ultrafiltration (UF) failure during peritoneal dialysis (PD) is the commonly used term for a situation, where netUF, i.e., the difference between the drained and the instilled volume is less than expected in the PD population. As the glucose concentration of the dialysis solution is an important determinant of net ultrafiltration (netUF) by osmosis, normal values are related to the glucose concentration of the dialysate. After a 4 h exchange with 1.36% glucose median netUF was –85 mL (95% confidence interval –454 to +286) in 83 prevalent patients (Pannekeet et al., 1995) and 635 mL (range 95–1305) in 80 prevalent patients after a similar exchange with a 3.86% glucose dialysis solution (Parikova et al., 2005). The extremely large interindividual variability contrasts with an intraindividual variability of about 20% (Imholz et al., 1978) and points to large differences in transport characteristics of the "peritoneal membrane" between individual patients. The International Society for PD published a guideline on UF failure in 2000, containing a definition of UF failure, based on the results of the above mentioned studies: the 3 × 4 rule (Mujais et al., 2000). It states that UF failure is present when netUF is less than 400 mL after drainage of a 4% (3.86 or 4.25%, depending on the pharmacopeia) dialysis solution with an intraperitoneal stay of 4 h. Although patients with UF failure according to this definition are often hypervolemic, overhydration is not included in the definition of UF failure, because overhydration is not only dependent on fluid removal, but also on fluid intake. Yet overhydration is probably the most important cause of cardiovascular death in PD patients (Krediet and Balafa, 2010). As the vast majority of patients with UF failure is hypervolemic, knowledge of the mechanisms for UF failure, its presence, development, diagnosis, treatment and prevention are important.

The aim of the present review is to give an update on pathways and mechanisms of peritoneal fluid transport, and the way functional characteristics can be used in patient's follow-up to identify the most important cause for UF failure in an individual patient and the mayor underlying morphological abnormalities.

PATHWAYS FOR PERITONEAL FLUID TRANSPORT

According to Starling's law fluid with dissolved crystalloids egress from circulating blood at the arteriolar side of microcirculatory capillaries to the interstitium of perfused tissues by the hydrostatic pressure, after which the majority is taken up at the venous part by the increased intravascular colloid osmotic pressure and partly into the lymphatic system. Crystalloid fluid administered into the peritoneal cavity increases the intraperitoneal pressure, which is also transmitted to the peritoneal interstitial tissue and will thereby lower the hydrostatic filtration gradient to some extent. The protein content of such solution is so low, that extensive reabsorption by the intracapillary colloid osmotic pressure occurs and also into the lymphatic system. This leads to a complete absorption of all intraperitonealy administered isotonic solutions.

The objective of PD is removal of excess uremic waste products and fluid from the body by drainage of an intraperitoneally administered solution, usually called the dialysate. This is only possible when the reabsorption of filtered fluid is minimal, allowing net transport of fluid and solutes from the tissues to the dialysate, which can subsequently be drained out of the peritoneal cavity. The magnitude of filtration is partly dependent on the systemic blood pressure, and more importantly on autoregulation of organ perfusion by the tonus of the precapillary sphincter in afferent arterioles. Peritoneal diffusion of solutes is another transport mechanism to this hydrostatically induced convection, because the peritoneal cavity is filled with a dialysis solution. The distribution of this electrolyte-containing fluid is not restricted to the peritoneal cavity, but extends into the interstitial tissue up to the vascular wall. This implies that diffusion of solutes from the vascular lumen to the dialysis fluid occurs, which is quantitatively more important than hydrostatic convection, because of the large concentration gradient between blood and dialysate. Addition of a low-molecular weight solute, e.g., glucose to dialysis fluid creates a crystalloid osmotic pressure gradient which is temporary, because the osmotic agent diffuses into the interstitium and from there back into the systemic microcirculation. Together with the hydrostatic pressure gradient, the glucose-induced crystalloid pressure gradient induces fluid transport from the microcirculation to the peritoneal cavity, usually called UF.

According to the 3-pore theory of transport through the microvascular wall (Rippe, 1993), interendothelial pores are the pathways involved in solute transport. These consist mainly of small pores with radii of about 40 Å. The number of large pores (radius > 250 Å) is so small that their contribution to fluid transport and that of low molecular weight solutes can be neglected. Low molecular weight solutes pass the small pores easily, because their molecular radii are about 3 Å and even that of β_2-microglobulin is only 16 Å. Consequently glucose that has a molecular radius of 3.12 Å, can only induce a limited crystalloid osmotic pressure gradient across the small interendothelial pores. This requires extremely high dialysate concentrations and lasts for a limited length of time, because of its absorption. We found that small pore water transport rates after the first one to two hours of a 3.86% glucose exchange averages 3 mL/min (Parikova et al., 2008). This value remains stable during the remaining 2–3 h (Parikova et al., 2008). The difference between the crystalloid and colloid pressure gradient during the last part of the dwell decreases from 16 to 2 mmHg during the last 2 h of a dwell (Parikova et al., 2008). This is a low value compared to the hydrostatic pressure gradient of 17 mmHg, the latter calculated as the difference between the pressure in peritoneal capillaries which averages 25 mmHg at the arteriolar side (Struijk et al., 1996) and the intraperitoneal pressure of 8 mmHg (Guyton, 1981). Given a net combined crystalloid and colloid osmotic pressure gradient in the initial phase of a 3.86% glucose exchange of about 50 mmHg (Parikova et al., 2008), the contribution of the

hydrostatic pressure gradient to small-pore fluid transport will increase from about 34% in the beginning to about 80% during the last 2 h of the dwell.

Besides transcapillary UF by the crystalloid osmotic and hydrostatic pressure gradients which both increase intraperitoneal volume, fluid egresses from the peritoneal cavity by colloid osmosis-induced backfiltration at a rate of 0.4 mL/min (Imholz et al., 1993) and by absorption into the lymphatic system. Assessment of the latter requires intraperitoneal administration of a macromolecule, which is so large that its diffusion can be neglected. Radiolabeled albumin and neutral dextrans have been employed in PD patients (Krediet et al., 1991; Heimburger et al., 1992). The obtained values are dependent on the use of either the disappearance rate of the macromolecule from the peritoneal cavity or its appearance rate in the circulation. Typical values for the disappearance rate are 1.0–1.5 mL/min in PD patients, while they average 0.2 mL/min for the appearance rate (Rippe et al., 1986). The difference is partly explained by underestimation of the appearance rate, because the albumin space is about twice that of the circulating volume, and also by overestimation by the disappearance rate by transmesothelial passage of the macromolecule (Krediet, 2004).

Transcapillary UF by crystalloid osmosis occurs not only through small interendotheliaal pores, but also by the intra-endothelial water channel aquaporin-1 (AQP-1; Stelin and Rippe, 1990; Ni et al., 2006). This transcellular membrane protein functions as an ultrasmall pore, allowing transport of water, but not of solutes, including glucose and Na^+. Consequently it functions as an ideal semipermeable pore with a refection coefficient of 1.0, while that to glucose of the small pores is only 0.03–0.04 (Imholz et al., 1994). AQP-1 therefore induces free water transport (FWT) by crystalloid osmosis. It explains the so-called sodium sieving, found in an old clinical observation in severely overhydrated patients with acute kidney insufficiency, who were treated with PD using dialysate with very high glucose concentrations (Nolph et al., 1969). These patients showed a decrease of the dialysate Na^+ concentration, while the plasma concentration remained unaltered. In retrospect this must have been caused by dilution due to high FWT rates.

Assessment of the dialysate/plasma (D/P) ratio of Na^+ after 60 min of a dialysis exchange has been proposed as a semiquantitative method for AQP-1 function (Mujais et al., 2000). Calculation of $FWT_{0-60\ min}$ in patients is also possible using sodium transport in that period: the drained volume is divided in the volume that accompanies Na^+ transport from the circulation to the dialysate which is assumed to have occurred through the small pores, the remaining part of the drained volume is considered to represent FWT (Smit et al., 2004; La Milia et al., 2005). Transcapillary UF during the initial 60 min can therefore be considered to be composed of small pore fluid transport (SPFT) and FWT. Calculations in patients show that SPFT accounts for 60% of UF and FWT for 40% (Parikova et al., 2005). These values are in close agreement to previous results of computer simulations (Rippe et al., 1991).

MECHANISMS AND CAUSES OF UF FAILURE

Peritoneal transport of a solute from the circulation to the dialysate-filled peritoneal cavity is dependent on its diffusion velocity mainly determined by molecular weight, and the number of perfused peritoneal microvessels so the number of pores available (Krediet et al., 1993). Comparison of the small pore radius with those of low molecular weight solutes points to the importance of the effective peritoneal surface area (EPSA), i.e., the number of perfused peritoneal capillaries in the determination of the mass transfer area (MTAC) of a solute. This representation of the maximal transport of a solute by diffusion at time zero is often replaced by the D/P ratio after drainage of the dialysate, which is usually performed after a dialysate dwell time of 4 h. Creatinine is usually used as marker solution, because no evidence of local peritoneal production or release is present. Therefore changes in the MTAC creatinine or D/P creatinine represent changes in EPSA. High values of MTAC or D/P creatinine indicate fast peritoneal solute transport including that of glucose, and thereby a rapid disappearance of the crystalloid osmotic gradient and impaired UF. Consequently an inverse relationship between MTAC creatinine and netUF is present, as shown in **Figure 1**. This is also the case for FWT, but for SPFT a positive relationship is present with MTAC creatinine, as shown in **Figure 2**. This can be explained by the limited dependence of SPFT on crystalloid osmosis, compared to that on the hydrostatic pressure gradient.

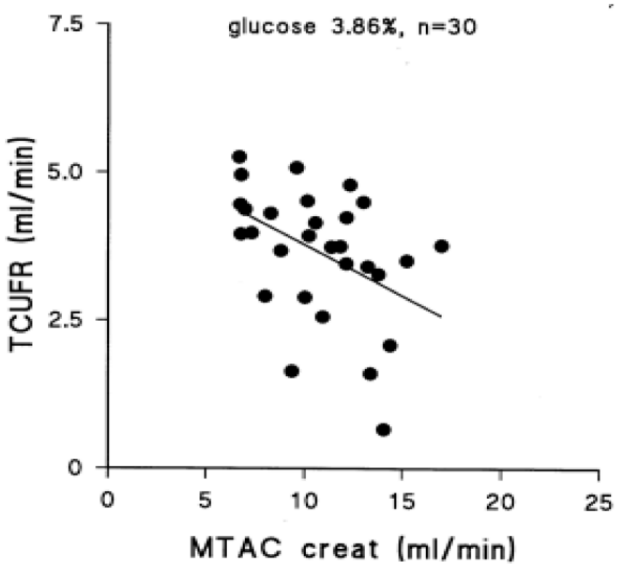

FIGURE 1 | The negative relationship between the transcapillary ultrafiltration rate during a 4 h exchange with 3.86% glucose dialysis solution and the mass transfer area coefficient of creatinine, representing the effective peritoneal vascular surface area. This figure was part of Figure 6.8 of chapter 6 in Krediet (2009). Published with permission of the author and of Springer Science and Business media.

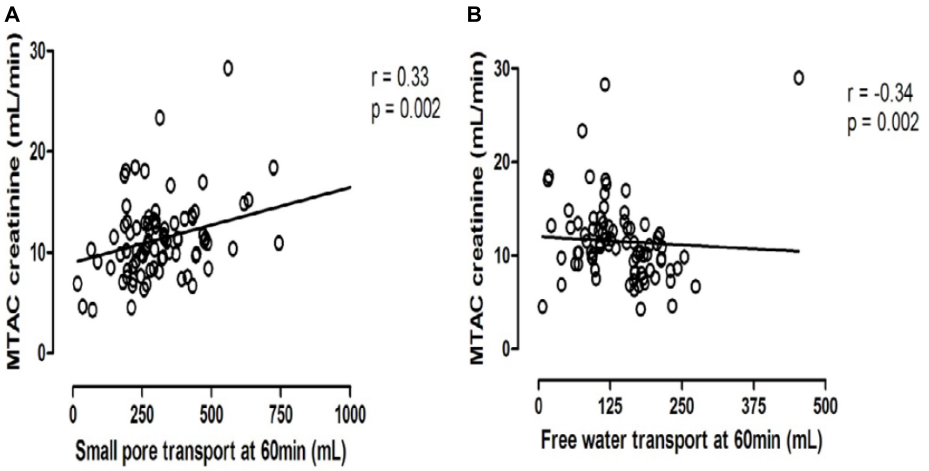

FIGURE 2 | The opposite relationships between the mass transfer area coefficient of creatinine and either small pore fluid transport **(A)** or free water transport **(B)**.

Ultrafiltration failure can be distinguished into early (<2 years) of PD and late UF failure (Sampimon et al., 2011). Early UF failure occurs in about 4% of incident PD patients and is often not a clinical problem, because most patients still have urine production, which protects them for overhydration. With the exception of a high lymphatic absorption rate, which is rarely present, a large EPSA is usually present, not caused by a large number of microvessels, but by increments in their perfusion. EPSA is not a static property of the peritoneal membrane, but fluctuates due to intraperitoneally produced or released vasoactive factors. Acute peritonitis is the best example. It is characterized by a low UF rate and a high MTAC creatinine, caused by peritoneal hyperemia (Krediet et al., 1987; Douma et al., 1998) and mediated by vasoactive substances, like prostaglandins and interleukin-6 (Il-6; Zemel et al., 1993). The higher dialysate than plasma values of these factors point to intraperitoneal production. Peritonitis is an acute and largely reversible condition (Van Diepen et al., 2015), but also microinflammation likely influences peritoneal solute transport, as evidenced by relationships between dialysate Il-6 and D/P creatinine (Pecoits-Filho et al., 2006; Lambie et al., 2013). No association is present with systemic inflammation (Lambie et al., 2013). The increased EPSA, associated with acute peritonitis and with chronic micro inflammation impairs UF, because of the rapid disappearance of the crystalloid osmotic gradient. Epithelial-to-mesenchymal transition of mesothelial cells (MMT) is present in *ex vivo* obtained mesothelial cells from peritoneal effluent of PD patients (Yanez-Mo et al., 2003). Histology studies of peritoneal tissues in PD patients also show this phenomenon. It occurs during the first two years of treatment and is associated with an increased EPSA (Del Peso et al., 2008) and high dialysate concentrations of vascular endothelial growth factor (VEGF; Aroeira et al., 2005). Previously an association between dialysate VEGF and MTAC creatinine has been shown in a cross-sectional analysis (Zweers et al., 1999). A clinical diagnosis of MMT without histologic confirmation is likely when early UFF is associated with high values of MTAC creatinine, effluent VEGF and cancer antigen 125, a marker of mesothelial cell mass or turn-over (Van Esch et al., 2004).

Late UFF develops in about 21% of patients who are treated with PD for >2 years (Sampimon et al., 2011). It involves both FWT and SPFT (Coester et al., 2014). FWT remains stable during the first 3 years of PD, but a subsequent decrease of $FWT_{0-60\ min}$ occurs to 67% of the initial value. This is accompanied by an increase of small solute transport that mirrors FWT: MTAC creatinine rises from 10 to 13 mL/min and glucose absorption after 4 h augments from 63 to 69% (Coester et al., 2014).

Very low values for FWT are present in patients with encapsulating peritoneal sclerosis. This is a rare, but severe complication of long-term PD, which occurs in 3% of incident PD patients in the Netherlands after a duration of 5 to 13, mean 8 years (Sampimon et al., 2011), but it occurs more often in Japan (Kawanishi et al., 2004). EPS is clinically characterized by signs of bowel obstruction and morphologically by a thickened peritoneal interstitium with sclerotic changes, leading to adhesion of bowel loops. Especially the deposition of collagen-1 is extremely dense and is associated with osmotic water transport, assessed semiquantitatively, despite a normal expression of AQP-1 (Morelle et al., 2015). Also quantitative values for $FWT_{0-60\ min}$ are very low: we found an interquartile range of 24 to 73, median 26 mL (Sampimon et al., 2014). $FWT_{0-60\ min} < 75$ mL predicted EPS with a sensitivity of 100% and a specificity of 81% (Lopes Barreto et al., 2018). Why EPS is associated with low FWT, is still unsolved. AQP-1 function may be impaired, but the deposition of interstitial collagen-1 is probably more important in the function of the crystalloid osmotic gradient, although the mechanism is still unsolved. The use of FWT in the follow-up of PD patients to identify those with progressive interstitial fibrosis has been proposed, but is hampered by the absence of a good reference method for quantification of peritoneal fibrosis (Krediet et al., 2016).

The time course of SPFT, the other constituent of transcapillary UF, is different. It shows a gradual decline to 46% of the initial value at 5 years (Coester et al., 2014). The influence of the crystalloid pressure gradient on this decrease can be neglected, compared to that of the hydrostatic pressure gradient, as shown previously. A reduction in the hydrostatic filtration pressure has been hypothesized, due to progressive vasculopathy (Krediet et al., 2018). This condition has first been described in the report of the peritoneal biopsy registry (Williams et al., 2002). Four grades are distinguished, ranging from subendothelial hyalinosis to luminal distortion and even obliteration. It is present in 70% of peritoneal biopsies after PD for more than 5 years. Vasculopathic blood vessels have a narrowed lumen and are therefore likely to lower the filtration pressure and thereby reduce SPFT, in contrast to FWT in EPS which is not related to vasculopathy (Morelle et al., 2015). Similar to diabetic microvascular disease, deposition of advanced glycosylation end products (AGEs) in the vascular wall may be the major culprit for the development of peritoneal vasculopathy. AGEs are irreversible covalently bound complexes between glucose molecules and proteins. *In vivo* they are formed within the vascular wall and lead to protein cross-links in it, thereby increasing its rigidity. AGEs are important in diabetic microangiopathy and impaired kidney function (Makita et al., 1991). AGE deposition is also present in peritoneal tissue of PD patients, especial submesothelial and in the vascular wall (Nakayama et al., 1997; Combet et al., 2000). An association between UF failure, peritoneal AGE deposition and the severity of vasculopathy has been described (Honda et al., 1999).

CONCLUSION

The development of UF failure in patients solely treated with conventional PD solution, is a major problem for the long-term use of this mode of renal replacement therapy in end stage renal disease patients without residual urine production. Peritoneal fibrosis and vasculopathy are the most important structural abnormalities, involved in its pathogenesis. FWT is especially dependent on the number of perfused peritoneal blood vessels and probably on the amount of fibrosis, SPFT is reduced by vasculopathy. A reduction of both FWT and SPFT is likely to occur in the majority of long-term PD patients. Those who develop EPS should be considered as a separate subgroup, in which FWT is much more impaired than SPFT. Measurement of peritoneal transport function should not only include netUF, but also separate determinations of FWT and SPFT to guide treatment options.

AUTHOR CONTRIBUTIONS

RK wrote this review.

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Urgent-Start Peritoneal Dialysis as a Bridge to Definitive Chronic Renal Replacement Therapy: Short- and Long-Term Outcomes

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Background: The peritoneal dialysis (PD) urgent-start pathway, without typical 2-week break-in period, was meant for late-referral patients able and prone to join PD-first program, with its main advantages such as: keeping the vascular system intact, preserving their residual renal function and retaining life-style flexibility. We compared the short- and long-term outcomes of consecutive 35 patients after urgent- and 94 patients after the planned start of PD as the first choice.

Methods: The study included all incident end-stage renal disease patients starting PD program between January 2005 and December 2015, classified into two groups: those with urgent (unplanned) and those with elective (planned) start. Urgent PD was initiated as an overnight automatic procedure (APD) with dwell volume gradually increased, and after 2–3 weeks, target PD method was established.

Results: The mean time between catheter implantation and PD start was 3.5 ± 2.3 in urgent and 16.2 ± 1.7 days in planned-start groups ($p < 0.00001$). 51% of the patients in the urgent-start group required PD during first 48 h after catheter insertion. Mean follow-up of 17.6 ± 11.09 months (median: 19.0) was in the urgent-start group and 28.6 ± 26.6 months (median: 19.5) in the planned-start group. The early mechanical complications were observed more often in the urgent-start group (29 vs. 4%, $p = 0.00005$). The only significant predictors of early mechanical complications were serum albumin ($p = 0.02$) and time between the catheter insertion and PD start. The first year patient survival and technique survival censored for death and kidney transplantation were not significantly different between groups. In Cox proportional analysis the independent risk factors for patient survival as well as for method and patient survival appeared Charlson Comorbidity Index CCI (HR 1.4; $p = 0.01$ and 1.24; $p = 0.02$) and time from catheter implantation to PD start with HR 5.11; $p = 0.03$ and 4.29; $p = 0.04$ for <2 days, while time >14 days lost its predictive value ($p = 0.07$).

Conclusion: Peritoneal dialysis may be a feasible and safe alternative to HD in patients who need to start dialysis urgently without established dialysis access, with an acceptable complications rates, as well as patient and technique survival.

Keywords: peritoneal dialysis, short-term outcomes, mechanical complication, technique survival, patient survival, long-term outcomes, infectious complications

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INTRODUCTION

Almost 3 million people worldwide are affected by end stage renal disease (ESRD), and the vast majority of those who cannot undergo renal transplantation are treated with hemodialysis (HD), while less than 10% are on peritoneal dialysis (PD) (Liyanage et al., 2015; Li and Kwong, 2017). Moreover, despite many advantages, comparable survival rates for both techniques and the efforts of PD societies, the numbers of PD patients are still declining, year by year (Mehrotra et al., 2016; Li et al., 2017). The problem is complex, with the main causes being: an inadequate education and training of the nephrologists, shortages of trained nurses, easy access to HD, lack of patients education, and, in some countries, financial issues. Another important aspect is that as much as 40–60% of the patients initiate dialysis urgently, with no dialysis access, due to a late referral (Perl et al., 2011; Blake et al., 2013).

Since the insertion of central venous catheter is readily available and much easier than PD catheter placement, HD becomes the typical initial modality in such situations and many remain on HD, taking the path of least resistance. This can be overcome by introducing a so-called PD urgent-start program, which has attracted increased interest in recent years (Povlsen and Ivarsen, 2006; Lobbedez et al., 2008; Sharma et al., 2008; Oliver et al., 2012; Arramreddy et al., 2014; Mahnensmith, 2014).

A program of this kind was introduced in the Department of Nephrology of the Medical University of Warsaw in 2004. The aim of our study was to compare the impact of planned and unplanned (urgent) initiation of PD therapy in ESRD patients on infectious and non-infectious complications rate, and patients as well as patient technique short- and long-term survival.

MATERIALS AND METHODS

All incident patients with ESRD starting a PD program in the Department of Nephrology at the Medical University of Warsaw between January 2005 and December 2015 were enrolled into the study. The protocol was approved by the Bioethical Committee of Medical University of Warsaw. All patients gave written informed consent in accordance with the Declaration of Helsinki.

In accordance with the International Society for PD (ISPD) (Li et al., 2016) and the European Renal Best Practice (ERBP) guidelines (Dombros et al., 2005), they were classified into two groups: those with urgent (unplanned) and those with elective (planned) start, depending on whether PD initiation took place during less than 14 days after peritoneal access creation or later.

In every late referral case the decision to initiate PD urgently was made individually in three steps. First, in patients with life-threatening metabolic disturbances (hyperkalemia, severe acidosis, severe volume overload, or marked uremia) the PD catheter implantation was preceded by 1–3 emergency HD procedures performed via temporary femoral vein access. Secondly, any medical or social contradictions to PD were carefully checked. In the last step, eligible patients were offered an informed choice of dialysis modality.

In those who opted for PD, a Tenckhoff catheter (straight or coiled) was inserted by open surgery under local or general anesthesia. PD was initiated as an overnight automatic procedure (APD) in supine position with 800–1000 mL dwell volume (for patients $<$, and $\geq$ 60 kg, respectively) and a dry day. The dwell volumes were gradually increased, and after 2–3 weeks patients remained on standard APD or were converted to continuous ambulatory PD (CAPD).

Follow up analyses of mechanical (leak, hernia, catheter migration, catheter obstruction, and bleeding) and infectious (peritonitis, exit site/tunnel infection) complications were performed after first 4 weeks, 90 days, and 12 months. Patients and technique survival rates were evaluated after first 90 days, 12 months, and at the end of the observation. The patients were observed for 3306 patient-moths (for entire group mean 25.6 $\pm$ 23.8, median 19 months); for planned-start group 28.6 $\pm$ 26.6, median 19.5 months, for urgent-start group 17.6 $\pm$ 0.9, median 19.0 months. Peritonitis and ESI/TI rates are expressed as episodes per year of treatment. Catheter migration as reflected by clinical suspicion was defined as lack/drop of ultrafiltration or problems with dialysate outflow. This suspicion was confirmed by X-ray of abdominal cavity; Catheter obstruction did not occur in our patients. Pericatheter leak (exit-site leak) was defined as fluid leak around the catheter exit-site. Abdominal leak on clinical suspicion seen as drop of ultrafiltration, asymmetric swelling of abdominal wall tissues and "orange peel" syndrome; was confirmed on radiological examination with contrast given to the catheter or during CT.

Statistical Analysis

Statistical analysis was performed with STATISTICA software package (version12), StatSoft Poland. Continuous variables with normal distribution were presented as mean (standard deviation [SD]) and compared between two groups using Student's *t*-test. Non-normal variables were expressed as median (interquartile range [IQR]) and compared with Mann–Whitney *U*-test. Categorical variables were presented as frequencies and percentages, and compared using Chi^2 test. The survival rates were analyzed using the Kaplan–Meier technique and log-rank test. The multivariate Cox proportional hazards model was used in survival analysis to adjust outcomes for confounding variables such as: estimated glomerular filtration rate (eGFR), serum albumin and blood hemoglobin concentration, Charlson Comorbidity Index (CCI), time between catheter insertion and PD start, any complication appearance during first 4 weeks of the treatment and the urgent start *per se*. The statistical difference was considered to be significant for $p < 0.05$.

RESULTS

Patient Characteristics

One hundred twenty nine incident patients (56% men), aged 50.8 $\pm$ 17.8 years entered the study. Among them, 35 (27%) started PD urgently and the remaining 94 (73%) in the planned manner. The main demographic and clinical data of the unplanned and planned beginners are presented in **Table 1**. There

TABLE 1 | The main demographic and clinical characteristics of the study groups.

Parameter	Urgent-start (*n* = 35)	Planned-start (*n* = 94)	*p*
Age (*years*)	51 ± 18.5	51 ± 17.7	NS
Sex (*% of men*)	49	52	NS
eGFR (*ml/min/1.73 m*^{2})	6.1 ± 2.9	8.1 ± 2.7	<0.001
Serum albumin (*g/dl*)	3.23 ± 0.36	3.55 ± 0.46	<0.01
Blood hemoglobin (*g/dl*)	9.3 ± 1.36	10.4 ± 1.27	<0.0001
Diabetes (%)	11	36	<0.0001
Cause of kidney disease (%)			<0.01
• diabetic kidney disease	9	25	
• glomerulonephritis	37	43	
• hypertensive nephropathy	9	11	
• unknown/other	45	21	
Charlson comorbidity index (CCI)	6 ± 3	5 ± 3	NS

were no significant differences between study groups in terms of age, sex, and CCI. The patients who started PD urgently had worse kidney function, lower serum albumin, and blood hemoglobin concentrations at the beginning of the treatment.

Dialysis Initiation

The mean time between peritoneal access creation and PD start was 3.5 ± 2.3 and 16.2 ± 1.7 days, in urgent- and planned-start groups, respectively ($p < 0.00001$). Fifty-one percent of patients in the urgent-start group required PD during first 48 h after catheter insertion, and in 3 of them with life-threatening clinical symptoms – 2–3 short emergency HD procedures via femoral access were performed before PD catheter placement. In the remaining patients who started the treatment urgently, the time to PD commencement ranged between 3 and 8 days.

Non-infectious Complications

All mechanical complications are presented in **Table 2**. The early ones were observed more often in the urgent-start group (29% of patients vs. 4%, $p = 0.00005$), however, with no need for surgical intervention or temporary transition to HD. The most frequent early complication was dialysate leakage, which occurred in four patients – all of them started PD within 48 h after catheter implementation. In these patients, PD was postponed for 3–5 days and subsequently resumed with low-volume dwells.

The late mechanical complications occurred in 33.5% of all studied patients, 20% in urgent-start and 31% in the planned-start group ($p = 0.15$). The rate of late non-infectious complications was similar in both groups, with the exception of PD catheter migration, more frequently seen in the planned-start group.

In the regression logistic analysis, serum albumin as continuous variable (0.18; CI: 0.04–0.77; $p = 0.02$) and time between the catheter insertion and PD start expressed as categorical variables: ≤48 h (1,79; CI: 0,45–7,18; $p = 0,02$), and >14 days (0.08; CI: 0.01–0.5; $p = 0.003$) occurred the only significant predictors of early mechanical complications. In regard to long-term follow-up, neither the time between the catheter insertion and PD start, the urgent start *per se* nor any early complication influenced the occurrence of late mechanical events.

Infectious Complications

There were no infectious complications in any studied patient during the first 4 weeks of PD treatment. During the whole observation at least one episode of peritonitis was observed—in 12 patients (34%) in the urgent-start group and in 31 (33%) patients in the planned-start group. There were no differences between the groups regarding peritonitis and/or exit-site/tunnel infection rates during the first year of PD therapy as well as during the whole observation period (**Tables 3**, **4**). In the regression logistic analysis none of analyzed parameters had an influence on peritonitis or exit-site/tunnel infection occurrence.

The Technique and Patients Survival

The patients were observed for 3306 patient-months, with mean follow-up of 17.6 ± 11.09 months (median: 19.0, range: 1.0–44 months) in the urgent-start group and 28.6 ± 26.6 months (median: 19.5, range: 1.0–103 months) in the planned-start group. The short- and long-term outcomes in studied patients are presented in **Table 5**. The analysis revealed that PD urgent-start was associated with reduced survival but only in the first 90 days of the therapy (86 vs. 99%; $p < 0.0001$), and at the end of observation the rates were similar. During the study period five patients died of peritonitis (1 from US group and 4 from PLS group), which creates quite a high mortality rate due to PD peritonitis in our population (5/56 episodes). A review of these cases revealed: all patients had high comorbidity: CCI 7–9; all episodes occurred > 12 months from PD start; in two cases polymicrobial peritonitis was secondary to bowel perforation; in one case due to Gram negative (*E. coli*); in one case due polymicrobial (*E. coli*, *Enterococcus* fecal, *Enterobacter cloacae*, *Candida* spp.) infection without proven bowel perforation; in one case it occurred in the course of sepsis secondary to the complications after surgical treatment of lower limb ischemia.

TABLE 2 | Rate of early (first 4 weeks) and late (>4 weeks) mechanical complications in the studied groups.

Complication	Urgent-start (*n* = 35)		Planned-start (*n* = 94)		*p*	
	Early	Late	Early	Late	Early	Late
Leakage	4 (11%)	5 (14%)	0	7 (7%)	<0.001	NS
Bleeding	3 (9%)	0	1 (1%)	0	<0.05	–
Catheter migration	3 (9%)	1 (3%)	3 (3%)	15 (16%)	NS	0.04
Catheter obstruction	0	0	0	0	–	–
Hernia	0	2 (6%)	0	12 (13%)	–	NS

TABLE 3 | Peritonitis rates and time to first episode in both studies groups.

Period	Urgent-start (n = 35)		Planned-start (n = 94)		p	
Evaluated parameter	**(A) Number of episodes/rate [episodes/year]**	**(B) Time to first episode (median) [months]**	**(A) Number of episodes/rate [episodes/year]**	**(B) Time to first episode (median) [months]**	**A**	**B**
First year of PD	6/0.17	6.8 ± 2.6 (7)	10 /0.10	8.4 ± 2.6 (8)	NS	NS
Whole observation	14/0.40	13.9 ± 10.5 (11.5)	42 /0.44	27.5 ± 21.3 (22)	NS	0.04

TABLE 4 | Exit-site/tunnel infections rates and time to first episode in both studies groups.

Period	Urgent-start (n = 35)		Planned-start (n = 94)		p	
Evaluated parameter	**(A) Number of episodes /rate [episodes/year]**	**(B) Time to first episode (median) [months]**	**(A) Number of episodes/rate [episodes/year]**	**(B) Time to first episode (median) [months]**	**A**	**B**
First year of PD	1/0.02	10.0 ± 2.8 (10)	6/0.06	8.3 ± 2.8 (9)	NS	NS
Whole observation	3/0.08	11.3 ± 3.1 (12)	14/0.1	23.6 ± 18.1 (14)	NS	NS

TABLE 5 | The short- and long-term outcomes in studied patients.

	Died		Receive transplant		Changed to HD		Stayed on PD		Technique survival**	
90 days	6 (4.6%)		1 (0.8%)		4 (3.1%)		118 (91.5%)		118 (97%)	
	US	PLS	US	PLS	US	PLS	US	PLS	US	PLS
	5* (14%)	1 (1.1%)	0	1 (1.1%)	0	4 (4.2%)	30 (86%)	88 (94%)	30 (100%)	88 (95.7%)
90 days–12 months	8 (6%)		17 (13%)		11 (9%)		93 (72%)		93 (89%)	
	US	PLS	US	PLS	US	PLS	US	PLS	US	PLS
	6 (17%)	2 (2%)	3 (9%)	14 (15%)	1 (3%)	10 (11%)	25 (71%)	68 (72%)	34 (97%)	84 (87%)
>12 months	29 (23%)		44 (34%)		29 (22%)		27 (21%)		27 (48%)	
	US	PLS	US	PLS	US	PLS	US	PLS	US	PLS
	13 (37%)	16 (17%)	10 (29%)	34 (36%)	6 (17%)	23 (25%)	6 (17%)	21 (22%)	29 (83%)	71 (76%)

*$p < 0.0001$; **Censored by death and kidney transplantation; US, urgent-start; PLS, planned-start.*

Thus, peritonitis was one of the contributing cause of death in these seriously ill patients. The causes of death are shown in **Table 6**.

At the end of observation only 27 (21%) of all studied patients (17% from urgent- and 22% from planned-start group) stayed on PD. The most frequent reason for PD cessation was kidney transplantation – 34% of all patients (29 and 34%, respectively), death – 23% of all patients (37 and 17%, respectively), and transition to HD – 23% of all patients (17 and 25%, respectively). The causes of transition to HD are presented in **Table 7**.

Cox proportional analysis was performed for patient survival, method survival and both in 90 days, 1 year and all follow-up period. The results are presented in **Table 8**.

During the first 90 days of dialysis therapy the only important negative predictor for patient survival appeared to be CCI (HR 1.6 [CI: 1.06–2.4]; p = 0.02); however, later on, both CCI and time from catheter implantation to PD start were found to be significant. The same applies for both method and patient survival: CCI (HR 1.24 [CI: 1.05–1.47]; p = 0.02), time from catheter implantation (continuous variable HR 0.66 [CI: 0.48–0.91]; p = 0.01) and ≤ 2 days (HR 4.29 [CI: 0.73–25.1];

TABLE 6 | Causes of death in patients with urgent- and planned PD start.

Time	Cause	US	PLS	Time	Cause	US	PLS
90 days	Cardiovascular	4	–	>12 months	Cardiovascular	1	6
	AIDS	–	1		Peritonitis	1	4
	Unknown	1	–		PAD	3	3
90 days–12 months	Cardiovascular	–	1		Malignancy	2	–
	Malignancy	1	–		Other/unknown	–	1

PAD, peripheral arterial disease; US, urgent-start; PLS, planned-start.

TABLE 7 | Causes of change to HD in patients with urgent- and planned PD start.

	Urgent-start (n = 35)		Planned-start (n = 94)	
Time	**Patients**	**Causes**	**Patients**	**Causes**
90 days	0		2 1	Loss of independence
			1	Patients decision
				Onco-surgery
90 days – 12 months	1	Peritonitis	2	Peritonitis
			2	Loss of
			2	independence
				Leakage
>12 months	2	Membrane	5	Peritonitis
	1	failure	3	Loss of
	1	Peritonitis	3	independence
	1	Leakage	2	Membrane failure
		Non-compliance		Abdominal surgery

p = 0.04) were found to be predictive during the whole analyzed period, although after 1 year – time >14 days lost its predictive value (p = 0.07). There were no predictors for method survival alone in any analyzed period.

DISCUSSION

In our nephrology clinic, the PD urgent-start pathway, without typical 2-week break-in period, was opened 14 years ago; its outline is presented in **Figure 1**. It was meant for late-referral patients, able and prone to join PD-first program, with its main advantages such as: keeping the vascular system intact, preserving their residual renal function and retaining life-style flexibility. There was also our hope that early unplanned PD may be a good method to increase use this option of RRT. Finally, for patients who ultimately decided to change the treatment for HD, PD treatment continued until arteriovenous fistula maturation enabled to avoid temporary vascular access catheter placement. In this paper we compare the short- and long-term outcomes of consecutive 35 patients after urgent- and 94 patients after the planned start, who have commenced PD as a first RRT in our unit during this period.

The results confirm that PD can be a safe method of introducing RRT in unplanned acute dialysis settings. In general they are comparable with those of other authors, presented in **Table 9** (Song et al., 2000; Banli et al., 2005; Povlsen and Ivarsen, 2006; Jo et al., 2007; Lobbedez et al., 2008; Yang et al., 2011; Casaretto et al., 2012; Ghaffari, 2012; Koch et al., 2012; Masseur et al., 2014; Alkatheeri et al., 2016; Bitencourt Dias et al. 2016, 2017; Jin et al., 2016; Pai et al., 2016; Wong et al., 2016; Xu et al., 2017; Wang et al., 2017; Nayak et al., 2018). However, there are many methodological differences among these studies concerning patients population, the technique of peritoneal catheter placement, or length of break-in period. In most of them the observation period was rather short (mostly 3–6 months), and only some of them have the control groups. In the presented study the median of the observation time was 19 months.

The biggest concern about acute PD start is the risk of early mechanical complications: dialysate leaks as well as the catheter dysfunction. It may be that an early rise in peritoneal pressure negatively affects the wound healing and facilitates leakage. This can be further intensified by hypoalbuminemia, relatively often present in ESRD patients who need urgent dialysis start. In the presented study a short break-in period together with low serum albumin occurred to be the independent predictors for early

TABLE 8 | Independent risk factors for patient and patient-method outcomes (Cox proportional analysis).

	90 days		12 months		Whole observation period	
Variable	**Patient survival**	**Patient and technique survival***	**Patient survival**	**Patient and technique survival***	**Patient survival**	**Patient and technique survival***
eGFR	1.0; CI, 0.88–1.13; NS	1.02; CI, 0.94–1.11; NS	0.84; CI, 0.62–1.15; NS	0.99; CI, 0.84–1.17; NS	1.0; CI, 0.88–1.13; NS	1.02; CI, 0.9–1.11; NS
Hb	0.8; CI, 0.53–1.1; NS	0.7; CI, 0.48–1.04; NS	1.46; CI, 0.75–2.87; NS	1.0; CI, 0.67–1.52; NS	0.77; CI, 0.53–1.11; NS	0.67; CI, 0.51–0.87; p = 0.003
CCI	1.6; CI, 1.06–2.4; p = 0.02	1.4; CI, 1.09–1.78; p = 0.007	1.4; CI, 1.02–1.79; p = 0.01	1.24; CI, 1.05–1.47; p = 0.02	1.43; CI, 1.2–1.66; p = 0.00002	1.2; CI, 1.08–1.32; p = 0.0003
Time ≤2 days	0.7; CI, 0.13–3.65; NS	8.53; CI, 0.77–94.89 p = 0.02	5.11; CI, 0.58–44.4; p = 0.03	4.29; CI, 0.73–25.1; p = 0.04	1.69; CI, 0.44–6.47; p = 0.07	1.4; CI, 0.56–3.51; NS
Time >14 days	0.6; CI, 0.16–1.86; NS	0.18; CI, 0.02–1.25; p = 0.01	0.08; CI, 0.004–1.67; p = 0.04	0.38; CI, 0.08–1.89; p = 0.07	0.21; CI, 0.08–0.58; p = 0.0006	0.56; CI, 0.28–1.12; p = 0.02

*CCI, Charlson Comorbidity Index; Time, time between catheter insertion and PD start. *Censored by death and kidney transplantation.*

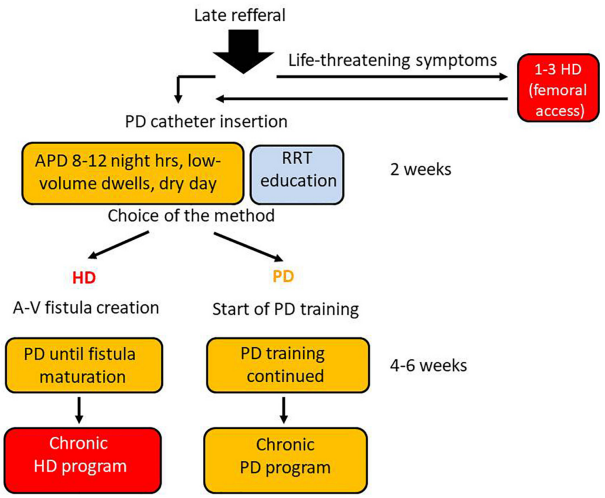

FIGURE 1 | PD urgent–start program realized in our nephrology unit. Every late referral patient is quickly but carefully evaluated. Those who have no contradictions to PD and give an informed consent for PD as a bridge to final therapy choice are qualified to the program. The patients with life-threatening clinical symptoms like: pulmonary edema, severe hyperkalemia or acidosis, are given 1–3 short HD treatments via acute femoral catheter, before peritoneal access creation. The peritoneal catheter is introduced by a surgeon from our team, and the low-volume 8–12 night hours APD is started, with concomitant RRT education program for the patient and his family. If – after receiving sufficient information – the patients decide to remain on PD, the PD training is started, while in case when he/she considers HD as a more suitable RRT option – a arteriovenous fistula is created and PD continued until matured.

dialysate leakage. The risk of this complication in patients who started PD urgently was 11% (vs. 0% in planned start group, $p < 0.001$), and its overall incidence was higher than described by some other authors (Povlsen and Ivarsen, 2006; Lobbedez et al., 2008; Sharma et al., 2008; Yang et al., 2011; Liu et al., 2014). There was also a higher incidence of bleeding into peritoneal cavity in urgent PD start group. However, after several days of peritoneal rest PD was resumed, and – unlike in some other studies – none of the patients needed a surgical intervention, and PD technique success at 3 months as well as during the whole observation was similar in both groups. The rates of late non-infectious complications were in both groups similar, with an exception of PD catheter migration, more frequently seen in the planned-start group. It did not affect the technique and patient survival either.

Contrary to the common perception that early use of peritoneal catheter may increase a risk of infectious complications such as exit-site/tunnel infections and peritonitis, we did not observe any of these in the early 4-week period of the treatment. The incidence rates during the whole observation period were in both studied groups similar. A shorter time to first peritonitis episode in urgent-start group shorter ($p = 0.04$) seems to be rather a consequence of a shorter observation period in this group. In the regression logistic analysis none of analyzed parameters had influence on peritonitis or exit-site/tunnel infection occurrence.

We found significantly higher mortality rate after first 90 days of the therapy in patients initiating PD urgently (14 vs. 1.1%, $p < 0.0001$). Although both groups were comparable in respect to age, many other factors may explain the reduced short-term survival in the unplanned starters (Lobbedez et al., 2008; Ivarsen and Povlsen, 2014). It is well-known that first 90 days of any dialysis therapy is a period of disproportionately high mortality and that in patients who start the treatment urgently and are often in a challenging clinical condition the outcomes are worst, with the percentages reaching even 30% in unplanned HD (where a part of which may be attributable to CVC (Khan et al., 1995; Collins et al., 2009; Mendelssohn et al., 2009; Baer et al., 2010; Panochia et al., 2016). Our urgent start patients were "sicker": had more advanced uremia, lower albumin and hemoglobin levels and worse general clinical status, with necessity of dialysis within first 48 h in 51% of them, including 3 (8.6%) patients with life-threatening uremic symptoms in whom 2–3 short emergency HD via femoral access were performed before PD catheter placement.

The higher mortality rate in the urgent-start group persisted after 1 year (17 vs. 2%) and at the end of observation (37 vs. 17%), although the difference did not reach the statistical significance, possibly because small numbers of studied patients. The only significant predictor of patient death during first 90 days of dialysis therapy appeared to be CCI (HR 1.6 [CI: 1.06–2.4]; $p = 0.02$), however, later on both: CCI and time from catheter implantation to PD start were found to be significant.

The studied group consists of unselected incident patients started PD in our unit and seems to be fairly typical patients population treated with PD (age, comorbidity, ∼30% urgent start). The mortality rate in this group in the entire observation period (mean: 25.6 ± 23.8, median: 19 months) was 23%, and 10.6% in the first year (4.6%–90 days, 6%–90 days–12 months). It seems to be comparable with reported mortality rates in dialysis populations (USRDS, ERA-EDTA Registry), which despite some improvement in the last decade, remains very high (∼20%) with a universal phenomenon of increased mortality early after dialysis initiation (Robinson et al., 2014). The mortality rate was distinctly, however not statistically significant higher (effect of the sample size?) in urgent-starters in all time intervals, and this is good established observation that urgent dialysis start is an important factor of poor prognosis (short and long-term) irrespective of dialysis modality and generally results from various aspects related to uremic complications and comorbidities, as well as dialysis issues (i.e., CVC in HD patients). In our study, in Cox proportional analysis the time ≤ 2 days from catheter implantation to dialysis start (which may be a marker of necessity of dialysis initiation – more advanced uremia, worse general clinical status), and CCI proved to be an independent risk factors for patient survival in the whole observation.

With death and renal transplantation being the censored events, the PD technique survival was excellent in both, urgent and planned start groups, being respectively: 100 and 96% at 3 months, 97 and 87% at 12 months, and 83 and 76% at the end of the observation. These percentages are higher than reported by

TABLE 9 | Studies published during lasts years on urgent PD start.

Reference	Patients (groups)	Insertion technique	Urgent-start intervention	Observation period	Mechanical complications	Infectious complications	Survival (patient survival, method survival)
Povlsen and Ivarsen, 2006	140 pts (52-US vs. 88-PLS)	Surgical	APD < 24 h after catheter insertion	3 months	Leakage : 7.7% in US vs. 0% in PLS. Catheter dysfunction: 15% in US vs. 5.8% in PLS	Peritonitis: 15% in US vs. 15% in PLS ESI: 3.9% in US vs. 3.8% in PLS	Technique survival: 87% for US, 90% for PLS
Yang et al., 2011	310 pts (226-US vs. 84-PLS)	Surgical	CAPD 48–64 h after catheter insertion	6 months	Leakage: 2.2% in US vs. 2.4% in PLS Catheter dysfunction: 1,3% in US vs. 0% in PLS	Peritonitis: 4% in US vs. 2.4% in PLS ESI: 1.3% in US vs. 0% in PLS	Not specified
Pai et al., 2016	149 pts (80-US vs. 69-PLS)	Surgical	6–13 days after catheter insertion	30 ± 25 months	–	Peritonitis: 1/65 patient-months in US vs. 1/95 patient-months in PLS	Drop out of PD 45 in early and 34 in delayed starters
Jin et al., 2016	178 pts (96-US vs. 82-US HD)	Surgical	PD start within 14 days after catheter insertion		Catheter malposition 3.1%	Peritonitis: 2.1% ESI: 0%	3 months survival 98% for PD and HD 1 year survival 92% for PD, 93% for HD
Xu et al., 2017	922 pts US	Surgical	50% within 1 day after catheter insertion	Median 31 months	Abdominal wall complications 4.8% Catheter complications 9.5%	–	36% pts continued to receive PD therapy
Wang et al., 2017	101 pts US	Surgical	2 days after catheter insertion	12 months	Leakage 10% in IPD vs. 3.9% in APD Catheter malposition 4% in IPD vs. 3.9% in APD	Infection 26% in IPD and 13.7% in APD	–
Nayak et al., 2018	56 pts (32-US vs. 24 PLS)	Surgical	≤48 h after catheter insertion	90 days	Leakage 9.4% in US vs. 0% in PLS Catheter migration 25% in US vs. 16.7% in PLS	Peritonitis: 9.4%???	Technique survival 91% for US, 96% for PLS
Casaretto et al., 2012	11 pts US	Laparoscopic	APD < 48 h after catheter insertion	90 days	No leaks, catheter dysfunction in 1 patient	No peritonitis	–
Koch et al., 2012	123 pts (66-US vs. 57-US HD)	Laparoscopic	APD during 12 h after catheter implantation	4.7 ± 2.0 months	Catheter dysfunction: 7.6% for PD vs. 5.3% for HD	Bacteremia 3% for PD vs. 21% for HD. Peritonitis: 3%, ESI: 4.5%	6 months survival 70% for PD, 58% for HD (NS)
Masseur et al., 2014	81 pts US	Laparoscopic	ADP – immediately to 3–6 days after catheter implantation	3 months	No leaks Catheter dysfunction in eight patients	No peritonitis	95%
Alkatheeri et al., 2016	30 pts US	Laparoscopic or percutaneous	APD – immediately (6 pts) to median 6 days after catheter implantation	Median 201 days	Leakage in 10%; Catheter dysfunction in 20%	Peritonitis – 1 (1:319 patient-months) ESI 2 (1:159 patient-months)	3 months patient survival 100%; technique survival 93%

(Continued)

TABLE 9 | Continued

Reference	Patients (groups)	Insertion technique	Urgent-start intervention	Observation period	Mechanical complications	Infectious complications	Survival (patient survival, method survival)
Song et al., 2000	59 pts US	Percutaneous	Immediately (<24 h)after catheter implantation: (I) gradual increase in exchange volume (II) Full exchange volume (2 L)	12 months	Leakage: 9.5% in Group I vs. 10.5% in Group II Catheter dysfunction: 4.8% in Group I vs. 5.3% in Group II	Peritonitis: 24% in Group I vs. 16% in Group II ESI: 9.5% in Group I vs. 5.3% in Group II	Catheter survival: Group I – 86%, Group II – 84%
Banli et al., 2005	41 pts US	Percutaneous	CAPD incremental since 6-th day after implantation	–	Leakage: 4.8%; Catheter dysfunction: 2.4%	Peritonitis: 2.4% ESI: 0%	–
Jo et al., 2007	51 pts US	Percutaneous	CAPD immediately after catheter implantation	12 months	Leakage: 2%; Catheter dysfunction: 12%	Peritonitis: 4% ESI: 4%	–
Ghaffari, 2012	27 pts (18-US vs. 9-PLS)	Percutaneous	Urgent: <2 weeks after catheter implantation Planned: 2–4 weeks after catheter implantation	3 months	Leakage: 33% in US vs. 11% in PLS Catheter dysfunction: 11% in US vs. 22% in PLS	Peritonitis: 1:110 patient-months in US vs. 1:42 patient-months in PLS ESI: 1:55 patient-months in US vs. 1:42 patient-months in PLS	–
Bitencourt Dias et al. 2016	76 pts (35-US vs. 6-PLS vs. 29-US HD vs. 6-PLS HD	Percutaneous	High volume PD < 48 h after catheter implantation	3 months	Leakage: 2.8% Catheter dysfunction: 20%	Peritonitis: 14.2% ESI: 8.6%	Patient survival – 80% Technique survival – 86%
Bitencourt Dias et al., 2017	51 pts US	Percutaneous	High volume PD < 72 h after catheter implantation	180 days	Leakage: 9.7% Catheter migration: 16%	Peritonitis: 0.5 patient/y ESI 17%	Patient survival – 82% Technique survival – 86%
Wong et al., 2016	81 pts US	Surgical or laparoscopy or percutaneous	Emergent – within 48 h of catheter implantation; Urgent – 48 h – 14 days after catheter implantation	12 months	Leakage: 5% Catheter dysfunction: 15%	Peritonitis – 16% (72/100 patient-years)	–
Lobbedez et al., 2008	60 pts (34 US PD vs. 26 US HD)	Not specified	APD 9.6 ± 10.3 days (median: 4 days) after catheter insertion	12 months	Leakage: 5.8%	The survival free of peritonitis: 73% at 6 months and 55% at 1 year	Patient survival: 83% for PD, 79% for HD PD technique survival – 90% after 6 months and 88% after 12 months

the other experienced urgent-start PD centers. In a French study by Lobbedez et al. (2008), which included 34 unplanned dialysis patients the actuarial PD technique survival was 90% at 6 months and 88% at 1 year. In the study done by Povlsen and Ivarsen, who compared outcomes of 52 urgent start with 52 planned-start patients, the 3-month PD technique censored survival rates were even lower, with corresponding values: 87 and 90% (Povlsen and Ivarsen, 2006).

CONCLUSION

Peritoneal dialysis may be a feasible and safe alternative to HD in patients who need to start dialysis urgently without established dialysis access, with an acceptable complications rates, as well as patient and technique survival. We found this method an important part of full RRT program in every reference dialysis unit, allowing for real free individual choice of the modality. However to be successful PD urgent start program needs adequate infrastructure, expertise, good organization with continuous availability of experienced surgeons or other doctors who place peritoneal catheters, dedication of the complete team, and a good RRT educational program, adjusted to the unplanned setting, to give the full possibility of unbiased final choice of the RRT method, that fits best to their lifestyle.

ETHICS STATEMENT

The study was exempted from the requirement of the Ethics Committee, as all the patients were in end-stage kidney disease and require renal replacement therapy in other planned or urgent manner. All of them singed the informed consent to start renal replacement therapy as required by the National Health found (routine informed consent for the procedure of increased risk).

AUTHOR CONTRIBUTIONS

EW, AG, and JM-R conceived the idea for the study and contributed to the design of the research. EW and JM-R performed the statistical analysis of the collected data and performed the analysis. EW, JM, and JM-R were involved in the preparation of the manuscript. EW, AG, and KG were involved in data collection. All the authors analyzed the data, edited, and approved the final version of the manuscript.

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frontiers in Physiology

REVIEW
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Biocompatible Peritoneal Dialysis: The Target Is Still Way Off

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Peritoneal dialysis (PD) is a cost-effective, home-based therapy for patients with end-stage renal disease achieving similar outcome as compared to hemodialysis. Still, a minority of patients only receive PD. To a significant extend, this discrepancy is explained by major limitations regarding PD efficiency and sustainability. Due to highly unphysiological composition of PD fluids, the peritoneal membrane undergoes rapid morphological and long-term functional alterations, which limit the treatment and contribute to adverse patient outcome. This review is focused on the peritoneal membrane ultrastructure and its transformation in patients with kidney disease and chronic PD, underlying molecular mechanisms, and potential systemic sequelae. Current knowledge on the impact of conventional and second-generation PD fluids is described; novel strategies and innovative PD fluid types are discussed.

Keywords: peritoneum, peritoneal dialysis, glucose, glucose degradation products, biocompatibility, transformation, transport, junctions

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PERITONEAL MEMBRANE ANATOMY AND PHYSIOLOGY

The peritoneum is a delicate structure covering the entire peritoneal cavity. The parietal peritoneum is composed of a single layer of mesothelial cells and a submesothelial zone, which contains blood vessels, lymphatic vessels, and nerves that are mainly organized in a three layer structure (Blackburn and Stanton, 2014; Schaefer et al., 2016a) (**Figure 1**). The parietal peritoneal capillary density is age-dependent, with a two times higher blood vessel density in infants than in older children. In adults, peritoneal blood vessel density slowly increases with age but remains below the density observed in infants (Schaefer et al., 2016a). The age-dependent changes in peritoneal vascularization during childhood may be explained by the rapid increase in body dimension in early life, which should reduce a constant number of capillaries in an increasing tissue volume, as it is the case for the number of glomeruli found in a given cone kidney biopsy in children of different ages (Feneberg et al., 1998). Similar findings were observed for the lymphatic density, which overall is much lower than blood vessel density. Submesothelial thickness steadily increases until the age of 18 years and is again lower in adults. Respective percentile curves for age-appropriate evaluation of the peritoneum have been established (Schaefer et al., 2016a). The extracellular peritoneal matrix contains bundles of collagens and mucopolysaccharides and a small number of cells such as fibroblasts and mononuclear cells, including sparse CD45 lymphocytes and CD68 macrophages (Schaefer et al., 2016a).

The visceral peritoneum covers the abdominal organs and their supply structures, the mesentery, but no detailed systematic analyses across age groups have been performed. The omental peritoneum consists of a calretinine and podoplanin positive mesothelial cell layer as the parietal peritoneum. It covers the adjacent adipose tissue, which contains isolated bundles of large vessels and a much lower numbers of capillaries than the parietal submesothelium. The omental and parietal peritoneal blood capillary microvessel density are correlated. Thus, omental tissue specimen should be informative regarding the

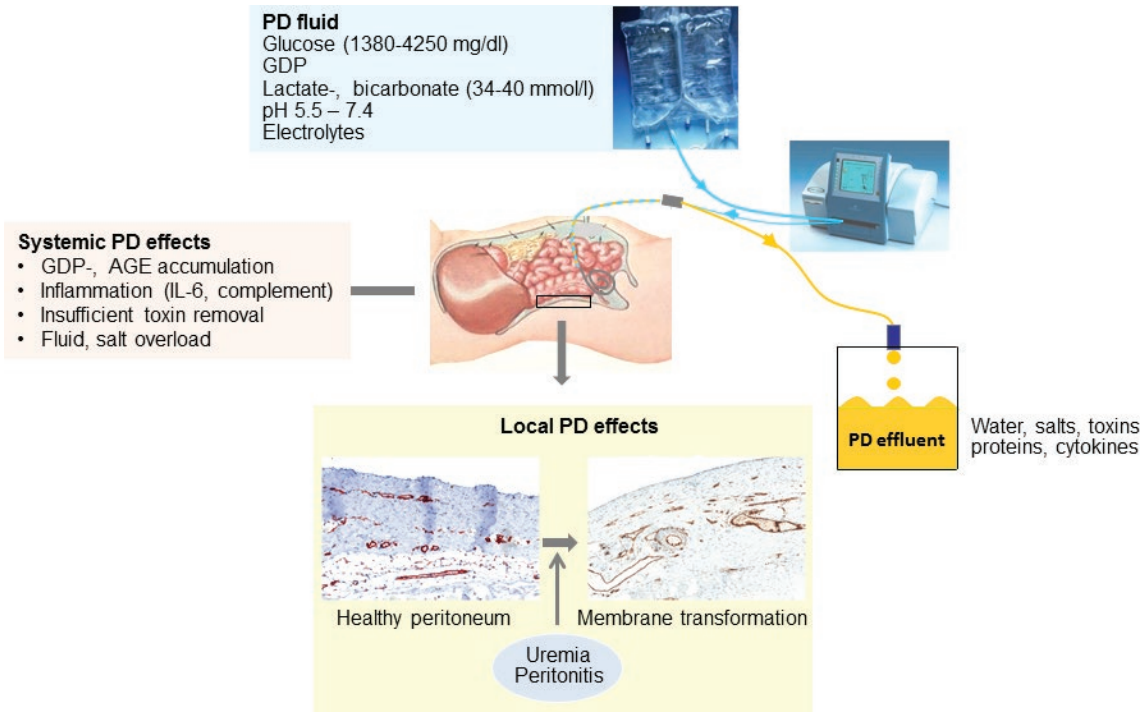

FIGURE 1 | Scheme of peritoneal dialysis application, peritoneal dialysis (PD) fluid composition, and local as well as systemic effects. Three vessel layer structure of the healthy peritoneal membrane as published previously (Schaefer et al., 2016a).

parietal peritoneal vessel density, at least in the non-diseased state (Schaefer et al., 2016a).

The peritoneum exerts numerous functions, many of which have already been recognized in the 19th century (Robinson, 1897; van Baal et al., 2017). The peritoneum maintains local homeostasis and provides protection from movement-induced frictions and adhesions by secretion of phospholipids, mainly phosphatidylcholine, together with surfactant proteins (SP-A, -B, -C) (Hills et al., 1998). In steady state, mesothelial cells produce 5–100 ml of peritoneal fluid containing complement factors (Tang et al., 2004; Zelek et al., 2016), immunoglobulins (Davies et al., 1990), defensins (Grupp et al., 2007), and immune cells like macrophages, lymphocytes, eosinophils, and mast cells (van Baal et al., 2017) that exert anti-infectious actions and regulate the inflammatory response (Isaza-Restrepo et al., 2018). *In vitro*, mesothelial cells migrate in an AQP-1-dependent manner (Ryu et al., 2012; Zhai et al., 2012), suggesting efficient wound healing capacity of superficial peritoneal erosions. Tissue remodeling is balanced by profibrotic cytokines and tissue inhibitors of metalloproteinase and by extracellular matrix degrading proteins such as metalloproteinases, gelatinase, and collagenase (Marshall et al., 1993; Ma et al., 1999). Plasminogen activator is responsible for a physiological fibrinolytic activity of peritoneum, and reduced concentrations following abdominal surgery promote adhesion formation (Holmdahl et al., 1996).

Angiogenesis in postnatal development is controlled by cytokines, including vascular endothelial growth factor (VEGF) and angiopoietins, and their receptors (Eklund and Saharinen, 2013). Angiogenesis is tightly regulated through a balance between activating and inhibiting signals (Fagiani and Christofori, 2013). Adult vasculature is quiescent, but blood vessels retain a high plasticity in order to respond to angiogenic signals after inflammation or injury. These angiogenic mechanisms should also be active in the peritoneum.

The omental fat tissue (Summers, 2006) generates numerous hormones and cytokines involved in immune responses and angiogenic and neurogenic factors (Chamorro et al., 1993; Goldsmith, 2001). It is a lipid store and pools immune cells, and it can adhere to neighboring peritoneum to embank local inflammation (Hall et al., 1998).

Altogether, the peritoneum is of clinical impact in various conditions such as postoperative adhesions (Hills et al., 1998; Arung et al., 2011), in patients with abdominal and gynecological carcinoma (Lemoine et al., 2016), and in patients with chronic kidney disease stage 5D requiring dialysis (CKD5D). This review focuses on the usage of the semipermeable peritoneum as a biological dialysis membrane, its transformation with peritoneal dialysis (PD), and current concepts and future prospects to improve PD efficacy and sustainability.

PERITONEAL DIALYSIS

PD is a life-saving, renal replacement therapy for a worldwide increasing number of patients with CKD5D. PD removes excess water and electrolytes as well as metabolic waste products by osmosis across a concentration gradient between the blood and the PD fluid and ultrafiltration-associated solvent drag (convection). PD is a cost-effective, home-based therapy and has significant advantages over

hemodialysis (HD), in particular, regarding quality of life. Early patient outcome is at least similar to patients on HD (McDonald et al., 2009; Waldum-Grevbo et al., 2015). Despite these benefits, only a small number of dialysis patients receive PD, in Europe about 13% and in the USA about 10% (Mehrotra et al., 2016; Kramer et al., 2018). This discrepancy is in part explained by major limitations of PD. Infectious complications, mainly peritonitis, and the PD fluid induced progressive deterioration of the PD membrane with chronic PD, lead to PD function deterioration and eventually technique failure. As with hemodialysis, the uremic toxin and water removal capacity is far below physiological renal function. Most of the patients require strict dietary control and pharmacological treatment, such as phosphate binders, but the vast majority of patients are salt, fluid, and toxin overloaded. In particular, dietary phosphate and sodium intake are inadequately compensated by PD and essentially contribute to high blood pressure (Ortega and Materson, 2011), CKD mineral bone disorder, and cardiovascular disease (CVD). Mortality rates of both hemodialysis and PD patients are 40-fold higher compared to the age-related healthy population; accelerated cardiovascular disease is the primary cause of death (de Jager et al., 2009).

PD FLUID COMPOSITION

The osmotic agent most frequently applied is glucose. PD fluids contain glucose at concentrations of 10- to 50-fold above physiological serum concentrations. Glucose creates an osmotic gradient with an osmolality of about 50–150 mOsmol/l above serum osmolality allowing for removal of water (called "ultrafiltration") and of electrolytes and toxins by ultrafiltration-associated convection. Correction of metabolic acidosis is achieved by uptake of a buffer compound, lactate or bicarbonate, present in the dialysate at concentrations of 34–40 mmol/l.

First-generation PD fluids contain 35–40 mM lactate buffer and have an acidic pH of 5.5. The low pH aggravates the detrimental effects of the high lactate concentrations on peritoneal mesothelial and leukocyte function (Topley et al., 1988, 1996). During heat-sterilization and prolonged storage, high amounts of glucose degradation products (GDP) are formed, e.g., methylglyoxal and 3,4-dideoxyglucosone-3-ene. Peritoneal GDP exposure correlates with peritoneal advanced glycation end products (AGE) deposition and increasing peritoneal transporter status function, with the latter reflecting the PD fluid-induced peritoneal transformation process (Nataatmadja et al., 2018). GDP are rapidly absorbed into the circulation and increase systemic AGE concentrations (Zeier et al., 2003; Schmitt et al., 2007). AGE bind to the AGE receptor RAGE and trigger various intracellular events, such as oxidative stress and inflammation, leading to cardiovascular complications (Stinghen et al., 2016). Skin tissue AGE concentrations are increased in PD as compared to HD patients and independently associated with CV morbidity (Jiang et al., 2012).

To prevent GDP formation and to achieve a neutral to physiological pH, second-generation PD fluids have been introduced 20 years ago. These separate the buffer compound, lactate and/or bicarbonate, from the glucose, which is kept at a very low pH to reduce GDP formation during heat sterilization and storage. Prior to administration, the compartments are mixed; the final pH of the ready-to-use fluid is 7–7.4. Depending on the manufacturing process, GDP formation is substantially reduced but still varies considerably between different brands (Erixon et al., 2006). Second-generation PD fluids significantly reduce systemic GDP load and circulating AGE concentrations. The impact of 10–20% reduction in serum AGE concentrations achieved with low versus high GDP PD fluids on PD patient outcome, however, is still uncertain (Zeier et al., 2003; Schmitt et al., 2007).

PD fluids with an alternative osmotic agent contain icodextrin, a much less resorbed osmotic agent derived from starch. It allows for a slow but persistent colloid osmotic ultrafiltration and therefore can be used for a single long dwell per day (Dousdampanis et al., 2018; Morelle et al., 2018). Icodextrin fluid is especially applied in patients with high peritoneal solute transporter status and improves patient's hydration status (Cho et al., 2013). Despite the absence of glucose and the very low GDP content, the acidic PD fluid has been associated with increased local and systemic inflammation (Parikova et al., 2003; Martikainen et al., 2005; Moriishi and Kawanishi, 2008; Velloso et al., 2014). Another alternative to glucose-based PD fluids is amino acid containing solutions, which are free of GDP and have an only slightly acidic pH of 6.7. For optimized nutrition of malnourished patients and to prevent increased serum nitrogen levels and metabolic acidosis (Dombros et al., 1990), they should be applied at a ratio of 1–4 with glucose-containing PD fluids (Tjiong et al., 2005). The nutritional effects are limited; stable isotope studies in adult CAPD patients yielded a 4% higher protein synthesis rate than patients treated with glucose-containing PD solution only (Tjiong et al., 2007). The biocompatibility of amino acid fluids remains uncertain, and experimental studies and findings in humans do not unanimously support the notion of improved peritoneal biocompatibility. Rats exposed to amino acid PD fluid had less peritoneal AGE deposition, lower VEGF levels, and a lower vessel density compared to rats treated with first-generation PD fluid (Mortier et al., 2004). *In vitro,* mesothelial cells exposed to amino acid PD fluid synthesized less HSP72, released more IL-6 and prostaglandin E2, and had superior viability as compared to acidic, high GDP fluid (Bender et al., 2008). Others, however, reported more mesothelial nitric oxide (NO) synthesis (Reimann et al., 2004). NO plays a key signaling role in numerous biologic processes, including control of vascular tone and permeability, and angiogenesis, *via* an interaction with VEGF (Papapetropoulos et al., 1997). Human peritoneal endothelial NO synthase expression and activity increase with time on PD and are related to endothelial VEGF upregulation and peritoneal vessel density (Combet et al., 2000).

Altogether, limited progress has been achieved during the past 50 years of PD treatment regarding PD fluid technology and mainly consists of reduction of the GDP content, pH neutralization, introduction of the bicarbonate buffer and of two alternative osmotic compounds. Glucose-based PD fluids still predominate, and PD treatment still confers major local peritoneal and systemic toxicity (**Figure 1**) (Schmitt and Aufricht, 2016).

PERITONEAL MEMBRANE TRANSFORMATION WITH CHRONIC PD

In patients with CKD5, at the time of catheter insertion, the peritoneum already exhibits minor but distinct alterations, including submesothelial thickening and vasculopathy, as compared to controls

with normal renal function (Williams et al., 2002). In diabetic patients, peritoneal changes at start of PD are even more pronounced and comprise mesothelial loss, mesothelial basement membrane thickening, vascular wall thickening, and inflammatory cell infiltration (Contreras-Velazquez et al., 2008). The latter and hypoalbuminemia are associated with technique failure and mortality rate. In pediatric CKD5 patients, an increase in parietal vessel density (Schaefer et al., 2018) was observed. In contrast, omental fat vessel density was found to be reduced in pediatric CKD5D, pointing to another distinct and early feature of CKD-related vascular disease (Burkhardt et al., 2016). Parietal peritoneal micromorphological changes are accompanied by vascular endothelial telomere shortening, mild inflammatory cell invasion, epithelial-to-mesenchymal transition (EMT), fibrin deposition, and TGF-β-induced SMAD phosphorylation (Schaefer et al., 2018). Compared to the subsequent PD-induced changes, morphological alterations are still mild and do not progress much in patients on HD (Williams et al., 2002).

In a landmark paper of Williams et al., severe transformation of the peritoneum was demonstrated with chronic PD in patients treated with acidic, high GDP fluids (Williams et al., 2002). These changes included progressive loss of the mesothelial cell layer, a massive increase in submesothelial thickness especially in patients with more than 4 years of PD, and rapidly progressing, severe peritoneal vasculopathy. Number of peritoneal vessels per peritoneal section length was increased at the time of PD-related surgery and in patients with PD membrane failure, i.e., insufficient peritoneal transport function, as compared to a small group of patients with normal renal function. The study group did not relate their histologic findings to PD function and patient outcome; however, resulting therapeutic complications of long-term PD have repeatedly been described. Peritoneal solute transport gradually increases with time on PD, particularly when increasing concentrations of glucose are applied (Davies et al., 1998, 2001). Ultrafiltration capacity declines and eventually results in long-term ultrafiltration failure, which is often characterized by impaired osmotic conductance to glucose and reduced free water transport (Krediet and Struijk, 2013). High solute transport predicts technique failure and is associated with poorer patient survival (Davies et al., 1998). Peritoneal protein clearance also increases during the course of PD, but to a relatively smaller extend (Struijk et al., 1991; Ho-dac-Pannekeet et al., 1997).

Introduction of neutral pH, low GDP fluids raised hope to prevent long-term deterioration of the peritoneal membrane, based on numerous *in vitro* and experimental *in vivo* studies. These studies suggested improved local host defense (Mortier et al., 2003), reduced mesothelial damage (Grossin et al., 2006) and EMT (Bajo et al., 2011), less peritoneal GDP and AGE deposition, less TGF-β and VEGF signaling, and less submesothelial fibrosis and angiogenesis, altogether resulting in better preservation of peritoneal ultrafiltration capacity (Mortier et al., 2004, 2005; Rippe, 2009). Respective clinical trials were less consistent. Compared to first-generation PD fluids, administration of neutral pH, low GDP fluids resulted in higher CA125 effluent concentrations (Haas et al., 2003; Szeto et al., 2007), a putative marker of mesothelial cell viability and lower hyaluronic acid and procollagen peptide concentrations, suggesting improved peritoneal membrane integrity (Williams et al., 2004). A declining incidence of encapsulating peritoneal sclerosis has been associated with low GDP fluid usage (Nakao et al., 2017). Residual renal function, a major predictor of patient outcome, was better preserved (Kim et al., 2008; Haag-Weber et al., 2010; Johnson et al., 2012b). While superior residual renal function during the first year of PD may be related to less-effective fluid removal and consequent volume expansion with neutral pH, low GDP fluid, the long-term effect could be related to lower renal GDP and AGE exposure (Cho et al., 2014; Yohanna et al., 2015). The Euro-Balance trial, a randomized, two times 12-week crossover trial, demonstrated improved residual renal function together with decreased peritoneal ultrafiltration with the pH neutral, low GDP fluid, as compared to the first-generation, acidic high GDP solution (Williams et al., 2004). The largest study up to now, the BalANZ trial yielded a lower risk of anuria and lower ultrafiltration and higher solute clearance rates with the low GDP fluid during the first 9 months of PD. Over the entire 2 study years, the increase in solute transport and ultrafiltration decline were less pronounced with the low GDP fluid, resulting in comparable peritoneal membrane function at the study end with either fluid (Johnson et al., 2012b). A recent meta-analysis confirmed that neutral pH, low-GDP solutions result in a higher D/P creatinine during the first 6 months of treatment as compared to acidic, high GDP fluids but not subsequently (Yohanna et al., 2015). Peritonitis incidence and severity were reduced in the BalANZ trial and in another randomized, parallel trial over 2 years (Johnson et al., 2012a; Farhat et al., 2017), whereas other randomized trials did not report such differences (Williams et al., 2004; Szeto et al., 2007). Of note, these studies all compared the neutral pH, lactate-buffered, low GDP fluid with the acidic, lactate-buffered, high GDP PD fluid. Consecutive 1-day and 12-week randomized crossover studies in children comparing physiological pH, pure bicarbonate-buffered, low GDP fluid with first-generation PD fluid demonstrated similar ultrafiltration rates and a similar to 10% lower small solute transport rate with the former, which is in contrast to the reduced ultrafiltration and increased solute transport rates reported with neutral pH, lactate-based, low GDP fluids (Schmitt et al., 2002; Haas et al., 2003).

Peritoneal biopsies are usually not performed within clinical routine and limited to occasion of abdominal surgery required for other reasons. On the other hand, they are well tolerated, even in small children, and highly informative. They provide information not only on acute inflammatory but also on chronic PD-induced peritoneal damage and should allow for a prognostic estimate of PD performance. Scientific impact of peritoneal tissue analysis is considerable. Based on findings in 100 patients with diseases not affecting the peritoneal integrity and 90 CKD5 patients at time of first PD catheter insertion, Schaefer et al. (2018) analyzed 82 children on PD with low GDP fluids and revealed unexpected findings. In patients with a median PD vintage of 4 months, peritoneal blood capillary density and number per section length doubled, endothelial exchange area increased, and the three-layer structure has turned to a rather homogenous vessel distribution. Hypervascularization further increased in the majority of patients after 9 months of PD and remained largely unchanged thereafter. Peritoneal vessel density independently predicted glucose and creatinine transport. Vasculopathy, already present at time of PD initiation significantly progressed. In contrast, lymphatic vessel density remained largely unchanged in all PD patient groups. Submesothelial thickening progressed slowly and was severe in patients on PD for more than 4 years. These changes were

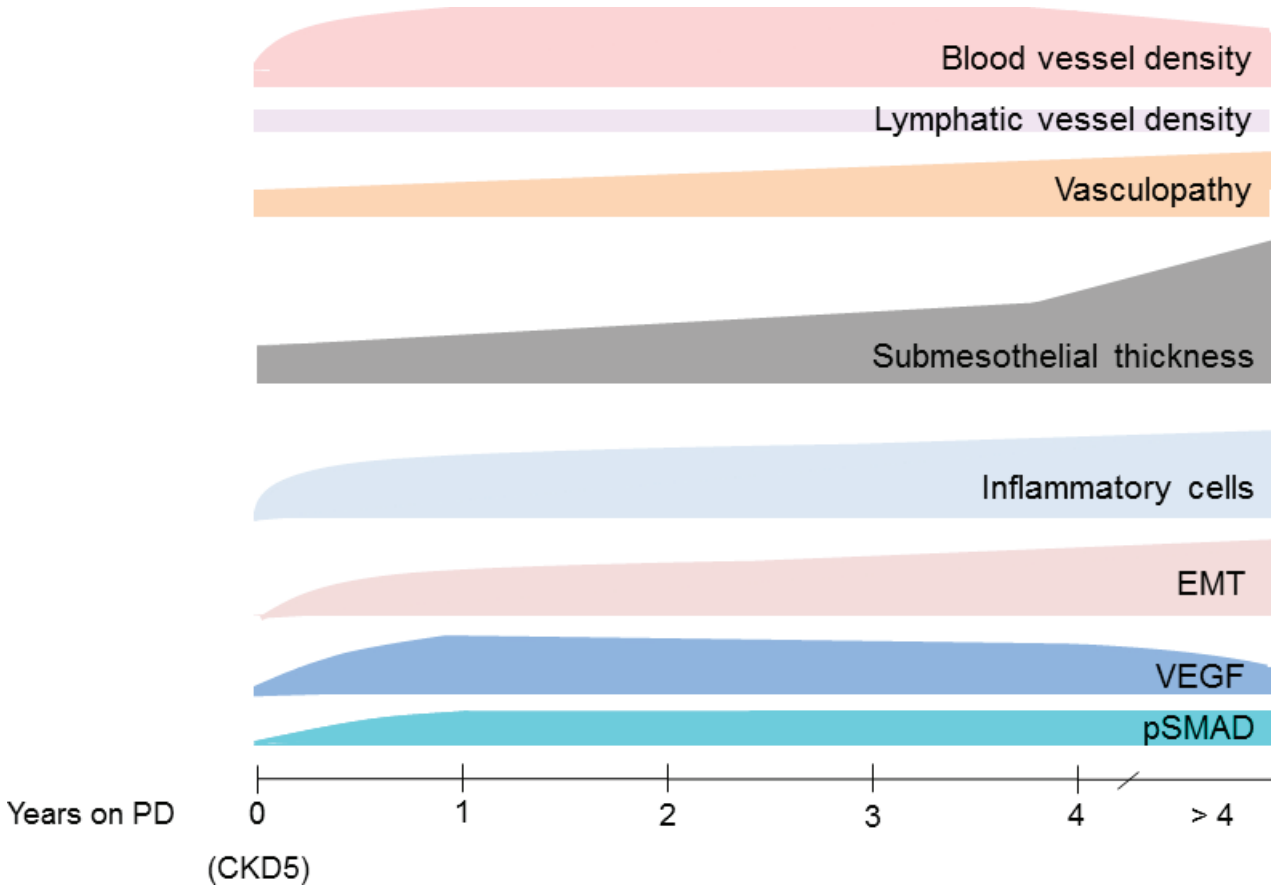

FIGURE 2 | Transformation of the peritoneum with time on PD treatment with neutral pH, low GDP PD fluids. Blood microvessel density substantially increases within few months of PD. It is closely correlated with endothelial surface area, which presents the primary barrier for transport across the peritoneal dialysis membrane. Percentage of patients with substantial inflammatory cell infiltration and EMT increases with time on PD. VEGF signaling is particularly induced within the first year of PD, the TGF-β signaling cascade (pSMAD) activation is delayed but remains high during long-term treatment (Schaefer et al., 2018).

accompanied by induction of VEGF- and TGF-β-induced SMAD phosphorylation, by EMT and inflammatory cells invasion (Schaefer et al., 2018) (**Figure 2**). This first study looking in detail in a larger number of patients into early and long term induced peritoneal changes and applying digital imaging analysis suggests that the assumption of significantly improved biocompatibility with neutral pH, low GDP fluids cannot be maintained (Blake, 2018). Still, conclusions need to be drawn with caution, and comparison with high GPD fluids is difficult. Children are uniquely suited for the analysis of specific CKD- and PD-related pathomechanisms, since, different from adults, they mostly suffer from underlying diseases not affecting the peritoneum (Harambat et al., 2012) and they are largely free of lifestyle and aging-related tissue damage. On the other hand, findings cannot necessarily be transferred altogether to the adult PD population. Angiogenesis may be regulated differently in growing children, and factors absent in children may have an impact on peritoneal pathomechanisms in elderly PD patients. Neutral pH, low GDP fluids have been recommended by the European Pediatric Dialysis Working Group in 2011 (Schmitt et al., 2011), and the majority of European children are now treated with low GDP fluids. PD vintage and body surface area adjusted dialytic glucose exposure matched comparison with high GDP fluid treatment thus far has been limited to a total of 30 children. After 1 year of PD, children on high GDP PD had a higher degree of vasculopathy and more submesothelial thickening (Schaefer et al., 2016b). Similar findings were reported in 24 adult Japanese patients on PD for about 4.5 years. Peritoneal AGE accumulation, submesothelial thickening, and vasculopathy were less severe with low GDP usage (Kawanishi et al., 2013). In a subsequent study from the same group including additional patients, the protective effect of low GDP fluid on vasculopathy was reconfirmed (Hamada et al., 2015). Del Peso et al. (2016) compared 23 low and 23 high GDP PD-treated patients matched for PD vintage, and the mean treatment duration was 2 years. The mesothelial cell layer was better preserved, and vasculopathy was less pronounced in the patients on low GDP PD. In children, better preservation of the mesothelium cell layer could not be demonstrated, possibly due to the fragility of the pediatric samples and related processing artifacts. Altogether, these findings suggest distinct benefits of second over first-generation PD fluids, but higher patient numbers are needed to draw firm conclusions, at best in combination with functional data.

No peritoneal tissues have been obtained from patients on amino acids or icodextrin solutions, clinical trials, however, suggest better preservation of the peritoneal transporter status when icodextrin solution is added to glucose-based high GDP regime (Davies et al., 2005).

In order to improve PD fluid biocompatibility, an in-depth understanding of molecular mechanisms of PD-induced membrane transformation and of related systemic effects of PD is required. Derived surrogate biomarkers of PD-induced pathomechanisms may allow predicting individual PD patient prognosis at an early stage and to guide dialysis therapy and establish therapeutic interventions. Up to now in clinical practice, PD biomarkers are largely limited to effluent cell count and cell differential. A number of potential surrogate parameters of peritoneal pathophysiology are on the horizon but still far from being established in clinical routine (Aufricht et al., 2017).

MOLECULAR MECHANISMS OF PD-INDUCED PERITONEAL TRANSFORMATION

Progressive destruction of the mesothelial cell layer, angiogenesis, and fibrosis and ultimately (life-threatening) peritoneal sclerosis (EPS) are due to an array of molecular mechanisms, which interact with each other. Peritoneal vessel density predicts peritoneal solute transport and overshooting vessel formation reduces ultrafiltration capacity, unless major fibrosis has developed, which reduces the osmotic conductance of glucose (Krediet et al., 2000). Experimental and human biopsy studies not only related peritoneal VEGF synthesis to peritoneal angiogenesis (De Vriese et al., 2001; Schaefer et al., 2018) but also shedded light on further aspects of the angiogenic machinery. Monoclonal VEGF antibody bevacizumab inhibits peritoneal angiogenesis and fibrosis in response to chlorhexidine (Ada et al., 2015). TNP-470, an endothelial cell cycle, and tumor angiogenesis inhibitor decreased peritoneal VEGF expression, EMT, vessel density, and fibrosis (Yoshio et al., 2004). Administration of endothelin-1 receptor antagonists in mice attenuated PD-induced EMT, angiogenesis, fibrosis, and peritoneal functional decline (Busnadiego et al., 2015). Similar findings were obtained for endostatin, an endothelial cell proliferation and migration inhibitor, in a mouse model of EPS (Tanabe et al., 2007) and for intraperitoneal rho-kinase inhibition in a rat model of peritoneal fibrosis (Peng et al., 2013). Rapamycin decreased mesothelial cell VEGF synthesis and VEGF-C and VEGF-D release *in vitro*; combined PD and rapamycin treatment in mice reduced peritoneal EMT and thickening and submesothelial blood and lymphatic vessel proliferation as compared with mice exposed to PD fluid only (Gonzalez-Mateo et al., 2015). Addition of Tie2 fusion protein sTie2/Fc blocking Angiopoietin 2 downstream signaling to PD fluid infused once daily in uremic mice dose dependently reduces PD-induced peritoneal angiopoietin 2 synthesis and peritoneal hypervascularization (Xiao et al., 2013). Thus, several different interventions within the angiogenic signaling cascades can substantially reduce PD-induced peritoneal membrane transformation. Thus far, however, such approaches have not been tested in the clinical setting of PD.

While experimental studies clearly demonstrated reduction of peritoneal angiogenesis with low compared to high GDP fluids (Mortier et al., 2004, 2005), the role of the buffer compound is less clear. *In vitro*, bicarbonate-buffered low GDP fluid induced less endothelial tube formation than the respective lactate-based fluid, due to an increase in angiopoietin 1/2 ratio, that is, a shift towards vessel maturation, and tyrosine kinase receptor (TEK) translocation to the endothelial cell membrane, where it co-localized with vascular-endothelial cadherin, which stabilizes vessels (Eich et al., 2017). TEK plays a pivotal role in the regulation of sprouting and maturation of the vessels (Eklund and Saharinen, 2013). The finding was supported by a larger cross-sectional area of peritoneal vessels in eight bicarbonate fluid treated, peritonitis free children, as compared to the vessel area in age and glucose exposure matched children treated with the respective second-generation lactate PD fluid. Vessel size is an indicator of maturation (Suri et al., 1998). Up to now, only one, small size randomized trial comparing lactate and bicarbonate-buffered, neutral pH, low GDP fluids has been accomplished and – in line with the experimental findings – demonstrated better preservation of ultrafiltration achieved per gram of dialytic glucose exposure and body surface area in pediatric patients over 10 months with the bicarbonate fluid (Schmitt et al., 2013).

PD fluid toxicity induced early and pronounced peritoneal inflammation involving invasion of the PD membrane with macrophages and leucocytes, and inflammatory cytokine release is another major driver of structural and functional deterioration (Lambie et al., 2013, 2016; Schaefer et al., 2018). IL-6 is secreted by mesothelial cells after induction by IL-1ß and TNF-α (Topley et al., 1993). Individual differences in dialysate IL-6 concentrations have been linked to genetic polymorphisms (Siddique et al., 2015). *In vitro* and in mice, IL-6 was linked to VEGF production and thus angiogenesis *via* STAT3 and SP4 transcriptional factors (Catar et al., 2017); effluent IL-6 and VEGF concentrations are correlated (Pecoits-Filho et al., 2002). In PD patients, dialysate concentrations of the proinflammatory cytokine IL-6 are associated not only with higher peritoneal transporter status, i.e., faster solute and toxin removal, but also with ultrafiltration decline and protein loss (Lambie et al., 2013). In experimental PD, the anti-inflammatory Cox-2 inhibitor, celecoxib, reduced peritoneal inflammation, angiogenesis, and fibrosis and preserved peritoneal membrane function (Fabbrini et al., 2009).

Another key element of peritoneal membrane transformation is epithelial (mesothelial) to mesenchymal transition (EMT), i.e., migration of mesothelial cells into the submesothelium and transition to a myofibroblast cell type. Lineage tracing studies furthermore suggest that myofibroblasts may also be derived from type I collagen-producing submesothelial fibroblasts (Chen et al., 2014). EMT is triggered by profibrotic and inflammatory stimuli cytokines (Yanez-Mo et al., 2003; Margetts et al., 2005; Loureiro et al., 2011; Bowen et al., 2013). Myofibroblasts secrete inflammatory, proangiogenic, and profibrotic cytokines and extracellular matrix components (Aroeira et al., 2007). In CKD5 patients, only single isolated EMT cells are present in the submesothelium, but their numbers rapidly increase with PD (Schaefer et al., 2018). In multivariate analysis, peritoneal EMT was independently associated with submesothelial thickness and with the microvessel number per mm tissue section. In experimental PD, EMT and associated peritoneal membrane damage can be inhibited by intraperitoneal

BMP-7, antagonizing TGF-β signaling (Loureiro et al., 2010). TGF-β signaling again is centrally involved in the peritoneal fibrotic process as shown in various animal models of PD (Margetts et al., 2001) and in humans (Zhou et al., 2016; Schaefer et al., 2018). TGF-β is secreted by resident (myo-) fibroblasts, with different fibroblast subgroups having different profibrotic properties. Glycopeptide Thy1-positive fibroblasts exhibit particular profibrotic and myofibroblast features (Kawka et al., 2017). MicroRNA (miR) array studies identified miR-21 and miR-31 to be highly expressed and induced by TGF-β in mesothelial cells and to correlate with mesenchymal transition *in vitro*. Micro ribonucleic acid-21 and miR-31 are upregulated in the peritoneum of PD patients, and their effluent concentrations are associated with icodextrin and low GDP fluid use and related to peritonitis count and effluent IFN-γ concentration. Altogether these findings suggest a great potential of these miRs as biomarker for membrane change in patients receiving PD (Lopez-Anton et al., 2017), respective large size clinical trials are needed.

SYSTEMIC IMPACT OF PD FLUID BIOINCOMPATIBILITY

Rather than mitigating CKD-associated pathomechanisms, such as inflammatory, carbonyl, and oxidative stress, PD, while partially replacing renal function, adds additional risk factors. CKD-associated vasculopathy, prevalent even in young CKD patients, is further accelerated by PD. Potential pathomechanisms include the peritoneal glucose uptake, the additional GDP, and consequent AGE load and PD-associated inflammation. In a cohort of almost 1,000 PD patients, intraperitoneal inflammation was the most important determinant of peritoneal solute transport but did not affect patient survival (Lambie et al., 2013). In contrast, systemic inflammation associated with comorbidity and independently predicted patient survival, suggesting independent peritoneal and systemic processes being active. Other studies point to a strong link between PD treatment and vasculopathy. Whole exome expression analyses of omental arterioles isolated from children with normal renal function, with CKD5D and while on low GDP PD revealed activation of metabolic processes in CKD5D arterioles and of inflammatory, immunologic, and stress-response cascades in arterioles of PD patients. The latter exhibited particular upregulation of the complement system and respective regulatory pathways, with concordant findings at the proteomic level. In independent validation cohorts, PD specimens had the highest abundance of omental and parietal arteriolar C1q, C3d, terminal complement complex and of phosphorylated SMAD2/3, a downstream effector of TGF-β. Furthermore, in the PD parietal arterioles, C1q and terminal complement complex abundance correlated with the level of dialytic glucose exposure, the abundance of phosphorylated SMAD2/3, and the degree of vasculopathy (Bartosova et al., 2018). The close correlation of vascular TGF-β-induced SMAD2/3 phosphorylation and the severity of vasculopathy is supported by recent genome wide association, and systems biology studies identified the TGF-β–SMAD pathway to be strongly associated with coronary artery disease (Zeng et al., 2016). The analysis of small arteries and precapillary arterioles at least 1 mm below the mesothelial surface and thus beyond the PD penetration level (Stachowska-Pietka et al., 2012) is of particular interest because they control peripheral resistance and microcirculation. Vasculopathy in this part of the arterial tree predicts left ventricular hypertrophy and cardiovascular events in hypertensive patients (Rizzoni et al., 2003; De Ciuceis et al., 2007). Concentrations of effluent complement protein have been linked to overall mortality in PD patients (Zelek et al., 2016).

NOVEL PD FLUID PROTOTYPES

Severe peritoneal damage still observed with low GDP fluids suggests that glucose per se has a major detrimental effect and that glucose sparing should mitigate PD-associated sequelae. Adding icodextrin and amino acid solution to a glucose-based PD regime improved glycated hemoglobin and lipid profile as compared to the glucose only PD regime, but deaths and serious adverse events, including several related to extracellular fluid volume expansion, have been reported to increase in the intervention group (Li et al., 2013). PD fluids with lower sodium concentration increased sodium removal and improved blood pressure but accelerated residual renal function decline (Blake, 2016; Rutkowski et al., 2016). In a small size crossover trial in adults and a pilot study in children, adapted automated PD, i.e., combining sequential short- and longer-dwell exchanges, with small and large dwell volumes, resulted in higher solute and fluid removal as compared to the standard regime with comparable dialysate fluid turn over and dialysis time (Fischbach et al., 2016). This concept awaits validation in extended clinical trials.

Replacing glucose by novel osmotic agents is a promising way to go in order to improve PD fluid biocompatibility. About 3.5% taurine-based PD fluid achieved equivalent ultrafiltration as glucose-based PD fluid and induced less mesothelial and fibroblast-like cell proliferation in rats (Nishimura et al., 2009). Hyperbranched polyglycerol containing PD fluid achieved similar solute and water transport rates in rats and induced less peritoneal membrane damage (Mendelson et al., 2013; Du et al., 2016), but data on the metabolism of polyglycerol in plasma and ramifications of plasma accumulation and tissue disposition with long-term use are scant. A recent study in obese type 2 diabetic ZSF1 rats over 3 months suggests less systemic adverse effects on the kidneys and the plasma oxidative status with hyperbranched polyglycerol fluid as compared to second-generation and icodextrin PD fluid (La Han et al., 2018).

A different approach to more biocompatibility PD fluids is addition of protective compounds counteracting peritoneal fluid toxicity (**Figure 3**). PD fluids result in cellular stress and also suppress the natural stress response mechanisms, e.g., exerted by heat shock proteins (HSP) (Macario and Conway de Macario, 2007). Glutamine, a non-essential amino acid, has been shown to restore the cellular stress response pathway HSP27/72, which is suppressed by PD fluids. Addition of the dipeptide alanyl-glutamine to first- and second-generation PD fluid improved mesothelial cell stress response and cell survival *in vitro* and *in vivo* (Kratochwill et al., 2012). In uremic rat and mouse models of PD, alanyl-glutamine reduced peritoneal thickness, and angiogenesis, and peritoneal αSMA, IL-17, TGF-β, and IL-6 (Ferrantelli et al., 2016). In a first clinical trial, effluent cell HSP72 expression was increased following a 4-h dwell with alanyl-glutamine supplemented first-generation PD fluid, and the effluent increased TNF-alpha release

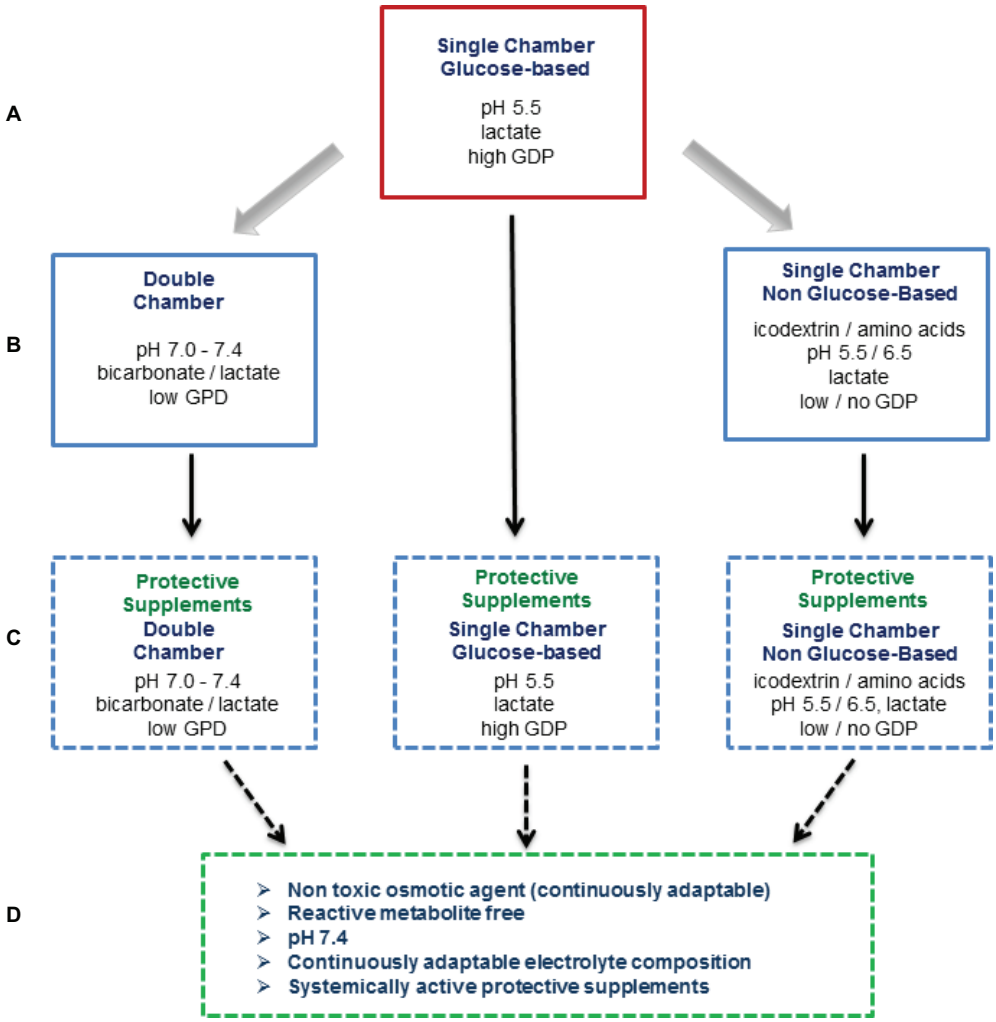

FIGURE 3 | Overview on currently applied and potential novel PD fluid types. For about 50 years, conventional PD fluids have been based on glucose as the osmotically active agent and are heat sterilized in single-chamber bags at acidic pH together with selected electrolytes (Na, Ca, Mg, and Cl) and a buffer (lactate), which results in major glucose degradation product (GDP) formation **(A)**. In the 1990s, second-generation PD fluids were developed. These multi-chamber bag systems substantially reduce GDP generation and allow for a physiologic buffer compound (bicarbonate) and a neutral pH of the ready-to-use PD fluid. At the same time, alternative osmotic compounds were introduced, an amino acid mixture and the oncotically active glucose polymer icodextrin **(B)**. At present, protective agents counteracting local and systemic PD fluid toxicity are being developed, with alanyl-glutamine supplemented PD fluids have shown promising effects in first clinical trials **(C)**. The fourth generation PD fluid type depicted reflects the vision of the ultimate future PD fluid **(D)**.

from LPS-stimulated peripheral blood mononuclear cells as compared to non-supplemented PD fluid. In post peritonitis patients, IL-6 and IL-8 effluent concentrations were reduced (Kratochwill et al., 2016). In a subsequent randomized crossover study, 41 patients were treated with alanyl-glutamine supplemented second-generation PD fluid over a period of 8 weeks each. Intraperitoneal alanyl-glutamine increased CA-125 appearance rate and effluent cell LPS-stimulated IL-6 release, and the peritoneal transport of uric acid, phosphate, and potassium was higher and the peritoneal protein loss was reduced (Vychytil et al., 2018). These studies demonstrate significant benefits of alanyl-glutamine enriched PD fluid on peritoneal membrane integrity, immune competence, and transport function. A phase 3 trial is now needed to translate these encouraging effects into hard clinical outcomes.

Experimental PD studies and a clinical pilot study in 4 patients suggest good tolerability of carnitine supplemented PD fluids and superior ultrafiltration than achieved with 2.5% glucose solutions, despite lower osmolarity of the carnitine-containing solution (Bonomini et al., 2011). Addition of L-carnitine to acidic, glucose-based PD fluids in 27 non-diabetic patients improved insulin sensitivity assessed by euglycemic hyperinsulinemic clamp studies (Bonomini et al., 2013).

Understanding the molecular mechanism of peritoneal transport, its regulation by CKD and PD and pharmacological modification should be another way to improve PD biocompatibility and efficacy. Peritoneal membrane function has been well described by the three pore model (Rippe, 1993). Thus far, only the molecular basis of the "ultra-small pores" could be identified in mice,

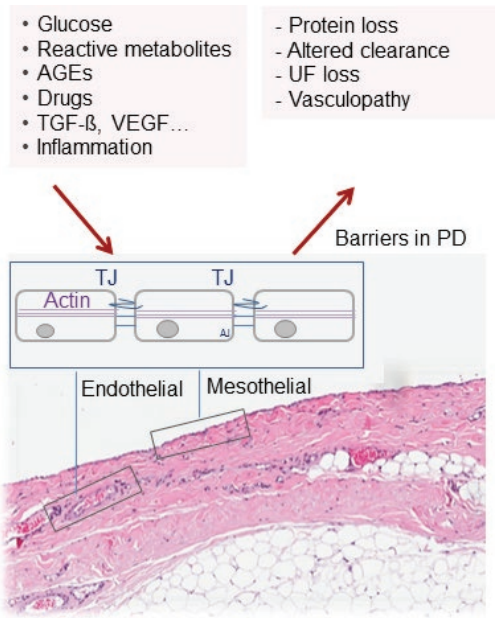

FIGURE 4 | Barriers for solute and water transport in PD. The endothelial and mesothelial cell monolayers form leaky membranes. Intercellular junctions define the selective permeability properties and thus paracellular bulk flow of small and large solutes together with water. An in-depth understanding of these key elements of PD should provide promising therapeutic targets to improve PD efficacy, biocompatibility, and sustainability.

aquaporin-1 (AQP-1), which exerts 50% of water transport (Ni et al., 2006). The molecular counterparts of "small pores" and "large pores," i.e., the mechanisms and regulatory machinery of the remaining 50% of the water transport, of solutes and size-dependent toxin removal are still unknown as are their modifications by uremia and PD. The primary transport barrier is the endothelium, and the role of the mesothelium is uncertain. Both capillary and mesothelial cell layers form leaky structures with similar *in vitro* transmembrane resistances but higher solute transport rates across the endothelial layer for 4–70 kDa dextrans (Horiuchi et al., 2009). Intercellular junctional complexes, including tight junctions, gap junctions, and desmosomes (Ito et al., 2000), define the selective permeability properties of the cell monolayer membrane and thus of bulk flow of small and large solutes together with water (**Figure 4**). Transcellular mechanisms imply transporters such as PiT for phosphate (Biber et al., 2013) and GLUT-1/2 and sodium glucose co-transporters such as SGLT-1/2 for glucose uptake (Debray-Garcia et al., 2016).

In-depth understanding of these key elements of peritoneal membrane transport function should provide promising therapeutic targets to improve PD efficacy, biocompatibility, and sustainability. Feasibility of this approach has been demonstrated for AQP-1. Dexamethasone twofold increased peritoneal AQP1 abundance and net ultrafiltration in rats without altering solute transport (Stoenoiu et al., 2003). AQP-1 agonist, AqF026, a chemical derivative of the aryl sulfonamide compound furosemide, increased water transport after 60 and 120 min of dwell time by 15–20% (Yool et al., 2013). Although this strategy is promising to remove more water to achieve euvolemia, it does not increase salt removal and may aggravate thirst in patients. Still, these studies elegantly demonstrate that understanding and modulating the peritoneal water, salt, and toxin transport mechanisms are a promising area of research, hopefully resulting in major improvement of PD patient outcome.

RÉSUMÉ

The recent findings on histomorphological alterations of the peritoneum with so-called biocompatible, neutral pH PD fluids are disappointing and raised the question whether current concepts of PD fluid biocompatibility are "dead" (Blake, 2018). These sobering findings, however, have to be balanced against patient-related outcome parameters. There is an early survival advantage for PD as compared to HD, together with advantages of quality of life and autonomy to this home-based, cost-effective therapy. A recent analysis of the ERA-EDTA registry, which comprehensively collects real-life data from European countries, suggests an increasing 5-year patient survival benefit of PD over HD over the last 20 years (van de Luijtgaarden et al., 2016). In Europe, low GDP fluids have been licensed about 20 years ago and have increasingly been applied since then. At present, it is unclear that which factors contribute to these encouraging trends, but it is tempting to speculate that the lower systemic GDP and AGE load associated with low GDP PD fluid use (Zeier et al., 2003; Schmitt et al., 2007), an improved local host immune defense system possibly resulting in less frequent and less severe episodes of peritonitis (Johnson et al., 2012c), and the better preservation of residual renal function (Cho et al., 2014) play a significant role. Further large-scale patient-related analyses are needed to delineate the specific PD-related risk factors and potential countermeasures, as well as the development of novel PD fluid types, which not only mitigate peritoneal damage but also systemic sequelae of chronic PD.

AUTHOR CONTRIBUTIONS

MB and CS performed the literature search and wrote the manuscript. Both authors approved the final version of the manuscript.

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Targeted Metabolomic Profiling of Peritoneal Dialysis Effluents Shows Anti-oxidative Capacity of Alanyl-Glutamine

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Readily available peritoneal dialysis (PD) effluents from PD patients in the course of renal replacement therapy are a potentially rich source for molecular markers for predicting clinical outcome, monitoring the therapy, and therapeutic interventions. The complex clinical phenotype of PD patients might be reflected in the PD effluent metabolome. Metabolomic analysis of PD effluent might allow quantitative detection and assessment of candidate PD biomarkers for prognostication and therapeutic monitoring. We therefore subjected peritoneal equilibration test effluents from 20 stable PD patients, obtained in a randomized controlled trial (RCT) to evaluate cytoprotective effects of standard PD solution (3.86% glucose) supplemented with 8 mM alanyl-glutamine (AlaGln) to targeted metabolomics analysis. One hundred eighty eight pre-defined metabolites, including free amino acids, acylcarnitines, and glycerophospholipids, as well as custom metabolic indicators calculated from these metabolites were surveyed in a high-throughput assay requiring only 10 μl of PD effluent. Metabolite profiles of effluents from the cross-over trial were analyzed with respect to AlaGln status and clinical parameters such as duration of PD therapy and history of previous episodes of peritonitis. This targeted approach detected and quantified 184 small molecules in PD effluent, a larger number of detected metabolites than in all previous metabolomic studies in PD effluent combined. Metabolites were clustered within substance classes regarding concentrations after a 4-h dwell. PD effluent metabolic profiles were differentiated according to PD patient sub-populations, revealing novel changes in small molecule abundance during PD therapy. AlaGln supplementation of PD fluid altered levels of specific metabolites, including increases in alanine and glutamine but not glutamate, and reduced levels of small molecule indicators of oxidative stress, such as methionine sulfoxide. Our study represents the first application of targeted metabolomics to PD effluents. The observed metabolomic changes in PD

effluent associated with AlaGln-supplementation during therapy suggested an anti-oxidant effect, and were consistent with the restoration of important stress and immune processes previously noted in the RCT. High-throughput detection of PD effluent metabolomic signatures and their alterations by therapeutic interventions offers new opportunities for metabolome-clinical correlation in PD and for prescription of personalized PD therapy.

Keywords: N(2)-L-alanyl-L-glutamine, oxidative stress, chronic kidney disease, metabolome, methionine sulfoxide

INTRODUCTION

Peritoneal dialysis (PD), as a home-based renal replacement therapy for patients with end-stage renal disease, offers an attractive (and in some aspects, superior) alternative to conventional center-based hemodialysis (Mehrotra et al., 2016; Pippias et al., 2016; van de Luijtgaarden et al., 2016; Li et al., 2017). The PD effluent contains endogenous and exogenous metabolites, proteins, nucleic acids, and free-floating cells, all available for sampling as a non-invasive liquid biopsy without burden to the PD patient. Metabolites are relatively stable and easily accessible end products of gene expression and protein activity and as the ultimate effector level of biological systems, they are predictive of cellular and tissue phenotype (Dettmer et al., 2007; Kuehnbaum and Britz-McKibbin, 2013; Aretz and Meierhofer, 2016). As such, metabolites are particularly attractive diagnostic components of PD effluent that may reflect or predict the complex clinical phenotypes and outcomes of PD patients (Herzog et al., 2018). However, the metabolome of PD effluents remains little explored.

In clinical practice, urea nitrogen, creatinine, and other selected components are measured in PD effluents and 24-h sampling of dialysate and urine, to monitor dialysis therapy efficacy, usually as part of a peritoneal equilibration test (PET) of standardized dwell time, to compare transperitoneal transport characteristics of surrogate molecules of different molecular weights. These parameters, however, reflect only a limited aspect of the complex metabolic changes produced in these patients by uremia and therapeutic intervention (Vaidya and Bonventre, 2006). Measurement of a wide range of metabolites should allow a more accurate assessment of the complex clinical phenotype of patients than allowed by the few conventional analytes.

Metabolomics techniques systematically measure multiple metabolites directly from complex biological samples (Mamas et al., 2011). Technological progress in high-resolution accurate mass (HRAM) spectrometry and full-scan capabilities theoretically permits simultaneous identification and quantitation of a great number of molecules. Capture of all sample data potentially allows unbiased, untargeted collection of information on as many *a priori* unknown metabolites as possible (Kell et al., 2005; Vanholder et al., 2015). The major drawbacks of the untargeted approach are the non-validated and at best semi-quantitative results. Despite the theoretical capability to analyze all metabolites, the important pre-analytical and technical differences of the analytes have to date hindered expansion of the PD effluent metabolome beyond ∼70 identified metabolites (Dunn et al., 2012; Guleria et al., 2014; Csaicsich et al., 2015; Vanholder et al., 2015; Aretz and Meierhofer, 2016; Kratochwill et al., 2016). The alternative targeted approach relies on a pre-defined list of candidate metabolites for which the method has been previously optimized and validated. The aim is to generate metabolic profiles under various conditions from defined cohorts of (PD) patients, generating metabolic fingerprints reflecting pathological progression stages and their modulation by therapeutic interventions.

PD fluid supplementation with glutamine, added as the stable, glutamine-releasing dipeptide, alanyl-glutamine (AlaGln), has demonstrated *in vitro* and *in vivo* restoration of cellular stress responses, immune modulation and reduced peritoneal fibrosis (Bender et al., 2010; Kratochwill et al., 2012; Herzog et al., 2014; Ferrantelli et al., 2016). Randomized controlled trials (RCTs) showed that improved cellular immune competence and restored stress responses in peritoneal cells by AlaGln supplementation of PD fluid can be translated into the clinical setting (Kratochwill et al., 2016; Herzog et al., 2017, 2018; Vychytil et al., 2018). These clinical trials allowed collection of PD effluent samples for characterization of PD-related mechanisms and their alterations by AlaGln supplementation.

In this study, we applied, for the first time, a targeted metabolomics approach to the study of PD effluent from stable patients on chronic PD therapy. The aim was to expand and validate the metabolome of PD effluent and to investigate the effects of AlaGln addition to PD fluid as a new therapeutic intervention, using a highly standardized, high-throughput method.

MATERIALS AND METHODS

Peritoneal Dialysis (PD) Effluent Samples

Peritoneal dialysis effluent samples were obtained during a prospective randomized, open-label, two-period, cross-over phase I/II study conducted at the Department of Nephrology of the Medical University of Vienna, Austria. The study protocol was approved by the local ethics committee of the Medical University of Vienna (EK 867/2010, EK 1167/2013, EK 2035/2015), registered at www.clinicaltrials.gov (NCT01353638), and performed in accord with the Declaration of Helsinki. The study design, clinical methods, eligibility criteria, randomization, patient characteristics, and adverse events have been previously described (Kratochwill et al., 2016). In brief, 20 stable PD

patients (13 male/7 female mean age 58 years) with a mean PD vintage of 2.4 years were treated per protocol. Patients were judged as clinically stable and had no severe concomitant disease (5 patients had a history of peritonitis more than 3 months prior to sample collection, 14 patients had residual renal urine output, and 6 patients were anuric). All patients provided written informed consent prior to trial participation.

Effluent samples were collected in standard 9 ml native collection tubes (Vacuette, Bio-Greiner-One, Kremsmünster, Austria) immediately after completion of instillation of the dialysis fluid into the patients' cavity (=time point 0) and 4 h later (=time point 4 h) after each of two standard PETs (La Milia et al., 2013), one using commercially available PD fluid (Dianeal, 3.86% glucose, Baxter, Deerfield, IL, United States) and a second with the same PD fluid supplemented with 8 mM AlaGln ("Dipeptiven" = N(2)-alanyl-L-glutamine 200 mg/mL, Fresenius Kabi, Bad Homburg, Germany), performed in randomized order. The two PETs were separated by a wash-out period (28–35 days) (**Supplementary Figure S1**).

PD Effluent Analyses

Peritoneal dialysis effluent was centrifuged (250 × *g*, 10 min) immediately following collection, and cell-free supernatant samples were aliquoted and stored at −80°C until further analysis.

Peritoneal dialysis effluent concentrations of glucose and creatinine were measured by validated standard methods in the clinical laboratory of the Vienna General Hospital. Creatinine concentrations were determined by a kinetic measurement of Jaffé reaction and corrected for high glucose levels by determination of a correction factor from measurements of unused PD fluid with the same method. PD effluent concentrations of glutamine and alanine were measured as previously described (Kratochwill et al., 2016) with the EZ:Faast amino acid kit (Phenomenex, Torrance, CA, United States). Amino acids were extracted by solid phase extraction, derivatized to chloroformates, dried, and reconstituted after addition of homoarginine, methionine-d3, and homophenylalanine as internal standards. Liquid chromatographic (LC) separation on an EZ:Faast AAA-MS column (250 mm × 2.0 mm) was followed by data acquisition by a triple quadrupole mass spectrometer (MS) (Waters Corp., Milford, MA, United States) and data processing by QuanLynx software v.4.1 (Waters).

Targeted Metabolomics in PD Effluent Samples

Metabolites (n = 188) in PD effluent samples were analyzed using the AbsoluteIDQ p180 kit (Biocrates Life Science AG, Innsbruck, Austria). Sample preparation, analysis, data processing and validation were performed according to the manufacturers' instructions. Internal standards, calibration standards and quality controls were dissolved and used immediately before use. Ten microliters individual PD effluents, calibration standards or quality controls were mixed with 10 µl internal standard. Phosphate buffered saline (Sigma-Aldrich, St. Louis, MO, United States) was used as blank. Samples dried 30 min at room temp. by nitrogen evaporator were derivatized with phenylisothiocyanate (in 50 µl of 1:1:1 ethanol:H_2O:pyridine for 20 min at room temp), then dried again for 60 min. Dried samples were extracted in 300 µl methanol containing 5 mM ammonium acetate for 30 min with shaking at 450 rpm. All mixtures were filter-centrifuged, and flow-throughs were captured for analysis. Amino acids and biogenic amines were separated on a C18 column and gradient-eluted [0.2% formic acid in water (v/v) to 95% 0.2% formic acid in acetonitrile (v/v)]. For flow injection analysis (FIA) of acylcarnitines, glycerophospholipids and hexoses, the mobile phase was prepared by mixing FIA reagent (manufacturer-provided) with methanol. Samples were analyzed by MS (6500 QTRAP, AB Sciex, Framingham, MA, United States) run in multiple reaction monitoring (MRM) mode for both LC and FIA analysis. Data processing and validation used MetIDQ software (Biocrates Life Sciences AG).

Statistical Analysis and Data Visualization

All statistical analyses and visualizations used R (v3.5.1[1]). Mean and median concentrations and corresponding standard deviations and interquartile ranges were calculated for all targeted metabolites from all samples. Correlation matrices for all metabolites were generated through hierarchical clustering by Euclidian distance. Correlation of PD effluent and plasma metabolite concentrations in 911 healthy subjects was from data of Goek et al. (2013). All features of the data set were subjected to Welch t-tests for changes in the following patient characteristics: (i) "Peritonitis History": preceded episodes of peritonitis, minimum 3 month before enrollment into the study; (ii) "Anuric": comparison of patients with residual renal function (RRF) and anuric patients; (iii) "Time on PD": continuous PD therapy for more or less than 12 months prior to study enrollment. The effect of the study intervention (8 mM AlaGln in PD fluid during a 4 h PET) was tested using paired t-tests. Due to the exploratory nature of this study, no power calculation was performed; the power calculation in the trial from which the samples were obtained was aimed for a power of 80% to detect a difference in means of 30% points, using a 0.05 two-sided significance level at an assumed standard deviation of within-subject period differences of 50% points for the primary outcome parameter (Kratochwill et al., 2016). All results were Benjamini-Hochberg-corrected for multiple testing. Custom metabolic indicators in the dataset were automatically calculated from metabolite concentrations by the MetIDQ software and were included in this analysis.

Data Availability

All datasets for this study are included in the manuscript and the **Supplementary Files**.

[1] http://www.r-project.org/

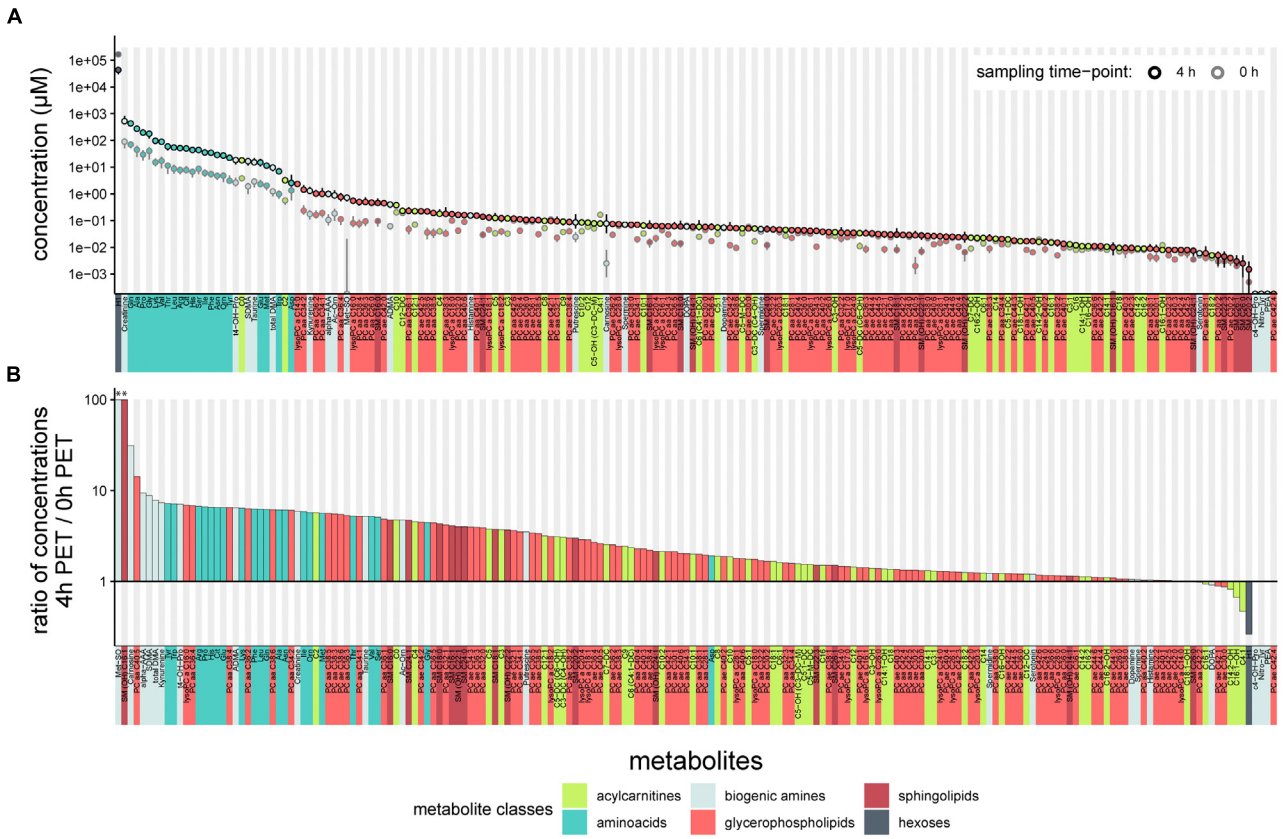

FIGURE 1 | Concentrations of 188 targeted metabolites in PD effluents after a standardized 4-h peritoneal equilibration test (PET). **(A)** Median metabolite concentrations (circles) in PD effluents (standard PD fluid with 3.86% glucose). Bars above and below circles indicate upper and lower quantiles; semicircles represent undetected metabolites. Of 188 metabolites, 184 were detected after a 4 h PET dwell (black-outlined circles) and 182 in samples at the 0 h PET time point (immediately taken after initial filling of the PD fluid; gray-outlined circles). **(B)** Ratios of PD effluent metabolite concentrations after 4 h as compared to the 0 h sampling time. Ratios > 1 indicate higher levels after 4-h dwell, ratios < 1 indicate decreased concentrations after 4-h dwell as compared to 0 h, or complete absence of the metabolite at both sampling time-points (c4-OH-Pro, Nitro-Tyr, PEA, PC ae C42:4). Methionine Sulfoxide (Met-SO) and sphingomyelin SM (OH) C16:1 (see astericks "**" above leftmost columns) were undetectable at initial sampling (0 h) and were assigned ratio values of 100. aa, in glycerophospholipids (GLP) both moieties bound by ester bonds to the glycerol backbone, ae, in GLP moieties bound by one ester and one ether bond to the glycerol backbone.

RESULTS

We first tested the feasibility of a targeted metabolomics approach in cell-free PD effluents. We used 10 µl effluent samples of standardized 4-h dwells from 20 patients included in a randomized controlled cross-over trial. PET samples were taken immediately after filling (0 h) and 4 h after filling (4 h). One hundred eighty four of the 188 total metabolites in the targeted panel were detectable in PD effluent samples (**Figure 1** and **Supplementary Table S1**), and metabolites of all six metabolite classes in the panel could be quantified. The most abundant metabolite in effluents at both sampling time points was "sum of hexoses (H1)" (reflecting the 3.86% glucose PD fluid used in the clinical trial for the PET). Creatinine was the second most abundant metabolite, followed by most of the free amino acids included in the targeted assay. All measured free amino acids were found in the upper quartile of all quantified metabolites, representing the most abundant metabolite class in PD effluents (**Figure 1A**).

One hundred seventy five metabolites were more abundant in PD effluents after the 4 h dwell than directly after filling, whereas only nine metabolites (H1, DOPA, four acylcarnitines: C4:1, C14:2-OH, C16:1, and C16:1-OH, and three glycerophospholipids: PC ae C30:0, PC ae C42:1, and PC aa C42:2) showed higher concentrations in the PD effluent directly after filling. As expected during PD therapy, hexoses showed the strongest decrease in concentration (−3.8-fold) after 4 h dwell time (**Figure 1B**). Methionine sulfoxide (Met-SO) and the sphingomyelin SM (OH) C16:1 showed the highest relative increase after 4 h dwell (since they were initially undetectable) and their ratios were manually set to 100 for graphic display (**Figure 1B**). Next highest ratios were those of carnosine (31.2), alpha-aminoadipic acid (alpha-AAA) (9.4), hydroxyproline (t4-OH-Pro) (7.1), symmetric dimethylarginine (SDMA) (8.8), and total-DMA (7.9). **Figure 1B** illustrates the descending order of D4/D0 metabolite concentration ratios.

The PD effluent metabolite concentrations measured by the p180 assay were validated by comparison to concentrations of

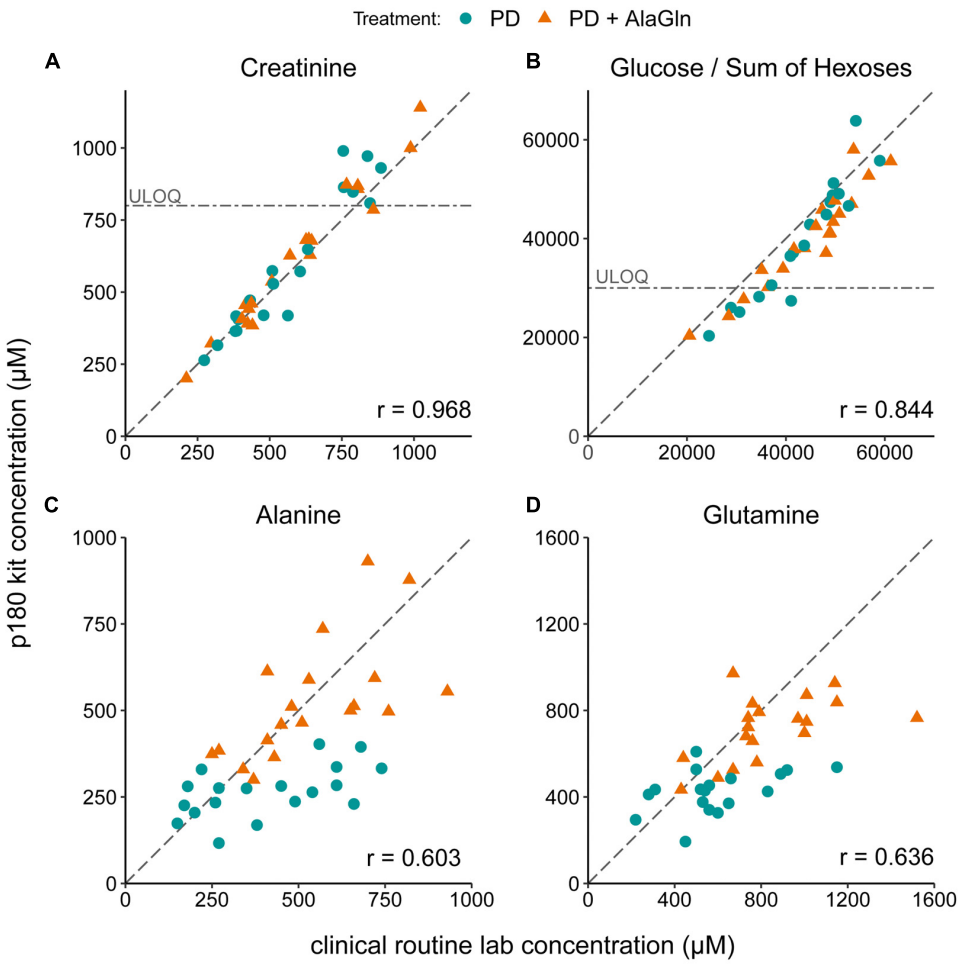

FIGURE 2 | Validation of metabolomic quantitation of four metabolites by correlation with routine clinical laboratory methods. Correlations of **(A)** creatinine, **(B)** glucose, **(C)** alanine, and **(D)** glutamine concentrations with the concentrations measured in the p180 metabolomics assay ("sum of hexoses": 90–95% glucose). Pearson's correlation coefficients (r) are indicated. Creatinine and glucose concentrations were partly above the upper limits of quantification (ULOQ). Dashed line: line of identity for each comparison.

selected metabolites measured in the same samples by routine clinical laboratory methods (**Figure 2**). Targeted metabolomics values of creatinine and glucose correlated very well with measurements by validated routine clinical laboratory methods, with Pearson's coefficients of 0.968 and 0.844, respectively (**Figures 2A,B**). Although creatinine and the sum of all hexoses (including glucose) concentrations were found above the kits' respective upper limits of quantification, linearity of the assay was preserved. Alanine and glutamine were originally assessed by a LC-MS-based method, comparable to the method used in the p180 assay but with only a one-point calibration. Correlation of both measurements was still obvious with correlation coefficients for alanine and glutamine of 0.603 and 0.636 (**Figures 2C,D**).

Associations and similarities between individual metabolites were sought through generation of a correlation matrix (**Figure 3**; see **Supplementary Figure S2** for a higher resolution image including metabolite names and correlation matrix of the 0 h PET data). Hierarchical clustering of the Euclidian distance between Pearson's correlation coefficients calculated for all metabolite pairs revealed strong positive correlation for the class of glycerophospholipids (GPLs), which also correlated with most of the sphingolipids (SLs). To some extent, acylcarnitines and amino acids (AAs) are clustered together as well, although their individual patterns show more variability probably reflecting their higher chemical diversity when compared to the GPLs. Indeed, AAs form distinct subgroups, likely reflecting chemical features, as indicated by the extreme positions of the unique AAs proline and glycine. The small amino acid glycine clustered with GPLs, but also positively correlated with most other AAs. Of the included metabolic classes, the biogenic amines showed the most diverse clustering behavior.

The PD effluent concentrations of individual metabolites reflect the sums of their plasma concentrations, their transport into the peritoneum and/or local production. To gain an impression of the systemic background of the observed metabolites, we compared PD effluent concentrations in our

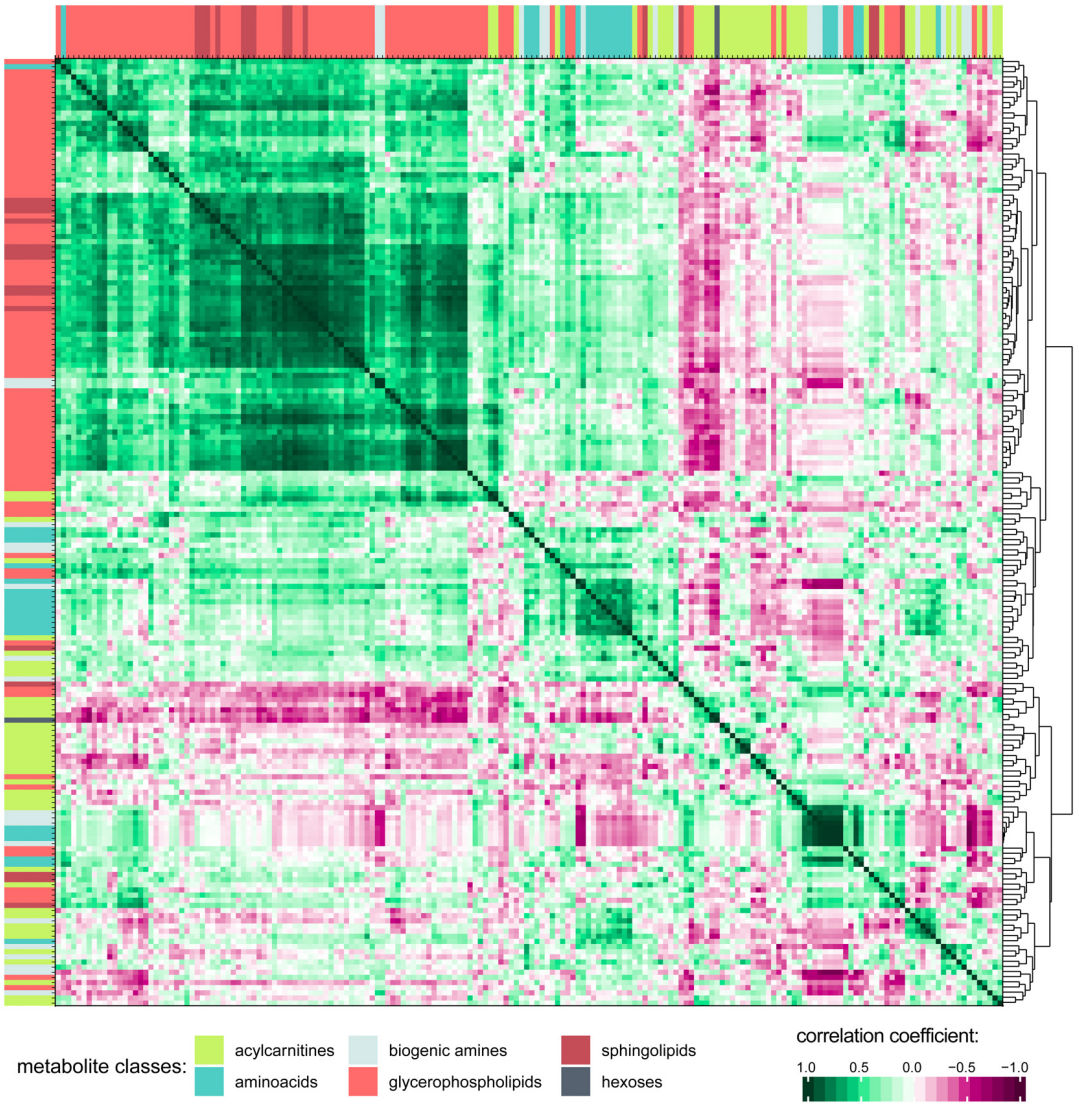

FIGURE 3 | Correlation matrix of all metabolites in PD effluent after a 4 h peritoneal equilibration test (PET) dwell. Pearson's correlation coefficients were calculated for all metabolite combinations. Only detectable (non-zero) metabolites are included in the correlation matrix. Hierarchical clustering was performed on the Euclidian distance between the metabolites' correlation coefficients. Metabolites are colored by substance class (**Supplementary Figure S2** shows this figure at higher resolution, and includes metabolite names).

RCT subjects with plasma concentrations measured in a large cohort of healthy individuals using the same method (Goek et al., 2013; **Figure 4**). These literature derived data served as an approximation of plasma levels of respective metabolites. This crude strategy became necessary as no serum samples of studied patients were available from the RCT. As expected, PD effluent concentrations of glucose (H1; 42.3 ± 13.8 mM) and creatinine (588.6 ± 240.1 μM) after 4-h dwells exceeded those in healthy plasma. In contrast, PD effluent concentrations of most AAs and biogenic amines after a 4-h dwell were slightly below or similar to healthy plasma levels. Post-4-h PD effluent concentrations of glutamic acid (Glu), citrulline (Cit), acetylornithine (Ac-Orn), and total DMA, as well as of three acylcarnitines (C5, C9, C10:2) exceeded healthy plasma concentrations, a perhaps surprising result given the high instilled volume of PD fluid plus the additional water osmotically absorbed from the patient circulation. PD effluent concentrations of GPLs and SLs were lower than healthy plasma levels.

We next examined the possible influences of patient factors or AlaGln supplementation of PD fluid during the PET in the RCT on individual PD effluent metabolites and custom metabolic indicators. Patient-related variables included (i) preceding episodes of peritonitis, (ii) residual renal function (RRF) and anuria, and (iii) elapsed time since PD treatment initiation (two groups continuously treated for $<$ or $\geq$12 months duration). Fifty one metabolites were significantly influenced by at least one tested variable (**Figure 5** and **Supplementary Table S2**). A history of peritonitis was associated with changes in four metabolites:

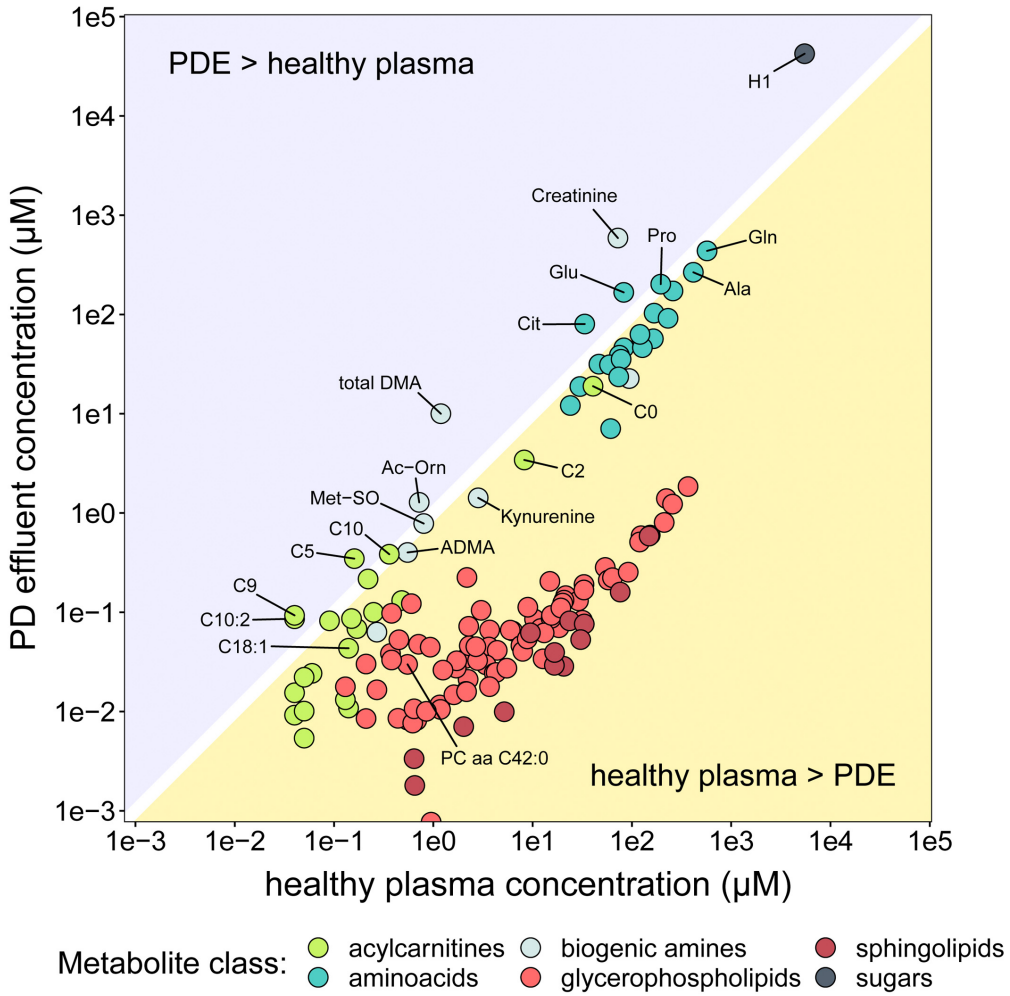

FIGURE 4 | Comparison of metabolite concentrations in PD effluent and healthy plasma. The *y*-axis (log scale) shows mean concentrations of PD effluent metabolites. The *x*-axis (log-scale) shows mean concentrations of plasma metabolites in 911 healthy individuals Goek et al. (2013). Only metabolites reported in the plasma dataset are shown (n = 140), colored by substance class.

elevation of three GPLs, most pronounced for PC aa C42:0 (significant after correction for multiple testing), and reduction of the ratio of non-dicarboxy-acylcarnitines to total acylcarnitines (total AC-DC/total AC), an indicator of ω-oxidation of fatty acids (**Figure 5B**). Anuria was associated with significant changes in all metabolite classes. PD effluent levels of two short-chain acylcarnitines, 14 GPLs and SLs and three AAs (phenylalanine, serine, valine) showed lower concentrations than those in patients with RRF. Elevated PD effluent creatinine concentrations were associated with both time on PD treatment and with anuria. Upon closer inspection, the increase in creatinine by prolonged treatment could be explained by those patients without RRF (**Figure 5C**). None of the PD patients treated for less than 1 year was anuric, whereas anuria characterized 40% of PD patients treated for more than 1 year (Kruskal–Wallis: $\chi^2 = 6.93$, $p = 0.049$; Dunn's *post hoc* test between groups: <1 year and RRF vs. ≥1 year and RRF $p = 0.1506$; ≥1 year and RRF vs. ≥1 year and anuric $p = 0.0440$; <1 year and RRF vs. ≥1 year and anuric $p = 0.0078$). PD effluent from patients treated with PD for more than 1 year contained higher concentrations of the biogenic amines Met-SO, SDMA and the total amount of DMA, in addition to creatinine. Levels of five acylcarnitines and one SL (sphingomyelin C22:3) were also increased with longer time on PD therapy (**Figure 5D**). AlaGln supplementation of the PD fluid used in the PET increased post-4-h dwell concentrations of alanine and glutamine in PD effluent (and calculated metabolic indicators that include alanine and glutamine) compared to those from the same patients after control PET with standard PD fluid (**Figure 5E**), but did not influence levels of other AAs, such as glutamic acid (paired *t*-test: $p = 0.33$) (**Supplementary Figure S3**). AlaGln-supplemented PD fluid increased post-dwell PD effluent concentrations of 8 GPLs, most prominently PC aa C40:1 (significant after correction for multiple testing), but decreased post-dwell levels of the long-chain acylcarnitine oleoylcarnitine, C18:1 (**Figures 6A,B**). AlaGln-supplemented PD fluid also showed decreased PD effluent Met-SO concentrations

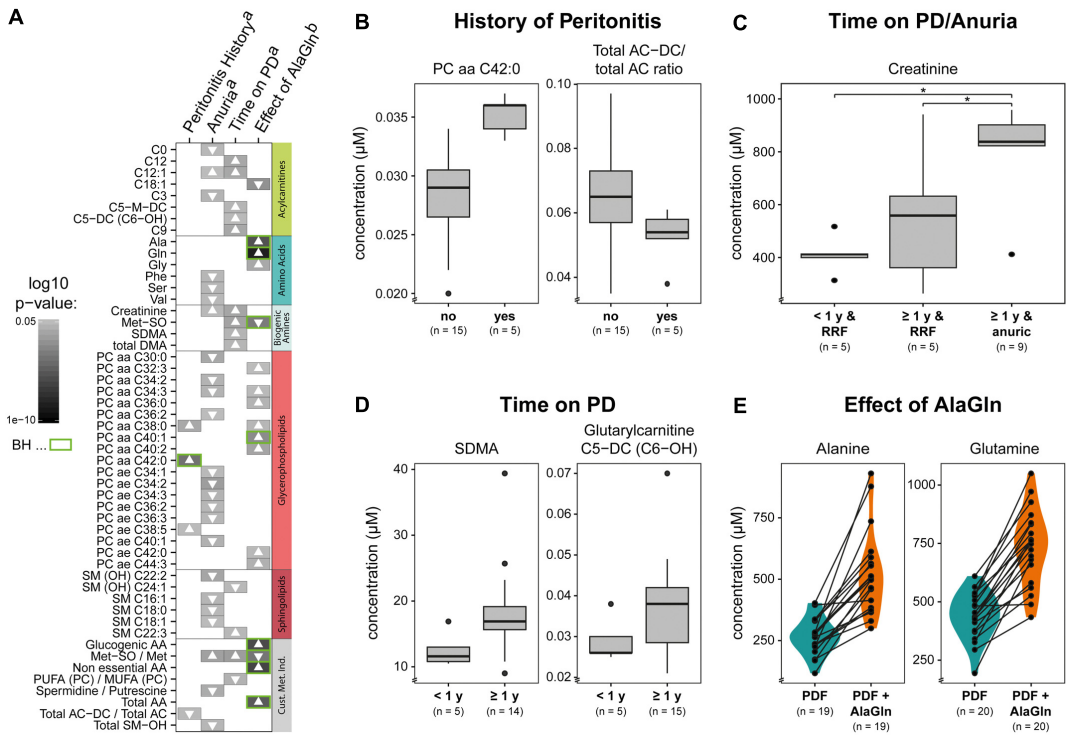

FIGURE 5 | Effects on PD effluent small molecule profiles of patient factors, therapy characteristics, and PD fluid supplementation with alanyl-glutamine (AlaGln). Data shown are from PD effluents obtained after a standardized 4 h peritoneal equilibration test (PET) dwell. **(A)** Effects of **(B)** peritonitis history, **(C)** anuria, **(D)** chronic PD duration (< or ≥1 year), and **(E)** effect of 8 mM AlaGln supplementation of PD fluid (vs. standard PD fluid in one 4 h PET). All metabolites with significant results in at least one comparison are shown panel **(A)** with selected comparisons presented in detail panels **(B–E)**. Results from all *t*-tests are reported as gray-scale tiles with darker colors representing higher significance (highest *p*-value 0.049), with Benjamini-Hochberg-corrected *p*-values (green: BH < 0.05) **(C)** Kruskal-Wallis: $\chi^2 = 6.93$, $p = 0.049$; Dunn's *post hoc* test between groups: <1 year and RRF vs. ≥1 year and RRF $p = 0.1506$; ≥1 year and RRF vs. ≥1 year and anuric $p = 0.0440$ (*); <1 year and RRF vs. ≥1 year and anuric $p = 0.0078$ (*); a, Welch *t*-tests; b, paired *t*-tests, to investigate the effect of AlaGln supplementation in the cross-over study. Met-SO, methionine sulfoxide; SDMA, symmetric dimethylarginine; total DMA, symmetric and asymmetric dimethylarginine; PUFA (PC)/MUFA (PC), ratio of polyunsaturated to mono-unsaturated glycerophospholipids; AC-DC, dicarboxy acylcarnitines; AA, amino acids; RRF, residual renal function.

and decreased Met-SO-to-methionine ratio (Met-SO/Met), an indicator of systemic oxidative stress. The effect of AlaGln in PD fluid was opposed to the effect of anuria and time on PD, where Met-SO and Met-SO/Met levels were significantly increased. The levels of free unmodified methionine remained constant (**Figure 6B**). In a subgroup analysis, the AlaGln supplementation-associated decrease of the Met-SO/Met ratio was independent of PD therapy duration [mixed design ANOVA, time on PD between patients ($p = 0.007$) and AlaGln treatment within patients ($p < 0.001$); interaction ($p = 0.825$)] (**Figure 6C**).

DISCUSSION

This study reports the first targeted metabolomics analysis of PD effluent. Our approach enabled detection of up to 184 small molecules in each patient's PD effluent obtained from standardized PETs during an RCT (Kratochwill et al., 2016) for characterization of the metabolic status during PD treatment. To our knowledge, this study presents the highest number of metabolites yet detected and quantitated in PD effluents.

Emerging technologies now allow acquisition, analysis and clinical utilization of omics level information in chronic kidney disease (CKD), end-stage renal disease (ESRD) and dialysis patients. Genomics, transcriptomics, proteomics, and metabolomics have already demonstrated value in CKD patient classification (Rossing et al., 2008; Rhee et al., 2010; Choi et al., 2011; Zhao, 2013; Mullen et al., 2014; Kottgen et al., 2018). Metabolites, as intermediates and end points of gene expression and protein activity and the sum of biotic and abiotic perturbations, represent the ultimate level of biological effectors (Kell et al., 2005; Kuehnbaum and Britz-McKibbin, 2013; Barrios et al., 2016).

Although effluents from PD patients are easily available biofluids, PD metabolomics remains in early stage investigation, with fewer than 80 metabolites identified to date in PD effluent (Dunn et al., 2012; Guleria et al., 2014; Tang et al., 2014; Csaicsich et al., 2015; Vanholder et al., 2015; Kratochwill et al., 2016). Considering the effort required to overcome the challenge of high abundance protein species posed by the PD effluent soluble proteome, and the low peritoneal cell number available for phenotyping, metabolites seem particularly attractive components of the PD effluent (Aufricht et al.,

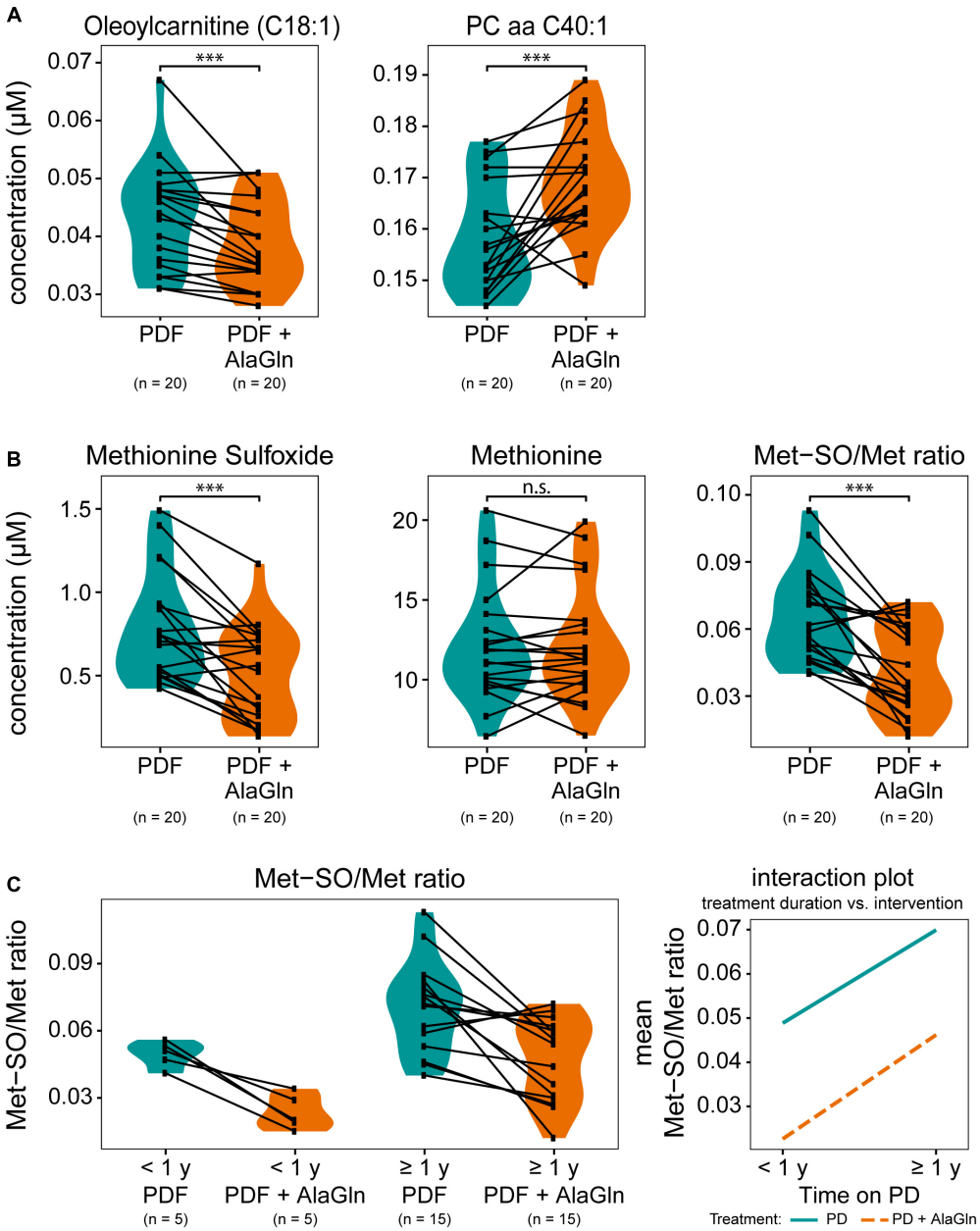

FIGURE 6 | Effects on selected small molecule concentrations of alanyl-glutamine (AlaGln) addition to PD fluid (PDF). Effects of standard PDF vs. 8 mM AlaGln supplemented PDF in 4 h PET effluents compared by paired *t*-tests (except **C**). **(A,B)** All *p*-values corrected for multiple testing by BH method; ***$p < 0.001$; n.s.: $p = 0.87$ **(C)** mixed design ANOVA: chronic PD duration (shorter or longer than 1 year) compared among patients and treatment intervention with AlaGln tested within individual patients; effect of PD treatment duration: $p = 0.007$; effect of AlaGln supplementation: $p < 0.001$; interaction: $p = 0.825$; Met-SO, methionine sulfoxide.

2017; Herzog et al., 2017, 2018). PD effluent metabolite levels reflect the sum of uremia-induced alterations of metabolism, nutrition, muscle/energy wasting and accumulation of toxic metabolites in dialysis patients, many likely functions of patient pathophysiological status, morbidity and mortality. PD effluent parameters currently monitored in clinical practice, including urea nitrogen and creatinine, reflect only limited aspects of the metabolic complexity of uremia and related therapeutic interventions (Vaidya and Bonventre, 2006; Zhao, 2013). Numerous additional small molecules may act as renal/uremic toxins, contributing directly to uremic pathophysiology, as they accumulate along with other low molecular weight substances in the setting of renal impairment (Barrios et al., 2016). Evaluation of a wider range of metabolites should therefore allow a more accurate assessment of the complex clinical phenotypes of PD patients.

The human metabolome comprises at least several thousand predicted and/or experimentally observed chemical entities

varying in abundance and physicochemical properties (incl. size, molecular weight, polarity, hydrophobicity) (Aretz and Meierhofer, 2016; Wishart et al., 2018). Our previous pilot study applying untargeted metabolomics identified 41 small molecules in PD effluents (Csaicsich et al., 2015), suggesting the untargeted approach failed to deliver the systems-level view of the metabolome suggested by its name. In contrast, the targeted approach seeks not to identify unknown metabolites, but instead, screens for a pre-defined list of metabolites with pre-optimized methods and appropriate standards allowing absolute quantification (Vanholder et al., 2015; Barrios et al., 2016; Kalim and Rhee, 2017).

In this study we analyzed small molecules in cell-free PD effluent sampled at the start and the end of a standardized 4 h PET using the p180 assay, a highly reproducible, high-throughput FIA/LC-MS method already validated by multiple laboratories with plasma and serum samples (Siskos et al., 2017). Applying this technology to 10 µl volumes of individual PD effluents, we detected and quantitated 184 metabolites of the 188 included in the targeted panel. Three of the four undetected metabolites, biogenic amines *cis*-hydroxyproline, nitrotyrosine, and phenylethylamine, are at or below detection limits in healthy plasma (Siskos et al., 2017).

We validated the p180 assay results in PD effluent by comparison to previously published RCT data obtained by routine clinical laboratory methods. PD effluent creatinine and glucose concentrations measured by routine methods and by the p180 assay exhibited correlation coefficients >0.8. Effluent concentrations of alanine and glutamine previously measured by a different LC-MS-based kit (Kratochwill et al., 2016) also correlated well with the p180 assay data. In contrast to the earlier used method, which employed one-point calibration, all free amino acids are quantitated via calibration curves in the p180 assay.

The lack of plasma metabolomic measurements prevented comparison of PD effluent metabolite concentrations with systemic values from the same individuals. We therefore compared our effluent data to previously published p180 plasma metabolomics data from a population-based trial with 911 healthy participants (Goek et al., 2013). We hypothesized that abundant, peritoneal membrane-permeant plasma metabolites would partially or completely equilibrate by the end of the 4-h PET dwell, whereas plasma metabolites enriched in PD effluent would represent molecules subject to preferential peritoneal membrane transport or clearance, molecules elevated in CKD or PD, and/or locally produced molecules. However, the inferable conclusions from these results are challenged by the limitation that plasma from non-uremic individuals serves only as a rough approximation. Uremia itself causes high plasma concentrations of many metabolites, like creatinine, and patients with renal failure are likely to have metabolomic signatures, distinct from normal plasma (Breit and Weinberger, 2016). Unfortunately, no datasets (tabulated raw data) from targeted metabolomics experiments in CKD patients were available in literature. Therefore, the performed comparisons cannot be used to reliably distinguish between peritoneal transport of a solute or local production in the respective patient but only as a first insight into the PD effluent metabolome in comparison to generalized systemic levels. In future studies it will be a prime goal to obtain metabolomic data from plasma and PDE samples from the same patients in parallel. Since glucose is the major constituent of the PD fluid used in this trial, it was expected to be more abundant in PD effluent than in healthy plasma samples. PD effluent concentrations of creatinine, the best established biomarker of renal failure progression, were also higher than plasma creatinine concentrations in the healthy cohort. Post-dwell effluent concentrations of most of the smaller metabolites (AAs and biogenic amines) were only slightly below healthy plasma concentrations, in contrast to the larger GPLs and SLs. However, post-dwell PD effluent concentrations of citrulline, acetylornithine (Ac-Orn), glutamic acid, total DMA and three acylcarnitines (C5, C9, C10:2) were higher than those in healthy plasma. Acylcarnitines are elevated in cardiovascular disease, insulin resistance, as well as in CKD, in which accumulation has been routinely attributed to impaired renal clearance. Acylcarnitines are formed from carnitine and acyl-CoA and serve as fatty acid transport cofactors across mitochondrial membranes. The esterification of acyl-CoA, overaccumulated in renal failure, with carnitine to generate acylcarnitines has been proposed to retard progression of renal disease (Kerner and Hoppel, 2000; Mai et al., 2013; Barrios et al., 2016; Breit and Weinberger, 2016; Strand et al., 2017). In our cohort of PD effluent samples we found seven short and medium-chain acylcarnitines significantly elevated in association with anuria and prolonged time on PD, all of which were previously correlated with eGFR decline in a large cross-sectional study (Goek et al., 2012). In that study glutarylcarnitine [C5-DC(C6-OH)] showed the strongest effect size. This metabolite, which in our dataset correlated with duration of PD therapy, was also reported to increase in plasma in parallel with CKD progression in another CKD cohort (Nkuipou-Kenfack et al., 2014).

Also detected in PD effluent, by our approach, were the more hydrophobic, long-chain acylcarnitines which are derived exclusively from fatty acid metabolism and are regarded as markers of mitochondrial fatty acid oxidation. As these accumulated long-chain acylcarnitines are not cleared by hemodialysis (HD) (Kalim et al., 2013; Makrecka-Kuka et al., 2017), we speculate that their removal during PD may represent a previously undescribed PD-specific advantage in clearance of accumulated metabolites from ESRD patients.

Amino acids and biogenic amines were among the most abundant metabolites in PD effluent and, with few exceptions, were highly enriched in effluent during the 4-h dwell time. Clustering in the cross-correlation matrix revealed distinct subgroups likely reflecting similar chemical properties. Kynurenine and tryptophan, the latter being degraded to hydroxykynurenine, are among the metabolites with the highest concentration increase after a 4 h PD dwell, confirming our previous untargeted analysis (Csaicsich et al., 2015). Interestingly, however, PD effluent kynurenine concentrations were not higher than those in healthy plasma, and were unassociated with any investigated clinical factors. PD effluent concentrations of phenylalanine, serine and valine were lower in anuric patients than in those with RRF. Indeed, anuria was associated with significant changes in all

metabolite classes, and was the driving factor for increased PD effluent creatinine concentrations.

Arginine metabolites ADMA, SDMA, citrulline, and ornithine have been previously described as plasma metabolome markers of CKD progression and eGFR decline (Ceballos et al., 1990; Goek et al., 2013; Shah et al., 2013; Duranton et al., 2014; Nkuipou-Kenfack et al., 2014; Breit and Weinberger, 2016). Our study also found PD effluent concentrations of SDMA and total-DMA (sum of ADMA + SDMA) significantly increased with longer time on PD therapy. SDMA levels were highly correlated with creatinine levels in a meta-analysis of 18 studies encompassing 2100 patients (Bode-Boger et al., 2006; Kielstein et al., 2006). ADMA concentrations have been reported to be elevated in plasma of PD patients, but significantly lower than in HD patients, likely reflecting superior ADMA clearance by PD (Kielstein et al., 1999; Cross et al., 2001; Mittermayer et al., 2005; Zhang et al., 2010). ADMA inhibition of endothelial nitric oxide synthase (eNOS), which converts arginine to citrulline and nitric oxide (NO), may explain the higher citrulline levels in PD effluent than in healthy plasma.

Peritoneal cavity glutamine levels during PD may be sub-physiological (Kratochwill et al., 2016), a deficiency that may be associated with increased peritoneal vulnerability due to inadequate cellular stress responses and impaired metabolic and immune competence (Wischmeyer, 2002; Roth, 2008; Kratochwill et al., 2012).

In the present study we tested whether the addition of alanyl-glutamine (AlaGln) to PD fluid influences metabolite concentrations in PD effluents. AlaGln supplementation of PD fluid has already been shown to improve survival of mesothelial cells, boost peritoneal immune-competence and counteract deleterious effects of PD therapy (Kratochwill et al., 2012, 2016; Ferrantelli et al., 2016; Herzog et al., 2017, 2018; Vychytil et al., 2018). The samples used in our current study were from a previously reported randomized controlled cross-over trial testing the safety and efficacy of addition of 8 mM AlaGln to PD fluid during a single 4-h PET dwell (Kratochwill et al., 2016). Consequently, PD fluid supplementation with AlaGln dipeptide resulted in significant increases of alanine and glutamine levels in PD effluent compared to standard PD fluid. These results were confirmed by our targeted metabolomics approach. Except for a minor increase in glycine levels, AlaGln addition did not influence levels of other AAs such as glutamic acid. Glutamine is both the most abundant free amino acid of plasma and classified as conditionally essential. Besides its role in nutrition, glutamine regulates immune cell functions, glucose metabolism and glutathione-mediated redox potential (Curi et al., 2007; Roth, 2008).

AlaGln in PD fluid increased post-dwell PD effluent concentrations of 8 GPLs and one acylcarnitine. In contrast, the long-chain acylcarnitine oleoylcarnitine (C18:1) was decreased in AlaGln-supplemented post-dwell PD effluent. This acylcarnitine has not been significantly associated with CKD progression in the above-mentioned metabolomic studies but has been described as a marker of uremic cardiovascular risk in a large HD cohort (Kalim et al., 2013). The effect of its decrease in PD effluent must await further study.

Reductions of methionine sulfoxide (Met-SO) and the Met-SO-to-methionine ratio were observed in effluents from patients treated with AlaGln-supplemented PD fluid. This reduction by AlaGln was highly significant and independent of time on PD therapy, whereas longer PD vintage *per se* significantly increased Met-SO. Post-translational methionine sulfoxidation usually leads to impairment or loss of protein function. Met-SO and its ratio to unmodified methionine have been studied as markers of oxidative stress in CKD (Thornalley and Rabbani, 2014; Breit and Weinberger, 2016). The importance of oxidative stress in CKD and PD has been extensively described (Himmelfarb et al., 2002; Ksiazek et al., 2007; D'Apolito et al., 2010; Sung et al., 2013). In healthy controls renal clearance of Met-SO is low, probably reflecting intact systemic metabolism to unmodified methionine by Met-SO reductases. Oxidative stress and generation of Met-SO increase with CKD progression, paralleled by additional accumulation of Met-SO due to loss of renal clearance (Thornalley and Rabbani, 2014; Breit and Weinberger, 2016). Increased free radicals as markers of oxidative stress in PD patients were associated with decline of RRF and with technique failure (Morinaga et al., 2012).

In summary, we have demonstrated the feasibility of targeted metabolomics in minute amounts of PD effluents as a new tool to analyze a wide range of small molecules in a single measurement. The analysis of absolute abundances, of ratios between concentrations at the end and the start of the PET dwell, and comparison to plasma reference concentrations showed that the investigated panel of metabolites associated with multiple metabolic pathways can provide information of potential clinical relevance. This includes assessment of the patient's uremic status and uremia-induced metabolic changes, of dialysis quality, of risk for PD-induced co-morbidities, and of success of therapeutic interventions. The assay shows excellent technical stability and allows comparison of metabolite concentration to external datasets, potentially increasing understanding of PD-related pathomechanisms. The behavior during a PD dwell of several known metabolites was confirmed, and other metabolites previously unknown in PD effluent were discovered to be enriched or associated with PD vintage. PD fluid supplementation with AlaGln in a randomized controlled cross-over trial reduced metabolomic markers of oxidative stress, indicating an anti-oxidative effect of the additive. The targeted metabolomics approach might represent a promising tool not only for basic and translational research but eventually also for clinical practice. Further studies will focus on defining metabolomic signatures of known and novel pathomechanisms in larger cohorts and in cohorts with extended PD duration using AlaGln-supplemented PD fluids.

AUTHOR CONTRIBUTIONS

FW analyzed and interpreted the data, and prepared the manuscript. RH performed the clinical trial, analyzed and interpreted the data, and prepared the manuscript. MB performed the clinical trial. AW and DK performed the MS analysis. MU analyzed the data. SA interpreted the data and

critically read the manuscript. AV performed the clinical trial and critically read the manuscript. CA conceived the study, interpreted the data, and critically read the manuscript. KK analyzed and interpreted the data, prepared the manuscript, and conceived the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01961/full#supplementary-material

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Conflict of Interest Statement: CA is cofounder of Zytoprotec GmbH, a spin-off of the Medical University Vienna that holds the patent "Carbohydrate-based peritoneal dialysis fluid comprising glutamine residue" (International Publication Number: WO 2008/106702 A1). RH, AW, MU, and KK are former employees of Zytoprotec GmbH. AV has received honoraria and travel grants from Baxter and Fresenius (manufacturers of dialysis solutions) unrelated to this trial. DK is CEO of ARCHIMED Life Science.

The remaining 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.

The handling Editor declared a past collaboration with the authors.

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Peritoneal Protein Clearance Is a Function of Local Inflammation and Membrane Area Whereas Systemic Inflammation and Comorbidity Predict Survival of Incident Peritoneal Dialysis Patients

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It is not clear whether the association of increased peritoneal protein clearance (PPCl) with worse survival on peritoneal dialysis (PD) is a consequence of either local or systemic inflammation or indicative of generalized endothelial dysfunction associated with comorbidity. To investigate this we determined the relationship of PPCl to comorbidity, membrane area (equivalent to low molecular weight peritoneal solute transport rate), local and systemic inflammation and hypoalbuminaemia, and for each of these with patient survival. 257 incident patients from three GLOBAL Fluid Study centers were included in this analysis. Clinical profiles were collected at baseline along with a peritoneal equilibration test, 24-h dialysate protein and paired plasma and dialysate cytokine measurements. Although peritoneal protein clearance was associated with increased age and severe comorbidity on univariate analysis, only dialysate IL-6, peritoneal solute transport rate, plasma albumin and cardiac comorbidities (ischaemic heart disease and left ventricular dysfunction) were independent explanatory variables on multivariate analysis. While peritoneal protein clearance and daily peritoneal protein loss were associated with survival in univariate analysis, on multivariate analysis only plasma IL-6, age, residual kidney function, comorbidity, and plasma albumin were independent predictors. Peritoneal protein clearance is primarily a function of peritoneal membrane area and local membrane inflammation. The association with comorbidity and survival is predominantly explained by its inverse relationship to hypoalbuminaemia, especially in diabetics.

Keywords: large pore flux, survival, mortality, hypoalbuminaemia, interleukin-6, peritoneal solute transport rate, peritoneal membrane, inflammation

INTRODUCTION

Peritoneal protein clearance (PPCl) has been shown to relate to comorbidity in several studies (Heaf et al., 2005; Szeto et al., 2005; Johansson and Haraldsson, 2006; Van Biesen et al., 2006; Perl et al., 2009; Sánchez-Villanueva et al., 2009; Balafa et al., 2011) and in many cases to worse survival on peritoneal dialysis (PD). An attractive explanatory hypothesis would be that increased PPCl, due to the increased flow of proteins through the large pore pathway of the peritoneal microvasculature might reflect systemic endothelial barrier dysfunction and thus be a biomarker of vascular comorbidity and worse survival (**Figure 1A**). However, data from the Netherlands group (Balafa et al., 2011) demonstrated that while baseline peritoneal albumin and protein clearances from a 4 h dwell with 3.86% glucose dialysate were associated with signs of comorbidity there was no measurable effect on patient survival. Thus the potential relationships between PPCl, comorbidity and survival remains to be fully determined, and to date the impact of local peritoneal inflammation has not been studied.

According to the three pore model, PPCl should be proportional to peritoneal solute transport rate (PSTR), given that both large and small pores are located within peritoneal capillaries. Local peritoneal but not systemic inflammation is the main predictor of PSTR at the commencement of dialysis, (Lambie et al., 2013) reflecting small pore area, but could additionally increase protein losses by causing a relative increase in large pore area. The purpose of this analysis was to distinguish between local versus systemic determinants of PPCl and determine how these relate to survival using a subgroup of centers in the Global Fluid Study in which peritoneal protein losses were measured.

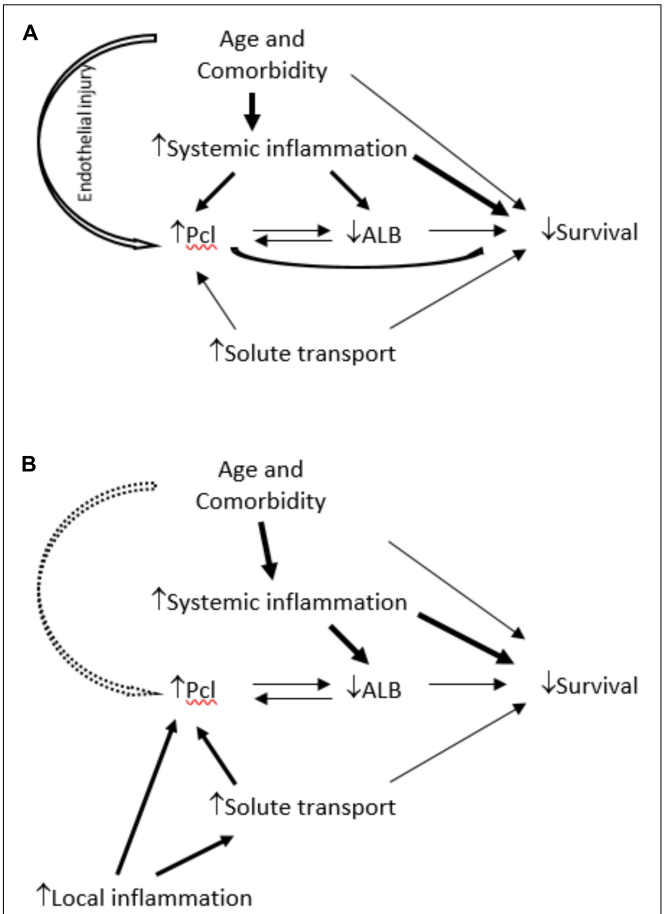

FIGURE 1 | In **(A)**, the link between comorbidity and PPCl is considered to be a consequence of three possible mechanisms: increased systemic inflammation, systemic endothelial dysfunction or its association with albumin – considered as potentially bidirectional because although increased protein clearance will cause greater protein losses there is also mathematical coupling. The model proposed in **(B)** light of these study findings, is that local not systemic inflammation is the predominant determinant of PPCl and an independent link with comorbidity, if present, is weak. The link between PPCl and survival is subordinate to that with plasma albumin, but the bidirectional association is not fully resolved.

MATERIALS AND METHODS

This was a subgroup analysis of the GLOBAL Fluid study cohort. A detailed description of the GLOBAL Fluid Study has been published previously (Lambie et al., 2013). In brief, it is an international, multi-center, prospective, observational cohort study where 10 centers from the United Kingdom, Canada, and South Korea sought to enroll all PD patients starting or already on PD between 2002 and 2008, with the only exclusion criterion being lack of informed consent and <18 years of age. For this analysis, three of the ten centers in the GLOBAL Fluid study were included, two in the United Kingdom, one in South Korea, as these centers routinely measured daily dialysate protein losses. All the incident patients in these three centers in the GLOBAL Fluid study during 2002 to 2008 were included. Ethical approval was obtained from the Multi-Center Research Ethics Committee for Wales covering the United Kingdom, and the Kyungpook National University Hospital Ethics Committee for the Korean center. Written informed consent was obtained from all subjects and they were followed until their death or the censor date, December, 2011. Two patients using an amino acid based solution were excluded from the analysis as the effects of this solution on local inflammatory cytokine production are unknown.

Prospective Collection of Routine Clinical Measurements

Samples of dialysate and plasma and extended clinical phenotype were collected prospectively when patients were undergoing routine planned clinical assessments of membrane function within three months of treatment start. The clinical characteristics, including membrane function, PPCl, biochemistry profiles, and comorbidity were estimated locally. Both of the two United Kingdom centers used the original 2.27% PET for the measurement of PSTR (D/P creatinine) and the South Korea center calculated the PSTR based on 4 h result from a 3.86% exchange.

TABLE 1 | Patient demographic and biochemical characteristics, peritoneal membrane function and systemic and dialysate inflammatory cytokines by center.

Center	G05 (n = 77)	G01 (n = 57)	K03 (n = 123)
Age (yr)	56.3 ± 15.3	57.1 ± 14.3	53.3 ± 14.6
Gender (M/F)	47/30	37/20	72/51
BMI (kg/m^2)	27.0 ± 5.4	28.2 ± 4.5	23.4 ± 2.9
Comorbidity Grade n (%)			
Low	23 (29.9%)	26 (45.6%)	49(40.0%)
Medium	43 (55.8%)	27 (47.4%)	73 (59.2%)
High	11 (14.3%)	4 (7.0%)	1 (0.8%)
DM (yes/no)	27/50	14/43	59/64
Day of PET (day)	42 ± 20	25 ± 19	38 ± 14
Alb (g/L)	35.8 ± 4.2	37.6 ± 4.7	33.4 ± 5.1
Hgb (g/L)	11.9 ± 1.5	11.4 ± 1.4	8.7 ± 2.4
Urine volume (ml)	1203 ± 785	1324 ± 813	1027 ± 620
PSTR	0.77 ± 0.14	0.60 ± 0.12	0.73 ± 0.10
CAPD/APD	59/18	57/0	123/0
Icodextrin (with/without)	20/57	0/57	16/107
Bicarbonate buffered solution (with/without)	2/75	28/29	21/102
PPCl (ml/day)	89.9 ± 33.6	89.7 ± 46.2	95.5 ± 48.3
Dialysate IL-1β AR (pg/min)	0 (0–0)	0 (0–2.12)	0 (0–0.32)
Dialysate TNF-α AR (pg/min)	0 (0–1.78)	3.30 (1.15–8.64)	1.69 (0.28–5.62)
Dialysate IL-6 AR (pg/min)	32.9 (8.8–59.6)	41.7 (23.1–90.6)	73.9 (30.7–135.0)
Dialysate IFN-γ AR (pg/min)	10.5 (0–46.4)	0 (0–15.2)	0 (0–3.3)
Plasma IL-1β (pg/ml)	0.12 (0.06–0.26)	0.01 (0–0.07)	0.05 (0–0.21)
Plasma TNF-α (pg/ml)	7.2 (5.7–8.7)	8.3 (6.7–9.7)	17.6 (15.4–22.0)
Plasma IL-6 (pg/ml)	1.5 (0.7–2.8)	0.8 (0.2–2.3)	2.0 (1.2–3.7)
Plasma IFN-γ (pg/ml)	1.0 (0.4–1.6)	0.7 (0–2.2)	2.2 (1.3–4.0)

BMI, body mass index; PSTR, Peritoneal Solute Transport Rate expressed as dialysate/plasma creatinine at 4 h; PCl, protein clearance; Comorbidity Grade – Davies Comorbidity grade as defined previously(Davies et al., 2002); AR, appearance rate.

Comorbidity was documented according to the externally validated Davies comorbidity score (Davies et al., 2002). Briefly, 7 comorbid domains were considered, including non-cutaneous malignancy, ischemic heart disease (IHD), peripheral vascular disease (PVD) (including cerebrovascular and renovascular disease), left ventricular dysfunction (LVD), diabetes mellitus (DM), systemic collagen vascular disease, and 'other' (any other condition known to reduce life expectancy in the general population). The comorbidity score for each patient was defined as the number of these domains affected. The comorbidity grade was then derived from the comorbidity score. Grade 0 (low risk) was a zero score, grade 1 (medium risk) was a score of 1–2, and grade 2 (high risk) a cumulative score of $\geq$3.

TABLE 2A | Univariate associations between PPCl, patient and membrane characteristics, plasma and dialysate inflammatory cytokines.

	Correlation Coefficient[a]
Age	0.15
BMI	0.05
Serum albumin	−0.47
PSTR	0.44
24 h urine volume	−0.02
Dialysate IL-1β AR	0.04
Dialysate TNF-α AR	0.14
Dialysate IL-6 AR	0.20
Dialysate IFN-γ AR	0.04
Plasma IL-1β	0.02
Plasma TNF-α	0.06
Plasma IL-6	0.13
Plasma IFN-γ	−0.01

a, Pearson correlation coefficient for normal distributed variables and Spearman correlation coefficient for non-normal distributed variables. BMI, body mass index; PP, pulse pressure; PSTR, Peritoneal Solute Transport Rate, (4 h dialysate/plasma creatinine); Ccr, creatinine clearance; IL, interleukin; TNF, tumor necrosis factor; INF, interferon; AR, appearance rate.

The peritoneal dialysate protein loss was measured from the collection of 24-h peritoneal dialysate effluent. A validated correction factor was used to calculate PPCl: 24 h dialysate protein loss/(serum albumin/0.4783), (Haraldsson, 1995) and expressed as mL/day.

Sample Analysis

Dialysate and plasma samples were stored locally at −80°C, and were sent to a central laboratory for measurements of IL-1β, TNF-α, IL-6 and INF-γ by electrochemiluminescence immune assay using the commercially available Pro-Inflammatory I 4-plex (Meso-Scale Discovery, Gaithersburg, MD, United States). For correlations and comparisons, dialysate appearance rate (AR) of cytokines was calculated from the dialysate concentration divided

TABLE 2B | Univariate associations between PPCl and patient characteristics.

		PPCl, ml/day mean ± SD
Gender	Male	96.3 ± 45.5
	Female	87.4 ± 41.1
Comorbidity grade	Low	79.4 ± 37.0
	Medium	99.6 ± 43.1
	High	109.7 ± 67.0
PD modality	CAPD	92.4 ± 44.6
	APD	94.5 ± 33.9
Icodextrin	Without	90.2 ± 35.5
	With	92.9 ± 45.1
Biocompatible solution	Without	88.1 ± 38.5
	With	93.6 ± 45.1
DM	Without	85.8 ± 39.59
	With	103.1 ± 48.12
IHD	Without	88.46 ± 39.93
	With	112.24 ± 55.72
LVD	Without	89.98 ± 40.34
	With	118.53 ± 66.01
Malignancy	Without	92.15 ± 43.8
	With	106.14 ± 46.83
PVD	Without	91.38 ± 43.86
	With	101.61 ± 43.43
Collagen disease	Without	92.69 ± 43.86
	With	78.86 ± 48.93

DM, Diabetes Mellitus; IHD, Ischaemic Heart Disease; LVD, Left Ventricular Dysfunction; PVD, Peripheral Vasculare Disease.

by dwell time. The median intra-assay coefficient of variation was 8.0.

Statistical Analysis

Continuous data were expressed as mean values ± SD for normally distributed variables; otherwise median (± inter-quartile range) was used unless they could be $\log_{10}$ transformed. One-way ANOVA or Student's *t*-test were used to examine differences in normally distributed continuous data or baseline categorical variables, while Mann-Whitney or Kruskal-Wallis tests were used for non-parametric variables. The relationship between PPCl and continuous variables was examined by Pearson correlation coefficient.

Mixed linear modeling was used to identify the determinants of PPCl with a random intercept for center to account for the observed center effects. Kaplan-Meier plots with log rank tests and Cox regression with robust standard errors were used for survival analysis. Collinearity was assessed using a variation inflation factor of >5 as a pree-specified cutoff to indicate a problem.

Significance was pre-specified for *p*-values <0.05 and 95% confidence intervals not crossing the value for no effect. All statistical analyses were performed using SPSS 20 (SPSS Inc., Chicago Ill., United States) apart from the mixed linear model, which was performed by MLwin software (Version 2.22, Center for multilevel modeling University of Bristol).

RESULTS

Patient and Membrane Characteristics

A total of 257 patients in the three centers were included in the study. **Table 1** displays the main baseline characteristics. There were significant differences between centers in a variety of variables, including BMI, comorbidity, PSTR, residual renal function and biochemical characteristics and all the plasma and dialysate cytokines levels. The dialysis regime was also different among centers.

Univariate Correlation to PPCl

The univariate correlations between PPCl, patient and membrane characteristics, as well as dialysate and plasma inflammatory cytokines are presented in **Table 2**. PPCl was positively related to age and PSTR (**Figure 2A**). A strong negative correlation was seen between PPCl and serum albumin, which is in part a function of mathematical coupling. No significant difference in PPCl was found according to icodextrin use, biocompatible solution use or modality (CAPD versus APD).

Both dialysate IL-6 appearance rate (AR) and less strongly plasma IL-6 were positively correlated to PPCl (**Figure 2B** and **Table 2**) Dialysate but not plasma TNF-α were related to PPCl. PPCl was associated both with the overall severity of comorbidity, (**Figure 2C**) and with specific comorbidities, namely IHD, DM, and LVD (**Table 2B**).

Multivariate Model for PPCl

To account for clustering by center, a random intercept term was used for center. Gender, age, comorbidity grade, Log_{10} dialysate IL-6 AR, Log_{10} plasma IL-6, and PSTR were included in the multivariate model.

With PPCl as the dependent variable, dialysate IL-6 AR, plasma albumin and PSTR were the independent explanatory variables (**Table 3**). Further models substituting specific comorbid domains instead of the overall comorbidity grade showed that IHD and LV dysfunction predicted higher PPCl, but not DM, PVD or other comorbidities (**Supplementary Tables 1, 2**). A sensitivity analysis using daily peritoneal protein loss as an alternative measure to peritoneal protein clearance showed a similar pattern but no correlation to plasma albumin, (**Supplementary Table 4**). Tests for collinearity demonstrated no significant effect, with the highest variance inflation factor of 1.49 for albumin. Variables included in models were selected *a priori*, i.e., on the basis of previous reported associations or biological plausibility, with no statistical criteria used for selection.

Survival Analysis

There were 115 deaths in the 257 patients during a median follow up of 37 months. In the Kaplan-Meier plot, the survival rate was compared between patients with PPCl split by the median value of the whole group. Higher PPCl was shown to be related to worse overall outcome and this was also true for daily peritoneal protein loss (**Figure 3**).

Using Cox regression, in the basic model which also included gender, renal Kt/V, local and systemic inflammation,

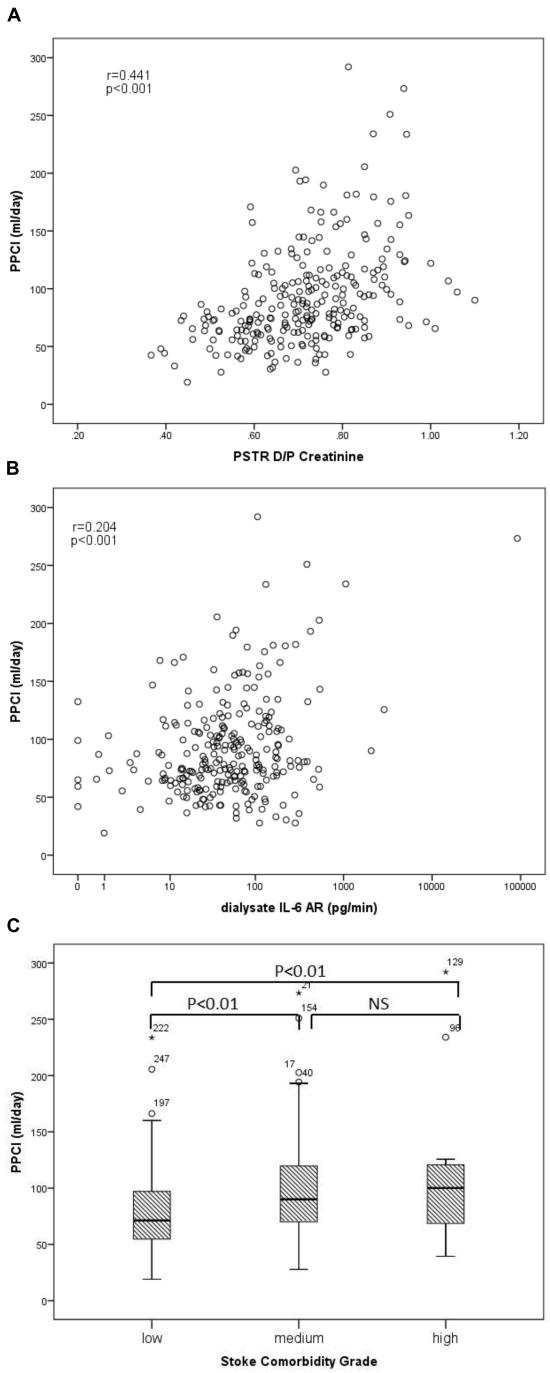

FIGURE 2 | Univariable associations of peritoneal protein clearance. **(A)** PPCl and PSTR (D/P creatinine), **(B)** PPCl and Dialysate IL-6 AR (pg/min), (r-correlation coefficient of univariable assosication), and **(C)** PPCl and comorbidity grade.

the independent predictors of survival were age, renal Kt/V, systemic IL-6, PSTR, and comorbidity. On adding PPCl into the model, the predictive value of PSTR was displaced by a significant association with PPCl. By further adding plasma albumin into the model, the significant prediction

TABLE 3 | Multivariable model for PPCl[a].

	β	95% CI	*P*-value
PSTR (for each 0.1 increase $D/P_{creatinine}$)	11.88	7.83–15.93	<0.001
Dialysate IL6 AR (for each 10 fold increase)	8.70	0.82–16.59	0.03
Plasma IL6 (for each 10 fold increase)	5.55	−10.05–21.15	0.49
Albumin (for each 1g/L increase)	−2.70	−3.76–1.63	<0.001
Age (year)	0.04	−0.28–0.35	0.82
Male gender	−0.45	−9.46–8.56	0.92
Comorbidity Grade 1 (compared with Grade 0)	6.72	−3.05–16.49	0.44
Comorbidity Grade 2 (compared with Grade 0)	10.01	−9.81–29.83	

a, mixed linear model with random intercept for center. PPCl, peritoneal protein clearance; PSTR, peritoneal solute transport rate; AR, appearance rate; IL-6, interleukin-6.

of mortality by PPCl was displaced, with age, renal Kt/V, plasma IL-6, comorbidity grade, and plasma albumin the independent predictors for survival (**Table 4**). Application of the likelihood ratio test confirmed that addition of PPCl to the models confirmed that it was only a significant predictor until plasma albumin, with which it is correlated was added to the model, resulting in an overall better prediction. The lack of association of PPCl with survival was not altered by substituting specific comorbidities into the model (see **Supplementary Table 3**). When daily protein loss was substituted for PPCl in the third model this was not a significant predictor (Hazard Ratio 1.06/g/day, (95%CI 0.96–1.17). A sensitivity analysis including hemoglobin as a covariate made no substantive difference.

DISCUSSION

The purpose of this sub-analysis of the GLOBAL Fluid study was to determine whether PPCl is a consequence of local peritoneal membrane inflammation or a reflection of vascular injury associated with systemic inflammation and comorbidity and, in turn understand how these associate with subsequent patient survival. This is the first time to our knowledge that both local and systemic inflammation, PSTR, PPCl, and comorbid conditions have all been measured at the same time. We demonstrated that PPCl is a function of local inflammation (as reflected by the product of effective membrane area and local dialysate IL-6 appearance rate) and not systemic inflammation in patients commencing PD. The association of comorbidity with PPCl is predominantly explained by the inverse correlation to plasma albumin. This is for several reasons including the reverse acute phase response associated with systemic inflammation, which is in part causal, as a greater protein loss will cause the albumin to drop, but predominantly due to mathematical coupling. The calculation of PPCl has albumin as its denominator which will be depressed

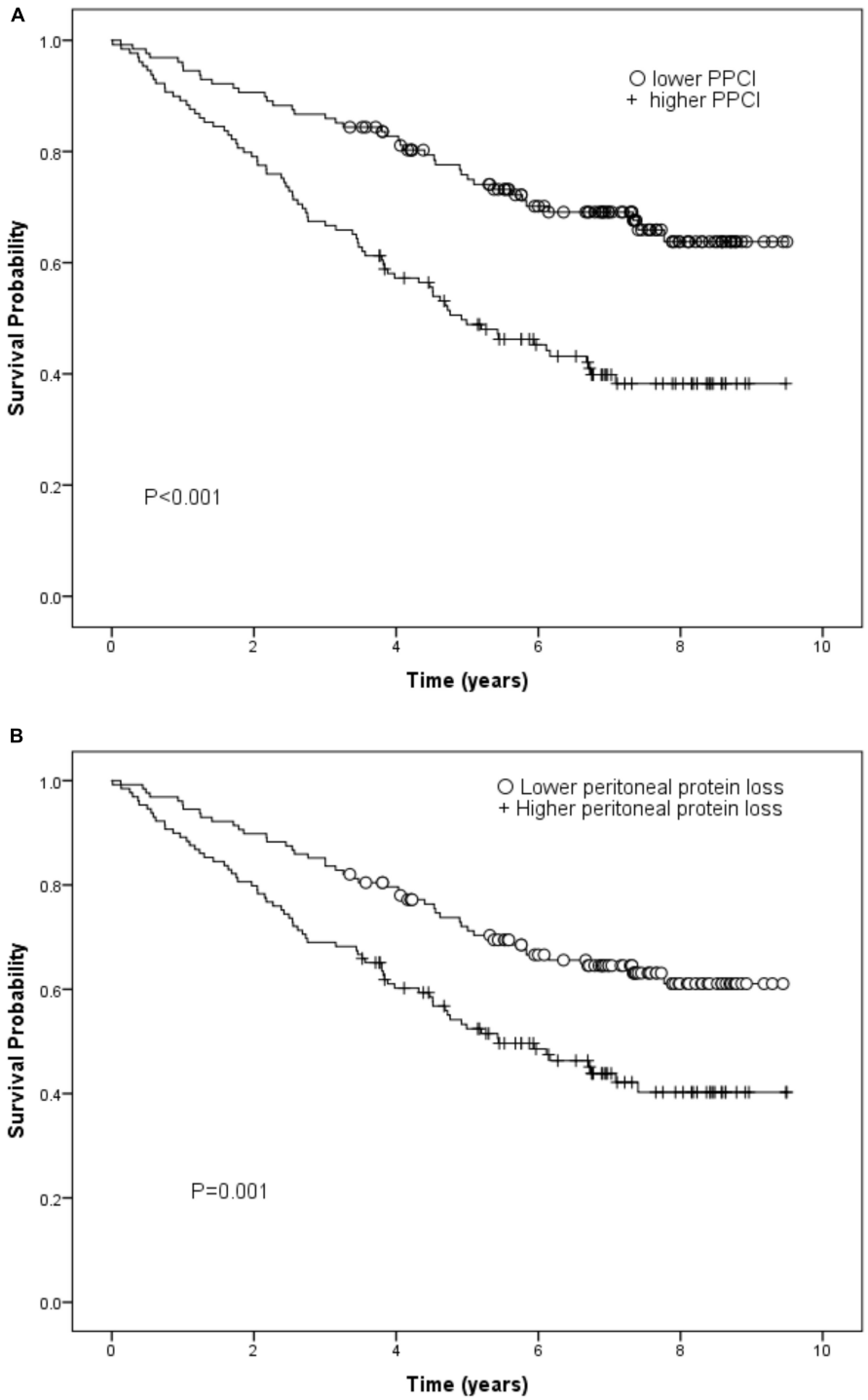

FIGURE 3 | (A) Kaplan-Meier curve for peritoneal protein clearance (PCl) (log rank test) and **(B)** Kaplan-Meier curve for daily peritoneal protein loss (log rank test).

in the presence of systemic inflammation. Thus PPCl is not an independent predictor of survival in this analysis which takes both systemic and local inflammation into account, suggesting that the previously reported associations were not due to an association with systemic inflammation or endothelial barrier dysfunction, as demonstrated in the revised causal diagram, **Figure 1B**.

Several studies have shown that membrane inflammation is associated with high PSTR, primarily a measure of the effective membrane area in incident patients (van Esch et al., 2004; Hwang

TABLE 4 | Cox regression models of patient survival stratified by center – with PPCl and plasma albumin as additional covariates.

	Base Model			Base Model + PPCl			Base Model + PPCl + Alb		
	HR	**95%CI**	***P*-value**	**HR**	**95%CI**	***P*-value**	**HR**	**95%CI**	***P*-value**
Age (per year)	1.075	1.05–1.1	<0.01	1.076	1.05–1.1	<0.01	1.077	1.05–1.1	<0.01
Gender (Female)	1.154	0.75–1.77	0.51	1.179	0.76–1.83	0.47	1.192	0.77–1.85	0.43
PSTR	9.912	1.8–54.44	<0.01	3.393	0.42–27.26	0.25	2.681	0.36–20	0.34
Plasma IL-6 (per 10 fold increase)	2.397	1.18–4.89	<0.05	2.239	1.06–4.71	<0.05	2.201	1.08–4.47	<0.05
Comorbidity grade 1	2.271	1.4–3.69	<0.01	2.063	1.26–3.38	<0.01	1.827	1.09–3.05	<0.05
Comorbidity grade 2	7.787	3.36–18.04	<0.01	7.198	3.22–16.1	<0.01	4.933	2.07–11.78	<0.01
Peritoneal IL-6 AR (per log order)	1.098	0.77–1.56	0.6	1.047	0.72–1.53	0.81	1.040	0.7–1.54	0.84
Renal Kt/V (per unit)	0.614	0.42–0.9	<0.05	0.634	0.43–0.93	<0.05	0.658	0.46–0.95	<0.05
PfopPCl (per ml/min)				1.005	1–1.01	<0.05	1.002	1–1.01	0.5
Plasma Albumin (per g/l)							0.924	0.87–0.98	<0.01

PPCl, peritoneal protein clearance; PSTR, peritoneal solute transport rate; AR, appearance rate; IL-6, interleukin-6; HR, hazard ratio, CI, confident interval.

et al., 2009; Lambie et al., 2013). However, until now it has not been clear how this relates to the variability in large pore flux. It is well known that the large pore flux (and thus PPCl) increases dramatically during peritonitis, i.e., in the presence of severe inflammation that includes an influx of neutrophils, but this has been less clear in the context of a stable, non-infected peritoneal cavity. A study in 40 prevalent CAPD patients found that the dialysate appearance of IL-6 correlated with albumin and IgG clearances, which was also related to PSTR at the same time (Pecoits-Filho et al., 2002). It was not clear from this study, however, whether the inflammation associated increase of PPCl was fully explained by an increase in membrane area. Our results indicate that local IL-6 production, increases PPCl not just via increased effective area (PSTR), but also through an increase in the density of large pores leading to a more leaky membrane.

Although the hypothesis that PPCl reflects generalized endothelial dysfunction is attractive, our findings from the multivariate analysis did not support this assumption. We demonstrated that comorbidity was not an independent predictor of PPCl (after controlling for local and systemic inflammation, PSTR and plasma albumin). This is consistent with previous findings in a longitudinal single center cohort study, (Yu et al., 2014) where no significant correlation between PPCl and comorbidity was observed, albeit in a highly selected cohort (restricted to patients on PD for more than 4 years). This longitudinal study also found that PPCl decreases with time for a given membrane area, despite the fact that comorbidity tends to accumulate with time on PD, suggesting that progressive membrane fibrosis is occuring (Yu et al., 2014). Furthermore, detailed analysis of the transcapillary escape rate of albumin, a measure of systemic endothelial barrier dysfunction, found that this was highly abnormal in PD patients, in keeping with an impaired glycocalyx in dialysis patients, (Vlahu et al., 2012) but found no relationship to either systemic inflammation or PPCl. Given that this was a study of prevalent patients who may have acquired membrane fibrosis its interpretation requires caution, but it is nevertheless further evidence that systemic impairment of endothelial barrier function is not a major determinant of PPCl (Yu et al., 2012). PPCl is therefore predominantly a function of peritoneal membrane inflammation and membrane surface area rather than a reflection of systemic endothelial injury.

In addition to the analyses testing our primary hypothesis, we undertook exploratory analyses of the association between different comorbidities and PPCl. Whereas DM was not independently associated with PPCl, this was the case for IHD and LV dysfunction (**Supplementary Table 2**). The explanation for this is not certain, but we did observe that patients with IHD had greater systemic inflammation [plasma IL-6 IHD 2.40 pg/ml (1.30–4.35) vs. DM 1.86 pg/ml (1.03–3.02)], whereas those with DM had a more marked reduction in plasma albumin. This agrees with the findings of the previously cited single center study, (Yu et al., 2012) incorporating a much wider biomarker profile, which found systemic inflammation was more pronounced in IHD whereas platelet activation and systemic albumin leak (not peritoneal) was more significant in DM, suggesting differential effects according to type of comorbidity on endothelial function. Other factors to be taken into account include the different kinetics of peritoneal protein loss as compared to systemic protein leak on plasma albumin (which can be recycled) and other factors that could theoretically increase peritoneal protein losses such as transcapillary hydrostatic pressure. One of the limitations of the present study is that it may not have been large enough to demonstrate different effects by comorbidity on PPCl and survival.

There were a number of other limitations of our study. Firstly, athough a validated correction factor was used to estimate total protein from serum albumin a direct measurement of total plasma protein may have given different results. It is possible that in extreme systemic inflammation, total serum protein may be underestimated and PPCl overestimated because while albumin is a negative acute-phase protein, other proteins increase in inflammation. Thus, the association between systemic inflammation and PPCl may have been amplified, as it may have been in previous studies. However, this does not alter the conclusions of this study, namely that PPCl is more associated with local than systemic inflammation. Secondly, albumin, the predominant plasma and dialysate protein, is able to pass through small pores (predominantly by convection) as well as large pores (Lindholm et al., 1987). Ideally, a series of proteins at different

molecular weights should be measured to precisely dissect out the contribution of the different pore sizes, a procedure that is logistically challenging for a large prospective epidemiological study such as this. Thirdly, proteinuria, another route of protein loss that can affect plasma albumin levels in PD patients, (Gama-Axelsson et al., 2012) would be measured as this may differ by comorbidity. Unfortunately, these measures were not available in this study. Fourthly, a larger study may have been able to show independent effects of more of the covariates on survival; for example a recent study (Mehrotra et al., 2015) of more than 10,000 new patients found a significant association between high solute transport and survival that was not observed here.

Finally, as for all observational studies, we cannot prove causality in the association of peritoneal inflammation with PPCl, although it fits a lot of the Bradford-Hill criteria for causality. Our survival analysis is primarily disproving a causal association suggested by previous studies so this caveat is less relevant. An interventional trial of an agent that turns off intraperitoneal inflammation would be required to investigate causality. The strengths of the present study include the multi-center multinational nature of the study whilst still allowing for clustering by center in the analysis, the large number of patients studied, length of follow up and adjustment for multiple confounding covariates.

Summary

In conclusion, we have shown that PPCl is predominantly determined by peritoneal membrane area and local inflammation. The predictive value of PPCl on survival is mainly through its coupling to hypoalbuminaemia rather than a direct reflection of endothelial injury. PPCl is not a good marker for systemic endothelial function but a reflection of local peritoneal membrane inflammation.

AUTHOR CONTRIBUTIONS

ZY undertook the primary analysis of the data and wrote the main draft of the paper. ML supervised the data analysis. JC and AW recruited the patents from Swansea (United Kingdom center). JC assisted with database development. J-YD recruited patients from the Korean center. NT and SD co-lead the Global Fluid Study. SD recruited patients from the Stoke-on-Trent Center.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00105/full#supplementary-material

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Impact of Glucose Exposure on Outcomes of a Nation-Wide Peritoneal Dialysis Cohort – Results of the BRAZPD II Cohort

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Background: Data investigating the association of glucose exposure with technique failure and patient survival are limited to retrospective cohorts and was never tested outside Asia and considering the presence of competing risks.

Methods: Prospective multicenter cohort study of incident peritoneal dialysis patients where the association of cumulative glucose exposure in 6, 12, and 24 months with patient survival and technique failure was tested using Cox regression analysis and competing risk analysis.

Results: We analyzed 4367 incident peritoneal dialysis patients with mean age 59.0 ± 15.8 years, 43.9% were diabetics, 46.7% males and 64.4% Caucasians. Glucose exposure was not associated with patient survival independent of the time of exposure and even after adjustments for confounders. In contrast, higher glucose exposure was associated with more technique failure in the Cox and competing risk models. The higher risk for technique failure was found in the subgroup exposed to the higher amount of glucose to a maximum of 86% in the model analyzing cumulative glucose exposure for 1 year.

Conclusion: Glucose exposure was associated with technique failure but not with patient survival.

Keywords: peritoneal dialysis, cohort, glucose, membrane, death, ultrafiltration failure

INTRODUCTION

Glucose has long been used as an osmotic agent in peritoneal dialysis (PD) solutions. Over decades, it was responsible to maintain hundreds of thousands of patients with end-stage renal disease alive and with relatively few symptoms (Li et al., 2017). However, glucose absorption during PD has also been implicated in the development of several metabolic and cardiovascular complications (de Moraes and Pecoits-Filho, 2009; Cho et al., 2010; de Moraes et al., 2015) that eventually lead to the description of higher rates of technique failure and mortality in some studies (Wu et al., 2012; Wen et al., 2015).

The negative impact of glucose exposure on solute transport was first described in the Stoke PD Study (Davies et al., 2001). The chronic exposure of the peritoneal membrane to glucose may cause structural and physiological changes that could potentially increase technique failure and mortality. Nevertheless, only a few relatively small studies evaluated the association of glucose exposure with hard outcomes and all in Asian patients. Wu et al. and Wen et al. in two retrospective studies found that higher glucose exposure was associated with technique failure and cardiovascular (CV) mortality (Wu et al., 2012; Wen et al., 2015). The association of patient mortality with glucose exposure are thought to be in part mediated by a negative impact of glucose on carbohydrate metabolism (Li et al., 2013; de Moraes et al., 2015). However, the incidence of obesity, metabolic syndrome and cardiovascular disease differs considerably across countries (Han and Lean, 2016) and no study of our knowledge investigated the association of glucose exposure on patient survival outside Asia.

Therefore, the aim of this study is to investigate the impact of glucose exposure on patient and technique survival of chronic PD patients of the largest cohort in Latin America.

MATERIALS AND METHODS

The BRAZPD II study was a major nation-wide cohort that included patients from December 2004 to January 2011. It collected clinical, demographic and laboratorial data of 9905 adult patients in peritoneal dialysis from 122 centers from all geographic regions across Brazil. This cohort covered around 65% of all PD patients in the country, all of them using standard Baxter® solutions, and icodextrin was not available in Brazil. All data were collected monthly using a specific software (PDNet) designed to the study (de Moraes et al., 2014).

Residual renal function (RRF) was classified at patient inclusion in the study as present (>100 ml/day) or absent. Longitudinal data on RRF and membrane profile were not available. The study protocol was approved by the Ethical Committee of the Pontificia Universidade Católica do Paraná (CEP-PUCPR 448) and the National Ethical Committee under the process 25000.187284/2004-01. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Study Population and Outcomes

The inclusion criteria were age >18 years old and start PD within the recruitment period. There were two events of interest: death for any cause and technique failure. The later was defined as a definite transfer to hemodialysis for any reason.

Glucose Exposure

Glucose exposure was calculated as the product of glucose concentration times the volume of each bag prescribed in a regular dialysis day. For example, in a CAPD patient prescribed with 4 exchanges of glucose 2.5% and 2 l of infusion volume, the glucose exposure would be (4 ml × 2000 ml) × 0.025 = 200 g of glucose/24 h. Then we calculated time-average glucose exposure and divided the population in quartiles.

In order to evaluate the impact of the cumulative glucose exposure on outcomes we created 3 models. These models represent 3 subgroups of patients based on the time that they remained on PD and the time-average glucose exposure during this period: patients on Model I, II and III remained on PD for a minimum period of time of 6, 12, and 24 months. The first quartile comprises patients with the lowest glucose exposure whilst the 4th are the patients with the highest glucose exposure (**Figure 1**).

Statistical Analysis

Continuous variables were expressed as mean ± SD or median and interquartile range, whilst categorical variables were expressed as frequencies and percentages. Patient survival and technique failure were analyzed by the traditional Cox regression and by the competing risk model as proposed by Fine and Gray. Patients alive at the end of the study were always treated as censored. For a better understanding of how we classified censoring and competing risks for both Cox and Fine and Gray models and for the events mortality and technique failure please see **Table 1**.

We then tested all potential confounders in an univariate analysis (with and without the presence of competing risks) and those that had a p-value < 0.10 were selected for the multivariate model. In addition, we also tested all variables selected for the multivariate model for collinearity (**Supplementary Tables S1 and S2**).

Assumptions for proportional hazards and proportional sub-distribution hazards were checked with residual plots. Sub-hazard distribution ratios were calculated as proposed by Fine and Gray. Statistical significance was set at the level of $p < 0.05$ and all analysis performed using the software STATA 14®.

RESULTS

We analyzed the outcomes of 4367 incident patients in PD that fulfilled the inclusion criteria. Mean age was 59.0 ± 15.8 years, 43.9% were diabetics, 46.7% males and 64.4% were White. The complete demographic and clinical of our patients are reported on **Table 2**. Kaplan Meier curves for patient survival and technique failure by quartiles are shown in **Figure 2**.

Patient Survival

There were 755 deaths, of which 278 were cardiovascular events, 246 sepsis not related to the therapy, 78 related to peritonitis, 60 for stroke, 14 for pulmonary edema, 1 for uremia and 78 unknown. The mortality rate was 8.2 (CI 95% 7.6–8.8) deaths per 1,000 patient-months. Regarding technique failure, peritonitis was the main cause of dropout (n = 319), followed by ultrafiltration failure (n = 85), catheter dysfunction (n = 49), refractory exit-site infection (n = 11) and 46 for other causes.

For patient survival, the variables identified in the univariate analysis to compose the multivariable model were body mass index, age, literacy, race, diabetes, peripheral artery disease,

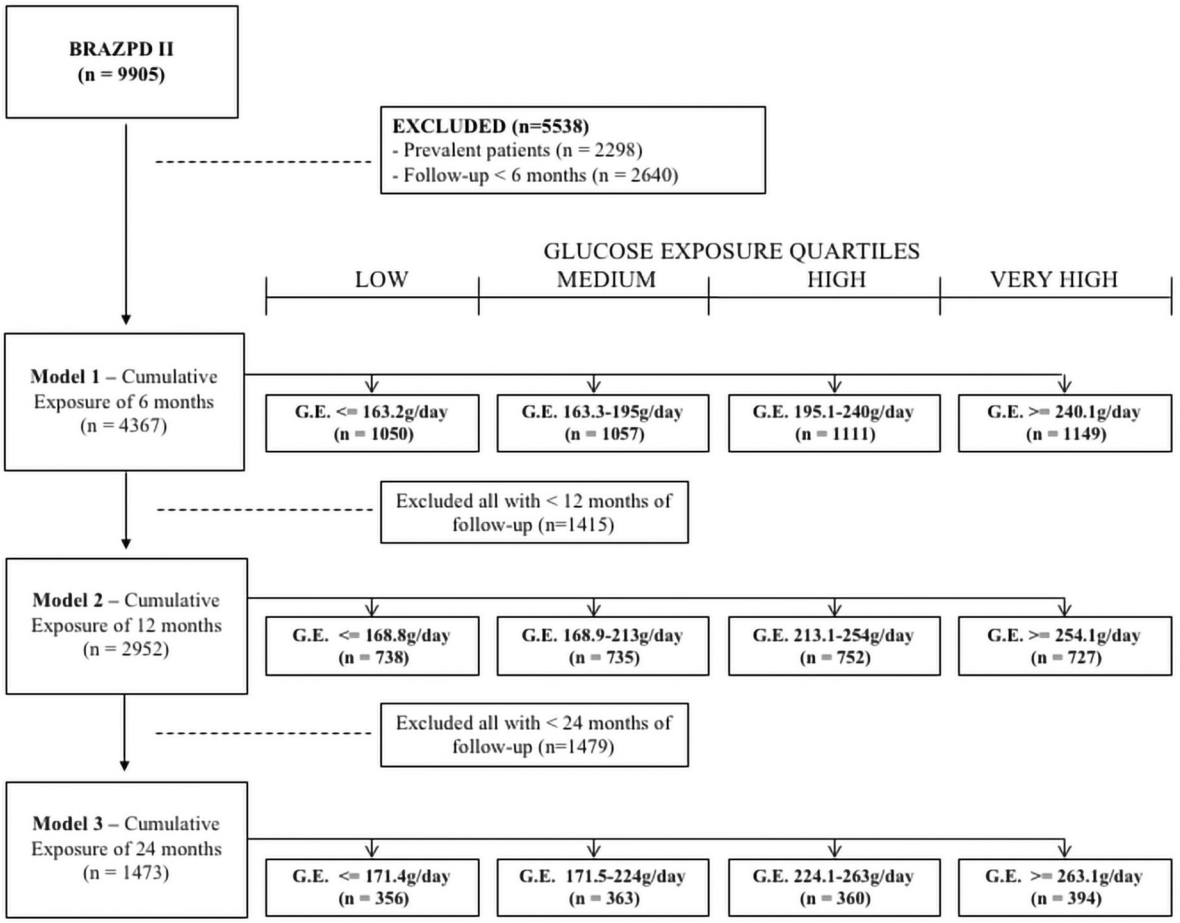

FIGURE 1 | Study design.

TABLE 1 | Definition used for censoring, events and competing risks.

	Event	Competing risk	Censored
Cox regression analysis	D	–	TF +T + L + R + A
	TF	–	D + T + L + R + A
Competing risk analysis	D	TF + T + L + R	A
	TF	D + T + L + R	A

D, death for any cause; TF, technique failure; T, transplantation; L, loss of follow-up; A, alive at the end of the study; R, recovery of kidney function.

TABLE 2 | Clinical and demographic characteristics of the study population.

Variable	
Age (years)	59.1 ± 15.8
Body mass index	24.7 ± 4.7
Coronary artery disease (yes)	917 (21.0%)
Diabetes (yes)	1,918 (43.9%)
Gender (Male)	2,041 (46.7%)
Initial Modality (CAPD)*	1,938 (44.4%)
Literacy (<4 years)	1,527 (35.0%)
Peripheral artery disease (yes)	884 (20.2%)
Pre-dialysis care (yes)	2,276 (52.1%)
Previous hemodialysis (yes)	1,780 (40.8%)
Race (White)	2,811 (64.4%)
Residual renal function (yes)	2,937 (67.2%)

**173 patients with missing information.*

history of previous hemodialysis and presence of residual renal function at baseline. For technique failure only age and history of previous hemodialysis met the criteria for inclusion in the multivariable model.

Glucose exposure was not associated with patient survival in any of the 3 models even after adjustments for confounders: Model 1 (SHR 0.95; CI 95% 0.86–1.06), Model 2 (SHR 0.87; CI 95% 0.75–1.01) and Model 3 (SHR 0.90; CI 95% 0.73–1.11) (**Figure 2**); see **Table 3**.

All models for patient survival were adjusted for body mass index, age, literacy, race, diabetes, peripheral artery disease, history of previous hemodialysis and presence of residual renal function at baseline (all these covariates included in the final model for being significantly associated with mortality in the univariate analysis).

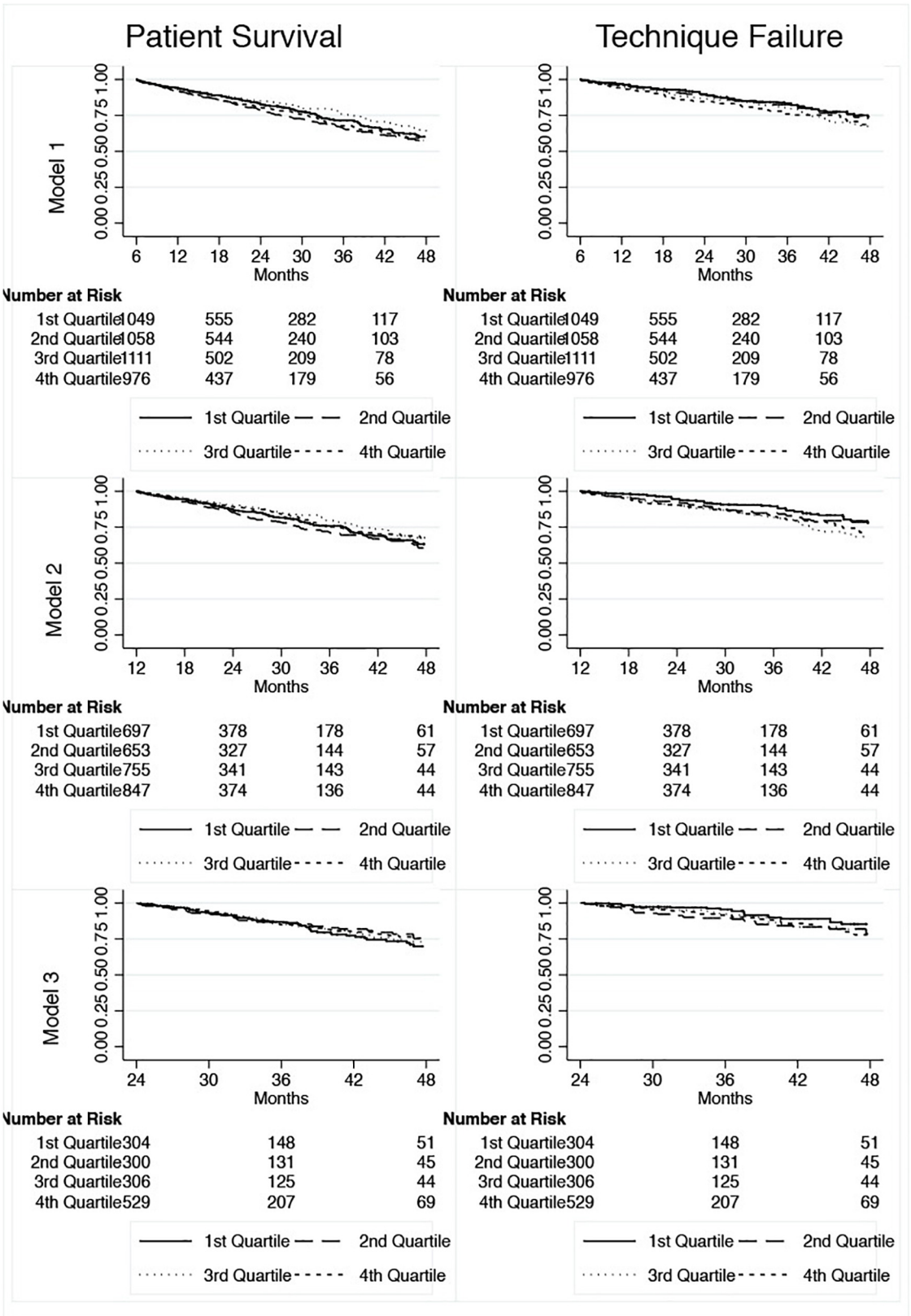

FIGURE 2 | Kaplan-Meier curves for patient survival and technique failure.

TABLE 3 | Multivariate analysis for patient survival.

Glucose exposure	COX		FINE AND GRAY	
	HR (95% CI)		HR (95% CI)	
	Univariate	Multivariate	Univariate	Multivariate
Model 1				
Medium	1.19 (0.99–1.45)	1.21 (1.00–1.47)	1.17 (0.97–1.42)	1.20 (0.96–1.40)
High	0.87 (0.70–1.07)	0.88 (0.67–1.03)	0.83 (0.68–1.03)	0.83 (0.61–0.94)
Very high	1.13 (0.92–1.39)	1.09 (0.84–1.28)	1.05 (0.86–1.29)	1.03 (0.79–1.20)
Model 2				
Medium	1.15 (0.90–1.46)	1.18 (0.91–1.46)	1.10 (0.87–1.40)	1.12 (0.86–1.37)
High	0.82 (0.63–1.06)	0.85 (0.55–1.25)	0.76 (0.59–0.98)	0.78 (0.52–0.88)
Very high	0.89 (0.69–1.14)	0.91 (0.66–1.21)	0.82 (0.64–1.05)	0.84 (0.63–1.05)
Model 3				
Medium	0.85 (0.57–1.27)	0.89 (0.62–1.29)	0.80 (0.54–1.19)	0.89 (0.62–1.29)
High	0.91 (0.61–1.34)	1.04 (0.65–1.35)	0.87 (0.59–1.27)	0.91 (0.64–1.31)
Very high	0.88 (0.62–1.24)	0.91 (0.49–1.14)	0.83 (0.59–1.16)	0.86 (0.49–1.04)

Adjusted for age, previous hemodialysis, race, literacy, peripheral artery disease, diabetes and baseline residual renal function.

Technique Failure

In contrast to patient survival, glucose exposure was associated with technique failure (**Figure 3**). Models were adjusted for age and history of previous hemodialysis, the only two variables associated with technique failure in the univariate analysis. In addition, the incidence of ultrafiltration failure as the cause of the technique failure doubled comparing patients from the first to the last quartile of glucose exposure (**Figure 4**).

Model I – Glucose Exposure During the First 6 Months of Therapy

The technique failure rate was 5.6 (CI 95% 5.1–6.0) events per 1,000 patient-months. In the Cox regression analysis, patients from the second, third and fourth quartile had, respectively, a hazard ratio of 1.11 (CI 95% 0.86–1.43), 1.38 (CI 95% 1.08–1.77), and 1.46 (CI 95% 1.13–1.88) compared to patients from the first quartile.

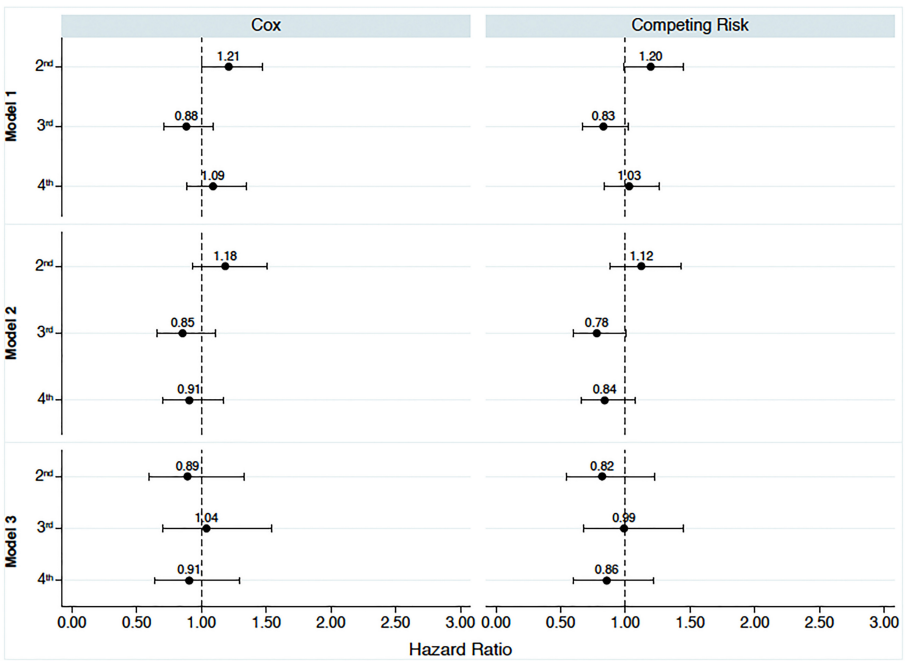

FIGURE 3 | Multivariate analysis for patient survival according to the quartiles of glucose exposure. 1st, first quartile; 2nd, second quartile; 3rd, third quartile; 4th, fourth quartile. Reference values available in **Figure 1**. *Model 1*: Time-average glucose exposure for the first 6 months of therapy; *Model 2*: time-average glucose exposure for the first I year; *Model 3*: time-average glucose exposure for the first 2 years of peritoneal dialysis.

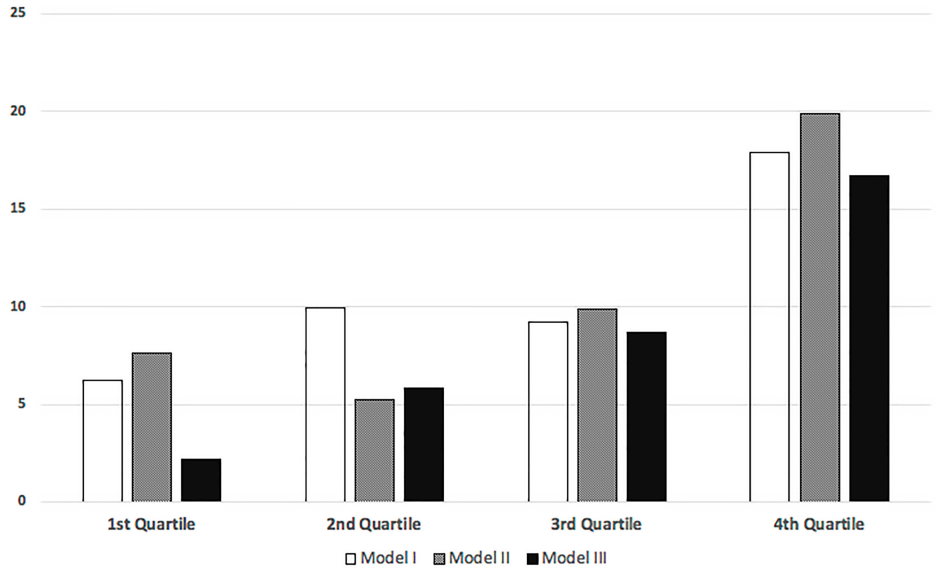

FIGURE 4 | Percentage of ultrafiltration failure as the cause of technique failure stratified by quartiles of glucose exposure.

TABLE 4 | Multivariate analysis for technique failure.

Glucose exposure	COX		Competing risk	
	HR (95% CI)		HR (95% CI)	
	Univariate	Multivariate	Univariate	Multivariate
Model 1				
Medium	1.13 (0.87–1.46)	1.11 (0.84–1.40)	1.08 (0.84–1.39)	1.06 (0.80–1.33)
High	1.36 (1.06–1.75)*	1.38 (1.09–1.79)*	1.35 (1.06–1.73)*	1.37 (1.08–1.77)
Very high	1.48 (1.15–1.91)*	1.46 (1.13–1.88)*	1.41 (1.09–1.81)*	1.39 (1.08–1.78)
Model 2				
Medium	1.42 (1.01–1.98)*	1.41 (1.05–1.94)	1.37 (0.98–1.91)	1.35 (0.98–1.87)*
High	1.84 (1.34–2.52)*	1.86 (1.44–2.58)*	1.84 (1.35–2.51)*	1.86 (1.27–2.52)*
Very high	1.64 (1.19–2.24)*	1.63 (1.24–2.28)*	1.62 (1.19–2.21)*	1.61 (1.08–2.22)*
Model 3				
Medium	1.76 (1.06–2.92)*	1.75 (1.05–2.90)*	1.75 (1.05–2.91)*	1.75 (1.04–2.95)*
High	1.53 (0.91–2.58)	1.60 (0.95–2.69)	1.50 (0.90–2.52)	1.55 (0.90–2.60)
Very high	1.70 (1.07–2.71)*	1.69 (1.06–2.69)*	1.69 (1.06–2.67)*	1.68 (1.04–2.68)*

*Adjusted for age and history of previous hemodialysis; *$p < 0.05$.*

Model II – Glucose Exposure During the First Year of Therapy

For 2,952 patients that survived for more than a year, 212 were transferred to HD due to unsuccessful treatment of a peritonitis episode, 56 for ultrafiltration failure, 31 for catheter dysfunction, 10 for refractory exit-site infection and 33 for other causes.

Comparing the four groups we found, in the Cox regression, that patients from the second, third and fourth quartile had a higher risk for technique failure compared to patients from the first quartile: 2nd (HR 1.41; CI 95% 1.01–1.96), 3rd (HR 1.86; CI 95% 1.36–2.55), and 4th (HR 1.63; CI 95% 1.19–2.23).

Model III – Glucose Exposure During the First 2 Years of Therapy

There were 1,439 patients with more than 2 years of PD and 158 were transferred to HD. Peritonitis remained as the main cause of TF ($n = 97$), followed by ultrafiltration failure ($n = 23$), catheter failure ($n = 11$), refractory exit-site infection ($n = 7$) and other causes ($n = 20$). The number of events was lower in this model but the 2nd (HR 1.74; CI 95% 1.05–2.90) and 4th (HR 1.69; CI 95% 1.06–2.69) quartile remained associated with TF. The 3rd quartile was not statistical significant (HR 1.60; CI 95% 0.95–2.69).

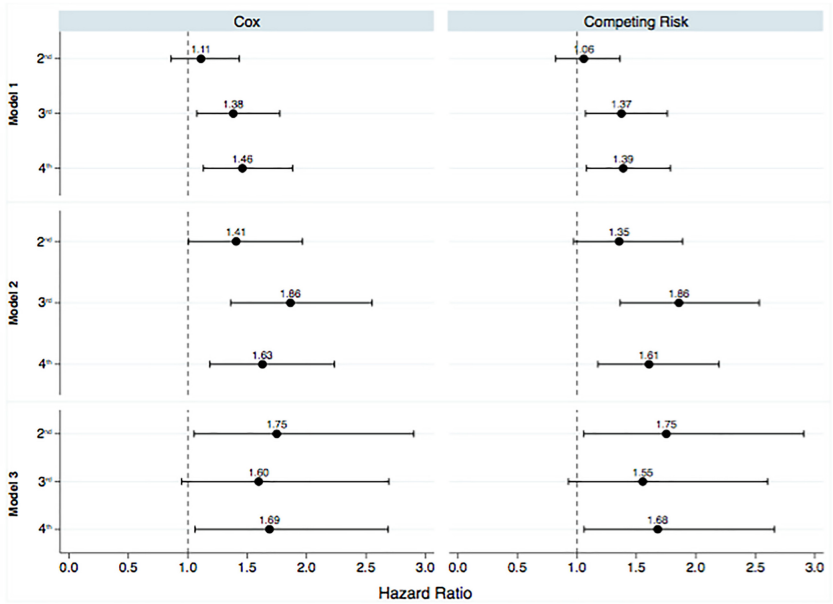

FIGURE 5 | Multivariate analysis for technique failure according to the quartiles of glucose exposure. 1st, first quartile; 2nd, second quartile; 3rd, third quartile; 4th, fourth quartile. Reference values available in **Figure 1**. *Model 1*: Time-average glucose exposure for the first 6 months of therapy; *Model 2*: time-average glucose exposure for the first I year; *Model 3*: time-average glucose exposure for the first 2 years of peritoneal dialysis.

When we considered the presence of competing risks all associations found in the Cox Regression remained significant (**Table 4** and **Figure 5**).

DISCUSSION

Peritoneal glucose exposure was first linked to abnormal changes in the peritoneal membrane transport in a cohort of 22 patients treated continuously for 5 years in early 2000s (Davies et al., 2001). These abnormalities cause an increase in the membrane permeability which could lead to a faster absorption of glucose reducing the osmotic power of the PD solution and consequently contributing to technique failure and increased risk of death for fluid overload (Grodstein et al., 1981; Churchill et al., 1998; Szeto et al., 2007). However, only few studies tested in large scale the impact of different levels of peritoneal glucose exposure on technique failure and patient survival (Wen et al., 2015). This is the largest cohort study to test the association of glucose exposure with patient survival and technique failure.

Peritoneal dialysis patients may absorb huge amounts of glucose depending of factors as the patient's membrane transport, dwell time and, mainly, the concentration of glucose in the dialysis solution. In early eighties, Grodstein et al. demonstrated an absorption of up to 350 g per day when CAPD patients were exposed to a high glucose load with 4.25% glucose PD solution (Grodstein et al., 1981). One of the consequences of the chronic exposure to glucose solutions are disturbances in the metabolism of carbohydrate as insulin resistance, dyslipidemia, higher needs of insulin in diabetic patients and even an elevation in fasting glucose and HbA1C in non-diabetic patients (de Moraes and Pecoits-Filho, 2009; de Moraes et al., 2011; de Moraes et al., 2015).

Higher amounts of glucose are often need for high transporters patients in order to increase ultrafiltration and the increased mortality of this subgroup of patients was in the past attributed at least in part to difficulties in the control of fluid overaload (Churchill et al., 1998). However, when fast transporters are properly managed with short cycles in automated PD such high mortality rates tend to disappear (Yang et al., 2008). The majority of our patients were treated on APD and this may have contributed to our finding of a lack of association between glucose exposure and patient survival. Nevertheless, we didn't capture data on volume status which would be valuable to understand our findings but at the same time very difficult to perform in large cohorts. In addition, we don't have data on cholesterol and HbA1c levels to evaluate their association with glucose exposure. At the same time, it is interesting to note that the majority of studies that investigated the impact of glycaemia or dyslipidemia control in PD patients were unable to found any association in terms of patient survival (Chawla et al., 2010; Baigent et al., 2011).

Finally, glucose exposure was associated to technique failure. The increased risk fora definite transfer to hemodialysis for patients receiving higher intraperitoneal glucose load varied from 36 to 84% depending of the model and subgroup analyzed (**Figure 2**). The progressive increase observed for ultrafiltration failure as the cause of technique failure reinforce the negative impact of the glucose exposure on the peritoneal membrane permeability (**Figure 3**; Fusshoeller, 2008). However, our findings are in contrast to the study of Yang et al. in a cohort of

193 Canadian patients (Yang et al., 2008). This likely reflect differences in the management of volume status, for example the peritoneal equilibration test was not routinely performed in some centers due financial limitations. Of note, icodextrin was not available during the study period and could have helped to improve the management of hypervolemia.

Our study have some limitations including all those related to any cohort study. In addition, the lack of data on peritoneal membrane transport, longitudinal data on residual renal function and other unmeasured confounders including biomarkers of cardiovascular disease should also be taken into account in the interpretation of our results. Nevertheless, this is the largest cohort study to evaluate the association of glucose exposure with technique failure and patient survival. Moreover, both events preclude the occurrence of each other and no previous study of our knowledge considered the presence of competing risks in the investigation of the effect of peritoneal glucose exposure in these outcomes.

In conclusion, peritoneal glucose exposure was not associated with mortality but significantly affected technique failure.

AUTHOR CONTRIBUTIONS

All authors contributed to conception and design of the study and to manuscript revision, read and approved the submitted version. VR and TPM organized the database. TPM performed the statistical analysis and wrote the first draft of the manuscript.

SUPPLEMENTARY MATERIAL

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Peritoneal Ultrafiltration in the Long-Term Treatment of Chronic Heart Failure Refractory to Pharmacological Therapy

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Introduction: Despite continuous improvement in the treatment, heart failure (HF) is a growing health problem and a major cause of mortality and morbidity in the world. There is some positive experience with the removal of the fluid excess via peritoneum in those patients, regardless of their renal function. The aim of this single center pilot study was to assess the efficacy of peritoneal ultra filtration (PUF) with a nightly 12-h exchange in the long-term treatment of refractory HF.

Methods: The study included patients with chronic HF resistant to updated HF therapy (pharmacological and devices if applicable), who had experienced at least three hospitalizations due to HF during the preceding year and were disqualified from heart transplantation. All of them were treated with nightly 12-h 7.5% icodextrin exchange.

Results: There were 15 patients (13 men), aged 72 ± 9 years, with charlson comorbidity index (CCI) 9 ± 1.2, NYHA class IV (11 patients) or III (4 patients), and eGFR 32 ± 11 ml/min/1.73m^2. They were followed up for 24 ± 8 months (range 12–43, median 26 months). During the 1st year, all patients improved their NYHA functional class from 3.7 ± 0.5 to 2.6 ± 0.5; P = 0.0005, with stable (34.3 ± 12.4, and 35.6 ± 16.5%, respectively) left ventricular ejection fraction (LVEF), and inferior vena cava (IVC) diameter decreased from 27.8 ± 2.7 to 24.4 ± 3.4 mm; P = 0.09. Daily diuresis increased from 867 ± 413 to 1221 ± 680 ml; P = 0.25, while the dose of furosemide could be reduced from 620 ± 256 to 360 ± 110 mg/d; P = 0.0005, however, the kidney function deteriorated, with eGFR drop from 32 ± 11 to 25.6 ± 13 ml/min/1.73m^2; P = 0.01). HF-related hospitalizations decreased from 8.9 ± 2.8 days/month to 1.5 ± 1.2 days/month (P = 0.003). Mechanical peritoneal dialysis complications occurred in five patients and infectious complications in four (peritonitis rate 1 per 72 patient-month). Patient survival was 93% at 1 year and 73% at 2 year. Technique survival was 100%.

Conclusion: In patients with refractory HF, PUF with one overnight icodextrin exchange appears to be a promising therapeutic option as an adjunct to pharmacological management of those who are not transplant candidates. It should be emphasized that the treatment can have a great impact on the quality of life and the total costs of treating these patients.

Keywords: chronic progressive heart failure, cardiorenal syndrome, peritoneal ultrafiltration, icodextrin, patient survival, HF-related hospitalizations

INTRODUCTION

Chronic progressive heart failure (HF) is an increasing public health problem of the 21st century and carries a poor prognosis. Paradoxically, improvement in diagnosis and treatment of ischemic heart disease and hypertension results in growing numbers of patients who survive acute cardiovascular events, at the expense of progressive HF, however, It's been estimated that only in Europe more than 10 million people suffer from chronic HF, and approximately 5% of that population have reached the end-stage stadium of the disease, refractory to available therapies (Dickstein et al., 2008; Jessup et al., 2009). For the selected patients a device therapy, surgery and transplantation may be offered, however, many of them are not suitable for these invasive procedures, mainly due to age and coexisting diseases. Survival of such patients is less than 50% at 6 months, and the treatment with many hospitalizations constitutes a remarkable financial burden and confers suffering and a poor quality of life (Dickstein et al., 2008; Damman et al., 2009; Jessup et al., 2009).

The reduced cardiac output and fluid redistribution result in the decreased perfusion of other organs, including kidneys. The compensatory mechanisms such as activation of the sympathetic nervous system, renin-angiotensin-aldosterone (RAA) axis and arginine vasopressin lead to enhanced renal water and sodium retention in an effort to preserve glomerular filtration rate (GFR), but in the long-term these mechanisms are deleterious for both heart and kidneys (Mullens et al., 2009). The coexistence of cardiac and renal dysfunction induces a vicious circle that leads to an aggravation of both pathologies, chronic cardio-renal syndrome (CRS) development, and refractoriness to the treatment with decreased delivery of diuretics to their effector sites in the nephron (Ronco et al., 2008; Damman et al., 2009; Mullens et al., 2009). This spiral results in a further water and salt retention, further decline in cardiac output, and ultimately hypotension, with pulmonary edema and death (Ronco et al., 2008; Mullens et al., 2009).

There is a growing evidence that peritoneal dialysis (PD), with its flexibility in techniques, regimens and solutions, may be a feasible measure for patients with chronic CRS and refractory volume overload (Gotloib et al., 2005; Sánchez et al., 2010; Nuñez et al., 2012; François et al., 2015; Lu et al., 2015; Kazory, 2017). There are two patient groups with chronic CRS who could benefit from PD or PUF: patients with end-stage renal disease (ESRD) and those with preserved significant residual renal function. In the first group PD is needed as a method of not only water but also uremic toxins removal, while in the second one mainly or exclusively for a relief of refractory congestion.

The aim of this prospective single center pilot study was to assess the usefulness of PUF with one nightly 12-h icodextrin exchange in the long-term treatment of patients without ESRD with HF refractory to optimal medical therapy disqualified from heart transplantation.

MATERIALS AND METHODS

Study Population

All consecutive patients with severe chronic HF (NYHA class III or IV) who fulfilled the inclusion criteria and were treated with PUF in our unit between January, 2005 and December, 2017 were enrolled into the study. The inclusion criteria were predefined as follows: (1) severe HF refractory to the optimal tolerable medical therapy, according to the current guidelines (maximal tolerable pharmacological therapy and implantable devices – ICD or CRT, if applicable); (2) contradictions for heart transplantation, (3) at least three hospitalizations for HF during last 12 months; (4) written informed consent. The exclusion criteria were: (1) inability of the patient or his assistant to cooperate; (2) presence of reversible causes of congestive HF (modifiable valvular heart disease, possible revascularization in coronary heart disease, hyperthyroidism, anemia, etc.); (3) infectious endocarditis; (4) presence of ESRD, (5) advanced malignant disease; (6) a history of myocardial infarction during last 90 days, myocarditis and pulmonary embolism during last 180 days.

The protocol was reviewed and approved by the Bioethical Committee of Medical University of Warsaw. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Study Protocol

Double-cuff Tenckhoff catheter was implanted surgically under local or general anesthesia depending on an anesthetist's decision. Four patients, before catheter implantation, required extracorporeal ultrafiltration (3–10 sessions, 1–2 L/day) due to massive water overload. All of the subjects were enrolled in a long-term program of PUF, initially with one nightly 12-h 7.5% icodextrin solution exchange. PUF was started with low-fill volume (1000 mL), which was gradually increased to 1500–2000 mL depending on individual patient's tolerance.

The baseline clinical and laboratory parameters and number of hospitalizations for HF in the preceding year were recorded. During follow-up, patients were evaluated after one, 6 and

12 and 24 months after PUF initiation. Body weight, urine output, eGFR by MDRD formula, NYHA class, hospitalization days, PD complications and adverse events related to the treatment were recorded. Echocardiographic parameters were evaluated every 6 months.

Statistical Analysis

All data were presented as mean ± standard deviation, median and interquartile range, and percentages, as appropriate. An analysis of differences between proportions was performed by means of Fisher's exact test, and general linear model was used to assess any repeated measurements of the same variable. Peritonitis rates were calculated as episodes per patient-month at risk and hospitalization rates as days per month. Patient survival was analyzed using an actuarial method. A value of $P < 0.05$ was considered statistically significant. All calculations were performed using STATISTICA software package (version 12), StatSoft Poland.

RESULTS

Baseline clinical characteristics of the studied group are presented in the **Table 1**.

The time between Tenckhoff catheter placement and PUF commencement (break-in period) was 7.8 ± 4.0 days (median 8, range from 2 to 15 days). Slightly longer break-in period was noticed in patients with ascitic fluid drainage (8.8 ± 4.2 days, median 9). Nine (60%) patients were capable for self-care, the others needed assistance for PD procedures. The mean daily peritoneal ultrafiltration achieved with the technique was 927 ± 143 mL (median 1000 mL; 600–1200 mL).

The patients were followed up for 360 patient-months, mean 24 ± 8 months (median 26; 12–43 months). The treatment with only one overnight icodextrin exchange was continued for 13 ± 6 months (median 11; 7–33 months). The leading reason of the changes in PUF prescription such as increasing the number of manual exchanges or switching the patient to automated PD was the need for more intensive fluid and/or solutes removal. In three cases the causes were non-medical: two patients developed progressive dementia and needed assistance, and one patient due to loss of partner changed to intermittent PD, performed in the hospital three times a week.

During the first 6 months of the treatment a relief in congestion was obtained, with concomitant improvement in sensitivity to furosemide, hence in all patients a switch from intravenous into oral forms and the dose reduction (from 620 ± 256 to 277 ± 117 mg/d, $P = 0.001$) was possible (**Table 2**). Significant increase in urine output (from 867 ± 413 to 1807 ± 464 mL/d, $P = 0.001$), and a mild improvement of eGFR (32 ± 11 vs. 36.6 ± 13.8 mL/min/1.73m^2, $P = 0.59$) were also noticed. However, during the next months the continuous deterioration of kidney function with urine output reduction was observed (**Table 2**).

During PUF almost all patients improved in their NYHA functional class by at least one. At 6 months there were 11 (73%) patients in NYHA class II and 4 (27%) in NYHA functional class III, at 12 months – 5 (33%) NYHA II, 9 (60%) NYHA III and 1 (7%) NYHA IV (**Table 3**). They also experienced an improvement, or at least stabilization in their LVEF, a decrease in IVC diameter, and a rise of MAP. This allowed for some pharmacological treatment modifications, particularly re-administer or further up-titration ACEi/ARB, mineralocorticoid receptor antagonists and hydrochlorothiazide to maximal tolerated doses (**Table 3**).

TABLE 1 | Baseline clinical characteristics of the study population.

Parameter	Value
Age	72 ± 9 years
Male sex	87%
Charlson comorbidity index	9 ± 1.2
Type of cardiomyopathy (% of patients):	
Ischemic	47%
Valvular	33%
Restrictive	20%
PM or ICD or CRT (% of patients)	73% (28/36/36%)

PM, pacemaker; ICD, implantable cardioverter-defibrillator; CRT, cardiac resynchronization therapy device.

PUF Complications

The mechanical complications occurred in five patients. In three cases it was an inguinal hernia, in two of them the surgical repair was performed, with a moderate HF decompensation in a postoperative period surgery, demanding a temporary filling volume decrease in one patient and a switch to automated PD in the other. There were also two cases of exit-site leakage. In one patient with early leakage, the catheter was surgically re-transplanted, and after 3 days of break-in period PUF was restarted in a supine position. In the second patient a simple decrease in filling volume brought the resolution. To prevent infection in patients with exit-site leakage, antibiotic prophylaxis with cephalosporins was used.

Peritonitis was the only infectious complication. Overall five episodes were observed, for an average one episode per 72 patient-months. The mean time to first peritonitis episode was 12.7 ± 6.5 months (median 11 months), and no episode occurred in the 1st days after PUF start. The leading microbiologic etiology agent was Gram positive cocci (3 – coagulase-negative staphylococci, 1 – *Streptococcus salivarius*), and in one case *Escherichia coli*.

The mean hospitalization days related to PUF complications was 4.3 ± 8.3 days per month in the first, and 2.4 ± 4.9 days per month in the 2nd year of PUF.

In the study period, no episode of exit site/tunnel infection occurred. The typical procedures for the exit site care with povidone iodine were used in all patients (ointment with mupirocin is routinely not used in Poland).

Morbidity, expressed as all cause hospitalization days per month decreased significantly after start of PUF (**Table 3**). During the 1st year of PUF only one patient died (a sudden death), and three others died during the 2nd year (two from sepsis, one suddenly).

TABLE 2 | Kidney function, urine output, furosemide dose, and body weight during the follow up.

	Baseline	6 months	12 months	24 months	*P*-value
eGFR (mL/min/1.73m^2)	32.0 ± 11.0	36.6 ± 13.8	25.6 ± 13.0	9.0 ± 13.0§	0.0001
Urine output (mL/day)	867 ± 413	1807 ± 464	1221 ± 680	480 ± 540§	0.0003
Furosemide dose (mg/d)	620 ± 256*	277 ± 117**	360 ± 110**	182 ± 192**	0.002
Body weight (kg)	78.5 ± 5.4	70.2 ± 5.3	69.0 ± 5.5	67.5 ± 5.3	0.0001

*eGFR, estimated glomerular filtration rate; *intravenous route of administration; **oral route of administration; §4 patients became anuric.*

TABLE 3 | Changes in echocardiographic parameters, MAP, NYHA class, all-cause, and HF-related hospitalizations during the follow-up.

	Baseline	6 months	12 months	24 months	*P*-value
LVEF (%)	34.3 ± 12.4	37.9 ± 10.8	35.6 ± 10.7	31.7 ± 9.8	0.1
IVC diameter (mm)	27.8 ± 2.7	22.4 ± 4.6	24.4 ± 3.4	26.8 ± 4.0	0.0003
MAP (mmHg)	58.8 ± 11.9	75.1 ± 11.0	76.0 ± 11.4	71.0 ± 11.2	0.0002
NYHA class (II/III/IV)	0/4/11	11/4/0	5/9/1	0/6/3	–
NYHA class (mean ± SD)	3.7 ± 0.46	2.3 ± 0.46	2.6 ± 0.5	3.3 ± 0.5	0.00006
Medications (% of patients):					
ACEi or ARB	27%	73%	55%	30%	
β-blockers	87%	100%	100%	90%	
MRI	20%	80%	73%	80%	
HCT	20%	53%	45%	22%	
All-cause hospitalizations (days/month)	8.9 ± 2.8	1.2 ± 1.3	2.74 ± 1.4	2.57 ± 1.2	0.00003
HF-related hospitalizations (days/month)	8.9 ± 2.8	0.49 ± 0.8	1.48 ± 1.2	1.89 ± 0.9	0.00003

LVEF, left ventricular ejection fraction; IVC, inferior vena cava; MAP, mean arterial pressure; NYHA, New York heart association; ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; MRI, mineralocorticoid receptor antagonists (spironolactone or eplerenone); HCT, hydrochlorothiazide.

At the end of observation 13 patients (87%) died, 9 (69%) of them survived > 24 months (mean 25.4 ± 8.0 months, (median 26 months) from the start of PUF. Most of patients died from a worsening of CHF (46%) or suddenly (31%), while 15% from sepsis and 8% from stroke. The technique survival censored for death was very good; 11 patients switched to full dose PD, three died while treated with PUF and one was lost to follow-up after 18 months of the treatment. The cumulative actuarial survival of the study population is presented on **Figure 1**.

DISCUSSION

The suggestion that PUF may offer clinically relevant benefits such as improvement in severe HF is not new. The first report of the successful use of PUF in this population was published in 1949, the second one in 1967 (Schneierson, 1949; Mailloux et al., 1967).

However, although many other studies have been performed since then, all of them were small, mainly of retrospective nature, done in heterogeneous populations, with patient inclusion criteria that were not always clear and with different treatment schedules. In this paper we present the results of an observational single study performed in a small but relatively homogeneous group of patients with refractory CHF and a long-term follow-up, where PUF with a one overnight icodextrin exchange was used as a rescue therapy. To make the group as homogeneous as possible, we excluded from the analysis patients treated with different PD solutions (dextrose based or mixed dextrose and icodextrin) as well as those with ESRD.

The major finding of our study was a spectacular, more than 80% decrease in hospitalization rates (for both the number of admissions and days spent in hospital), observed already after the 1st months of the treatment. This is in accordance with results of other studies, reporting at least 50% reduction in hospital admissions (Gotloib et al., 2005; Nakayama et al., 2010; Sotirakopoulos et al., 2011; Cnossen et al., 2012; Koch et al., 2012; Nuñez et al., 2012; Ruhi et al., 2012; Rizkallah et al., 2013; Bertoli et al., 2014) and with a recently published systematic review (Lu et al., 2015), where this was considered the most significant effect PUF. Such a marked decrease in the number of hospitalizations in our study may be partly due to the fact that in Poland it is not possible to administer iv furosemide in outpatient setting. Although all the patients were critically ill, with congestion depended to very high doses of iv diuretics, most of them started PUF without prior extracorporeal ultrafiltration. In other studies, extracorporeal ultrafiltration (or even hemodialysis) was used obligatorily or at least in selected patients to relief congestion, and some patients started PUF in an outpatient setting (Gotloib et al., 2005; Nakayama et al., 2010; Cnossen et al., 2012; Bertoli et al., 2014). Our observation suggests that most of the patients can safely and effectively initiate PUF without preceding extracorporeal ultrafiltration.

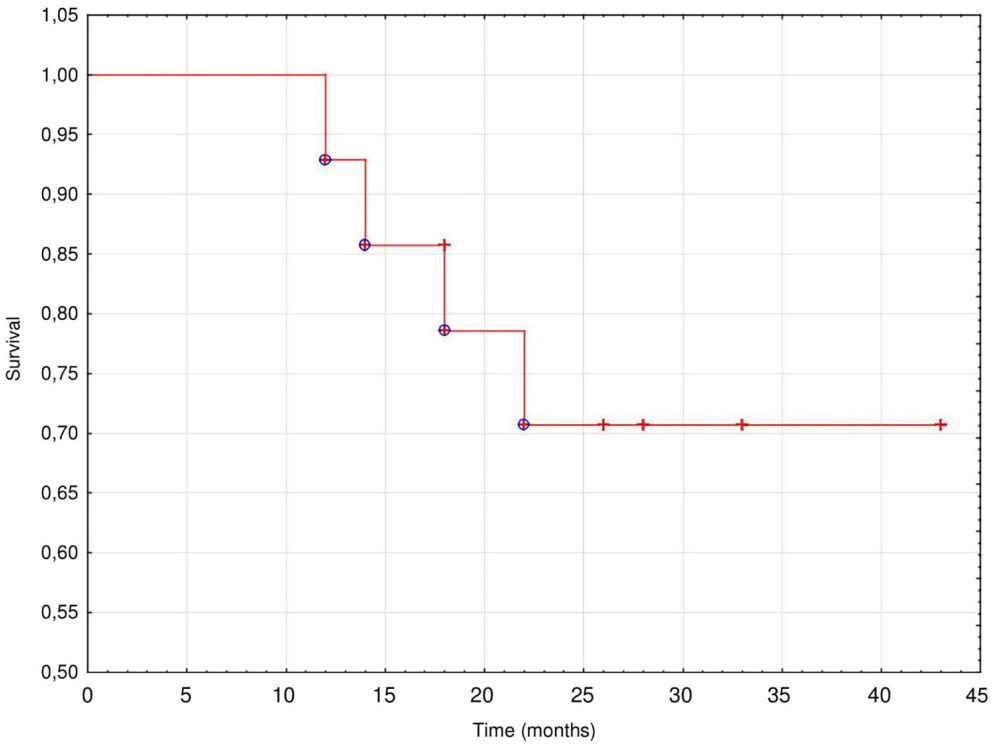

FIGURE 1 | Cumulative actuarial patients' survival.

Peritoneal ultrafiltration was associated with a rapid, significant and long-term improvement in clinical status as demonstrated by the reduction in NYHA functional class, a feature observed in most published studies (Gotloib et al., 2005; Nakayama et al., 2010; Sánchez et al., 2010; Sotirakopoulos et al., 2011; Cnossen et al., 2012; Koch et al., 2012; Nuñez et al., 2012; Ruhi et al., 2012; Rizkallah et al., 2013; Bertoli et al., 2014). It was initially related with a significant, but transient improvement in LVEF. Indeed, the removal of excess fluid via ultrafiltration may improve cardiac output due to changes in the Frank-Starling curve, an increased left ventricular diastolic inflow, and an improvement in lung compliance (François et al., 2015; Kazory, 2017); however, only in some studies marked recovery of systolic left ventricular function was observed (Gotloib et al., 2005; Takane et al., 2006), while in others, the change was not significant (Ruhi et al., 2012; Rizkallah et al., 2013; Bertoli et al., 2014). This slow decrease in LVEF observed in our study was well compensated by PUF since the patients remained in better NYHA classes even after 24 months of the therapy, despite the progressive loss of RRF with urine output reduction. Our hypothesis is that an abrupt initial reduction in preload burden and ventricular end-diastolic volume with PUF leads to a significant rise in LVEF and the arterial pressure with better organs perfusion. This can explain a renal function improvement observed during the 1st months of the therapy. However, an increased afterload partially offsets the higher LVEF by rising the end-systolic volume.

In some reports pulmonary artery systolic pressure (PAPs) assessed by echocardiography was performed to evaluate the reduction of congestion after PUF/PD implementation (Nakayama et al., 2010; Sánchez et al., 2010; Koch et al., 2012; Nuñez et al., 2012; Bertoli et al., 2014). In our study, the improvement in volume status after the start of the PUF resulted in decrease in inferior vena cava diameter (IVCd). The measurement of IVCd with an assessment of its respiratory variability was chosen as a parameter to estimate the pressure in the right atrium, one of the non-invasive parameters in the diagnosis of pulmonary hypertension with an independent predictive value (Kalogeropoulos et al., 2014). In addition, it has been confirmed that increased IVC diameter itself identifies HF patients with adverse outcomes (Pellicori et al., 2013). The value of IVCd changes alone as a parameter in indirect assessment of right ventricle function and monitoring the response to PUF therapy warrants further evaluation.

The higher MAP allowed for the pharmacological HF treatment optimization, which in turn, together with a decongestion with PUF, resulted in a significant clinical improvement with the long-term reduction in the NYHA class. Unfortunately, the data on pharmacological treatment are not provided in the available studies.

Peritoneal ultrafilration therapy has also allowed for a restoration of sensitivity to diuretics and a rise in diuresis. It may be due to a better delivery of diuretics to their effector sites in the nephron along with intrarenal decongestion and a decreased stimulation of RAA axis and sympathetic nervous

system (François et al., 2015; Kazory, 2017). Moreover, the controlled drainage of ascites might reduce intra-abdominal pressure, which has been demonstrated to improve renal function in HF (Mullens et al., 2009). The increase in daily diuresis comparable to that observed in our study was reported only by a few authors (Basile et al., 2009; Nakayama et al., 2010), others observed no change (Sánchez et al., 2010; Nuñez et al., 2012; Bertoli et al., 2014) or even its significant drop after 6 months of the treatment (Koch et al., 2012). It may be due to the selection bias, and other factors such as, for example, UF before the start of the PUF. Two studies, one prospective and one retrospective, have suggested that in non-ESRD patients PUF may preserve residual renal function (Nuñez et al., 2012; Bertoli et al., 2014). However, it could be due to a relatively short observation period, in both of them limited to 1 year. We observed a similar effect during the 1st year of the treatment in our patients, but later on their renal function deteriorated quickly and some of them became even anuric, requiring a full dose of dialysis.

Introduction of PUF resulted in effective ultrafiltration approximating 1000 mL/day, which together with augmented urine output resulted in a significant reduction in body weight, similar to described in some (Sotirakopoulos et al., 2011; Cnossen et al., 2012; Nuñez et al., 2012), but not all studies (Nakayama et al., 2010; Sánchez et al., 2010; Bertoli et al., 2014). However, interpreting this finding is difficult. While in the 1st months of the treatment it apparently was related to relief of congestion and improved volume status, in the later period it could as well indicate a progressive malnutrition typical for severe chronic diseases. Therefore, more objective assessment of body composition such as bioimpedance should be employed, as in the study by Gotloib et al. (2005).

It should be stressed that the low all-cause hospitalizations rates (including those related to comorbid conditions and PUF itself) persisted during the entire follow-up, despite a gradual worsening of HF and renal function. One third of studied patients died suddenly, but the quality of their last year of life, spent at home and with less clinical symptoms, seemed to be much better, although not measured by any standardized questionnaire. This huge reduction in hospital admissions, despite the cost of PUF, may also have a marked financial aspect, as shown by Sánchez et al. (2010).

In our study 2-year patient survival rate of 73% was similar, even slightly better than that reported in other studies (Sánchez et al., 2010; Rizkallah et al., 2013; Bertoli et al., 2014). Taking into account the poor prognosis of these patients resulting from HF and a high comorbidity burden our data give some hope for an improvement not only in the quality of life but also in survival compared with patients receiving conservative therapies.

We are fully aware of the limitations of our study, being a pilot, single-center, retrospective and observational study with relatively small sample size, therefore the results should be interpreted with caution. However, single center character allows implementation of consistent, standardized approach to the management and surveillance during the treatment of PUF. Moreover, we included all the consecutive patients and followed them for a median of 26 months, much longer than in other studies (Sánchez et al., 2010; Cnossen et al., 2012; Koch et al., 2012; Nuñez et al., 2012; Ruhi et al., 2012). It should be also stressed that to perform study on PD patients always is associated with problems with sample size due to prevalence of this modality among dialyzed patients.

CONCLUSION

Peritoneal ultrafiltration appears to be a reasonable strategy for the treatment of refractory HF as a rescue therapy in patients who are not eligible for heart transplantation when the optimal medical treatment failed. It offers a relevant clinical benefit due to a significant reduction in hospitalization rate, better quality of life, and perhaps even some survival advantage. With the progression of CKD incremental PD can be implemented as a self-care or an assisted procedure. There are many questions that remain unanswered such as when PUF should be considered. Perhaps not randomized, but larger observational clinical trials should be designed and performed to provide more information and establish the best protocol for the management of refractory HF in patients with concomitant chronic kidney disease. Cost-benefit analyses and reimbursement policies should also be implemented.

AUTHOR CONTRIBUTIONS

EW conceived and designed the study, collected of the data, performed the statistical analysis, wrote the manuscript, and prepared its final version. SN designed the study. AG collected the data and analyzed the data. JM-R conceived and designed the study, wrote the manuscript, and prepared its final version. JM wrote the manuscript and prepared its final version. All the authors approved the final version of the manuscript.

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Peritoneal Dialysis Vintage and Glucose Exposure but Not Peritonitis Episodes Drive Peritoneal Membrane Transformation During the First Years of PD

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The impact of peritoneal dialysis (PD) associated peritonitis on peritoneal membrane integrity is incompletely understood. Children are particularly suited to address this question, since they are largely devoid of preexisting tissue damage and life-style related alterations. Within the International Peritoneal Biobank, 85 standardized parietal peritoneal tissue samples were obtained from 82 children on neutral pH PD fluids with low glucose degradation product (GDP) content. 37 patients had a history of peritonitis and 16 of the 37 had two or more episodes. Time interval between tissue sampling and the last peritonitis episode was 9 (4, 36) weeks. Tissue specimen underwent digital imaging and molecular analyses. Patients with and without peritonitis were on PD for 21.0 (12.0, 36.0) and 12.8 (7.3, 27.0) months (p = 0.053), respectively. They did not differ in anthropometric or histomorphometric parameters [mesothelial coverage, submesothelial fibrosis, blood, and lymphatic vascularization, leukocyte, macrophage and activated fibroblast counts, epithelial-mesenchymal transition (EMT), podoplanin positivity and vasculopathy]. VEGF and TGF-ß induced pSMAD abundance were similar. Similar findings were also obtained after matching for age and PD vintage and a subgroup analysis according to time since last peritonitis (<3, <6, >6 months). In patients with more than 24 months of PD vintage, submesothelial thickness, vessel number per mmm section length and ASMA fibroblast positivity were higher in patients with peritonitis history; only the difference in ASMA positivity persisted in multivariable analyses. While PD duration and EMT were independently associated with submesothelial thickness, and glucose exposure and EMT with peritoneal vessel density in the combined groups, submesothelial thickness was independently associated with

EMT in the peritonitis free patients, and with duration of PD in patients with previous peritonitis. This detailed analysis of the peritoneal membrane in pediatric patients on PD with neutral pH, low GDP fluids, does not support the notion of a consistent long-term impact of peritonitis episodes on peritoneal membrane ultrastructure, on inflammatory and fibrotic cell activity and EMT. Peritoneal alterations are mainly driven by PD duration, dialytic glucose exposure, and associated EMT.

Keywords: peritoneal dialysis, peritonitis, peritoneal membrane, glucose, glucose degradation products, TGF-ß, VEGF, EMT

INTRODUCTION

Peritoneal dialysis (PD) provides a cost effective renal replacement therapy independent of a vascular access, greater individual freedom and at least equal patient outcome within the first years of treatment as compared to hemodialysis (van de Luijtgaarden et al., 2016). It is the preferred dialysis modality in young children and is increasingly applied in adults (Mehrotra et al., 2016). PD fluids, however, expose the peritoneal membrane to glucose concentrations 10–50 fold above physiological concentrations and depending on the manufacturing process, to high amounts of toxic glucose degradation products (GDP), to lactate and an acidic pH in single chamber PD solutions. With extended exposure, the peritoneum undergoes profound transformation, including progressive mesothelial cell loss, submesothelial fibrosis, angiogenesis, and vasculopathy. At the time of PD failure, submesothelial blood and lymphatic vessel number is increased (Williams et al., 2002; Braun et al., 2011). These morphological changes result in a gradual increase in small solute transport rates and loss of ultrafiltration (UF) capacity, requiring exposure to an additional glucose load, which within a vicious circle ultimately results in UF and PD failure (Davies et al., 1998, 2001).

Separation of the glucose from the buffer compound, lactate or bicarbonate, allowed introduction of neutral pH, low GDP fluids and raised hope to prevent long term deterioration of the peritoneal membrane, based on numerous *in vitro* and experimental *in vivo* studies (Mortier et al., 2003, 2004, 2005; Grossin et al., 2006; Rippe, 2009; Bajo et al., 2011). Clinical trials revealed higher CA125 effluent concentrations (Haas et al., 2003; Szeto et al., 2007), a putative marker of mesothelial cell viability, and lower hyaluronic acid and procollagen peptide concentrations, suggesting improved peritoneal membrane integrity (Williams et al., 2004). A recent analysis of the peritoneal membrane in children at the time of PD catheter insertion, as well as while on chronic PD with neutral pH, low GDP PD fluids, however, revealed doubling of peritoneal microvascularisation and endothelial exchange area within a few months of PD initiation. These findings independently predicted small solute transport rates. Submesothelial fibrosis progressed less fast (Schaefer et al., 2018). These changes were accompanied by induction of VEGF and TGF-β induced SMAD phosphorylation, by epithelial-mesenchymal transition (EMT), and by inflammatory cell invasion (Schaefer et al., 2018). Lymphatic vessel density and podoplanin positivity – markers of emerging encapsulating peritoneal sclerosis (Braun et al., 2011) – remained largely unchanged. These findings raised questions regarding the assumption of significantly improved biocompatibility as reflected by better preservation of peritoneal membrane integrity and transport function, with neutral pH, low GDP fluids (Blake, 2018). They also emphasized the need for an in-depth analysis of the underlying pathomechanisms, with an ultimate goal of improving PD efficacy and sustainability.

Peritonitis episodes remain a common complication of PD (Andreoli et al., 1999; Mehrotra et al., 2016) and have independently been associated with poorer technique and patient survival (Davies et al., 1996; Boudville et al., 2012; Ye et al., 2017). In an early study comprising 233 PD patients on acidic, high GDP fluids, single peritonitis episodes had no effect on peritoneal small solute transport and UF, whereas recurrent peritonitis episodes increased the D/P creatinine and reduced the UF capacity. Functional changes correlated with the severity of infection as assessed by the cumulative dialysate leukocyte count (Davies et al., 1996). A more recent study with the same fluid type and close monitoring of transport function after the first peritonitis episode suggested recovery of peritoneal small solute transport within 2 weeks post peritonitis, but only subtotal recovery of UF (Ates et al., 2000). In a cohort of 137 patients treated with both low and high GDP fluids and 92 patients with a history of a single episode of peritonitis, the latter exhibited significantly and persistently increased small solute transport, and decreased large molecule transport and UF rates (van Diepen et al., 2015); subgroup analyses with regard to the PD fluid type were not performed.

Data on the impact of peritonitis on peritoneal membrane ultrastructure are few. In Di Paolo et al. (1986) demonstrated peritonitis associated degeneration of the mesothelium and alterations of the connective tissue, which partly resolved within several months of the peritonitis episode. In rodents, acute peritonitis induced loss of mesothelial cells, EMT, and fibrosis (Katz et al., 2012; Balogh et al., 2015). Peritoneal overexpression of the inflammatory cytokines interleukin 1-beta and TNF-alpha, which are upregulated earliest in patients with acute peritonitis (Zemel et al., 1994), induced VEGF and angiogenesis, increased solute permeability and reduced UF (Margetts et al., 2002). We now provide a detailed analysis of the impact of peritonitis episodes on peritoneal membrane integrity, cellular infiltration, EMT, and key cytokine abundance in children treated with neutral pH, low GDP peritoneal dialysis solutions. Children are largely devoid of preexisting comorbidities such as age and life-style related tissue and vascular alterations, mainly

suffer from congenital disorders mostly limited to the kidneys and urinary tract (Harambat et al., 2012) and therefore are particularly positioned to facilitate the study of PD treatment and peritonitis induced changes of the peritoneum.

MATERIALS AND METHODS

Biopsy Sampling

Parietal peritoneal biopsy samples from 25 centers were obtained within the scope of the International Pediatric Peritoneal Biobank study. Sampling followed a standardized protocol as described previously (Schaefer et al., 2016). All samples were fixed with needles on cork and instantaneously stored in formalin. After transfer to Heidelberg, parietal tissue samples were embedded in paraffin and underwent immunostaining and digital imaging analysis. Case report forms were provided online via the International Peritoneal Dialysis Network[1] or by mail and entered into the central data base. The protocol was approved by local ethical boards and performed in accordance with the local national Medical Association's professional code of conduct (Landesärztekammer Baden-Württemberg) and the declaration of Helsinki. Oral and written consent was obtained from parents and patients as appropriate. The study was registered at www.clinicaltrials.gov (NCT01893710). Between February 2011 and January 2018, 87 parietal peritoneal samples were obtained from 84 children on neutral pH, low GDP PD fluid (45 with pH 7.4 bicarbonate fluid, 15 with pH 7.0 lactate fluid, 24 with pH 7.4 lactate/bicarbonate fluid). The effect of the low GDP PD treatment on peritoneal membrane integrity and function has been published previously (Schaefer et al., 2018). This analysis focused on the distinct effects of peritonitis episodes. Three patients underwent a repeat biopsy, 2 of whom had experienced a single episode of peritonitis before the first sampling. These biopsies were included in the present analysis. The median patient age was 5.7 (2.7, 13.5) years, and PD vintage was 16.9 (7.3, 36.0) months. Daily dialytic glucose exposure was calculated from the most recent, stable PD regime. Two patients with candida peritonitis were excluded from the analysis.

Immunostaining

All specimens underwent hematoxylin-eosin (H&E) and acid fuchsin orange (AFOG) staining according to standard protocols. Immunostaining was performed with the following antibodies: calretinine, podoplanin (D2-40), CD31, CD45, CD68, ASMA (alpha smooth muscle actin), FSP-1, and cytokeratin. All slides were scanned using Nanozoomer Digital pathology system and analyzed by digital microscopy using Aperio Image Scope version 11. Mesothelial cells integrity was assessed on H&E, calretinine, and podoplanin stained slides and quantified in a semi-quantitative way (0 = no cells present, 1 = 1–24% of the surface covered, 2 = 25–49% of the surface covered, 3 = 50% of cells present, 4 = 51–75%, 5 = 76–95%, 6 = complete coverage). Submesothelial thickness was measured on H&E and CD31 stained slides on at least five different points. Vessels were stained by CD31, lymphatic vessels by D2-40. Quantification was performed by Microvessel algorithm (Aperio Precision Image Analysis), to calculate the number of blood vessels; podoplanin positive lymphatics were subtracted from the CD31 positive vessels (stains both, blood, and lymphatic vessels). Submesothelial microvessel number/mm was calculated as absolute number of vessels in the submesothelial area per 1 mm length of surface peritoneum. Vasculopathy was quantitated as described previously (Honda et al., 2008; Schaefer et al., 2018). Endothelial surface area relative to peritoneal volume was calculated for the total vessel density, blood vessel density, and lymphatic vessel density in a following way: the endoluminal perimeter of CD31/podoplanin stained endothelium *section thickness *number of vessels divided by the analyzed peritoneal area *section thickness ($\mu m^2/\mu m^3$). ASMA positivity and CD45 lymphocyte and CD68 macrophage infiltration was quantified in a semi-quantitative way (0–3 score). AFOG stained slides were evaluated for fibrin deposits. EMT cells were determined as previously described using calretinin staining (Yanez-Mo et al., 2003) and findings reconfirmed by cytokeratin and FSP1 co-staining.

Statistics

Data distribution was assessed graphically and by Shapiro–Wilk test. Data are presented as median with interquartile range (IQR). To assess the differences between the groups, ANOVA or Kruskal–Wallis test were used based on the data distribution. Because of the low sample size, matching by the coarsened exact matching method (Blackwell et al., 2009) was performed for age, PD duration and glucose exposure. The exposure variable was history of peritonitis. Patients without peritonitis were matched to patients with any positive number of previous peritonitis episode. Multivariable linear regression analyses in a forward entry, starting with a univariate analyses were performed to test the PD characteristics with the peritoneal morphology in patients with and without peritonitis; log transformation was performed in case of unequal data distribution. Analyses were performed by STATA13 software (StataCorp, College Station, TX, United States), two-sided tests were performed.

RESULTS

Patient Population

Out of 82 patients included in the analysis, 37 patients had a history of peritonitis, and 16 out of the 37 had two or more peritonitis episodes. Time interval between the last peritonitis episode and tissue sampling was 9 (4, 36) weeks. The organisms causing peritonitis were *Staphylococcus* (*aureus*, *warneri*), *Enterococcus faecalis*, *Brevundimonas vesicularis*, *Enterobacter* (*cloacae*, *asburiae*), *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Escherichia coli*, *Streptococcus pyogenes*, and *Pseudomonas* (*stutzeri* and *aeruginosa*). In seven episodes the dialysate culture remained negative and in three episodes

[1] www.pedpd.org

TABLE 1 | Patient and PD treatment characteristics and blood biochemistry at time of biopsy.

	No peritonitis	Previous peritonitis	*p*-value
Patients (n)	47	38	
Age (years)	6.2 (2.7, 11.8)	5.3 (2.4, 13.5)	0.87
Gender (% female)	48%	44%	0.75
BSA (m^2)	0.8 (0.5, 1.1)	0.8 (0.5, 1.4)	0.57
PD duration (months)	12.8 (7.3, 27.0)	21.0 (12.0, 36.0)	0.053
Glucose exposure (g/m^2/day)	100 (91, 160)	105 (95, 127)	0.75
Urine output (ml/24 h)	800 (213, 1225)	500 (150, 1100)	0.51
Anuric (%)	19	39	0.064
Creatinine (mg/dl)	8 (4.2, 11.27)	5.05 (3.34, 8.61)	0.22
Albumin (g/l)	37 (34, 40)	35 (31, 41)	0.51
BUN (mg/dl)	57 (38, 67)	42 (21, 57)	0.09
Hemoglobin (g/dl)	10.2 (9.4, 11.5)	10.8 (9.5, 11.7)	0.66
Calcium (mmol/l)	2.4 (2.35, 2.5)	2.4 (2.3, 2.5)	0.33
Phosphorus (mmol/l)	1.73 (1.44, 2.05)	1.65 (1.38, 1.93)	0.53
PTH (pmol/l)	18.3 (12.5, 42.9)	21.4 (10.3, 40.5)	0.75
Bicarbonate (mmol/l)	24 (21, 26)	23 (21, 27)	0.96

BSA, Body surface area; BUN, blood urea nitrogen; PTH, Parathyroid hormone.

TABLE 2 | Characteristics and morphological findings of the patients treated with low GDP PD with and without history of peritonitis.

	No peritonitis	Previous peritonitis	*p*-value
Tissue samples (n)	47	38	
Mesothel absent	42%	43%	0.87
Mesothel score (0–6)	1.5 (0, 3.5)	1 (0, 3)	0.33
Submesothelial thickness (μm)	352 (264, 475)	419 (264, 550)	0.49
Microvessel density (/mm^2)	184 (112, 325)	169 (102, 251)	0.50
Submesothelial microvessel number (/mm)	60 (33, 138)	80 (30, 115)	0.60
Lymphatic vessel density (/mm^2)	24 (12, 48)	29 (21, 43)	0.49
Diffuse podoplanin staining	25%	26%	0.94
Blood cap. vessel density (/mm^2)	176 (74, 270)	143 (66, 266)	0.52
Total endothelial surface area ($\mu m^2/\mu m^3$)	10.0 (7.0, 18.3)	9.6 (5.7, 13.3)	0.35
Lym. endothelial surface area ($\mu m^2/\mu m^3$)	1.9 (0.9, 4.2)	2.0 (1.3, 3.6)	0.75
Blood cap. end. surface area ($\mu m^2/\mu m^3$)	8.1 (5.4, 12.9)	6.8 (2.1, 13.0)	0.25
L/V ratio	0.4 (0.2, 0.5)	0.4 (0.3, 0.5)	0.76
ASMA positivity (%)	47%	59%	0.27
ASMA score (0–3)	0 (0, 1)	1 (0, 2)	0.081
CD45 positivity (%)	66%	65%	0.97
CD45 score (0–3)	1 (0, 2)	1 (0, 2)	0.075
CD68 positivity (%)	60%	70%	0.18
CD68 score (0–3)	1 (0, 1.5)	1 (0, 2)	0.075
Fibrine positivity (%)	21%	22%	0.93
EMT presence	33%	41%	0.49
EMT (cells/mm^2)*	30.0 (15.0, 79.6)	18.0 (5.0, 60.3)	0.22
VEGF (% submesothelial area)	33.6 (17.6, 57.0)	32.4 (20.3, 42.9)	0.35
pSMAD (% submesothelial area)	14.2 (5.4, 27.0)	20.6 (8.7, 28.6)	0.19

*Cap., capillary; end., endothelial; submes., submesothelial; lym., lymphatic; L/V ratio, lumen diameter/vessel ratio; ASMA, alpha smooth muscle actin; EMT, epithelial to mesenchymal transition. VEGF, vascular endothelial growth factor. *Only cell scores from EMT positive patients included.*

the organism was not reported. Patient and PD treatment characteristics are given in **Table 1**. At the time of biopsy, the PD vintage was 8 months shorter in the peritonitis free patients, while dialytic glucose exposure and anthropometric parameters were similar in both groups.

Histological Findings

Despite a longer PD duration, patients with a history of peritonitis did not differ in any of the histomorphometric parameters from peritonitis free patients (**Table 2**). The extent of mesothelial cell loss, submesothelial fibrosis and

hypervascularization (i.e., blood and lymphatic vessel density per mm^2 peritoneal surface area and per mm submesothelial tissue section length) were comparable, as were the respective endothelial surface areas available for peritoneal fluid and solute transport. Both patients groups had a similar lumen over vessel ratio. This L/V ratio was lower as previously reported for children with normal renal function and children with CKD5 (Schaefer et al., 2018), i.e., the PD patients exhibited significant peritoneal vasculopathy. The relative proportion of patients with ASMA positive, activated submesothelial fibroblasts, CD45 positive leukocytes, and CD68 positive macrophages were comparable, the differences in respective semiquantitative cell scores did not reach statistical significance in unadjusted and multivariable adjusted models (**Table 2** and **Supplementary Table S1**). EMT cells, key cells in the peritoneal transformation process, and key cytokines associated with peritoneal angiogenesis, VEGF, and with fibrosis, TGF-ß induced pSMAD, were not different in the two groups.

After matching groups for age, PD duration and PD related daily glucose exposure and age, all 23 histomorphometric, cellular, and inflammatory parameters were comparable (**Table 3**).

To account for potential temporal differences, a subgroup analysis was performed according to the time since last peritonitis (<3, <6, >6 months), and again, all findings were comparable in both groups.

When stratifying patients according to the number of peritonitis episodes experienced, the mesothelium was significantly less well preserved in patients with two and more peritonitis episodes and the L/V ratio (e.g., vasculopathy) was more severe (**Table 4**). These patients, however, were on PD for a longer period of time and the difference did not persist in multivariable analysis when PD duration was included as an independent variable (**Supplementary Table S2b**).

Patients who have been on PD for at least 24 months and had a history of peritonitis had a higher submesothelial thickness and a higher number of vessels per mm section

TABLE 3 | Comparisons of age, PD-vintage and dialytic glucose exposure matched low GDP PD patients with and without history of peritonitis.

	No peritonitis	Previous peritonitis	*p*-value
Patients (n)	24	24	
Age (years)	4.0 (1.8, 9.4)	3.3 (1.5, 10.1)	0.71
Gender (% female)	46%	58%	0.39
BSA (m^2)	0.6 (0.4, 1.2)	0.6 (0.5, 1.0)	0.88
Urine output (ml/24 h)	1050 (400, 1425)	500 (300, 1100)	0.45
Anuric (%)	29	47	0.27
PD duration (months)*	11.3 (8.5, 21.4)	12.0 (8.5, 22.4)	0.66
Glucose exposure (g/m^2/day)	97 (89, 132)	100 (85, 108)	0.64
Mesothel absent	46%	38%	0.53
Mesothel score (0–6)	0.5 (0.0, 3.5)	1.0 (0.0, 3.0)	0.91
Submesothelial thickness (µm)	304 (200, 358)	413 (250, 500)	0.24
Microvessel density (/mm^2)	200 (107, 325)	170 (97, 318)	0.82
Submesothelial microvessel number (/mm)	59 (32, 75)	82 (30, 116)	0.21
Lymphatic vessel density (/mm^2)	39 (23, 56)	33 (22, 46)	0.41
Diffuse podoplanin staining	33%	23%	0.42
Blood cap. vessel density (/mm^2)	176 (71, 328)	139 (66, 362)	0.72
Total endothelial surface area ($\mu m^2/\mu m^3$)	10.0 (7.7, 19.0)	10.2 (5.9, 16.4)	0.82
Lym. endothelial surface area ($\mu m^2/\mu m^3$)	3.4 (1.8, 5.7)	2.6 (1.3, 4.4)	0.30
Blood cap. end. surface area ($\mu m^2/\mu m^3$)	8.0 (4.1, 12.8)	6.7 (3.3, 15.7)	0.89
L/V ratio	0.4 (0.2, 0.5)	0.4 (0.3, 0.5)	0.28
ASMA positivity (%)	54%	58%	0.77
ASMA score (0–3)	1 (0, 1)	1 (0, 2)	0.55
CD45 positivity (%)	83%	71%	0.30
CD45 score (0–3)	1 (1, 1.5)	1 (0, 2)	0.89
CD68 positivity (%)	67%	79%	0.33
CD68 score (0–3)	1 (0, 1.5)	2 (1, 2)	0.11
Fibrine positivity (%)	25%	25%	1.00
EMT presence	46%	42%	0.77
EMT (cells/mm^2)**	49 (20, 198)	21 (8, 65)	0.34
VEGF (% submes. area)	32.2 (19.2, 63.1)	35.0 (20.3, 51.1)	0.50
pSMAD (% submes. area)	18.1 (6.2, 29.1)	20.3 (7.3, 26.7)	0.65

*Cap., capillary; end., endothelial; submes., submesothelial; lym., lymphatic; L/V ratio, lumen diameter/vessel ratio; ASMA, alpha smooth muscle actin; EMT, epithelial to mesenchymal transition. VEGF, vascular endothelial growth factor. *At time of biopsy. **Only cell scores from EMT positive patients included.*

TABLE 4 | Comparisons of PD patients according to the number of previous peritonitis episodes.

	No peritonitis	1 peritonitis	≥ 2 peritonitis	*p*-value
Tissue samples (n)	47	22	16	
Age (years)	6.1 (2.6, 11.3)	6.2 (1.6, 16.4)	6.7 (3.1, 13.2)	0.96
Gender (% female)	46%	55%	38%	0.58
BSA (m^2)	0.8 (0.5, 1.1)	0.9 (0.5, 1.5)	0.8 (0.6, 1.1)	0.74
PD duration (months)*	12.4 (7.3, 24.5)	13.8 (9.0, 26.0)	32.9 (21.0, 70.0)	0.018
Glucose exposure (g/m^2/day)	99.7 (91.6, 177.6)	101.8 (96.3, 117.1)	111.9 (94.8, 153.8)	0.62
Mesothel absent	39%	27%	71%	0.037
Mesothel score (0–6)	1.5 (0.0, 4.0)	2.0 (0.0, 3.0)	0.0 (0.0, 1.0)	0.045
Submesothelial thickness (μm)	352 (258, 450)	305.3 (267, 500)	510 (300.2, 767.3)	0.21
Microvessel density (/ mm^2)	194 (115, 336)	159 (95, 211)	192 (150, 271)	0.15
Microvessel number (/mm)	61.3 (32.8, 155, 3)	50.4 (27, 82.1)	96.2 (79.9, 179, 4)	0.059
Lymphatic vessel density (/mm^2)	24.4 (12.0, 48.2)	22.7 (18.5, 35.4)	33.3 (28.3, 46.3)	0.44
Diffuse podoplanin staining	22%	30%	29%	0.71
Blood cap. vessel density (/mm^2)	175.7 (74.1, 269.5)	128.6 (63.2, 190.0)	188.1 (123.9, 406.2)	0.32
Total end. surface area ($\mu m^2/\mu m^3$)	10.1 (7.4, 18.7)	9.0 (5.2, 11.8)	10.2 (8.5, 15.5)	0.17
Lymphatic end. surface area ($\mu m^2/\mu m^3$)	1.9 (0.9, 4.2)	1.7 (1.2, 3.7)	2.5 (1.6, 2.9)	0.94
Blood cap. end. surface area ($\mu m^2/\mu m^3$)	8.1 (5.4, 12.9)	4.6 (2.1, 8.9)	7.7 (4.0, 15.7)	0.20
L/V ratio	0.4 (0.3, 0.5)	0.5 (0.4, 0.5)	0.3 (0.2, 0.4)	0.018
ASMA positivity (%)	48%	59%	53%	0.68
ASMA score (0–3)	0.0 (1.0, 1.0)	1.0 (0.0, 2.0)	1.0 (0.0, 3.0)	0.42
CD45 positivity (%)	67%	68%	65%	0.97
CD45 score (0–3)	1.0 (0.0, 2.0)	1.5 (1.0, 2.0)	1.0 (0.0, 2.0)	0.76
CD68 positivity (%)	57%	77%	65%	0.25
CD68 score (0–3)	1.0 (0.0, 2.0)	1.5 (1.0, 2.0)	1.0 (0.0, 2.0)	0.15
Fibrine positivity (%)	22%	18%	24%	0.91
EMT presence	33%	41%	41%	0.72
EMT (cells/mm^2)**	35.0 (10.0, 95.0)	23.0 (6.1, 45.3)	15.0 (1.0, 60.3)	0.45
VEGF (% submes. area)	33.6 (17.6, 57.0)	33.3 (20.3, 42.2)	28.7 (21.1, 49.5)	0.64
pSMAD (% submes. area)	15.3 (5.8, 27)	13.6 (6.6, 26.2)	25.8 (12.2, 33.6)	0.068

*Cap., capillary; end., endothelial; submes., submesothelial. L/V ratio, lumen diameter/vessel ratio; ASMA, alpha smooth muscle actin; EMT, epithelial to mesenchymal transition. VEGF, vascular endothelial growth factor. *At time of biopsy. **Only cell scores from EMT positive patients included.*

length, but did not differ in the vessel density per section area compared to patients with a similar dialysis vintage and no history of peritonitis (**Table 5**). They were also more likely to have activated ASMA positive submesothelial fibroblasts compared to peritonitis free patients. In multivariable analyses, adjusting for PD duration and glucose exposure, the difference in ASMA positivity remained significant ($p = 0.012$).

In multivariable analyses comprised of data from all biopsies and after adjusting for age, previous peritonitis, dialytic glucose exposure, PD duration and presence of EMT, submesothelial thickness was independently associated with PD duration and the presence of EMT ($p = 0.002/0.036$). Glucose exposure independently predicted peritoneal vessel density and EMT submesothelial microvessel number per mm tissue section ($p = 0.078$ and 0.027) (**Supplementary Tables S2a–c**). In subgroup analyses, after adjusting for age, glucose exposure, PD duration and EMT, submesothelial thickness was independently associated with EMT in peritonitis free patients ($p = 0.04$), and with duration of PD in patients with previous peritonitis ($p = 0.01$) (**Supplementary Tables S2d**,**e**).

DISCUSSION

This is the first detailed analysis of the long term impact of peritonitis on peritoneal membrane integrity in a substantial number ($n = 82$) of pediatric patients on chronic PD. Patients with and without a history of peritonitis did not differ in any of the histomorphometric parameters, nor in inflammatory cell invasion, EMT, or cytokine expression. These findings suggest that, PD fluid associated toxicity in contrast to peritonitis episodes drives peritoneal membrane transformation.

Experimental *in vivo* studies have previously demonstrated major peritoneal inflammatory and fibrotic changes with bacterial and LPS induced peritonitis (Hautem et al., 2017). Repeated peritoneal equilibration tests suggest rapid recovery of peritoneal solute transport in most patients after a single episode of peritonitis (Davies et al., 1996; Ates et al., 2000), but persistent changes with repeated peritonitis episodes (Davies et al., 1996), ultimately associating with worse technique and patient outcome (Boudville et al., 2012; Ye et al., 2017). Most of these data were obtained in patients treated with acidic, high GDP PD fluids. In a recent RCT, patients

TABLE 5 | Comparisons low GDP PD patients with and without history of peritonitis, who were on PD for at least 24 months.

	No peritonitis	Previous peritonitis	*p*-value
Tissue samples (n)	14	16	
Age (years)	7.4 (4.4, 16.7)	13.2 (6.7, 18.0)	0.35
Gender (% female)	42%	38%	0.82
BSA (m^2)	0.7 (0.6, 1.3)	1.0 (0.8, 1.3)	0.20
PD duration (months)*	37.6 (30.3, 48.0)	52.0 (32.9, 73.0)	0.30
Glucose exposure (g/m^2/day)	124.4 (64.3, 216.2)	119.8 (111.9, 146.2)	0.82
Mesothel absent	50%	76%	0.32
Mesothel score (0–6)	0 (0, 2)	0 (0, 1.5)	0.42
Submesothelial thickness (μm)	314 (223.3, 511.0)	527 (308.5, 844.0)	0.058
Microvessel density (/mm^2)	157.4 (97.9, 278.7)	192.4 (149.7, 236.6)	0.96
Submesothelial microvessel number (/mm)	43.4 (26.0, 66.5)	85.9 (53.1, 179.4)	0.014
Lymphatic vessel density (/mm^2)	53.5 (14.5, 59.0)	25.1 (14.9, 39.5)	0.41
Diffuse podplanin staining	25%	41%	0.37
Blood cap. vessel density (/mm^2)	96 (65, 204)	189 (126, 224)	0.57
Total endothelial surface area ($\mu m^2/\mu m^3$)	8.2 (5.5, 15.2)	9.7 (8.5, 11.5)	0.93
Lym. endothelial surface area ($\mu m^2/\mu m^3$)	4.1 (1.1, 5.8)	1.7 (0.9, 2.5)	0.14
Blood cap. end. surface area ($\mu m^2/\mu m^3$)	7.3 (2.3, 9.0)	7.5 (6.8, 10.9)	0.62
L/V ratio	0.5(0.2, 0.5)	0.4 (0.2, 0.5)	0.52
ASMA positivity (%)	33%	71%	0.017
ASMA score (0–3)	0.0 (0.0, 1.5)	2.0 (0.0, 3.0)	0.56
CD45 positivty (%)	50%	76%	0.23
CD45 score (0–3)	0.5 (0.0, 1.0)	1.0 (1.0, 2.0)	0.79
CD68 positivity (%)	67%	76%	0.73
CD68 score (0–3)	1.0 (0.0, 1.0)	1.0 (1.0, 2.0)	0.14
Fibrine positivity (%)	8%	18%	0.47
EMT presence	25%	53%	0.13
EMT (cells/mm^2)**	20.0 (5.0, 20.0)	11.3 (3.0, 26.6)	0.92
VEGF (% submes. area)	46.6 (16, 49.7)	28.7 (22.2, 42.9)	0.44
pSMAD (% submes. area)	11.7 (9.2, 18.2)	25.8 (8.5, 33.6)	0.11

*Cap., capillary; end., endothelial; lym., lymphatic; submes., submesothelial. L/V ratio, lumen diameter/vessel ratio; ASMA, alpha smooth muscle actin; EMT, epithelial to mesenchymal transition. VEGF, vascular endothelial growth factor. *At time of biopsy. **Only cell scores from EMT positive patients included.*

treated with neutral pH, low GDP PD fluid experienced less frequent and less severe episodes of peritonitis as compared to patients treated with high GDP fluids (Johnson et al., 2012). Albeit, not analyzed in that trial, the suggestion has been made that the impact of peritonitis episodes on peritoneal membrane integrity and transport function may be less pronounced in patients treated with low GDP fluids. These assumptions, however, could not be reconfirmed in a recent meta-analysis (Cho et al., 2014). Peritoneal biopsies taken from 5 patients treated with high GDP fluids during acute peritonitis and one to 4 months thereafter demonstrated persistent mesothelial alterations and submesothelial sclerotic lesions (Di Paolo et al., 1986).

A more recent study comparing peritoneal morphology of 23 patients on low and 23 patients on high GDP fluid, suggested better preservation of the mesothelial cell layer and less submesothelial fibrosis and vasculopathy with low GDP fluid. These differences, however, were lost when previous peritonitis episodes were taken into account, suggesting that potential benefits of the low GDP fluid are superimposed by untoward peritonitis effects (Del Peso et al., 2016). Our findings in pediatric patients who used low GDP PD fluids do not support the notion of peritonitis related peritoneal sequelae. In parietal peritoneal tissue from patients who had experienced previous peritonitis episodes, we did not observe any differences in histomorphological features, inflammatory cell invasion, in VEGF, and in TGF-ß induced pSMAD abundance and in the degree of EMT, as compared to peritoneal specimens from peritonitis free patients. EMT cells secrete VEGF and TGF-ß (Aroeira et al., 2005, 2007) and thus represent key mediators of the peritoneal transformation process (Yanez-Mo et al., 2003; Lopez-Cabrera, 2014), albeit their origin is debated (Chen et al., 2014). Matching for age to rule out preexisting differences in age related peritoneal thickness and vascularization (Schaefer et al., 2016), as well as for PD vintage and dialytic glucose exposure, reconfirmed the findings. Likewise, analyses according to the time interval since the last peritonitis episode, and the number of peritonitis episodes did not demonstrate any significant differences. In the small subgroup of patients with more than 2 years of PD, peritonitis positive patients exhibited some differences. The likelihood of activated fibroblast positivity persisted in multivariable analysis, suggesting that in patients on long term PD, peritonitis episodes may be associated with some

enhanced peritoneal profibrotic activity, but the statistical power of this subgroup analysis is limited. In the multivariable analysis of the entire cohort, peritonitis did not predict any of the key morphological peritoneal parameters.

In contrast to the minor differences attributable to peritonitis history, PD duration and dialytic glucose exposure independently predicted the peritoneal membrane histomorphology. In multivariable analyses of all patients, PD duration predicted submesothelial thickness, and glucose exposure predicted peritoneal vessel density. PD treatment induced EMT (Aroeira et al., 2007) was associated with both submesothelial fibrosis and vessel density. History of peritonitis and the number of previous peritonitis did not predict key histomorphological outcome parameters. In subgroup analyses according to peritonitis history, submesothelial thickening was independently associated with EMT in peritonitis free patients, and with duration of PD in patients with previous peritonitis. The independent association of both submesothelial fibrosis and submesothelial vessel density with the presence of EMT cells points to the key role of EMT in the peritoneal transformation process. EMT, in turn, represents a potential biomarker, and therapeutic target (Aufricht et al., 2017).

Of note, during the past few decades, prevention and treatment of peritonitis has increasingly been guided by evidence based and repeatedly updated international recommendations (KDQQI guidelines) (Li et al., 2016). Not only has the incidence of peritonitis declined (Campbell et al., 2015), but peritonitis episodes are usually recognized early and broad spectrum antibiotic treatment covering most of the bacteria is initiated promptly. None of the centers reported unsuccessful treatment of the peritonitis episodes.

Our study has important limitations. Although this is the largest study on peritonitis induced changes of the peritoneal membrane thus far, the number of patients is not extensive, and thus the sensitivity is low. Moreover, and despite current recommendations (Mujais et al., 2000; National Kidney Foundation, 2006), only a minority of the contributing centers performed PET and therefore the impact of peritonitis episodes on peritoneal membrane function could not be studied comprehensively. On the other hand, strength of the study is the patient population as children are devoid of life style and aging related preexisting tissue damage and the predominant underlying diseases of these patients, such as congenital abnormalities of the kidneys and urinary tract, do not affect peritoneal integrity. This allows for a more sensitive and specific analysis. Whether growing children have a greater plasticity and thus a greater potential to compensate for peritonitis induced transient peritoneal damage is unknown.

In conclusion, our detailed analysis of the peritoneal membrane in pediatric patients on maintenance PD with neutral pH, low GDP fluids, does not support the notion of a consistent long-term impact of peritonitis episodes on the peritoneal membrane ultrastructure, on inflammatory and fibrotic cell activity and EMT. Peritoneal alterations are primarily driven by PD duration and dialytic glucose exposure.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

MB and BS contributed to the conception of the study, collected specimens and patient data, performed immunostainings and digital image analyses, and wrote the manuscript. FL contributed to the histological and digital imaging analyses. KV, PS, CT, RC, MD, GM-L, RB, AZ, PR, and BW collected specimens and clinical data and contributed to the manuscript. BW and FS contributed to the conception of the study. AU contributed to the conception of the study, performed the statistical analysis, and wrote the manuscript. CS conceptualized the study, contributed to specimen sampling, histological and digital imaging analyses and statistical analyses, and wrote the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00356/full#supplementary-material

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The Peritoneal Surface Proteome in a Model of Chronic Peritoneal Dialysis Reveals Mechanisms of Membrane Damage and Preservation

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Peritoneal dialysis (PD) fluids are cytotoxic to the peritoneum. Recent studies have shown that alanyl-glutamine (AlaGln) modulates the cellular stress response, improves mesothelial cell survival, reduces submesothelial thickening in experimental models of PD, and in clinical studies improves PD effluent cell stress and immune responses. However, the mechanisms of AlaGln-mediated membrane protection are not yet fully understood. Here, we explore those mechanisms through application of a novel proteomics approach in a clinically relevant *in vivo* model in rats. Experimental PD was performed for 5 weeks using conventional single-chamber bag (SCB) or neutral dual-chamber bag (DCB), PD fluid (PDF), with or without AlaGln supplementation, via a surgically implanted catheter. Rats subjected to a single dwell without catheter implantation served as controls. The peritoneal surface proteome was directly harvested by detergent extraction and subjected to proteomic analysis by two-dimensional difference gel electrophoresis (2D-DiGE) with protein identification by mass spectrometry. An integrated bioinformatic approach was applied to identify proteins significantly affected by the treatments despite biological variation and interfering high abundance proteins. From 505 of 744 common spots on 59 gels, 222 unique proteins were identified. Using UniProt database information, proteins were assigned either as high abundance plasma proteins, or as cellular proteins. Statistical analysis employed an adapted workflow from RNA-sequencing, the trimmed mean of *M*-values (TMM) for normalization, and a mixed model for computational identification of significantly differentially abundant proteins. The most prominently enriched pathways after 5 weeks chronic treatment with SCB or DCB, PDFs belonged to clusters reflecting tissue damage and cell differentiation by cytoskeletal reorganization, immune responses, altered metabolism, and oxidative stress and redox homeostasis. Although the AlaGln effect was not as prominent, associated enriched pathways showed mostly regression to control or patterns opposite that of the PDF effect. Our study describes the novel peritoneal surface proteome through combined proteomic and bioinformatic analyses,

and assesses changes elicited by chronic experimental PD. The biological processes so identified promise to link molecular mechanisms of membrane damage and protection in the *in vivo* rat model to pathomechanisms and cytoprotective effects observed *in vitro* and in clinical PD.

Keywords: N(2)-alanyl-L-glutamine, cytoprotective additive, *in vivo* proteomics, mesothelial cells, peritoneal immune response, PD rat model, animal model

INTRODUCTION

Peritoneal dialysis (PD) is a life-saving home-based renal replacement therapy which, despite improved preservation of residual renal function, remains underutilized due to its limitations of peritonitis and peritoneal fibrosis, leading to membrane, and technique failure (Davies, 2013). Chronic exposure to PD fluid (PDF) causes injury to the mesothelial cell layer of the peritoneal wall that serves as the dialysis membrane. Included among the contributors to the attendant chronic inflammation are deficient induction of cytoprotective cell stress and repair pathways and local immune dysfunction (Kratochwill et al., 2009, 2011; Bender et al., 2011).

Peritoneal dialysis fluid in single chamber bags (SCB) contain all components of the solution in a single compartment. The pH around 5.2 is a compromise between lower formation of glucose degradation products (GDP) at lower pH and damage to the peritoneum including infusion pain. In dual chamber bags (DCB), in contrast, glucose is separated from buffer components during heat sterilization (Garcia-Lopez et al., 2012). The initial acid pH minimizes formation of GDP, while the combined solution instilled into the patient's peritoneal cavity is restored to neutral pH. Although DCB PDFs cause reduced *in vitro* damage to cells (Topley et al., 1996; Jorres et al., 1998; Del Peso et al., 2015), these fluids may also be less potent inducers of beneficial cell stress, and repair pathways (Schmitt and Aufricht, 2016). This deficiency can result in chronic inflammation, complement activation and increased vascularity, likely underlying the persistent lack of clinical evidence for DCB PDF superiority (Bartosova et al., 2018; Schaefer et al., 2018). Taken together, data from the last 20 years of PD research support the idea that enhancement of beneficial cell stress and repair mechanisms (while in parallel countering chronic inflammation) holds greater promise for reduction of peritonitis and peritoneal fibrosis than does reduction in PDF toxicity.

Alanyl-glutamine (AlaGln) is a substance that has the potential to accomplish this goal. Our group has demonstrated *in vitro* that AlaGln modulates the cellular stress response and improves survival of mesothelial cells (Kratochwill et al., 2012). A first-in-human clinical trial showed that glutamine deficiency during clinical PD is linked to peritoneal pathomechanisms, such as impaired stress response and host defense (Kratochwill et al., 2016). A pilot trial indicated improved PD effluent cell function with regards to stress and immune responses due to priming of effluent cells by AlaGln and reducing basal chronic inflammation (Herzog et al., 2017). The recently conducted multicenter phase II trial confirmed protective effects of AlaGln at the level of surrogate markers of peritoneal membrane status and immune competence (Vychytil et al., 2018). Promising results in this trial regarding decreased peritoneal protein loss require in depth analysis of the potential membrano-protective mechanism.

Evaluating the molecular mechanism of the effect of AlaGln on peritoneal membrane tissue would require biopsies taken from patients after extended periods of treatment with AlaGln-supplemented PDF (and from matched controls). In the meantime, chronic animal models help improve our understanding of peritoneal transport and of peritoneal immune and inflammatory molecular processes in PD therapy and support the selection process for suitable surrogate markers. Indeed, Ferrantelli et al. (2016) has reported reduced submesothelial thickening and decreased vascularization by AlaGln in animal models of PD. Whereas in these studies the focus was on individual candidate markers of chronic inflammation, proteomic analysis would provide a holistic view of the complex molecular dynamics influencing the peritoneal membrane and an attractive approach to understand the molecular mode-of-action of AlaGln.

A recent proteomics study provided unprecedented insight into the composition of PD effluent and the molecular mode-of-action of AlaGln in a single dwell with conventional SCB PDF (Herzog et al., 2018). Nevertheless, this information is a global heterogeneous mix of the plasma proteome and the secretome of peritoneal cell populations, containing greater numbers of leukocytes than of mesothelial cells.

In contrast to human studies relying mostly on PD effluent, animal models allow sampling of the peritoneal wall surface for proteomic analysis. Here, we apply a proteomic approach in a clinically relevant *in vivo* model to characterize the peritoneal surface proteome and analyze the effect of AlaGln addition to PDF on molecular processes in response to chronic PDF exposure.

MATERIALS AND METHODS

Standard chemicals were purchased from Sigma - Aldrich (St Louis, MO, United States) if not specified otherwise.

In vivo PD Model in Rats

Male Wistar rats (13 weeks, 300 g, Harlan; CPB, Horst, Netherlands) were housed under normal conditions, with water and food *ad libitum*. The study was approved by the animal care committee of the Vrije Universiteit of Amsterdam. Rats received, under isoflurane anesthesia and analgesia, a subcutaneously implanted catheter to the peritoneal cavity ("rat-o-port", Access Technologies, Norfolk Medical, Skokie, IL, United States) for

daily installation of PDF. Following 7 days of healing and daily instillation of 2 ml physiological saline (NaCl 0.9%) with 1 U/ml heparin the rats received for 5 weeks daily injections of 10 ml PDF fluid (**Figure 1**). Rats were exposed to either glucose-based, lactate buffered, acidic pH, SCB PDF (Dianeal PD4 3.86%, Baxter Healthcare, Deerfield, IL, United States; n = 15) or the same

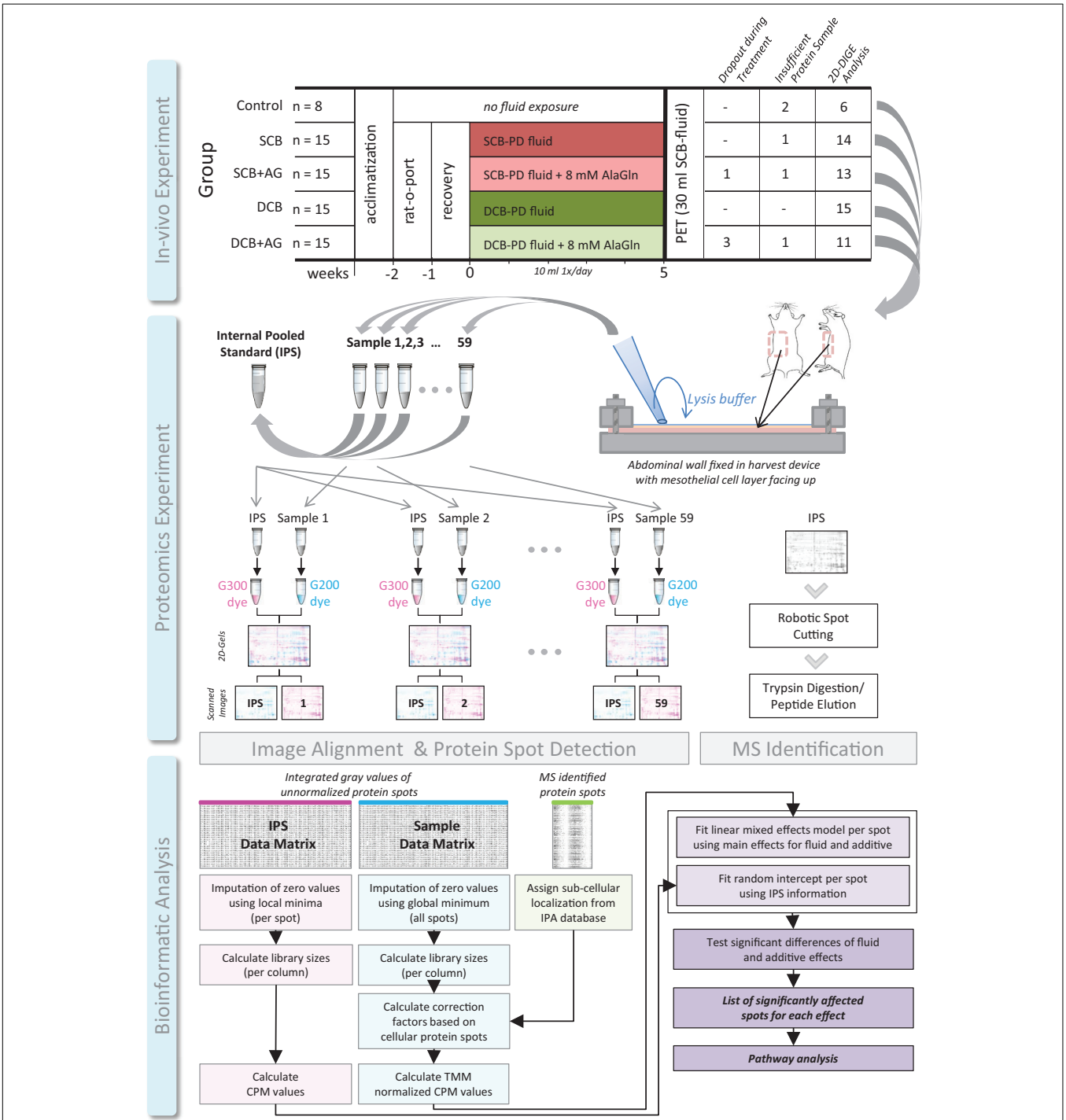

FIGURE 1 | Experimental workflow in three stages. *In vivo* experiment with 5 experimental groups. SCB, single-chamber bag; DCB, dual-chamber bag; AG, alanyl-glutamine; rat-o-port, surgically implanted catheter for PDF instillation; PET, peritoneal equilibration test. Proteomics experiment with steps sample lysis and harvest, preparation of the IPS and labeling of samples, 2D-DiGE, and fluorescent image scanning. IPS, internal pooled standard, G200, and G300 fluorescent dyes used for difference gel electrophoresis. Bioinformatic analysis, following the presented integrated workflow. CPM, counts per million relative signal calculation strategy adapted from RNA-sequencing workflows; TMM, trimmed mean of *M*-values normalization strategy adapted from RNA-sequencing workflows.

PDF with added 8 mM AlaGln (Dipeptiven, Fresenius-Kabi, Bad Homburg, Germany) (SCB+AG; n = 15) or to glucose-based, bicarbonate/lactate buffered, low GDP, neutral pH, DCB PDF (Physioneal 40 3.86%, Baxter Healthcare, n = 15) or the same neutral pH PDF with added 8 mM AlaGln (DCB+AG; n = 15). Rats without an implanted catheter (n = 8) served as controls and were not subjected to daily PD. Control animals were kept under the same conditions in parallel and were subjected to the PET and harvest on the same day.

Clinical condition of the animals was monitored daily, and weight was recorded weekly. On the last day of the experiment rats received a 90 min peritoneal equilibration test (PET) under anesthesia. 30 ml PDF (Dianeal PD4 3.86%) were instilled, and the abdomen was periodically moved to ensure complete wetting of the peritoneal membrane. After 90 min the PD effluent was drained via a standard venous catheter (Venflon Pro; Becton Dickinson, Franklin Lakes, NJ, United States) placed in the lower right abdomen into the peritoneal cavity, the rats were sacrificed and the abdominal walls were harvested.

Peritoneal Surface Proteome Isolation

Using a specifically designed harvesting device (**Supplementary Figure S1**), peritoneal surface proteins were isolated directly from the abdominal walls. The contra-lateral parietal peritoneal wall of the implanted catheter was removed and fixed with the mesothelial cell layer (luminal) side facing up and washed twice (250 mM sucrose, 10 mM Tris–HCl, pH 7.0) to remove residual plasma on the surface. Lysis buffer (500 µl, 250 mM sucrose, 10 mM Tris–HCl, pH 7.0, 0.25% Triton-X 100) was applied to the surface for 2 min, followed by very gentle scraping with a sterile disposable cell scraper to facilitate cell lysis. The resulting protein lysate was collected by tilting the device and pipetting into a collection tube.

For proteomics analyses, protein samples were precipitated (100% acetone, overnight, −20°C), washed with ethanol and resuspended in 200 µl (30 mM Tris, pH 8.5, 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM EDTA, 1 tablet of Complete Protease Inhibitor (Roche, Basel; Switzerland), and 1 tablet of phosphatase inhibitor (PhosSTOP, Roche) per 100 mL. Total protein concentration was determined according to the manufacturer's instructions (2D Quant kit; GE Healthcare, Uppsala, Sweden).

Two-Dimensional Difference Gel Electrophoresis (2D-DiGE)

An internal pooled standard (IPS) was prepared containing 47 samples in equal parts and was used in all individual gels. Proteins were labeled with fluorescent dyes (Refraction-2D Labeling Kit; NH DyeAGNOSTICS, Halle, Germany) according to the manufacturers' protocol with minor modifications. In brief, 30 µg protein of all individual samples (n = 59) was mixed with 0.2 nmol reconstituted G-Dye200 and the IPS was mixed with 0.2 nmol G-Dye300 (27 µg per gel). Labeling of the IPS was performed in one batch to achieve a uniform standard. Following the labeling (30 min, on ice in the dark) the reaction was stopped (1 µl of stop solution) and incubated for another 10 min on ice in the dark. For every individual gel, the labeled sample and the labeled IPS were mixed together shortly before rehydration.

Isoelectric Focusing

First dimensional protein separation with isoelectric focusing was conducted on a Protean IEF Cell (BioRad, Hercules, CA, United States) with 57 µg of total protein (consisting of 30 µg labeled sample and 27 µg labeled IPS) per immobilized pH gradient (IPG) strip [ReadyStrip IPG Strips 24 cm pH 3-10 non-linear (BioRad)]. The rehydration mix containing both sample and IPS was brought to a final volume of 450 µl and a final concentration of 5 M urea, 0.5%w/v CHAPS, 0.5% v/v Pharmalyte (GE Healthcare), and 12 µl/ml of DeStreak reagent (GE Healthcare). The strips were overlayed with mineral oil (Bio-Rad) and actively rehydrated at 50 V for 12 h and afterward focused by increasing the voltage step by step up to 8000 V within 17 h. The procedure was carried out at 20°C using a current limit of 30 µA per strip. Focused strips were stored at −80°C until further use.

Horizontal Gel Electrophoresis

The second dimension of protein separation was performed on a HPE-FlatTop Tower (Serva Electrophoresis GmbH, Heidelberg, Germany) following the manufacturer's manual using precast non fluorescent gels (HPE Large Gel NF 12.5% Kit, Serva). The proteins in the focused IPG strips were reduced and alkylated by consecutive incubation in equilibration buffer (Serva) with 1.8 g/5ml urea mixed with 50 mg/5ml dithiothreitol (DTT) for the first and with 125 mg/5ml iodoacetamide (IAA) for the second 25 min of incubation. The gels were placed onto the cooling plates with equal amounts of cooling contact fluid (Serva) beneath the gel and anode or cathode buffer soaked electrode wicks (Serva) were placed on the gels' edges (2 mm overlapping). The equilibrated IPG strips were applied on the gels and the electrophoresis was performed in five steps at 15°C according to the manufacturer's manual. The protocol started with 100 V, 28 mA, 4 W for 30 min, followed by 200 V, 52 mA, 12 W for another 30 min. Then 300 V, 80 mA, and 20 W were applied for 10 min. After this step, the IPG strips were removed and the run continued with 1500 V, 160 mA, and 120 W for 3 h 50 min. In a last step 1500 V, 180 mA, and 160 W were applied for another 50 min. Following electrophoresis, the gels were washed (H_2O) and stored at 4°C until fluorescence image acquisition.

Fluorescence Image Acquisition and Data Analysis

Gel images with fluorescent signals were acquired using a laser scanner (ThypoonTrio, GE Healthcare) at the labeling kit manual's recommended excitation and emission wavelengths (G-Dye200: ex/em 554/575 nm; G-Dye300: ex/em 648/663 nm). The photomultiplier voltage was adjusted for near saturation of the most abundant spots.

2D Gel Image Analysis

Gel images were analyzed using the Delta2D 4.2 software (Decodon GmbH, Greifswald, Germany) with the algorithm designated for DiGE experiments. IPS images were aligned by

pair-wise warping, and spot detection was carried out on a fused image of all gels.

Protein Spot Identification by Matrix-Assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry (MS)

Preparative gels for protein identification were loaded with 200 μg of IPS protein, of which 25 μg were labeled as described above. Gels were stained with Coomassie Brilliant Blue (CBB). Following electrophoresis protein spots were fixed overnight (10% acetic acid, 40% ethanol) followed by 3 washing steps (5 min, H_2O). After overnight incubation with CBB staining solution [8% (w/v) ammonium sulfate, 2% (w/v) orthophosphoric acid (85%), 20% (v/v) methanol, and 1% (v/v) CBB stock solution 2.5% (w/v – Coomassie Brilliant Blue G250 dissolved in H_2O)] the staining was intensified with incubation for 30 min in 20% ammonium sulfate (in H_2O) and briefly destained with 10% glycerol, 20% methanol in (in H_2O). All incubation steps were carried out on a horizontal rotary shaker.

Protein spots were automatically excised (EXQuest Spot Cutter; BioRad). Excised gel plugs were washed [100 mM NH_4HCO_3, 100 mM NH_4HCO_3/ethanol (1:1) and acetonitrile] until destained. After reduction (10 mM DTT in 25 mM NH_4HCO_3) for 1 h at 56°C, the samples were alkylated (55 mM IAA in 25 mM NH_4HCO_3) for 45 min at room temperature in the dark. Following another wash step (100 mM NH_4HCO_3 and acetonitrile), samples were digested with 0.39 μg trypsin in 50 mM NH_4HCO_3 overnight at 37°C. The cleaved peptides were eluted from the gel plugs with sonication in acetonitrile/H_2O/trifluoroacetic acid (TFA) (50:45:5). Eluates were dried by vacuum centrifugation (Concentrator plus, Eppendorf, Hamburg, Germany) and the peptides were redissolved with 0.1% TFA and desalted with C18 material (C18-ZipTip columns, Millipore; Billerica, MA, United States) according to the manual. Briefly, the material was wetted with acetonitrile and equilibrated with 0.1% TFA. Peptides were loaded onto the column, followed by washing with 0.1% TFA and direct elution onto two spots of the MALDI target (Thermo Fisher Scientific, Bremen, Germany) with α-cyano-4-hydroxycinnamic acid (10 mg/ml; CHCA; LaserBio Labs, Sophia-Antipolis Cedex, France) in acetonitrile/0.1% TFA.

Mass spectrometric (MS) analyses were performed on a matrix-assisted laser desorption ionization (MALDI) LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) operated in positive mode. MS spectra were acquired in a mass range from m/z 600–4000 with a resolution setting of 100,000 at m/z 400. Acquisition parameters were: automated spectrum filter off, automated gain control on, crystal positioning system on and 5 scans/step. 10 MS spectra were acquired for each spot. For tandem mass spectrometry (MS/MS) the mass spectrometer was operated in a data-dependent mode. Utilized parameters included precursor ion isolation in the linear ion trap, 3 mass units isolation width, 3 normalized collision energies (CID; 30, 35, and 40%), activation q of 0.25 and activation time of 30 ms. The 15 most prominent ions were sequentially isolated for collision-induced dissociation (CID) fragmentation in the linear ion trap.

The acquired raw MS data files were processed (Mascot Distiller 2.7.1.0; Matrix Science, London, United Kingdom) and searched against the rat SwissProt database (Rat_20170830) using Mascot. Additional search parameters were, enzyme: trypsin/P; allowed missed cleavages: 2; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); peptide tolerance, 5 ppm; MS/MS tolerance, 0.8 Da; charge state, 1+, 2+, or 3+. The ions score was set to 20 and standard scoring was chosen. Single peptide identifications, which were not supported by another spot, were checked manually by the following criteria: reviewed/unreviewed, molecular weight and *p*I, length of peptide, score, and annotation of b- and y-ions. One protein was excluded from the results list due to wrong MW, no identified y-ions and unreviewed status.

Scaling and Normalization Approach for Derivation of Protein Spots Associated With the Effect of PDF Exposure and AlaGln Addition

Spot intensity data for each gel were exported from the Delta 2D software and imported in R as a matrix containing all intensity data for sample channels, as well as internal standard channels.

The image analysis workflow supported in the Delta 2D software ensures a data matrix that is free from missing values. The detection limits of the fluorescence dyes and the laser scanner and rounding errors result in the presence of zero values in the data matrix. This impedes further raw data processing using linear mixed model analysis (LIMMA), because the LIMMA requires log-transformed data. Zero values in the data matrix were therefore replaced with the lowest observed value in the complete matrix (sample channel) or the lowest value observed for the protein spot (IPS channel).

To account for inter-individual variation introduced by plasma proteins, a statistical workflow from RNA sequencing (RNAseq), based on trimmed mean of *M*-values (TMM) (Robinson and Oshlack, 2010) was adapted for 2D-DiGE data. Analogous to varying library size in RNAseq, the plasma-type protein spots disproportionately influence the abundance calculation of cellular-type protein spots, an effect that can be observed in many omics-type data. By scaling the spot data based on library sizes calculated only from cellular-type spots, instead of all spots as in the standard workflow, this effect was removed from the data (**Figure 1**).

Separate linear mixed effects models were fit to the normalized log-transformed spot data of each protein, taking into account the effect of PDF exposure (fluid effect) with or without AlaGln (additive effect) as opposed to sham treatment using fixed effects, along with a random intercept term representing normalization by the IPS. Fold-changes associated with individual effects were extracted from the

linear model. For data visualization, random intercepts were subtracted from TMM-normalized values. Significance values were derived from group comparisons utilizing *t*-tests with the obtained *p*-values as well as BH-corrected *q*-values given in **Supplementary Table S2**.

Statistical Analysis and Data Visualization

All statistical analyses and visualizations used R (v3.5.1[1]). Ingenuity Pathway Analysis (IPA 7.0, Qiagen[2]) was used to identify pathways and predicted up/down regulation patterns significantly affected by differentially abundant proteins, calculating a *p*-value for each functional pathway using a one-tailed Fisher exact test. Pathways with *p*-values <0.05, after correction for multiple hypothesis testing with the Benjamini-Hochberg (BH) procedure, were considered significantly enriched. The IPA z-score assesses the match of observed and predicted up/down regulation patterns and serves a predictor for the activation state.

Data Availability

All 2D-DiGE datasets for this study are included in the manuscript and the **Supplementary Files**. The raw instrument files of MALDI-MS identifications will be made available by the authors, without undue reservation, to any qualified researcher. The R code that was used to generate the analysis is disclosed as **Supplementary Material**.

RESULTS

Experimental Groups

Sixty-four of 68 rats completed the proposed protocol. Four animals failed to complete the chronic dialysis regiment, and in one animal the PET could not be conducted. Due to leakage of the harvesting device, the samples obtained from a few animals were either insufficient in volume or contaminated and thus not used for 2D-DiGE analysis. Final sample group sizes subjected to proteomic analysis were 6 controls, 14 in the SCB group, 13 in the SCB+AG group, 15 in the DCB group, and 11 in the DCB+AG group (**Figure 1**; see **Supplementary Table S1** for details on dropouts and samples with insufficient volume).

Protein Separation and Identification Workflow

Analysis of sample homogeneity based on raw spot intensity data revealed experimental variation in the data that exceeded the level typically observed in 2D-DiGE data (e.g., from cell culture samples). Although the overall technical quality of the gels was excellent (**Supplementary Figure S2**), the harvested samples clearly included materials originating not only from mesothelial cells but also plasma protein contaminants. The gel images exhibit a spot pattern that resembles both cellular-type samples, as evidenced by major spots being cytoskeletal proteins, but also plasma-type samples indicated by prototypical high abundance spots. Nevertheless, spots attributable to albumin show clearly reduced abundance compared to typical gels from serum (**Figure 2A**).

In contrast to a standard workflow in which only significantly altered protein spots are forwarded to MS identification, in this experiment abundance alterations in cellular proteins would not be detectable without subtraction of plasma protein contaminants. We therefore aimed to identify as many spots as possible of the total protein spot pattern in order to generate a comprehensive map of the peritoneal surface proteome. From the total pattern of 744 protein spots, 625 were above the threshold of intensity and spot quality and were therefore cut and analyzed by MALDI-MS. In 505 of these spots (80%), proteins were successfully identified (**Figure 2A**), linking to 222 unique protein IDs (**Supplementary Table S2**).

Information on subcellular localization of all identified proteins was extracted from IPA, allowing protein assignment either to the group of high abundance plasma proteins (plasma type) or to the group of cellular proteins (cellular type) (**Supplementary Table S2**). The proportion of cellular and plasma proteins is shown in **Figure 2B**. Computing this proportion, considering spot abundance (integrated gray volume) confirms that these spots can indeed shift the abundance distribution in favor of plasma protein-type spots. The data also show that spots without successful protein identification were on average of very low abundance.

To account for inter-individual variation introduced by plasma proteins, a statistical workflow that has been introduced for RNAseq data, but is equally suitable for matrix from any omics-type experiment, based on TMM (Robinson and Oshlack, 2010) was adapted for 2D-DiGE data. Standard 2DE preprocessing typically subtracts the amount of protein volume measured on the internal standard channel from the corresponding measurement on the sample channel. This assumes that the two amounts are measured on the same scale. This assumption however, does not hold if the proportion of plasma proteins differs between samples but not – as by design – between internal standards (i.e., spot volumes are expressed relative to different amounts of plasma protein). Consequently, adjusting for varying plasma protein content needs to be performed before information from the internal standard can be used to reduce variability arising from systematic differences between gels. Analogous to varying library size in RNAseq, spots on 2D gels that contain plasma-type proteins that are in the state of transcellular migration (or adhere to peritoneal surface denuded from mesothelial cells) and are thus "dissolved" in the tissue, may disproportionately influence the abundance calculation of cellular-type protein spots. By scaling the spot data based on library sizes calculated only from cellular-type spots, this influence was removed from the data during the TMM normalization step. The TMM normalization step (with and without the utilization of the linear model) was compared to a standard 2D-DiGE workflow which was prone to yield more statistically significant spots for the comparisons of the fluid effect and at the same time was less sensitive to the subtle changes of the additive effect (**Supplementary Figure S3**). We fit a random

[1]http://www.r-project.org/

[2]http://www.ingenuity.com

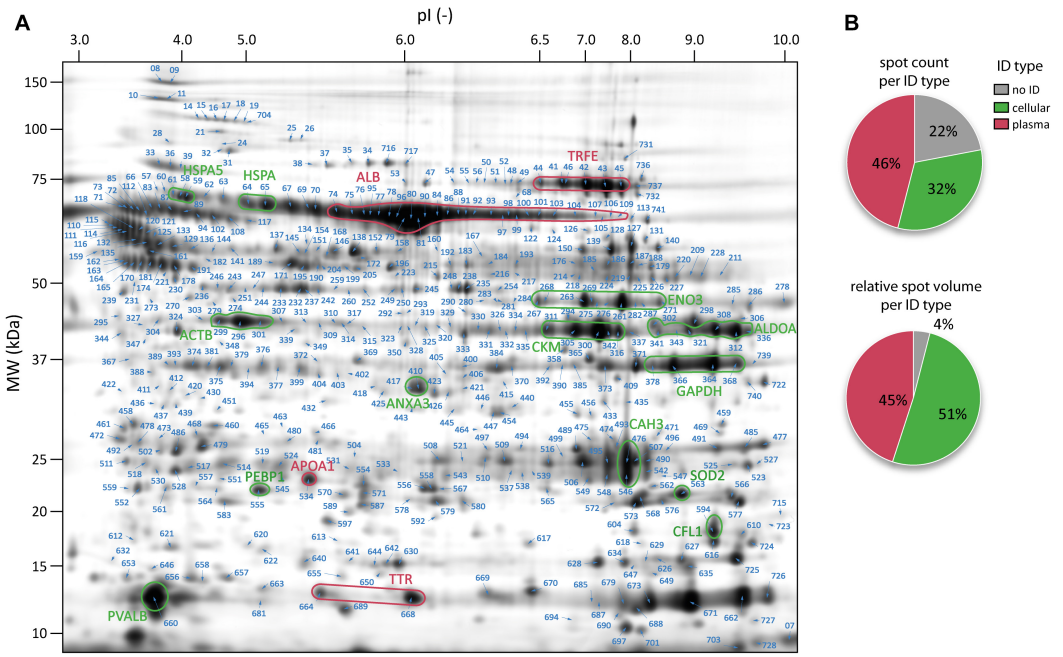

FIGURE 2 | Map of the peritoneal surface proteome. **(A)** Representative 2D gel (Fusion Image) of peritoneal surface proteome of rats. All identified spots are indicated with an arrow and their spot label. Prototypical intense cellular and plasma spots are marked and labeled with the gene name. Cellular proteins are highlighted in green and plasma proteins in red. **(B)** Pie-charts showing the spot counts (upper panel) and relative spot volumes (lower panel) for cellular and plasma proteins.

intercept for each protein using measurements from the sample channel and the internal standard. Essentially this is equivalent to subtracting the internal standard measurement from the sample measurement – as with standard processing – with the added benefit that within a linear model definition we have added flexibility to model additional error components, obtain meaningful estimates of treatment effects via linear contrasts, and get diagnostics about the model fit and error terms by means of variance components estimates.

Evaluation of Changes in Surface Proteome by Chronic PD

Trimmed mean of *M*-values-normalized data were subjected to a linear mixed effects model, considering the effect of PDF (for SCB and DCB groups), and the effect of the AlaGln additive (for SCB+AG and DCB+AG groups) as well as experimental covariates such as the internal standard signal comigrating with every applicable spot on each 2D gel. Using this model, with a threshold of $p < 0.05$, out of all 744 spots 452 protein spots (with 152 unique IDs) were significantly altered by SCB PDF (**Figure 3A**; numeric values for spot intensities have to be obtained from **Supplementary Table S2**) and 504 protein spots (with 167 unique IDs) were significantly altered by DCB PDF. 413 of these protein spots with 138 unique IDs were significantly changed by both DCB PDF and SCB PDF. The mixed model analysis identified 63 protein spots with 40 unique IDs as significantly changed in the group treated with AlaGln addition to SCB PDF, and 85 protein spots with 30 unique IDs as significantly changed in the group treated with AlaGln addition to DCB PDF. Hierarchical clustering of significantly altered proteins clearly separated the experimental groups (**Figure 3B**, top 50 molecules shown). Applying a more stringent threshold of $p < 0.01$ (highlighted in **Figure 3** and in **Supplementary Table S2**) to reduce false positives slightly lowered numbers of proteins significantly changed by PDF effect (by 14% for SCB and by 11% for DCB groups), and to a greater degree for the effect of AlaGln addition (by 72% for SCB and by 78% for DCB groups), indicating an effect magnitude for PDF markedly higher than for AlaGln addition. Consistent with this finding, initially detected changes in numerous proteins remained significant after correction for multiple testing (BH corrected $p < 0.05$: 424 spots for SCB and 494 spots for DCB groups), whereas no spots remained significant for the effect of AlaGln supplementation.

Pathway Analysis of PD-Related Proteome Changes

To avoid interpretation of false positive candidate molecules, while preserving coverage of relevant biological processes and pathways, a second level of statistical analysis was applied. UniProt accessions of significantly altered spots, together with their respective *p*-, *q*- and fold-change values (see **Supplementary Table S2** for details) were forwarded to pathway analysis, condensing the spot information on the level of unique protein coding gene names (molecules). The analysis of significantly enriched canonical pathways was BH-corrected for multiple hypothesis testing, and only proteins passing both abundance and pathway thresholds were interpreted.

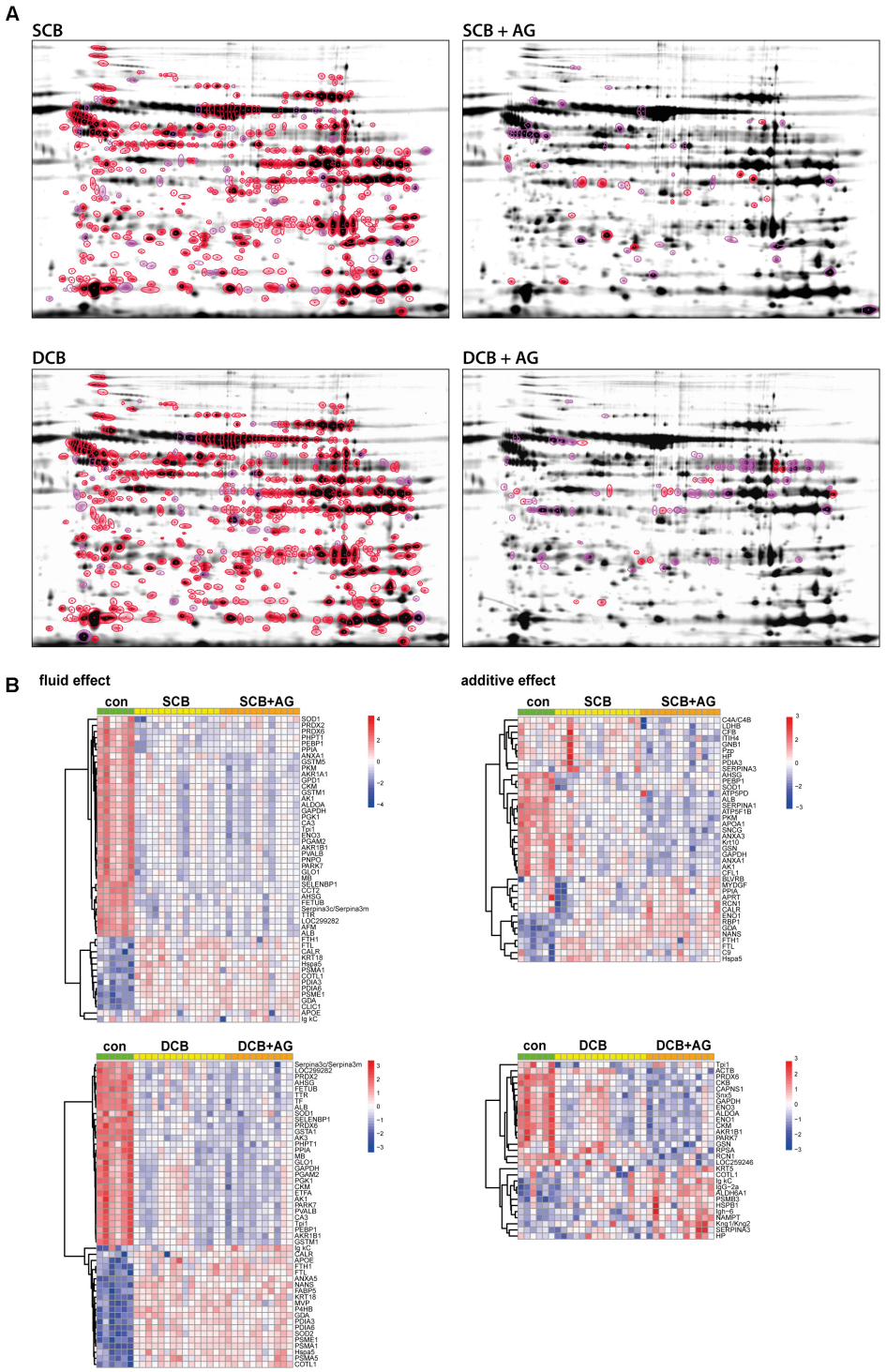

FIGURE 3 | Proteins affected by chronic treatment with PDF and addition of AlaGln **(A)** Representative 2D gel fusion images for each treatment provide an illustration of spot positions, numbers of significant spots, and the technical quality of the gels. They do not represent the dynamic range of intensities of the respective spots. Numeric values for spot abundance are calculated from integration of fluorescent values and taking into account the internal standard for the respective spot. Upper left, SCB; upper right, SCB + AlaGln; lower left, DCB; and lower right, DCB + AlaGln; highlighting significantly altered proteins with $p < 0.01$ in red and with $p < 0.05$ in pink. **(B)** Heatmap showing the top 50 significant ($p < 0.05$) spots with identification for each coefficient (upper left, fluid effect SCB; lower left, fluid effect DCB; upper right, additive effect SCB + AlaGln; and lower right, additive effect DCB + AlaGln). Values are based on TMM-normalized log2 data, with random intercepts subtracted and spots averaged via mean for each molecule (unique protein). Clustering of molecules is based on Pearson-correlation with average agglomeration. Rows were centered and scaled.

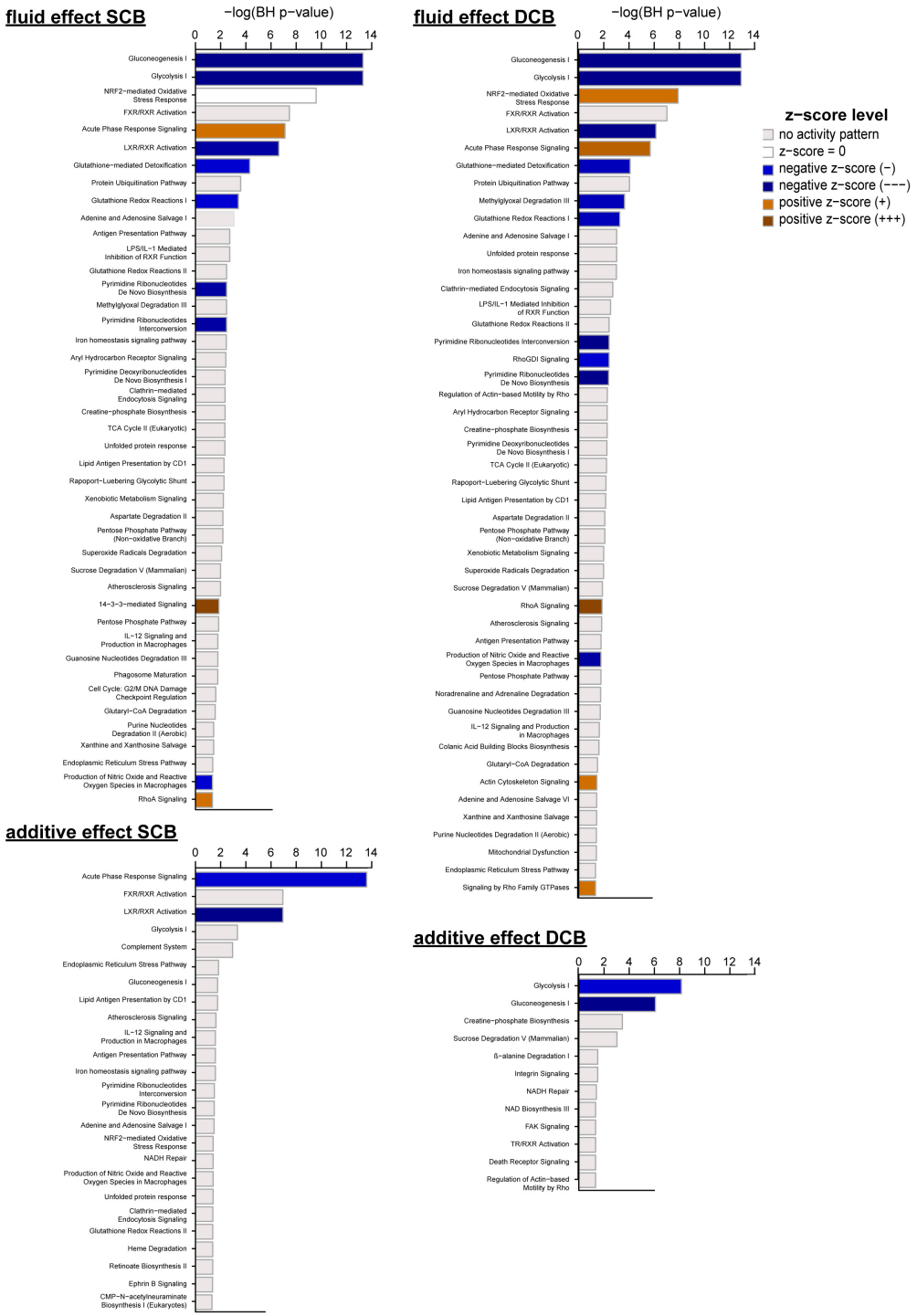

FIGURE 4 | Canonical pathways enriched after chronic treatment with PDF and addition of AlaGln. Significantly enriched canonical pathways from IPA, passing a threshold of $p < 0.05$ after correction for multiple hypothesis testing using the Benjamini-Hochberg (BH) procedure (numeric data are given in **Supplementary Table S3**). Fluid effect SCB, fluid effect DCB, additive effect SCB, and additive effect DCB denote the effects calculated from the mixed model analysis for protein spot data, which were used for generating the lists of significantly affected molecules for pathway analysis.

Enriched canonical pathways for effects of PDFs and additive addition were extracted and compared for level of enrichment (BH-corrected $p < 0.05$), enrichment ratio and activation z-score (**Supplementary Table S3**). **Figure 4** shows the enriched canonical pathways for the individual effects sorted by enrichment p-value (see **Supplementary Figure S4**

for hierarchical clustering of pathways based on significance of enrichment for all canonical pathways with BH-corrected p value < 0.05).

Those canonical pathways for which at least one z-score could be calculated for the four effects (SCB and DCB fluid effects, additive effects for SCB+AG, and DCB+AG), were grouped in functional clusters of typical PD-associated pathomechanisms (metabolism, immune response, cytoskeletal reorganization, and signaling, oxidative stress and redox homeostasis) as well as on molecular features based on shared genes and/or proteins (**Table 1**).

Comparison between SCB and DCB arms of the experiment showed largely overlapping processes and activation states. Concordance was most pronounced for metabolism and immune response pathway clusters, with almost identical activation z-scores for involved molecules. Differences in activation pattern (z-scores) and involved molecules were more pronounced for the clusters "cytoskeletal reorganization and signaling" and "oxidative stress and redox homeostasis". Activated "14-3-3-mediated signaling" was found only for the SCB PDF effect, whereas "RhoGDI signaling", "Signaling by Rho Family GTPases" and "Actin Cytoskeleton Signaling" were activated only for the DCB PDF effect.

The effects for AlaGln additive to both PDFs were not as strong as the fluid effects, indicated by a lower number of significant spots, enriched pathways and calculated z-scores. Interestingly, none of the pathways in the cluster "cytoskeletal reorganization and signaling" that were enriched in SCB and/or DCB PDF effects retained significant enrichment for the additive effect. Only the "NRF2-mediated oxidative stress response" pathway reached significance in the cluster "oxidative stress and redox homeostasis".

The pathways in the "immune response" cluster that remained significantly changed for the AlaGln additive effect included "LXR/RXR activation" and "acute phase response signaling", with the latter being inhibited by AlaGln.

Figure 5A shows an activation heatmap of enriched pathways. The canonical pathway "acute phase signaling" is presented as an example of a significantly enriched pathway in the comparison of both the SCB fluid and additive effects. Downstream of its common trunk (**Figure 5B**) the SCB PDF effect (**Figure 5C**) and SCB+AG effect (**Figure 5D**) show distinct activation patterns. However, in the presence of AlaGln that pathway was not activated, as indicated by counter-regulation of the same proteins.

DISCUSSION

We report here the first proteomic analysis of peritoneal surface lysates in a chronic rat model of PD. Proteomics is a particularly attractive approach to understand complex molecular dynamics influencing the peritoneal membrane and to obtain a systems biology view of the molecular mechanisms of membrane damage and preservation during standard PD and following cytoprotective interventions.

Peritoneal dialysis effluent has been previously exploited for indirect investigation of the peritoneal membrane and its secreted proteins. Recent, extensive characterization of PD effluents by a depletion and enrichment proteomics approach (Herzog et al., 2018) yielded signals dominated by extracellular proteins or detached and necrotic mesothelial cells, and complicated by non-resident peritoneal cell populations. Investigating a clean population of resident mesothelial cells in the clinical setting would require peritoneal biopsies in combination with single-cell proteomics techniques currently unavailable. Therefore, initial studies of *in vivo* models must be performed on more complex preparations of peritoneal membrane tissue, such as the optimized lysates prepared with a specifically designed harvesting device for the current study. This novel type of sample likely represents the currently best available peritoneal surface material, comparable only to patient biopsies not easily obtained in the context of experimental interventions beyond the requirements of clinical care (Schaefer et al., 2016).

Rats and mice do certainly not perfectly represent human PD patients. Once daily infusion in the *in vivo* model is contrasted by multiple dwells in the clinical situation. Although uremic rodent models are already available, the use of these models implicitly complicates the experiment by adding technical and biological variation and requiring additional control groups. Nevertheless, rodents exposed to PDF are accepted models to reflect chronic membrane damage and preservation during PD. Recently, glutamine-containing PDF in chronic rodent models showed improved cytoresistance of mesothelial cells in the acute setting, and reduced peritoneal fibrosis and attenuation of IL-17 dependent pathways (Bender et al., 2010; Ferrantelli et al., 2016). The results obtained from rats presented here were generated in an independent replicate experiment to that of Ferrantelli et al. (2016) carried out in the same facility and following identical protocols. Our chronic *in vivo* model of PD led to reproducible submesothelial thickening of the peritoneum (data not shown).

Our proteomics analysis of peritoneal surface lysates has generated the first comprehensive map of the *in vivo* peritoneal surface proteome. We argue that this minimally studied proteome characterizes a functional anatomical entity, analogous to the dermis of the skin or the kidney cortex. As 2D-DiGE analysis preferentially detects high abundance proteins, the ∼500 spots identified in this novel biological sample represent the major protein components of the peritoneal surface. Our relative abundance calculation suggested that identified gel spots represented approximately 95% of sample protein content. Gel images from peritoneal surface isolates resembled a cellular-like pattern, with the typical albumin spot region of significantly lower intensity than seen in typical serum-like patterns. Nevertheless, evidence of plasma proteins persisted in the peritoneal surface proteome data. However, the high reproducibility and technical quality of the internal standard gels suggested that the 2D gel electrophoresis (2D-DiGE) procedure itself was not the source of the variability.

We conclude that the peritoneal surface proteome reported here is not a pure mesothelial cell proteome. Potential explanations for this observation include insufficient washing of the tissue samples, adhesion of plasma proteins to the surface or even below the surface. In case of insufficient washing, the surface lysate sample would be contaminated by residual plasma/fluid.

TABLE 1 | Enriched and activated/deactivated canonical pathways for the effects of chronic treatment with PDFs and additive grouped by PD-associated pathomechanisms.

	SCB fluid effect				SCB+AG additive effect				DCB fluid effect				DCB+AG additive effect			
Ingenuity canonical Pathways	***p*-value (BH)**	**Ratio**	**z-score**	**Molecules**	***p*-value (BH)**	**Ratio**	**z-score**	**Molecules**	***p*-value (BH)**	**Ratio**	**z-score**	**Molecules**	***p*-value (BH)**	**Ratio**	**z-score**	**Molecules**
Cytoskeletal reorganization and signaling																
Actin cytoskeleton signaling	6.92E-02	0.022	2.24	CFL1, EZR, ACTB, GSN, MSN	1.68E-01	0.009		CFL1, GSN	**3.55E-02**	0.026	1.63	CFL1, EZR, ACTB, ARPC3, GSN, MSN	9.33E-02	0.009		ACTB, GSN
RhoGDI signaling	8.51E-02	0.023	−2.00	CFL1, EZR, ACTB, MSN	1.26E-01	0.011		GNB1, CFL1	**3.80E-03**	0.040	−0.38	CFL1, EZR, ACTB, ARPC3, ARHGDIA, ARHGDIB, MSN	2.39E-01	0.006		ACTB
Signaling by Rho family GTPases	7.94E-02	0.020	2.00	CFL1, EZR, ACTB, VIM, MSN	1.79E-01	0.008		GNB1, CFL1	**4.57E-02**	0.024	1.34	CFL1, EZR, ACTB, ARPC3, VIM, MSN	2.60E-01	0.004		ACTB
RhoA signaling	**4.47E-02**	0.032	1.00	CFL1, EZR, ACTB, MSN	2.94E-01	0.008		CFL1	**1.38E-02**	0.040	0.45	CFL1, EZR, ACTB, ARPC3, MSN	2.15E-01	0.008		ACTB
14-3-3-mediated signaling	**1.38E-02**	0.037	0.45	YWHAE, YWHAB, PDIA3, YWHAZ, VIM	2.96E-01	0.007		PDIA3	6.61E-02	0.029	1.00	YWHAE, PDIA3, YWHAZ, VIM				
Immune response																
Acute phase response signaling	**7.76E-08**	0.068	1.13	ALB, TTR, FTL, APOA1, SOD2, TF, APCS, AHSG, C9, SERPINA3, SERPINA1, RBP1	**2.51E-14**	0.068	−1.63	C4A/C4B, ALB, HP, FTL, APOA1, ITIH4, AHSG, C9, CFB, SERPINA3, SERPINA1, RBP1	**2.09E-06**	0.063	1.13	ALB, TTR, HPX, FTL, APOA1, SOD2, TF, AHSG, SERPINA3, SERPINA1, RBP1	8.32E-02	0.011		HP, SERPINA3
Production of nitric oxide and reactive oxygen species in macrophages	**4.47E-02**	0.026	−1.34	APOE, ALB, APOA1, APOA4, SERPINA1	**3.98E-02**	0.016		ALB, APOA1, SERPINA1	**1.70E-02**	0.031	−1.63	APOE, ALB, APOA1, APOA4, PPP1R7, SERPINA1				
LXR/RXR activation	**2.40E-07**	0.083	−1.90	APOE, ALB, TTR, APOA1, APOA4, TF, AHSG, C9, SERPINA1, GC	**1.15E-07**	0.058	−1.89	C4A/C4B, ALB, APOA1, ITIH4, AHSG, C9, SERPINA1	**7.24E-07**	0.083	−2.53	APOE, ALB, TTR, HPX, APOA1, APOA4, TF, AHSG, SERPINA1, GC				

(Continued)

TABLE 1 | Continued

	SCB fluid effect				SCB+AG additive effect				DCB fluid effect				DCB+AG additive effect			
Ingenuity canonical Pathways	***p*-value (BH)**	**Ratio**	**z-score**	**Molecules**	***p*-value (BH)**	**Ratio**	**z-score**	**Molecules**	***p*-value (BH)**	**Ratio**	**z-score**	**Molecules**	***p*-value (BH)**	**Ratio**	**z-score**	**Molecules**
Metabolism																
Gluconeo genesis I	**5.01E-14**	0.385	−2.53	PGK1, ENO1, PGAM1, ENO3, ENO2, PGAM2, GAPDH, ALDOA, MDH1, MDH2	**1.74E-02**	0.077		ENO1, GAPDH	**1.26E-13**	0.385	−2.53	PGK1, ENO1, PGAM1, ENO3, ENO2, PGAM2, GAPDH, ALDOA, MDH1, MDH2	**8.32E-07**	0.154	−2.00	ENO1, ENO3, GAPDH, ALDOA
Glycolysis I	**5.01E-14**	0.385	−2.53	PGK1, ENO1, PGAM1, ENO3, PKM, ENO2, PGAM2, GAPDH, ALDOA, Tpi1	**4.68E-04**	0.115		ENO1, PKM, GAPDH	**1.26E-13**	0.385	−2.53	PGK1, ENO1, PGAM1, ENO3, PKM, ENO2, PGAM2, GAPDH, ALDOA, Tpi1	**7.08E-09**	0.192	−1.34	ENO1, ENO3, GAPDH, ALDOA, Tpi1
Pyrimidine ribonucleotides *de novo* biosynthesis	**3.39E-03**	0.085	−2.00	AK1, ANXA1, NME2, CMPK1	**3.24E-02**	0.043		AK1, ANXA1	**4.17E-03**	0.085	−2.00	AK1, ANXA1, NME2, CMPK1				
Pyrimidine ribonucleotides interconversion	**3.39E-03**	0.089	−2.00	AK1, ANXA1, NME2, CMPK1	**3.16E-02**	0.044		AK1, ANXA1	**3.80E-03**	0.089	−2.00	AK1, ANXA1, NME2, CMPK1				
Oxidative stress and redox homeostasis																
Glutathione-mediated detoxification	**4.90E-05**	0.161	−1.34	GSTM1, GSTM5, GSTM3, GSTA1, GSTP1					**8.13E-05**	0.161	−1.34	GSTM1, GSTM5, GSTM3, GSTA1, GSTP1				
Glutathione redox reactions I	**3.89E-04**	0.167	−1.00	GSTM1, GSTA1, GSTP1, PRDX6					**5.37E-04**	0.167	−1.00	GSTM1, GSTA1, GSTP1, PRDX6	**9.33E-02**	0.042		PRDX6
NRF2-mediated oxidative stress response	**2.63E-10**	0.075	0.00	AKR7A2, GSTM1, AKR1A1, FTL, SOD2, ERP29, GSTM5, PRDX1, ACTB, GSTM3, VCP, GSTA1, SOD1, GSTP1, FTH1	**3.98E-02**	0.015		FTL, SOD1, FTH1	**1.26E-08**	0.070	1.34	AKR7A2, GSTM1, AKR1A1, FTL, SOD2, ERP29, GSTM5, ACTB, GSTM3, VCP, GSTA1, SOD1, GSTP1, FTH1	**2.39E-01**	0.005		ACTB
Methylglyoxal degradation III	**3.39E-03**	0.158		AKR7A2, AKR1A1, AKR1B1					**2.24E-04**	0.211	−1.00	AKR7A2, AKR1A1, AKR1B1, AKR1B10	**8.91E-02**	0.053		AKR1B1

The table shows canonical pathways for which at least one z-score could be calculated for the four effects (SCB and DCB fluid effects, additive effects for SCB+AG, and DCB+AG – calculated in the mixed model analysis), grouped in functional clusters of typical PD-associated pathways. p-value, The p-value of the overlap was calculated by Fisher's exact test and corrected by the Benjamini-Hochberg (BH) procedure for multiple testing. The p-values < 0.05 after BH correction are marked in bold font. Ratio, The ratio of pathway enrichment, based on observed vs. expected number of molecules. z-score, The z-score assesses the match of observed and predicted up/down regulation patterns and serves a predictor for the pathway activation state, with positive values indicating activation, and negative values indicating deactivation. Molecules, The molecules observed to be significantly affected by each of the effects, as calculated in the mixed model and that could be assigned to an individual canonical pathway.

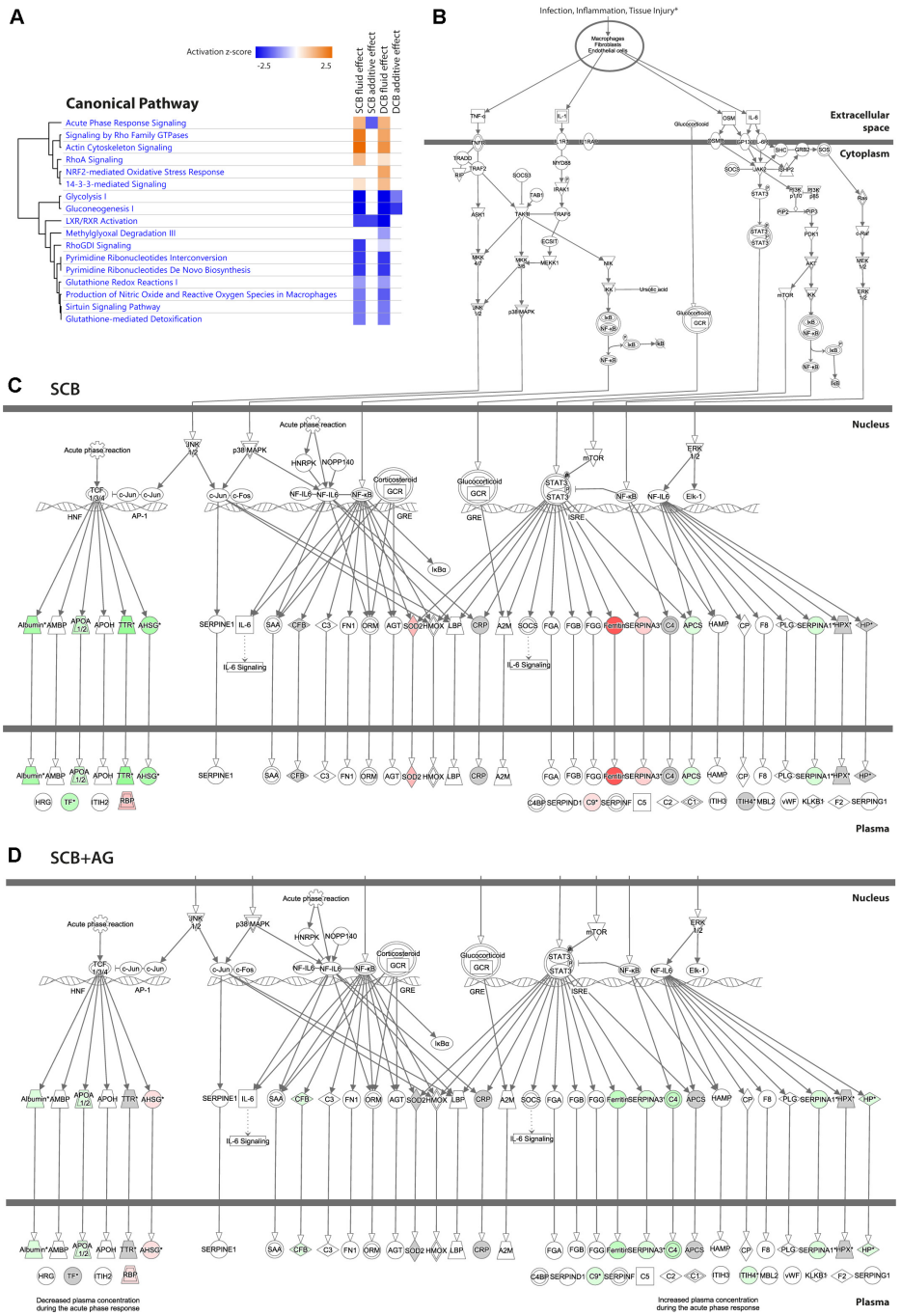

FIGURE 5 | Effect of chronic treatment with PDF and addition of AlaGln on individual pathways, incl. example of "acute phase response signaling". **(A)** Activation heatmap of enriched canonical pathways from IPA for the four effects calculated from the mixed model analysis. **(B)** Common trunk of the canonical pathway "acute phase signaling", presented as an example of a significantly enriched pathway in the comparison of both the SCB PDF and additive effects. **(C)** Regulation pattern for the SCB PDF effect. **(D)** Regulation pattern for the SCB+AG effect. Red protein symbols denote upregulated proteins, green protein symbols denote downregulated proteins. Gray protein symbols denote proteins that were identified but not found significantly differentially regulated. Gray protein symbols denote proteins that were not identified in the proteomics experiment.

PDE resembles diluted plasma and exhibits a stable composition of major (high abundance) components (Herzog et al., 2018). However, some serum proteins were significantly altered in our experimental groups, indicating systematic effects. Serum proteins tend to bind to cell surfaces and receptor molecules as scaffolding proteins, individually and in complexes, as extensively investigated in the setting of uremia (Duranton et al., 2012), such that gentle washing fails to remove them completely. Sub-surface

plasma proteins may represent proteins retained in the intercellular space or transported via transcellular transport in the top layer of the tissue, a poorly understood process previously described in PD (Balafa et al., 2011). Plasma protein association in surface tissue might also reflect PDF-induced increase in peritoneal tissue vascularization closer to the peritoneal surface (Williams et al., 2002; Schaefer et al., 2018). Taken together, we can systematically rule out that the observed plasma proteins are mere contamination but can reflect specific pathophysiologic conditions. Any pre-analytic manipulation of the material by depleting plasma proteins might therefore introduce additional unwanted artifacts masking potentially relevant changes of the peritoneal surface proteome.

For this challenge, we devised a method to estimate the proportion of plasma protein using external information about protein and express spot volume in terms of its proportion of the overall protein volume that is not of plasma origin. The TMM procedure is well established to normalize library sizes between samples of RNAseq experiments (Abbas-Aghababazadeh et al., 2018). This is conceptually similar to the processing step that normalizes overall protein amount between gels (Chawade et al., 2014). For our experiment, we have modified this procedure to normalize the overall abundance of non-plasma proteins between samples. Using this statistical workflow that compensated for experimental covariates by normalization and scaling of gel data, along with application of a linear mixed model, we were able to discriminate the effects of chronic PDF exposure and of those of AlaGln addition compared to a single control PET dwell with PDF.

Single-chamber bag and dual-chamber bag peritoneal dialysis fluids produced similar changes in regulation of protein abundance compared to control. Pathway analyses based on proteins differentially expressed after treatment with different fluids are consistent with previously described deleterious effects of PDF. The most prominently enriched processes and pathways after 5 weeks of chronic treatment with SCB or DCB PDFs were attributable to the clusters "cytoskeletal reorganization processes" (reflecting tissue damage and cell differentiation), "immune response", "altered metabolism" (including but not limited to glucose), and "oxidative stress and redox homeostasis", suggesting that the chronic rat model represents a good surrogate for chronic clinical PD. Although proteomic modification by the AlaGln effect was not as prominent as that produced by the PDF effect (as indicated by fewer significantly altered protein spots), the AlaGln-associated enriched processes were more closely related to repair processes. Importantly, the protein expression associated with several of the enriched processes changed or even reversed in the AlaGln-treated group, suggesting inactivation by AlaGln of otherwise activated pathways.

Single-chamber bag and dual-chamber bag peritoneal dialysis fluids revealed a similar but non-identical pattern in the cluster of cytoskeletal reorganization pathways, suggesting the cluster's coverage not only of tissue damage from bioincompatible fluid toxicity, but also actin cytoskeleton signaling related to tissue transformation and cellular *trans*-differentiation. Interestingly, changes in several small GTPase pathways ("Signaling by Rho Family GTPases", "RhoGDI and RhoA Signaling") correlated more strongly with DCB than with SCB PDF. Only RhoGDI and RhoA were significant with SCB PDF. RhoGDI signaling was inhibited, whereas other GTPase pathways were activated, despite similar protein patterns, suggesting the observed signaling changes likely represent the effector level, with different upstream regulators eliciting a common phenotype. Also interesting was that 14-3-3-mediated signaling was altered only by SCB PDF. 14-3-3-mediated signaling pathways include several upstream regulators of GSK-3β, activated in immortalized mesothelial cells by the acidic PDFs, Dianeal and Extraneal, but not by the neutralized PDFs, Physioneal and Balance (Rusai et al., 2013). These findings might inform future mechanistic experiments upstream of GSK-3β. The cytoskeletal reorganization pathways were not significantly enriched as a function of AlaGln addition, perhaps reflecting normalization of these processes by AlaGln, or inadequate sensitivity of the proteomic technologies used here. In the case of this "cytoskeletal reorganization" cluster, performance of the harvest PET with the same fluid used for chronic treatment might have been a better choice.

In the cluster of immune response pathways, acute phase response signaling was the central pathway activated by both SCB and DCB PDFs. The pattern of regulation shows upregulation acute phase response activators and downregulation of acute phase response inhibitors, with one prominent exception of retinol binding protein 1 (RBP1, the intra-cellular counterpart of RBP4) which has been identified in almost all studies in PDE, including our own (Herzog et al., 2018). Among activated proteins are components of the complement cascade (also significantly enriched but without calculated z-score). The similarity of SCB and DCB PDFs in acute phase pathway activation patterns is consistent with studies in human biopsies from children on PD with biocompatible fluids (Schaefer et al., 2018). However, the acute response upregulation present at time of harvest might not be maintained at longer equilibration times, since PET duration of the animal experiment was shorter than the typical human dwell time. Indeed, levels of positive acute phase response proteins increase as early as 4–5 h following a single inflammatory stimulus. While this reasoning applies to the control animals, the upregulation observed in the animals undergoing 5 weeks of PD must be considered a chronic effect.

The effect of AlaGln addition is the inverse of the effects of SCB and DCB PDF, leading to inactivation of the acute phase response signaling pathway. In this pathway STAT3 appears to be a signaling hub that is influenced by PDF and AlaGln, perhaps reflecting the differential O-GlcNAcylation (of e.g., STAT proteins) associated with PDF (Herzog et al., 2014). The affected parts of the acute phase response signaling pathway include several complement proteins, and indeed the same effect can be observed for this pathway. Complement system activation was recently reported to be activated by DCB PDFs in correlation with vasculopathy, as shown by transcriptomic and proteomic analysis of pediatric peritoneal biopsies (Bartosova et al., 2018). Down-regulation of the complement system supports the clinical finding that AlaGln addition improves biomarkers of peritoneal health even when combined with biocompatible fluids (Vychytil et al., 2018).

In the cluster of "metabolism" pathways, we found deactivation of glycolysis-associated pathways due to significant downregulation of several glycolytic enzymes in response to SCB and DCB PDF. This is particularly interesting, as SCB and DCB PDF are compared to control and therefore the observed downregulation is between a one-off PET with SCB PDF and chronic treatment for 5 weeks. The downregulation could reflect regulatory adaptation to the chronic high glucose environment and is consistent with earlier *in vitro* studies in which glycolytic enzymes were upregulated following a single exposure to glucose-based PDF, but downregulated compared to glucose exposure alone (Lechner et al., 2010). Future studies should determine if this downregulation is relative to the normal peritoneum (without PET) or represents regression to control levels. The similar SCB and DCB PDF results may reflect their identical glucose concentrations, although an influence of GDP content might have been anticipated. AlaGln addition to DCB PDF down-regulated key enzymes (ENO1, ENO2, GAPDH, and ALDOA) more broadly than when added to SCB PDF (ENO1 and GAPDH) only. This downregulation could indicate adaptation of glycolytic flux with added glutamine, however, the observation of differential effect in DCB PDF needs to be investigated in further studies.

The regulated pathways of the redox cluster include those associated with glutathione turnover, as well as oxidative stress responses linked to the inflammasome and GDP degradation. In particular, the NRF2-mediated oxidative stress response pathway was significantly enriched under both SCB and DCB PDF conditions, but appeared activated only by DCB PDF, based on the differential abundance pattern. The NRF2 pathway was further enriched for the AlaGln effect under SCB conditions, whereas AlaGln was without significant effect on the NRF2 pathway in the presence of DCB PDF. The reason for this difference is unclear, but suggests worthwhile investigative paths addressing the influences of buffer and GDPs. NRF2 is required for L-1β secretion and NLRP3 inflammasome activation (Jhang and Yen, 2017), and the NLRP3 inflammasome can contribute to peritonitis in PD patients (Hautem et al., 2017). This IL-1R-dependent effect was blocked by the IL-1R antagonist, anakinra, which latter also limited damage during PDF-induced sterile inflammation (Kratochwill et al., 2011). The neutral pH DCB solution contains significantly lower amounts of toxic GDP. The methylglyoxal degradation pathway was significantly enriched under both SCB and DCB PDF conditions. However, only after chronic treatment with DCB was this pathway's activity reduced following a PET with GDP-rich SCB PDF.

Alanyl-glutamine has been shown to mediate beneficial effects when added to PDFs in cell culture and clinical studies (Kratochwill et al., 2012, 2016; Herzog et al., 2017). The first long-term data of AlaGln addition were recently obtained in a multicenter phase II trial (Vychytil et al., 2018), showing improved immune competence as assayed by *ex vivo* stimulated cytokine release of peritoneal leukocytes, and peritoneal membrane protection as indicated by increased CA-125 levels. The fact that CA-125 is the only established biomarker for membrane status in PD reflects our inadequate understanding of membrane damage and protection (Aufricht et al., 2017). *In vitro* mechanistic work suggests a role of protein post-translational modification with O-GlcNAc in mesothelial cell cultures exposed to AlaGln-supplemented PDF (Herzog et al., 2014), but AlaGln effects on the peritoneal membrane have not yet been studied directly. Future studies with human biopsy material will be needed to validate the relevance of our findings in the clinical setting of PD.

The presence of serum proteins among the pathways altered by AlaGln was not expected, since the initial goal of this harvesting procedure was to obtain a pure mesothelial cell sample. However, the systematic effects of PDF and of AlaGln on the lysate levels of these proteins suggests the possibility that their presence may indicate more than plasma contamination or insufficient pre-lysis washing, perhaps even enabling study of paracellular protein transport.

The PET before harvest of the peritoneal surface lysate used SCB PDF in all arms of the chronic experiment, including control, DCB, and SCB. The options for the experiment were performing the PET with same fluid as the chronic treatment or with the same fluid in all groups. In this study, we decided for the latter. The advantages are the absence of matrix effects and improved the ability to compare isolated effects of the chronic treatment phase. The approach may have blunted differences between the solutions. The differences demonstrated may therefore be even more pronounced without PDF fluid type switch. This fluid type switch during the PET may also have induced short term changes. The 90 min PET is probably too short for changes in the protein profile of the surface tissue, but some effluent proteins such as cytokines may be sensitive to this switch, and due to rapid mesothelial secretion could influence the between-group differences generated during the chronic phase. The exposure of control animals to glucose-based PDF for the first time in our protocol may have increased induction of the stress response (Kratochwill et al., 2016). This condition resembles a first exposure of cultured cells to PDF and might explain why *in vitro* biocompatibility experiments often fail to replicate clinical outcomes. In one of the first proteomics studies we indeed showed that mesothelial cells exhibit a more adequate stress response to first SCB PDF exposure than to a subsequent repeat exposure (Kratochwill et al., 2009). Comparison of chronic treatment in rats to control may therefore be analogous to comparison of the stress response in patients on chronic PD compared to their initial exposure to PDF (when the stress response is physiologically active).

Further limitations of the experimental setting include the absence of a catheter in the control group. Our model only compares the effect of chronic PD (incl. a catheter) to the state of the healthy peritoneum. Including a catheter control would have enabled separating the effects of the fluid from the effects of the catheter alone but would also have doubled the amount of comparisons. Finally, the animals were not uremic, as nephrectomy would have increased the experimental complexity and variability and would have required additional controls. Investigating the effect of uremia on the observed peritoneal surface processes may be enabled by this study in combination

with clinical material, such as biopsies (Bartosova et al., 2018) or PD effluent (Herzog et al., 2018) from patients with varying levels of uremia.

This study is the first description of the peritoneal surface proteome during chronic PD in a well-established rat model of PD. Combined proteomic and bioinformatic investigation of the effects of different PDFs and of AlaGln supplementation in a chronic rat model proved feasible, despite considerable biological variation in this *in vivo* model. The proteomic method allowed extensive characterization of the peritoneal surface composition and biological processes occurring during chronic PD. By normalizing signals from cellular proteins separately from plasma proteins, assessment of PDF exposure-induced changes in the stress proteome of resident peritoneal mesothelial cells was feasible. Chronic PDF exposure in the *in vivo* rat model activated pathways associated with tissue damage and cell differentiation, immune responses, altered metabolism, and oxidative stress. AlaGln addition attenuated deleterious processes associated with membrane damage, and activated processes linked to membrane protection, consistent with recent *in vitro* and clinical findings.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the European Union Guideline on Animal Experiments. The protocol was approved by the animal care committee of the Vrije Universiteit of Amsterdam.

AUTHOR CONTRIBUTIONS

MB contributed to the *in vivo* experiment, sample preparation, data analysis and interpretation, and manuscript preparation. RH contributed to the sample preparation, data analysis and interpretation, and manuscript preparation. FK contributed to the statistical data analysis. AL and AW contributed to the 2D-DiGE and MS analysis. MU contributed to the data analysis. RB contributed to the supervision of *in vivo* experiments and critical reading of the manuscript. SA contributed to the data interpretation and critical reading of the manuscript. CA contributed to the study concept, data interpretation, and critical reading of the manuscript. KK contributed to the study concept, data analysis and interpretation, and manuscript preparation.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest Statement: CA is co-founder of Zytoprotec GmbH, a spin-off of the Medical University Vienna that holds the patent "Carbohydrate-based peritoneal dialysis fluid comprising glutamine residue" (International Publication Number: WO 2008/106702 A1). RH, AL, AW, MU, and KK are former employees of Zytoprotec GmbH.

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Abnormalities in Glucose Metabolism, Appetite-Related Peptide Release, and Pro-inflammatory Cytokines Play a Central Role in Appetite Disorders in Peritoneal Dialysis

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Background: Appetite disorders are frequent and scantly studied in peritoneal dialysis (PD) patients and are associated with malnutrition and cardiovascular complications.

Objective: We investigated the relationship between uremic insulin resistance, pro-inflammatory cytokines, and appetite-related peptides release (ARPr) with eating-behavior disorders in PD patients.

Methods: We included 42 PD patients (12 suffering anorexia, 12 obese with high food-intake, and 18 asymptomatic) and 10 controls. We measured blood levels of ARPr including orexigens [neuropeptide-Y (NPY), ghrelin, and nitric-oxide], anorexigens [cholecystokinin, insulin, corticotropin-releasing factor, leptin, and adiponectin (Ad)], and cytokines (TNF-α, sTNFα-R2, and IL-6) both at baseline and after administering a standard-food stimulus (SFS). We also measured the expression of TNF-α, leptin and Ad-encoding mRNAs in abdominal adipose tissue. We compared these markers with eating motivation measured by a Visual Analog Scale (VAS).

Results: Anorexics showed both little appetite, measured by a VAS, and low levels of orexigens that remained constant after SFS, coupled with high levels of anorexigens at baseline and after SFS. Obeses showed higher appetite, increased baseline levels of orexigens, lower baseline levels of anorexigens and cytokines and two peaks of NPY after SFS. The different patterns of ARPr and cytokines pointed to a close relationship with uremic insulin resistance. In fact, the euglycemic–hyperglycemic clamp

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reproduced these disorders. In anorexics, TNF-α fat expression was increased. In obese patients, leptin expression in fat tissue was down-regulated and showed correlation with the appetite.

Conclusion: In PD, appetite is governed by substances that are altered at baseline and abnormally released. Such modulators are controlled by insulin metabolism and cytokines and, while anorexics display inflammatory predominance, obese patients predominantly display insulin resistance.

Keywords: appetite peptides, pro-inflammatory cytokines, insulin resistance, fat tissue gene expression, peritoneal dialysis, euglycemic–hyperglycemic clamp

INTRODUCTION

Malnutrition is a severe and frequent (20–40%) complication in peritoneal dialysis (PD) patients. Its cause is not entirely known but it has been associated with the accumulation of pro-inflammatory cytokines, as well as metabolic and eating behavior disorders (EBD) (Owen et al., 1999). EBD in PD patients may range from anorexia to obesity with high food intake. Anorexia is the main obstacle to achieve an adequate nutritional status, while obesity is frequently associated with protein malnutrition, inducing a Kwashiorkor-like syndrome (Aguilera et al., 2004b).

In renal patients there is an accumulation in plasma of uremic toxins., PD is an essential daily life-saving treatment for end-stage renal failure and involves the exchange of solutes and the excess of water between blood and dialysis solution across the peritoneal membrane, which results in gradual reduction of uremic solutes and toxins (Raby and Labéta, 2018; Nigam and Bush, 2019). Reduced appetite (anorexia) is an early and usual symptom of uremic syndrome (Carrero et al., 2007, 2008). Anorexia in dialysis patients has been historically considered as a sign of uremia due to "inadequate" dialysis. Additionally the inflammation may also play a role in the genesis of appetite disorders in these patients (Aguilera et al., 2004b; Chazot, 2009).

Appetite is regulated by numerous organs, including the liver, the gastrointestinal tract (GIT) and the brain. The hypothalamus, is the main regulating region of the brain for appetite and energy homeostasis, involving counter-regulation of appetite by orexigenic molecules, including neuropeptide-Y (NPY), ghrelin and nitric-oxide and anorexigenic molecules, such as proopiomelanocortin (POMC), cholecystokinin (CCK), insulin, corticotropin-releasing factor (CRF), leptin, adiponectin (Ad), and pro-inflammatory cytokines (Yu and Kim, 2012).

It has been shown that low NPY serum levels are associated with anorexia in PD patients (Aguilera et al., 1998, 2004a; Yu and Kim, 2012). NPY is a 36-amino acid peptide and is the most abundant and widely distributed neuropeptide in the human brain (Adrian et al., 1983). In the periphery, NPY is expressed primarily in sympathetic ganglia, the adrenal medulla, and in platelets (Larhammar, 2001; Hirsch and Zukowska, 2012). The main effects of NPY are increased food intake and decreased physical activity. It also increases the proportion of energy stored as fat and blocks nociceptive signals to the brain. In regards of its role in obesity, it has been shown that an increase in NPY is caused by high levels of glucocorticosteroids through directly activating type II glucocorticosteroids receptors and indirectly, by abolishing the negative feedback of CRF on NPY synthesis and release (Brothers and Wahlestedt, 2010).

Corticotropin-releasing factor is a 41-aa neuropeptide secreted by neurons in the paraventricular nucleus of the hypothalamus (Vale et al., 1981), this has a role in the regulation of ACTH release and also the major physiological regulator of the hypothalamic–pituitary–adrenal (HPA) axis (Smagin et al., 2001; Sabzevari et al., 2019), and has an important role in the control of food intake (Tanaka et al., 2009). CRF has been measured in peripheral plasma in several diseases, stress situations, and HPA disorders and it has been suggested that the major proportion of plasma CRF has a hypothalamic origin (Suda et al., 1985; Cunnah et al., 1987; Ellis et al., 1990; Wittert et al., 1991; Donald et al., 1994; Catalán et al., 1998).

The pathogenesis of the wide spectrum of appetite disorders in uremia is unknown. The accumulation of appetite-related peptides (ARPs) and pro-inflammatory cytokines (Aguilera et al., 1993) upon renal failure may alter the hunger-satiety cycle. Both ARPs and cytokines might interact and potentiating each other, producing a wide range of eating behavior responses. In this context, it has been shown that CCK and interleukin-1 (IL-1) may synergize to worsen anorexia and that TNF-α may interfere with the orexigenic effect of neuropeptide-Y (NPY) (Xu et al., 1988; Daun and McCarthy, 1993).

Given the proposed definition of "malnutrition, inflammation, and atherosclerosis" (MIA) syndrome, dialysis patients may suffer from two types of malnutrition: Type-I malnutrition, in reference to a severe form of cachexia due to high plasma levels of inflammatory mediators that produce systemic catabolic effects, being anorexia one of the most important symptoms; and Type-II malnutrition, that is usually mild and reversible, and that is barely mediated by inflammation (Stenvinkel et al., 2000).

The other important groups of features that regulate appetite are associated with glucose and insulin metabolism (Isganaitis and Lustig, 2005). It is well known that uremic state is associated with insulin resistance (Zoccali et al., 2005) and a clear relationship between insulin release, glucose metabolism, ARP-release (ARPr) and pro-inflammatory cytokines has been established (Kalra et al., 2008). IL-1 and TNF-α induce insulin resistance and the sense of satiety that acts on the hypothalamus (central anorexia) (Isganaitis and Lustig, 2005). Some ARP and cytokines retained during renal failure contribute to the perpetuation of insulin resistance and to the establishment of

chronic appetite disorders (Aguilera et al., 2004a; Zoccali et al., 2005). In this sense, fat tissue plays a crucial role in insulin metabolism, controlling the release of peptides and cytokines (Isganaitis and Lustig, 2005; Axelsson, 2008). The abnormalities in leptin, Ad or resistin fat gene expression indicate that the influence of fat tissue on such metabolism is particularly affected by uremia (Kalra et al., 2008).

We were interested in defining the relationship between ARPr, circulating cytokines, insulin resistance, fat gene expression and EBD in PD patients. This population is of particular interest, since PD patients are exposed to constant peritoneal glucose absorption, which may worsen disorders of glucose metabolism and directly induce central anorexia (Aguilera et al., 2004b; Kalra et al., 2008). In some cases, the tendency toward high plasma levels of anorexigen peptides is thought to reflect the disequilibrium between orexigens and anorexigens (Aguilera et al., 2004b). However, not all PD patients suffer anorexia under similar conditions, suggesting that factors like peritoneal glucose absorption and insulin metabolism, or dialysis clearance, influence on appetite control. Accordingly, we hypothesize that disturbances in the secretion and/or renal retention of ARP and inflammatory cytokines, all of which act on fat tissue and are associated with insulin resistance, induce different EBD, ranging from anorexia to obesity with high food intake.

MATERIALS AND METHODS

We included 42 clinically stable PD patients (20 males and 22 females), 20 with continuous ambulatory peritoneal dialysis (CAPD) and 22 with automated PD. The causes of chronic renal failure were nephrosclerosis in 15 patients, glomerulonephritis in 11, polycystic kidney disease in 8, systemic disease in 4 and unknown in 4. Of these 42 PD patients: 12 suffered anorexia, 12 were obese with high food-intake and 18 were asymptomatic. We excluded diabetic patients, and those suffering from neoplasias, chronic or acute infections, liver, and rheumatoid diseases. We also included 10 healthy controls. This research was carried out in accordance with Good Clinical Practice Guidelines, applicable regulations as well as the ethical principles that have their origin in the Declaration of Helsinki. All of included patients signed a written consent.

Dialysis Adequacy and Nutritional Markers

Urea-Kt/V and nPNA (normalized protein nitrogen appearance) were calculated by standard methods (Selgas et al., 1993). Long-term nutritional markers in plasma/serum were analyzed: creatinine, albumin, cholesterol (colorimetric method, Hitachi 704) and transferrin were assayed by immunonephelometric methods (Boering Nephelometric-Terminal S.A., Spain) and serum iron (Hitachi 911). Medium-term nutritional markers were also assessed, plasma prealbumin and retinol-binding protein (RBP) immunonephelometric method). Serum growth hormone (GH) was determined by ELISA (AIA 1200; Tosoh Corporation, Tokyo, Japan), normal value <5 ng/mL. Serum IGF-I was determined by radioimmunoassay, RIA (Nichols Institute Diagnostics, San Juan Capistrano, CA, United States), normal range between 83 and 450 ng/mL. The short-term nutritional markers analyzed in serum were: urea nitrogen, phosphate, and potassium. The mean daily dietary intake was determined from the individual 24-h food records over a 3-day period (Food composition tables, Wander-Sandoz Nutrition, Barcelona, Spain; Jiménez Cruz et al., 1990).

Anthropometric parameters explored were: triceps skin-fold (TSF), mid-arm circumference (MAC), and mid-arm muscle circumference [MAMC (cm) = MAC (cm) – 3.14 × TSF (cm)]. The TSF was determined using a caliper (Holtain Ltd., Crosswell, Crymych, Dyfed, United Kingdom). Body composition was determined through bioelectric impedance (BI: multi-frequency, Maltron BF 905, United States).

Eating Motivation Analysis

To evaluate eating motivation in the patients, we use Visual Analog Scale (VAS), which includes five questions. The results were given in a horizontal scale (0–100) (Hylander et al., 1997). Anorexia was defined by three criteria: low eating motivation measured by VAS, that was considered when the mean of all answers was <60; low food intake (nPNA < 1.1 g/kg/day and daily dietary assessment <35 kcal/kg/day); and low nutritional markers (according to KDOQI clinical practice guideline of nutrition) (Kopple, 2001; Aguilera et al., 2004b).

Obesity with high food intake was considered when the BMI was higher than 30 kg/m^2, and eating motivation (VAS) and daily food intake was high (Hylander et al., 1997).

According to body composition, nutritional status, eating motivation and the knowledge derived from eating behavior disorders studied in other medical areas (Robins et al., 1988; American Psychiatric Association, 2013), we divided our patients into three groups: suffering anorexia (n = 12), obesity (n = 12) with high food intake or without EBD (n = 18). Finally, we included a control group of 10 health volunteers.

We evaluate the appetite peptide modulators at the baseline (fasting condition), as well as 30, 60, and 90 min after the ingestion of a standard 750 mL nutritional supplement (Fresubin^{TM}, Fresenius, Medical Care, Germany). They were analyzed according to manufacturer recommendation. These peptides included:

Hormones and Peptides Related to Insulin Resistance

(1) Glucose: assayed by the hexokinase reaction (Boehringer Mannheim, Germany). The normal fasting range from 90 to 120 mg/dL.
(2) Insulin (Sorin; Biomedica, Saluggia, Italy): normal range 10–15 μU/mL.
(3) C-peptide (Medigenix; Diagnostics Fleurus, Belgium): normal range 0.5–3 ng/mL.
(4) Glucagon (ICN Biomedicals, Irvine, CA, United States): normal range 70–90 pg/mL.
(5) Gastric inhibitory peptide (GIP; ELISA, Peninsula Laboratories, Inc., Belmont, CA, United States): normal range 35–52 pg/mL.

Insulin Resistance (IR) Test

(1) Insulin resistance (IR) was estimated using the homeostatic model assessment (HOMA-IR). HOMA-IR = Fasting insulin (μU/mL) × fasting glucose (mg/dL)/405. Low-IR values indicate high insulin sensitivity, whereas high-HOMA values indicate low insulin sensitivity (insulin resistance) (Matthews et al., 1985).

(2) Euglycemic hyperinsulinemic clamp studies were performed to discriminate the effect of hyperglycemia and hyperinsulinemia separately on ARPr. We followed the methodology of DeFronzo et al. (1979).

Anorexigenic Peptides

(1) Cholecystokinin, the 26–33 unsulfated fragment (ELISA, Peninsula Laboratories, Inc., Belmont, CA, United States): normal values 12–20 pg/mL.

(2) Leptin (RIA, Linco Research, St. Louis, MO, United States): normal range 3–7.8 ng/mL.

(3) Adiponectin (Ad; RIA, Linco Research, St. Charles, MO, United States): normal values in our population were 25–33 μg/mL.

(4) Corticotropin-releasing factor (CRF; ELISA, Easia Medigenix Diagnostics S.A., Belgium): normal range 20–40 pg/mL.

Orexigenic Peptides

(1) Neuropeptide Y (NPY; ELISA, Peninsula Laboratories, Inc., Belmont, CA, United States): normal range 220–370 pg/mL.

(2) Nitric oxide (NO) measured as serum nitrate NO_3, a final metabolite of NO, by capillary electrophoresis: normal range in 109 healthy volunteers was between 90 and 110 μmol/L.

(3) Ghrelin, the ^{125}I-Ghrelin (RIA, Linco Research, St. Charles, MO, United States): normal range 900–2,500 pg/mL.

Cytokines Acting on Appetite Control

(1) Tumor necrosis factor (TNF-α) and IL-1, were determined by ELISA (Easia Medigenix, Diagnostics S.A., Belgium), normal ranges were 3–20 pg/mL and 1–16 pg/mL, respectively.

(2) IL-6 (R&D Systems, Minneapolis, MN, United States: normal range 0.25–1.5 pg/mL).

(3) TNF-α receptor-2 (sTNFα-R2; BioSource Europe, Nivelles, Belgium: normal range 1–7.8 ng/mL).

Quantitative RT-PCR Analysis in Abdominal Fat Tissue

Gene expression was assayed in abdominal subcutaneous adipose tissue obtained from elective surgery (controls) and PD catheter placement when patients started PD or during PD catheter exchange. The samples were placed into warm saline, snaps frozen in liquid nitrogen and stored at −80°C. For quantitative RT-PCR analysis frozen tissue samples were lysed in TRI-Reagent (Ambion, Inc., Austin, TX, United States) using a Polytron homogenizer to extract total RNA. Complementary DNA was obtained from 1 μg of RNA by reverse transcription according to manufacturer's recommendations using High Capacity RNA to cDNA kit (Applied Biosystems, Cheshire, United Kingdom) as described previously (Sandoval et al., 2016). Quantitative PCR was carried out in a Light Cycler 480 using the FastStart Universal Probe Master (ROX) (Roche Applied Science) with the following program: 50°C, 2 min; 95°C, 10 min; 60 cycles of 95°C, 15 s and 60°C, 10 s; 4°C; and specific primers sets for human adiponectin, leptin, TNF-α and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) mRNAs (**Supplementary Table S1**) (Ruiz-Carpio et al., 2017). Data normalized to the housekeeping gene TBP were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Results are given as the mean ± SD and range. The comparisons between groups were performed using a non-parametric test, the Mann–Whitney rank-sum *U* test. Spearman regression analysis and "*t*" student tests were used for paired and non-paired data. To analyze the statistical differences between the groups, the ARPr serum curves and the VAS (tables and graphic curves) we used the variance multi-factor analysis (ANOVA). **Figure 4**, box plots show the 25th and 75th percentiles, median, minimum and maximum values of five independent experiments. The symbols represent the statistical differences between the groups. In figures, statistic differences between the groups data were performed using triple factor ANOVA test and Mann–Whitney rank sum *U* test using the SPSS statistic package version 14.5 (Chicago, IL, United States) and GraphPad Prism version 4.0 (La Jolla, CA, United States). A "*p*" value less than 0.05 was considered statistically significant.

RESULTS

The demographic and basal biochemical characteristics of the subjects are shown in **Table 1**. The most relevant findings were that anorexic patients were older and had significantly lower levels of nutritional markers and residual renal function (creatinine clearance). No differences were found in the frequency of EBD between CAPD vs. automated techniques. In CAPD group, seven from twelve patients were anorexics, six obese and eight were asymptomatics. Similarly, there were no differences in the distribution of EBD by gender. Six from 12 women were anorexics, seven obese, and nine were asymptomatics.

Visual Analog Scale (VAS)

The analysis of the VAS applied to the different groups indicated that the anorexic patients had a lower eating motivation and obese the higher eating desire (**Table 2**).

Insulin Resistance Markers

Supplementary Figures S1A–E show the changes in glucose, insulin, glucagon, C-peptide and GIP after Fresubin^{TM} intake. PD patients showed higher baseline levels of these markers and "lazy" curves (especially in obese and anorexics) than controls.

TABLE 1 | Baseline differences between the groups (PD patients).

Parameter	Anorexic	Obese	Asymptomatic	Controls	*P*
Age (years)	66.4 ± 10(a,d)	56.3 ± 7.1(b)	49.7 ± 14(a,c)	43 ± 4.7(b,d)	(a,b,c,d) < 0.05
DP duration (m)	36.8 ± 32.3	23 ± 11.5	45.5 ± 46.7		NS
CCr (mL/min)	0.5 ± 0.45(a)	1.42 ± 1.01(b)	1.38 ± 1.39(c)	101 ± 7(a,b,c)	(a,b,c) < 0.00
nPNA (g/kg/día)	0.87 ± 0.21(a)	1.1 ± 0.25	1.14 ± 0.11(a)		(a) < 0.05
KT/V de urea	2 ± 0.25	1.98 ± 0.33	2.17 ± 0.33		NS
Serum Urea (mg/dL)	152 ± 21(a)	159 ± 45(b)	146 ± 51(c)	28 ± 4(a,b,c)	(a,b,c) < 0.00
Cr (mg/dL)	10.4 ± 2(a)	11.3 ± 2(b)	10.5 ± 3(c)	1 ± 0.2(a,b,c)	(a,b,c) < 0.00
Cholesterol (mg/dL)	174 ± 57.4	211 ± 55.6	188 ± 56	184 ± 30	NS
Albumin (g/dL)	3.7 ± 0.08(a,b)	4 ± 0.2(a)	3.9 ± 0.4	5 ± 0.4(b)	(a,b) < 0.05
Transferrin (mg/dL)	209 ± 36	262 ± 47	205 ± 50.7	303 ± 57.2	NS
Prealbumin (mg/dL)	26 ± 7(a,b)	31 ± 2.9(a)	31 ± 7.5	34 ± 3(b)	(a,b) < 0.05
RBP (mg/dL)	8.4 ± 3(a,b)	11.5 ± 3(a)	13 ± 2(b)	5.3 ± 1.2	(a,b) < 0.05
IGF-I (ng/mL)	257.5 ± 122(a)	370 ± 142.7(a)	365 ± 224.6	205 ± 91.2	(a) < 0.05
GH (ng/mL)	3.4 ± 3.8	4 ± 4.8	2.2 ± 1.4	1.7 ± 1.7	NS
TSF (cm)	9.5 ± 4(a,b)	24.2 ± 11.4(a)	22.3 ± 9.4(b)	19.9 ± 10.1	(a,b) < 0.05
BSF (cm)	4 ± 0.6(a,b,c)	18.6 ± 7.3(a)	11.4 ± 8.6(b)	13.7 ± 9.5(c)	(a,b,c) < 0.05
AMMC (cm)	24.2 ± 1.4(a,b)	27.4 ± 2(a,c)	23.9 ± 13(c)	36.1 ± 13(b)	(a,b,c) < 0.05
Diet survey (kcal/day)	1277 ± 467.4(a,b,c)	2320 ± 179.4(a)	2006 ± 351(b)	2089 ± 339(c)	(a) < 0.01 (b,c) < 0.05
Fat (kcal/day)	60.4 ± 28.9(a,b)	102 ± 23.2(a,c)	98 ± 22(b)	74.7 ± 15(c)	(a,b,c) < 0.05
Proteins (kcal/day)	63 ± 18(a)	85.7 ± 16.6(a)	83.8 ± 13.7	74.5 ± 21.8	(a) < 0.05
Carbohydrate (kcal/day)	98 ± 41(a,b,c)	227 ± 71(a)	155.5 ± 27(b)	248.8 ± 67(c)	(a,b,c) < 0.01
BIP. BMI (kg/m^2)	23 ± 2(a)	31.1 ± 3(a,b)	25 ± 2	24 ± 1.5(b)	(a,b) < 0.05
BIP. Lean (kg)	22.8 ± 3.7(a,b)	30.4 ± 4.5(a)	25.4 ± 2.8	28.6 ± 5.7(b)	(a,b) < 0.05
BIP. Fat (kg)	18 ± 5.4(a)	25 ± 8.8(a,b)	13.5 ± 5.3(b)	18.7 ± 4.2	(a,b) < 0.05
IPB. Water (L)	35.7 ± 5.3(a)	43.7 ± 7.7(a)	37.7 ± 5.4	36.6 ± 5.8	(a,b) < 0.05
TNF-α (pg/mL)	121 ± 43.8(a,b,c)	40 ± 11.6(a,c)	38.2 ± 16(b,c)	16 ± 4(c)	(a,b) < 0.01 (c) < 0.001
IL-1 (pg/mL)	6.12 ± 0.8(a,b,c)	2.1 ± 0.43(a,c)	2.2 ± 1.34(b,c)	1 ± 0.8(c)	(a,b,c) < 0.001
IL-6 (pg/mL)	9.3 ± 3(a,b)	11 ± 5.2(c,d)	5.4 ± 2(a,d)	2 ± 1.2(b,c)	(a,b,c) < 0.05 (c) < 0.01
Adiponectin (pg/mL)	29.2 ± 4.2(a)	20.1 ± 7.8(b)	30.3 ± 7.7(c)	9.8 ± 2.4(a,b,c)	(a,b,c) < 0.01
HOMA-IR	7.8 ± 1.9(a,b)	10.4 ± 5.1(c,d)	3.5 ± 0.8(a,c)	2.48 ± 0.04(b,c,d)	(a) < 0.05 (b,c,d) < 0.01

Letters represent the statistical differences between the groups (read in horizontal). CCr, creatinine clearance. Cr, serum creatinine. RBP, retinol protein binding. IGF-I, insulin growth factor type I. GH, growth hormone. TSF, tricipital skin fold. BSF, bicipital skin fold. AMMC, arm muscular mean circumference. BIP, bioelectric impedance. BMI, body mass index. (a–d) Represents the statistical differences between the groups. Statistical comparisons were made group by group (Mann–Whitney U test).

TABLE 2 | Eating motivation measured with VAS in PD patients suffering EBD.

VAS Patients	Anorexics (*n* = 12)	Obese (*n* = 12)	Asymptomatics (*n* = 18)	Controls (*n* = 10)	*P*
Desire to eat before lunch	60 ± 6.1 (a,b)	76.6 ± 6 (a)	67.8 ± 6.9	72.8 ± 3.9 (b)	(a,b) < 0.01
Desire to eat after lunch	8.6 ± 2.2 (a)	21.6 ± 4 (a)	13.2 ± 5	13.5 ± 8.5	(a) < 0.05
Hunger before lunch	60 ± 6.1 (a,b,c)	78.3 ± 6 (a)	68.6 ± 4.7 (b)	74.3 ± 4.5 (c)	(a) < 0.01 (b,c) < 0.05
Hunger after lunch	8 ± 4.4 (a,b)	21.6 ± 4 (a,c)	12.8 ± 5.5 (c)	17.1 ± 4.8 (b)	(a,b,c) < 0.01
Fullness before lunch	28 ± 8.4 (a,b)	18.8 ± 2.5	12.5 ± 4.2 (a)	11.8 ± 4.1 (b)	(a,b) < 0.01
Fullness after lunch	81 ± 5.4 (a)	59.1 ± 19.6 (a,b)	77 ± 5.6 (b)	77 ± 5.6 (a)	(a,b) < 0.05
Prospective consumption before lunch	59 ± 5.5 (a,b,c)	78.3 ± 4 (a)	71.4 ± 3.7 (b)	75.7 ± 4.5 (c)	(a,c) < 0.001 (b) < 0.01
Prospective consumption after lunch	6 ± 2.2 (a,b,c)	25 ± 5.4 (a,b,d)	12.3 ± 2.7 (b,c)	13.5 ± 4.7 (c,d)	(a-b) < 0.001 (d) < 0.01
Palatability	60 ± 7 (a,b,c)	75 ± 5.4 (a)	71.4 ± 4.7 (b)	74.3 ± 5.3 (c)	(a-c) < 0.01

VAS, Visual Analog Scale is measured in a horizontal scale, maximum value 100. EBD, eating behavior disorder. PD, peritoneal dialysis. Letters represent the statistical differences (read in horizontal) Mann–Whitney U test.

Anorexigenic Peptide Release After Food Intake

The anorexic patients had high basal and elevated plasma CCK levels 30 min after Fresubin^{TM} intake (**Figure 1A**), in parallel with the peak rise in glucose and insulin (**Supplementary Figures S1A,B**). The CCK increment in obese patients was clearly weaker and retarded. In regard to leptin, obese patients showed highest values and flat curve. No single group showed changes in leptin levels (**Figure 1B**) after food stimulus. In relation to adiponectin, obese patients had the lowest baseline levels from PD groups. All groups showed a postprandial decrease without peaks. On the contrary, the control group

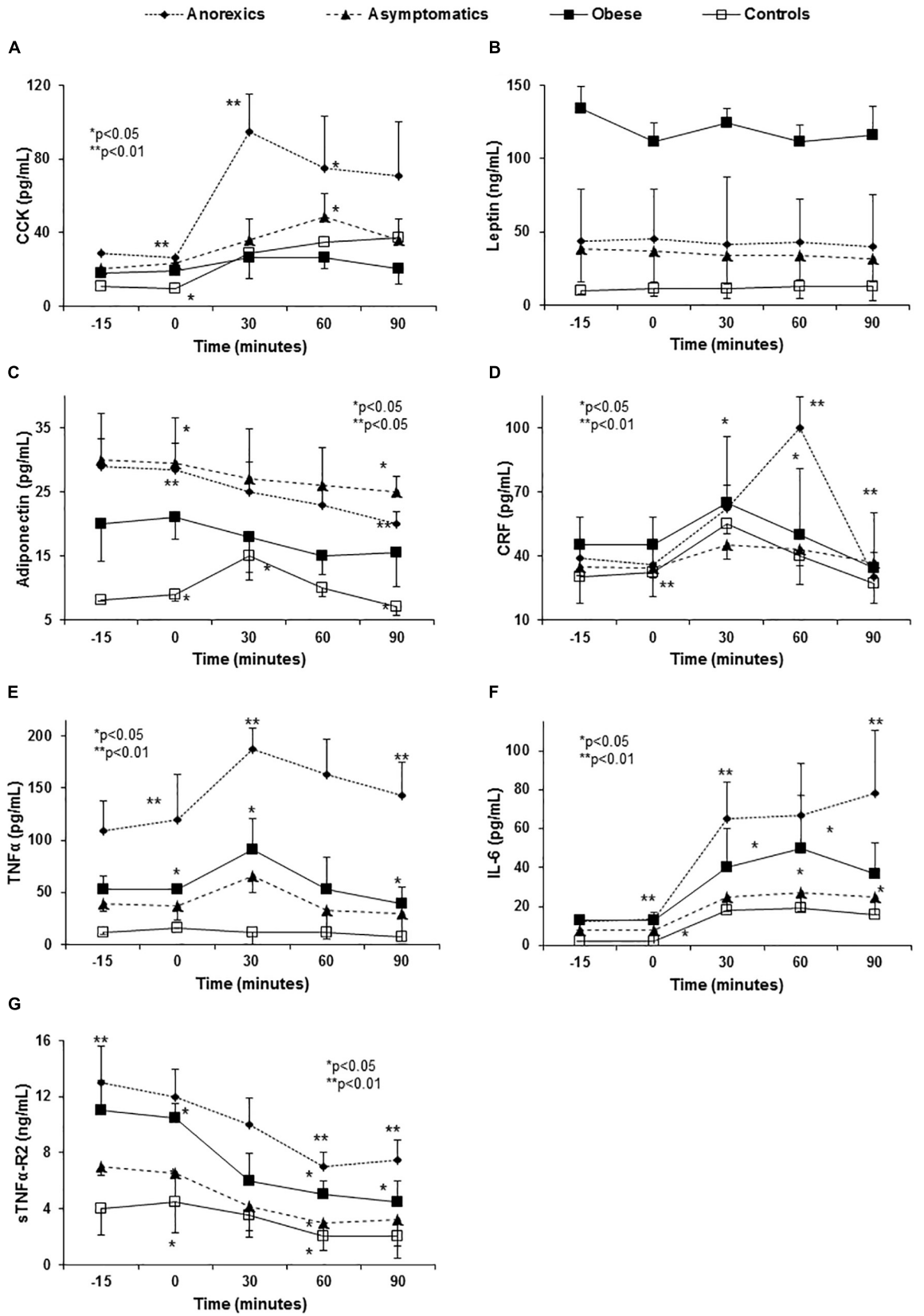

FIGURE 1 | Continued

FIGURE 1 | Abnormal anorexigen peptide and pro-inflammatory cytokine release in PD patients with eating behavior disorders. Panel **(A)** shows the CCK release after food consumption. Anorexic patients show a very important increase 30 min after Fresubin^{TM} intake that fell slightly at 60 and 90 min without reaching the basal CCK values. This peak explains the poor appetite (VAS) and the early satiety showed by these patients after food intake (**Tables 1–3**). The remaining groups had flat curves, except for the controls that maintained the peak values at 30 and 60 min. Obese patients did not show differences along of the curve. $P < 0.0001$ global statistical difference between the groups (three factor ANOVA test). Panel **(B)** shows flat curves of leptin without any modification over time. All PD patients had high basal leptin plasma levels especially the obese group. $P < 0.01$ (three factor ANOVA test). Panel **(C)** shows high adiponectin levels in PD patients, especially in the anorexic and asymptomatic patients when compared with the controls. All PD groups show a progressive and significant decrease over time and the control subjects show a peak at 30 min that fell at 90 min. Panel **(D)** shows the changes in plasma CRF levels after food intake. In normal conditions, CRF levels peak at 30 min and then decrease to values in the normal range after 90 min (controls) $p < 0.001$ (three factor ANOVA test). However, anorexic patients show an important elevation at 60 min possibly perpetuating the lack of appetite started by the CCK peak at 30 min **(A)** and the high basal adiponectin levels **(C)**. $P < 0.01$ (three factor ANOVA test). Panel **(E)** shows the changes in TNF-α plasma levels after standard food intake. Anorexic patients show the highest basal plasma levels and they increase to nearly 200 pg/mL at 30 min, returning to the basal levels after 90 min. Obese and asymptomatic patients have parallel curves with peaks at 30 min and returning to basal levels after 60 min. $P < 0.01$ (three factor ANOVA test). Panel **(F)** shows the changes in IL-6 and the important peak developed by the anorexics patients at 30 min that continued to rise slowly to a maximum after 90 min. In the remaining groups IL-6 peaked at 30 min but it decreased at 60 and 90 min. Panel **(G)** shows the changes in sTNFα-R2 for which the anorexic patients again had the highest values that decrease after eating, while maintaining the highest values over the entire time of the curve. However, after its levels fall at 60 and 90 min, they remained constant. Obese and asymptomatic patients and the controls showed parallel falls. $P < 0.001$ (three factor ANOVA test).

showed an important peak at 30 min that declined at 60 and 90 min (**Figure 1C**).

Corticotropin-releasing factor, other ARP that increased after eating, showed the highest basal levels in obese, with a peak at 30 min and a mild decrease after 90 min. By contrast, the CRF levels in anorexic patients peaked at 60 min with an important decrease after 90 min reaching similar values to the remaining groups (**Figure 1D**).

Orexigen Peptide Release After Food Intake

Anorexic patients had lower basal levels of NPY and there was a significant decrease in NPY 90 min after eating (**Figures 2A–C**). The obese patients had higher basal NPY levels and they experienced a significant increase 30 min after eating and remaining stable for up to 90 min (**Figure 2A**).

Postprandial changes in NO_3 plasma values were evident and PD patients showed higher basal levels than the controls (**Figure 2B**). All groups showed an important decrease at 30 min after eating and except in obese patients in whom maximal levels were evident after 60 min, NO_3 levels peaked after 90 min.

In regard to ghrelin, anorexic patients showed a dramatic fall in ghrelin 30 and 60 min after food consumption (**Figure 2C**) in comparison with the remaining groups. All groups showed similar values of ghrelin at 90 min after food stimulus.

Cytokine Release After Food Stimuli

Anorexic patients had higher basal TNF-α and IL-6 levels with important "peaks" 30 and 60 min after food consumption that did not fall after 90 min (**Figures 1E,F**). The curves from obese and asymptomatic patients displayed intermediate values between those of anorexic and control patients. The values for soluble TNF-α-receptor-2 (sTNFα-R2) were again highest in the anorexic patients and they decreased after eating albeit these patients maintained the highest values at all times (**Figure 1G**).

Glucose Clamp (Euglycemic and Hyperglycemic)

We found important differences in insulin sensitivity between PD patients and controls. The obese and anorexic patients showed higher insulin resistance compared with asymptomatic and controls (**Supplementary Table S2**).

Induction of Appetite-Related Peptide and Cytokine Release Disorders With Euglycemic Clamp

The constant administration of insulin to maintain stable levels of glucose in the euglycemic clamp permitted us to evaluate the influence of exogenous insulin and glucose administration on ARPr, bypassing the GIT. Both stimuli (glucose and insulin) were necessary and crucial to induce ARPr reproducing the abnormal patterns found in anorexic patients (peaks of CCK and CRF, **Figures 3A,C**), cytokines (peaks of TNF-α, sTNFα-R2, and IL-6) after eating in anorexic patients. Importantly, TNF-α and sTNFα-R2 secretion was consistently higher in anorexic patients (**Figures 3D–F**), suggesting that TNF-α was not able to auto-regulate its fat receptor (sTNFα-R2) and release. Moreover, in anorexics CCK and CRF maintained their elevated plasma levels at the end of the curves. Similarly, obese patients showed NPY peak with orexigenic action which had not declined after 90 min of follow-up (**Figure 2D**). NO_3 and ghrelin showed a dramatic decline at 60 and 90 min (**Figures 2E,F**) reproducing the similar patterns showed in anorexic and obese patients after food stimulus, except by the last part of the curves.

Relationship Between Cytokines, ARPr, and Insulin Resistance Marker

Given the known influence of circulating pro-inflammatory cytokines on insulin-glucose metabolism and their eventual relation with ARPr, we study their possible association as a framework. In the whole group, we found a positive linear correlation between plasma TNF-α levels and IL-1 (0.75, $p < 0.005$), as well as between IL-1 and GIP (0.46, $p < 0.05$), IL-1 and HOMA-IR (0.43, $p < 0.05$), and TNF-α and HOMA-IR (0.5, $p < 0.05$). In obese patients, there was a positive linear correlation between TNF-α and leptin (0.56, $p < 0.05$) and there appeared to be a linear correlation with *Ad* although this was not statistically significant (−0.43, $p < 0.07$, NS). These results suggest that there may be a feedback that would perpetuate a metabolic framework that would influence the feeding behavior.

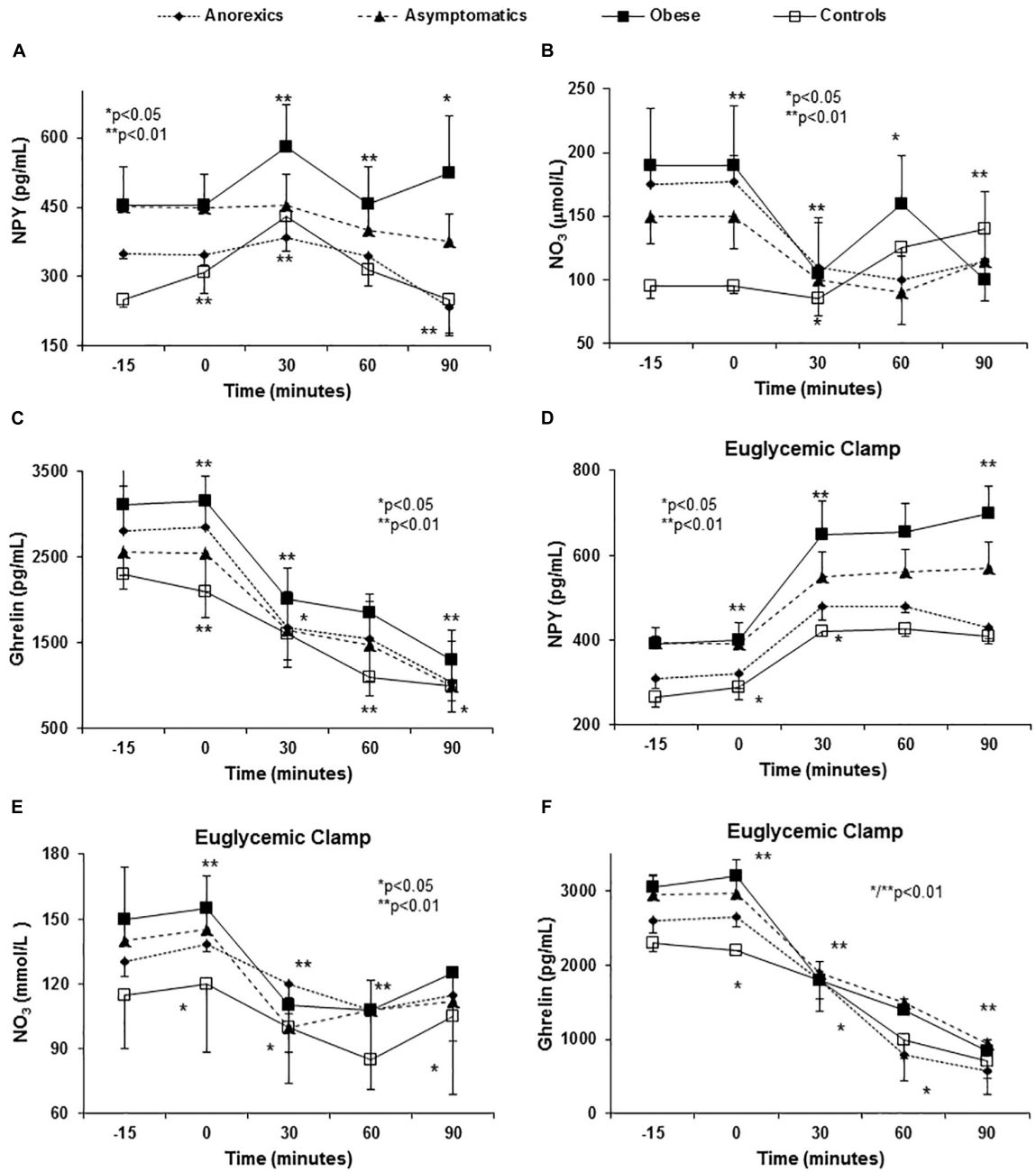

FIGURE 2 | Abnormal orexigenic peptide release (spontaneous and after euglycemic clamp) in PD patients with eating behavior disorders. All PD patients have higher NPY plasma levels (the most orexigenic peptide known) than controls **(A)**. $P < 0.01$ (three factor ANOVA test). In the control group, NPY levels peaked at 30 min followed by a decrease at 60 and 90 min to values below the baseline. Importantly, obese patients show a peak (the highest) at 30 min with a mild fall at 60 min and a rebound at 90 min. The appetite desire shows important correlation with these peaks (**Table 2**). Asymptomatic and anorexic patients show almost "flat curves." With regards NO (represented by NO_3), we found important baseline differences between PD patients and controls. After food intake, all patients show an important fall in plasma NO_3 levels at 30 min, except asymptomatic patients that show this fall at 60 min with a rebound at 90 min. Obese patients show an early fall, rebound at 60 min and a second fall at 90 min. Anorexic patients had a similar curve as the rest but they did not show a second fall **(B)**. $P < 0.01$ (three factor ANOVA test). The basal ghrelin plasma levels were higher in obese and lower in anorexic patients and an important fall occurs in all patients that is most pronounced in the anorexic patients **(C)**. $P < 0.01$ (three factor ANOVA test). Variance multi-factor analysis (ANOVA). Euglycemic clamp reproduced the abnormalities in orexigenic peptide release found in PD patients. After glucose and insulin infusion, the change in NPY in obese patients shows an important peak at 30 min that was maintained during the study **(D)**. $P < 0.001$ (three factor ANOVA test). Controls and the anorexic patients had lower values than the baseline with a virtually flat curve. The basal plasma NO_3 levels were higher in the PD patients than in the controls, showing a strong fall to reach similar values as in the controls at 30 min **(E)**. $P < 0.01$ (three factor ANOVA test). The plasma NO_3 levels were very similar between the groups at later times. With regards ghrelin, the obese patients have higher plasma levels and the anorexic patients the lowest. There was a decrease in NO_3 after glucose and insulin infusion that followed a very similar pattern in all groups. $P < 0.01$ (three factor ANOVA test). $P < 0.01$ (three factor ANOVA test). The plasma NO_3 levels were very similar between the groups at later times. There was a decrease in NO_3 after glucose and insulin infusion that followed a very similar pattern in all groups. With regards ghrelin **(F)** the obese patients have higher plasma levels and the anorexic patients the lowest. $P < 0.01$ (three factor ANOVA test).

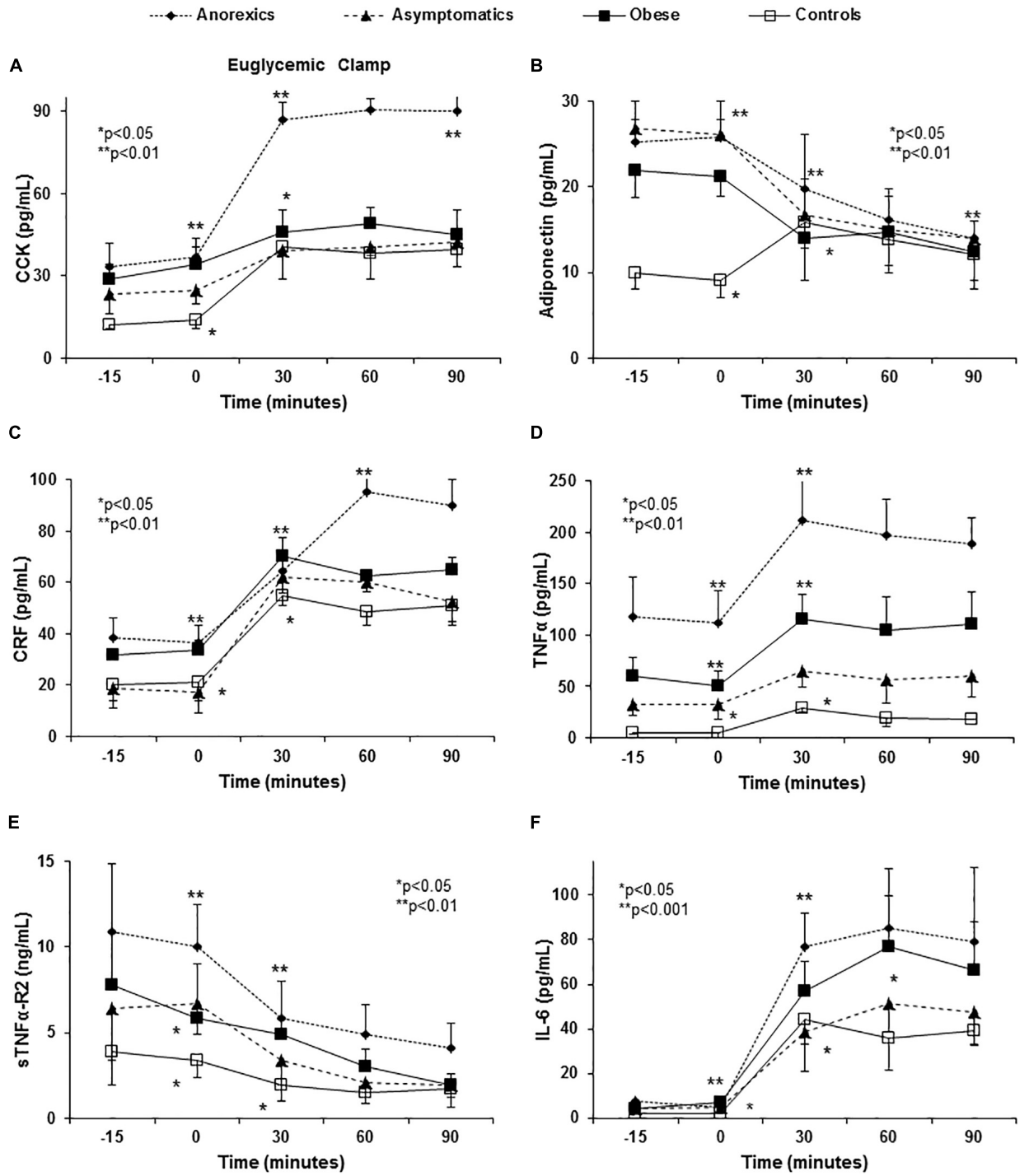

FIGURE 3 | Euglycemic clamp reproduced the abnormalities in anorexigenic peptide pro-and inflammatory cytokine release found in PD patients. A euglycemic clamp permits us to evaluate the effect of insulin and exogenous glucose administration on ARPr, by-passing the gastrointestinal tract. The hyperglycemia and hyperinsulinemia induced are sufficient to abnormally release CCK **(A)**. $P < 0.001$ (three factor ANOVA test). Anorexic patients maintain their CCK peak when compared with the remaining groups. With regards adiponectin, PD patients had higher plasma levels when comparison to the controls **(B)**. $P < 0.001$ (three factor ANOVA test). The obese patients show lower values than the anorexic and asymptomatic patients. All PD patients showed an important fall between 30 and 90 min. By contrast, controls had elevated levels that peaked at 30 min and then fell at 60 and 90 min. Another important anorexigen substance, CRF, also shows important changes and in anorexic patients high levels were reached at 60 min that were maintained until 90 min. The remaining groups show intermediate peaks **(C)**. $P < 0.01$ (three factor ANOVA test). The changes TNF-α, IL-6, and sTNFα-R2 after glucose and insulin infusion are shown in **(D–F)**. Euglycemic clamp reproduced the different curves from anorexic, obese and asymptomatic patients and controls found after eating. However, the ends of the curves (60 and 90 min) were flat, possibly due to the stable and high insulin and glucose levels maintained by the euglycemic clamp. $P < 0.001$ (three factor ANOVA test).

The Relationship Between ARPr and Cytokines May Promote Uremic Anorexia

In anorexic patients, there was a positive linear correlation between basal IL-1 and CCK (0.45, $p < 0.05$), while TNF-α showed a negative correlation with ghrelin (−0.66, $p < 0.01$). Similarly, NPY showed a negative linear correlation with basal IL-1 (−0.52, $p < 0.05$) and TNF-α (−0.51, $p < 0.05$), and a positive relationship with CRF (0.51, $p < 0.01$). Finally, basal leptin levels were negatively correlated with ghrelin (−0.54, $p < 0.01$). After food consumption, NPY was negatively correlated with basal IL-1 after 30 and 90 min (−0.64, $p < 0.01$ and −0.61, $p < 0.01$, respectively).

The Relationship Between ARPr and Insulin Resistance May Promote High Food Intake in Obese Patients

In this group, we found a positive linear correlation between basal serum leptin and basal BMI (0.6, $p < 0.01$), fat mass (by BIA: 0.67, $p < 0.01$), NO_3 (0.51, $p < 0.05$), HOMA-IR (0.66, $p < 0.01$), and *Ad* (−0.54, $p < 0.01$). Likewise, NPY was positively correlated with ghrelin (0.45, $p < 0.05$). After food stimuli, the NPY levels after 90 min showed a positive linear relationship with all points of the leptin curve at baseline, 30, 60, 90 min, (0.51, $p < 0.05$; 0.67, $p < 0.01$; 0.67, $p < 0.01$; 0.63, $p < 0.01$, respectively).

Relationship Between ARPr, Cytokine, and Appetite Desire (VAS)

Appetite desire was positively correlated with NPY and negatively correlated with CCK (basal and post-stimuli, **Figures 2A** and **1A**, respectively, **Tables 3**, **4**) and TNF-α in the whole group, suggesting a cause–effect relationship between these molecules and VAS. In anorexic patients TNF-α and CCK showed an inverse correlation with appetite desire, especially 30 min after eating (**Table 3**). TNF-α was associated with the sensation of fullness and poor palatability, as well as to poor prospective food consumption 90 min later. In obese patients NPY showed the strongest relationship with VAS, especially 30 min after food intake, and it was associated with high palatability and a stronger desire for prospective consumption 90 min after eating (**Table 3**).

Appetite-Related Gene Expression in Abdominal Fat Tissue

The TNF-α gene was over-expressed in all uremic patients, and anorexic patients showed higher mRNA expression than other groups (**Figure 4A**). Importantly, TNF-α gene expression was positively correlated with TNF-α plasma levels (0.66, $p < 0.05$). Obese patients had the lowest fat leptin expression, which was negatively correlated with plasma leptin levels (−0.54, $p < 0.0.5$), indicating a negative feedback that down-regulated the expression of this gene (**Figure 4B**). All patients showed lower global Ad fat expression than controls, and the lowest expression was evident in obese patients (**Figure 4C**).

Relationship Between Fat Appetite-Related Gene Expression and Insulin Resistance

There was a positive linear correlation between plasma insulin (**Supplementary Figure S1B**) and fat TNF-α expression (0.55, $p < 0.01$), as well as a negative relationship between plasma insulin and fat leptin expression (0.34, $p < 0.05$) or fat Ad expression (0.44, $p < 0.05$) (**Figures 4A–C**). Similarly, HOMA was positively related with fat TNF-α expression (0.64, $p < 0.01$) and it was negatively related to fat leptin expression (0.5, $p < 0.01$).

Relationship Between Fat Appetite-Related Gene Expression and Appetite Desire (VAS)

Table 5 shows the relationships between TNF-α, leptin and Ad gene expression, and appetite, measured by VAS, in anorexic and obese patients. The participation of TNF-α and its association with VAS was predominant in anorexic patients.

TABLE 3 | Relationship between anorexigens/orexigens and VAS in anorexics PD patients.

VAS Peptides (#)	CCK- 0 min	CCK- 30 min	CCK- 60 min	CCK- 90 min	NPY- 0 min	NPY- 30 min	NPY- 60 min	NPY- 90 min	TNF-α[&]
Desire to eat before lunch	−0.34*	−0.6**	−0.52**	−0.4*	0.36*	0.33*	0.38*		−0.56**
Desire to eat after lunch		−0.43*			0.46*	0.4*			
Hunger before lunch	−0.41*	−0.66**	−0.47*	−0.5**			0.34*		−0.66**
Hunger after lunch		−0.55**	−0.52**	−0.53**					
Fullness after lunch				0.55**	−0.46*	−0.6**		0.51**	0.52**
Prospective consumption before lunch				−0.48*	0.48*	0.34*		0.47*	−0.48*
Prospective consumption after lunch			−0.4*				0.56**		
Palatability	−0.5*	−0.53**		−0.75**		0.42*		0.42*	−0.6**
Hunger 2 h before lunch		−0.38*	−0.4*	−0.6**			0.34*		−0.5**
Satiety 2 h after lunch		0.53**		0.42*					

*VAS, Visual Analog Scale. PD, peritoneal dialysis. #, means the time after the food stimulus; &, baseline. *$p < 0.05$, **$p < 0.01$.*

TABLE 4 | Relationship between anorexigens/orexigens and VAS in obese PD patients.

VAS Peptides (#)	CCK-0 min	CCK-30 min	CCK-60 min	CCK-90 min	NPY-0 min	NPY-30 min	NPY-60 min	NPY-90 min	TNF-α[&]
Desire to eat before lunch		−0.41**		−0.38*	0.5**	0.58**		0.4*	
Desire to eat after lunch	−0.38*		−0.38*	−0.48*		0.54**			
Hunger before lunch	−0.5*	−0.36*	−0.58**	−0.35*	0.4*	0.38*	0.55**		−0.31*
Hunger after lunch		−0.38*	−0.39*	−0.33*	0.33*	0.54**		0.5**	
Fullness after lunch	0.43*				−0.34*	−0.34*			
Prospective consumption before lunch					0.57**	0.6**	0.37*	0.53**	−0.33*
Prospective consumption after lunch			−0.37*			0.53**	0.51*	0.53**	
Palatability	−0.46*		−0.56**			0.41*		0.63**	−0.37*
Hunger 2 h before lunch				−0.35*		0.51**	0.56**	0.7**	
Satiety 2 h after lunch			0.35*					−0.44*	

*VAS, **V**isual Analog Scale. #, means the time after the food stimulus**;** &, baseline. Linear correlations**:** *p < 0.05, **p < 0.01.*

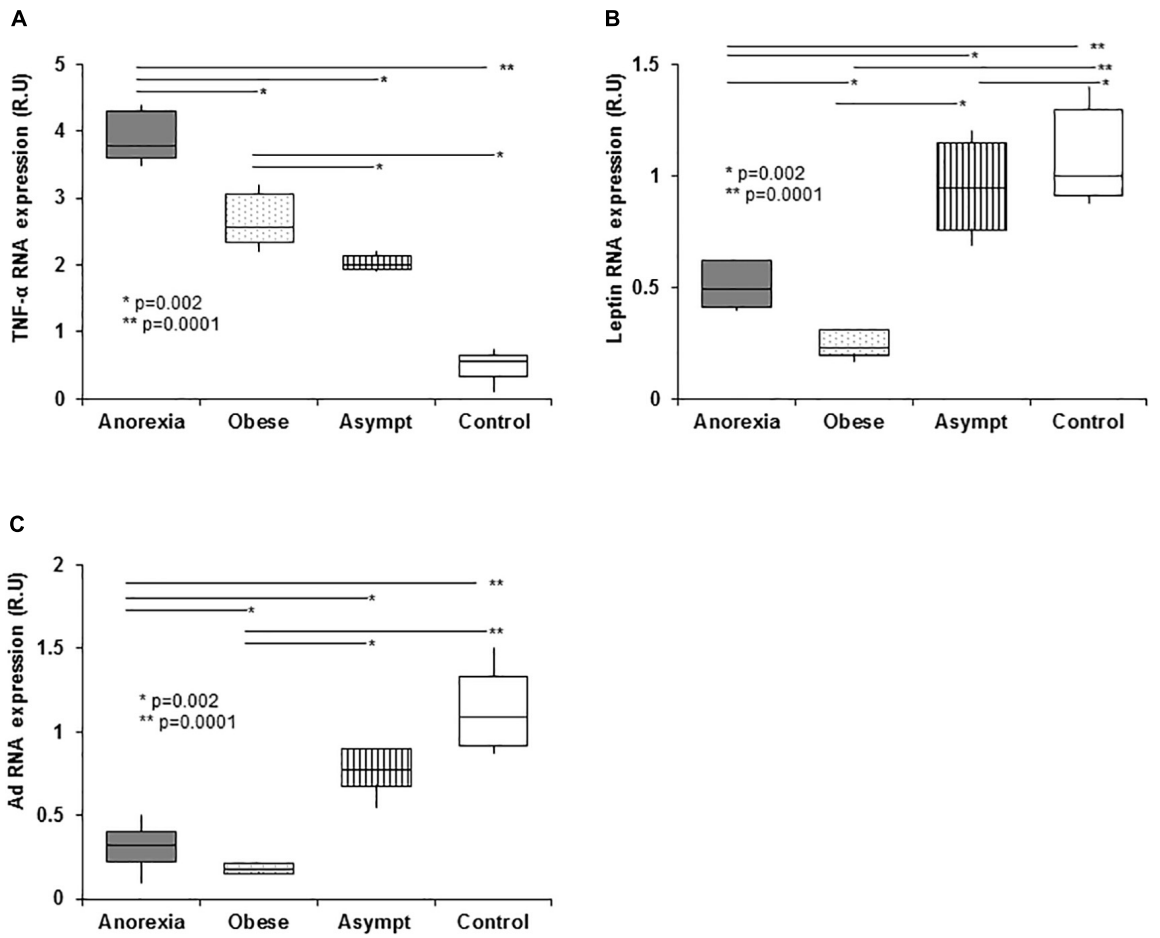

FIGURE 4 | Expression of genes related to eating behavior disorders in PD patients in abdominal subcutaneous fat (qPCR). PD patients show higher fat tissue TNF-α expression than controls. The highest TNF-α expression was in the anorexic patients, with obese and asymptomatic patients showing intermediate expression. The high TNF-α plasma levels present in anorexic patients did not inhibit the TNF-α expression in fat suggesting that in uremia, fat tissue is an important source of pro-inflammatory cytokines **(A)**. With regards leptin expression in fat tissue, the obese patients show an important downregulation of leptin gene expression in fat when compared to the remaining groups. This down expression may be associated with a negative feedback loop induced by the high plasma TNF-α levels. Anorexic and asymptomatic patients express intermediate levels of leptin between obese patients and controls **(B)**. Panel **(C)** shows the adiponectin expression, which was lower in the fat from PD patients than in controls. As expected, obese patients expressed adiponectin the weakest and again, anorexic and asymptomatic patients had expression levels intermediate to those of obese patients and controls. Box plots show the 25th and 75th percentiles, median, minimum and maximum values of five independent experiments. The symbols represent the statistical differences between the groups (ANOVA one-way and Mann–Whitney rank sum *U* test).

DISCUSSION

There are three important findings arising from this study:

(1) We have identified well-defined patterns of EBD in dialysis patients, defined by the VAS and nutritional markers (**Table 1** and **Supplementary Table S2**).
(2) The presence of ARPr disorders associated with insulin resistance and systemic inflammation (**Tables 3**, **4** and **Figures 1–4**).
(3) The close relationship between EBD, abnormal ARP, and cytokines release (**Tables 3**, **4**) and cytokines fat-tissue gene expression (**Table 5** and **Figure 4**).

Characterization of Appetite Disorders in PD Patients

Visual Analog Scale has been used to measure eating motivation and validated in PD, HD, and kidney transplant patients (Hylander et al., 1997). Employing this scale, we demonstrate that patients suffering anorexia showed the minimal score of hunger, lower palatability and eating desire, in conjunction with a sensation of greater fullness, contrasting with the features found in obese patients. Importantly, our patients showed a dietary preference for carbohydrates.

Uremic Insulin Resistance

As it has been demonstrated by others (Zoccali et al., 2005; Kalra et al., 2008), we found different grades of insulin resistance in our PD patients that were related with different EBD.

Baseline Results

Our PD patients showed high spontaneous baseline plasma levels of anorexigenic peptides and cytokines, including C-peptide and GIP, which have an anorexigenic effect mediated by insulin-glucose metabolism. Importantly, anorexic patients have the highest plasma levels of anorexigens including TNF-α and low NPY levels (orexigen), as we previously described (Aguilera et al., 1998, 2001). IL-1 and IL-6 are another cytokines associated with loss of appetite (McCarthy et al., 1995). According to our results, patients with anorexia have the lowest RRF and the highest cytokine concentration, suggesting that RRF is a more important determinant of appetite through this *via* than dialysis dose.

Results Following Food Intake

We clearly identified distinct alterations in ARPr pattern in studied groups after food intake. Given these results, we explored the influence of exogenous insulin and glucose administration, together or separately (hyperglycemic and euglycemic clamp), on ARP and cytokine release bypassing the GIT.

Anorexigen Substances

Cholecystokinin is a potent anorexigen with peripheral and central actions that is implicated in the pathogenesis of *anorexia nervosa*, cancer, senile and alcoholic anorexia (Dupré et al., 1973). In PD patients with anorexia, the plasma levels of CCK were elevated after eating. CCK is also retained in dialysis patients and it is not modified by PD or HD (Peikin, 1989). Previously, we did not find high CCK plasma levels in anorexic patients prior to food stimulus (Aguilera et al., 1998). Here, we identified a "peak" of plasma CCK 30 min after eating (**Figure 1A**), which may be responsible for the early sensation of fullness. This CCK "peak" was also found in patients with anorexia nervosa and although the exact cause is unknown, this phenomenon is potentially reversible when the nutritional status is recovered (Owyang et al., 1979). Modifications to peripheral insulin activity when nutrition or systemic inflammation improves might explain this phenomenon. By contrast, obese patients showed a delayed CCK "peak" and a similar abnormality may be found in non-uremic patients with bulimia nervosa, where the CCK "peak" is delayed and is 50% lower than those in control (Harty et al., 1991).

Again, the positive linear correlations found between CCK and IL-1, IL-1, and GIP, and GIP and insulin (other anorexigen) may perpetuate the anorexia and the insulin resistance suffered by our patients (Daun and McCarthy, 1993). In fact, a synergic effect between IL-1 and CCK inducing anorexia has been described, while IL-1 and GIP are factors that stimulate insulin release by the pancreas (Geracioti and Liddler, 1988). The euglycemic clamp replayed the same CCK and cytokines release patters (**Figures 3A,D–F**). These results suggest that insulin metabolism

TABLE 5 | Relationship between fat gene expression and appetite desire (VAS).

	Anorexics			Obese patients		
VAS/gene expression	**TNF-α**	**Leptin**	**Adiponectin**	**TNF-α**	**Leptin**	**Adiponectin**
Desire to eat before lunch	−0.66**	−0.55**	−0.54**		0.37*	
Desire to eat after lunch	−0.43*				0.45*	
Hunger before lunch	−0.4*	−0.55*	−0.34*			−0.4*
Hunger after lunch						
Fullness after lunch	0.7**			0.37*		
Prospective consumption before lunch		−0.6**			0.56**	
Prospective consumption after lunch		−0.46*	−0.37*			−0.5*
Palatability	−0.5**					
Hunger 2 h before lunch	−0.41*			−0.35*	0.44*	
Satiety 2 h after lunch		0.5*				

*VAS, Visual Analog Scale. Linear correlations: *$p < 0.05$, **$p < 0.01$.*

and high grade of systemic inflammation may be key in the induction of anorexia in PD patients.

Leptin is an adipose tissue hormone that modulates appetite and insulin activity in target cells, inducing insulin resistance (Klein et al., 1996). Although, we did not find changes in leptin levels, in non-renal population plasma leptin appears to increase 4 h after food intake (Klein et al., 1996). Unfortunately, we have no data from this time interval.

Adiponectin, in sharp contrast to leptin, plasma *Ad* levels are negatively correlated with body fat, decreasing with obesity and increasing with weight loss (Liu et al., 2003; Coll et al., 2007). Moreover, hyperadiponectinemia can reduce the food intake in rats (Coope et al., 2008). Here, we found high plasma *Ad* levels in PD patients, although, the lowest values were registered in obese patients as described elsewhere in non-renal obese (Coll et al., 2007). In normal conditions, *Ad* levels increase 30 min after food intake and decrease over the following 60 and 90 min. In all our patients the decrease was delayed, the obese patients maintaining the lowest values. It is generally accepted that *Ad* is negatively related to insulin levels due to its anti-insulin resistance effects (Isganaitis and Lustig, 2005). However, in uremia, the negative feedback between insulin secretion and *Ad* may be partially broken since adiponectin is retained (Díez et al., 2005). Indeed, the exogenous administration of insulin and glucose in the euglycemic clamp strongly regulated *Ad* in all studied groups (**Figure 3B**). Therefore, the differences in *Ad* release in these patients appear to be mediated by insulin resistance contributing indirectly to EBD in PD patients.

Corticotropin-releasing factor is produced by hypothalamic neurons that exert a central and peripheral anorexigenic effect, inhibiting that of NPY (Kalra et al., 2008). While there were no basal differences between PD patients, 60 min after eating anorexic patients displayed an important peak of CRF that was correlated with VAS. In addition to insulin, one of the most important regulators of CRF activity is plasma cortisol, therefore impaired cortisol suppression may explain EBD in uremia (Deshmukh et al., 2005). Unfortunately, we did not study the circadian variations in plasma cortisol and ACTH. In accordance with our findings, an important disruption in insulin release that may be associated to disorders in CRF secretion and *vice-versa* has already been observed in patients suffering chronic renal failure (Feneberg et al., 2002). Again euglycemic clamp reproduced the CRF release patters in all groups suggesting a deep dependence of insulin and glucose levels (**Figure 3C**).

Orexigenic Substances

In normal condition a "peak" of NPY appeared 15–30 min after food intake and may be responsible for hunger persistence, possibly augmenting the sensation of gastric emptiness (Weisman et al., 1998). This explains the popular idea that small snacks before the main meal stimulate appetite. Importantly, this NPY "peak" was absent in our patients with uremic anorexia (**Figure 2A**), who conversely showed a significant decrease in NPY 90 min after eating that fell below the basal values, explaining the early and late lack of appetite seen in anorexics. NPY "peaks" were evident 30 and 90 min after eating in obese patients, which could explain the repetitive and greater amount of food ingested. This post-prandial NPY "peak" rebound at 90 min has been described previously in bulimic patients (Rosenbaum et al., 1997). Moreover, our obese group showed relatively high NPY levels, although within the normal range, which could be associated with high appetite desire. Recent studies suggest the existence of disorders in hunger-hypothalamic receptor sensibility in the non-uremic obese population (Rosenbaum et al., 1997; Weisman et al., 1998). NPY and leptin levels are significantly higher in Zuker (*Ob/Ob*) rats, inducing repetitive and compulsive food intake due to the failure of leptin to inhibit NPY release (Stricker-Krongrad et al., 1994). In uremic status, hypothalamic receptor disorders have been poorly studied, although the parallels in the glucose, insulin and NPY curves and their complex relationship may underlie the excessive and sustained NPY release. Obesity and uremia status may be the maximal expression of this disorder since both are associated with the hyperinsulinemia and insulin resistance that might perpetuate the abnormal NPY release. The euglycemic clamp results support the role of insulin metabolism on NPY release (**Figure 2D**).

But not only carbohydrate intolerance appears to affect appetite in uremia. Cytokines also may be affected by insulin metabolism. We recently demonstrated an inverse relationship between TNF-α and NPY in PD patients, and we speculated that TNF-α may inhibit its orexigenic effect (Xu et al., 1988; Aguilera et al., 1998). We also found a negative linear correlation between NPY and IL-1, supporting the MIA hypothesis (Aguilera et al., 1998). Together, ARPr, TNF-α, IL-6, and s-TNFα-R2 showed important modifications after food stimulus. This parallelism invites to think that pro-inflammatory cytokines may synergize and perpetuate EBD as anorexia in our patients.

Nitric oxide is another important appetite stimulator (Xu et al., 1988; Vallance et al., 1992; Squadrito et al., 1994) and experimentally, a decrease in NO production inhibits appetite (Squadrito et al., 1994). A potent NO-synthase inhibitor accumulated in uremic patients (Vallance et al., 1992) and as a consequence, one might expect dialysis patients to have lower plasma NO_3 values than controls. However, it did not occur. This apparent contradiction may be explained by the uremic retention of inactive forms of NO, represented by NO_3, by the NO relationship with the pool of arginine (its substrate), ornithine, pro-inflammatory cytokines capable of inhibiting NO synthase, endothelin-1 and insulin metabolism (Yan et al., 2008). NO stimulates the insulin release and the equilibrium between NO and endothelin-1 with a predominium of endothelin-1 inducing insulin resistance (Yan et al., 2008). Moreover, high plasma levels of endothelin-1 have been reported in uremics (Roccatello et al., 1997).

On the other hand, modification of NO after food intake and after the euglycemic clamp highlights the role of insulin and uremic insulin resistance on hunger-satiety control (**Figure 2E**). Moreover, the high TNF-α plasma levels shown by anorexic patients may explain the dramatic fall in NO_3 after food stimuli due to decreased NO synthesis perpetuating the early anorexia (Yan et al., 2008).

Ghrelin is another orexigen GIT hormone that is secreted in response to stomach empty. It decreases after food intake,

constituting a peripheral negative feedback and increase after weight loss, in fasting condition and insulin induced hypoglycemia (Theander-Carrillo et al., 2006). Ghrelin plasma levels in uremia are generally elevated, although anorexic patients show relatively lower values (Aguilera et al., 2004a). We confirmed our previous findings, that PD patients maintain higher ghrelin plasma levels than controls (**Figure 2C**). Moreover, we confirmed the strong inverse relationship between ghrelin levels and glucose or insulin release after food intake, and that after euglycemic clamping ghrelin maintained its levels in parallel with the insulin and glucose levels (**Figure 2F**).

Not only insulin resistance and inflammation participate in appetite regulation, but the expression of genes in abdominal fat associated with insulin metabolism can also regulate appetite. Fat TNF-α over-expression (**Figure 4A**) is not inhibited by its high plasma levels and in fact, the levels of sTNFα-R2 are maintained, indicating that fat tissue is an important source of pro-inflammatory cytokines in uremia. By contrast, obese subjects have the lowest leptin and *Ad* fat expression (**Figures 4B,C**) indicating their down-regulation in fat, possibly due to negative feedback through their high plasma, as described by elsewhere (Nordfors et al., 1998).

Our results permit to explain the different eating behaviors in PD patients according to different degrees of insulin resistance and systemic inflammation. The euglycemic clamp exactly reproduces the insulin sensitivity and abnormal ARP and cytokines release patterns in anorexics and obese, indicating that glucose-insulin metabolism definitely triggers these appetite disorders.

Finally, other abnormalities as sex hormones, brain neurotransmitters or receptors acquired disorders in uremia (i.e., MC4-r), may contribute to the diversity of EBD in this population (Xu et al., 1988; Cheung et al., 2005; Carrero et al., 2008).

CONCLUSION

In PD patients, EBD are modulated by an abnormal baseline levels of ARP and cytokines, which are abnormally released after food intake and highly dependent of insulin metabolism. The renal ARP retention, the excess of pro-inflammatory cytokines and uremic carbohydrate intolerance, with predominance of inflammation in anorexics or hyperinsulinemia in obese patients, may explain the tendency to develop one or other EBD.

ETHICS STATEMENT

The present study adjusts to the Declaration of Helsinki and was approved by the Ethics Committee of the Hospital Universitario la Princesa, Madrid, Spain. Informed written consent was obtained from all the patients.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution equally to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00630/full#supplementary-material

FIGURE S1 | Insulin resistance markers release in PD patients with eating behavior disorders. Panel **(A)** shows the glucose release after standard food intake (Fresubin^{TM}). PD patients, especially obese patients, show an important and sustained elevation of glucose that reaches values between 150 and 165 mg/dL. In the control group glucose peaks at 30 min, decreasing to normal values at 60 min and then declining at later periods (90 min). $P < 0.001$ (three factor ANOVA test). Panel **(B)** shows a similar pattern of insulin release as glucose. In obese the insulin curve was highest and maintained around 150 μU/mL a long the time (90 min). All PD patients showed "lazy curves" with insulin levels between 50 and 90 μU/mL, whereas controls show a final decrease in insulin at 90 min. $P < 0.001$ (three factor ANOVA test). Panel **(C)** shows the glucagon plasma levels in the different groups. Controls show a mild elevation at 60 min with a significant decrease at 90 min. In PD patients the levels remain constant ("flat curves") except for the peak detected in obese patients at 30 min. $P < 0.01$ (three factor ANOVA test). Panel **(D)** shows the C-peptide curves and again, obese patients have the highest values in the curve. The controls show a peak at 60 min with a significant decrease at 90 min. $P < 0.001$ (three factor ANOVA test). Panel **(E)** shows the GIP levels in the groups studied. PD patients display an important elevation at 30 and 60 min (anorexics) with no decrease at later periods, whereas the lowest values were in the controls maintaining the peak at 60 and 90 min. Obese patients showed an important and sustained elevation of glucose that reaches values of 150–165 mg/dL. Control group showed a glucose peaks at 30 min, decreasing to normal values at 60 min and then declining at 90 min. In whole PD patients, a similar pattern of insulin release and glucose was found "lazy curves." Obese showed the highest insulin values with fat curves at later periods, whereas controls show a final decline in insulin curve at 90 min. In regard to glucagon, patients showed elevated baseline levels than controls, especially in obese and anorexics with a small peak at 30 and 60 min, respectively. Controls show a mild elevation at 60 min with a significant decrease at 90 min. The C-peptide a pancreatic insulin reserve marker, showed the highest values in obese. Controls show a peak at 60 min with a significant decrease at 90 min. **Figure 1E** shows the changes in GIP levels after food stimulus. Anorexic PD patients display an important elevation of GIP at 30 and 60 min which is maintained at later periods, whereas controls showed lowest values with peak at 60 and decreasing at 90 min. $P < 0.01$ (three factor ANOVA test).

TABLE S1 | Gene primers.

TABLE S2 | Euglycemic (insulin sensitivity) and hyperglycemic clamp study (insulin secretion).

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Long Peritoneal Dialysis Dwells With Icodextrin: Kinetics of Transperitoneal Fluid and Polyglucose Transport

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Background and objective: During peritoneal dialysis (PD), the period of effective net peritoneal ultrafiltration during long dwells can be extended by using the colloidal osmotic agent icodextrin but there are few detailed studies on ultrafiltration with icodextrin solution exceeding 12 h. We analyzed kinetics of peritoneal ultrafiltration in relation to icodextrin and its metabolites for 16-h dwells with icodextrin.

Design, setting, participants, and measurements: In 20 clinically stable patients (mean age 54 years; 8 women; mean preceding time on PD 26 months), intraperitoneal dialysate volume (V_D) was estimated from dilution of ^{125}I-human serum albumin during 16-h dwell studies with icodextrin 7.5% solution. Sodium was measured in dialysate and plasma. In 11 patients, fractional absorption of icodextrin from dialysate, dialysate, and plasma amylase and high and low (Mw <2 kDa) Mw icodextrin fractions were analyzed.

Results: Average V_D increased linearly with no difference between transport types. At 16 h, the cumulative net ultrafiltration was 729 ± 337 ml (range −18 to 1,360 ml) and negative in only one patient. Average transcapillary ultrafiltration rate was 1.40 ± 0.36 ml/min, and peritoneal fluid absorption rate was 0.68 ± 0.38 ml/min. During 16 h, 41% of the initial mass of icodextrin was absorbed. Plasma sodium decreased from 138.7 ± 2.4 to 136.5 ± 3.0 mmol/L ($p < 0.05$). Dialysate glucose G2–G7 oligomers increased due to increase of G2–G4 metabolites while G6–G7 metabolites and higher Mw icodextrin fractions decreased. In plasma maltose and maltotriose (G2–G3 metabolites) increased while higher Mw icodextrin oligomers were almost undetectable. Dialysate amylase increased while plasma amylase decreased.

Conclusions: Icodextrin resulted in linear increase of V_D with sustained net UF lasting 16 h and with no significant difference between peritoneal transport types. In plasma, sodium and amylase declined, G2–G3 increased whereas larger icodextrin fractions were not detectable. In dialysate, icodextrin mass declined due to decrease of high Mw icodextrin fractions while low Mw metabolites, especially G2–G3, increased. The ability of icodextrin to provide sustained UF during very long dwells – which is usually not possible with glucose-based solutions – is especially important in anuric patients and in patients with fast peritoneal transport.

Keywords: end-stage kidney disease, peritoneal dialysis, ultrafiltration, osmotic agent, polyglucose metabolites, amylase

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INTRODUCTION

Peritoneal dialysis (PD), the leading home-based renal replacement therapy for patients with end-stage kidney disease (ESKD), associates with clinical outcomes comparable to or even better than those of in-center hemodialysis (Li et al., 2017). However, fluid overload is a common problem that is associated with poor clinical outcomes in all ESKD patients including those undergoing PD (Ng et al., 2018). Restoring water balance by adequate ultrafiltration (UF) is therefore a key target for dialysis treatment.

In PD, peritoneal transcapillary UF is typically induced using the crystalloid osmotic agent glucose and the increased dialysate tonicity results in water flow through endothelial aquaporin-1 water channels and inter-endothelial small pores into the dialysate. However, the osmotic gradient decreases due to the rapid absorption of dialysate glucose to the vascular circulation, leading to reduced and eventually negative net UF – during the overnight dwell in continuous ambulatory PD (CAPD) and the long daytime dwell in automated PD (APD) – especially in patients with fast peritoneal solute transport rate (Krediet et al., 1987; Agrawal and Nolph, 2000; Rippe and Levin, 2000; Krediet and Mujais, 2002; Qi et al., 2011; Morelle et al., 2018). Other limitations of glucose-based solutions are metabolic complications linked to glucose absorption such as hyperglycemia, hyperinsulinemia, and hyperlipidemia (Grodstein et al., 1981; Boeschoten et al., 1988) and unphysiological features (low pH, hyperosmolality, and high concentrations of lactate, glucose, and glucose degradation products) that are harmful to the peritoneal membrane (Liberek et al., 1993).

These limitations can be avoided by using the colloid osmotic agent icodextrin, which induces a more long-lasting water flow occurring predominantly through the small pores of the peritoneal capillaries (Morelle et al., 2018). Icodextrin is a mixture of starch-derived glucose polymers, linked by α1–4 (90%) and α1–6 (10%) glucosidic bonds (Alsop, 1994; Mistry, 2011), with molecular weight (MW) predominantly (>85%) ranging between 1,638 and 45,000 Daltons (Da) and only 6% having a MW less than 1,638 Da. Since the colloid osmotic pressure created by icodextrin is almost constant, UF is sustained throughout a long dwell (Mistry et al., 1987, 1994; Davies, 1994; Ho-dac-Pannekeet et al., 1996; Krediet et al., 1997; Morelle et al., 2018).

Absorption of icodextrin from the peritoneal cavity into blood is slow and occurs mainly by convective pathways *via* the lymphatics (Davies, 1994). Icodextrin is hydrolyzed in plasma by circulating intra- and extracellular α-amylase to low molecular weight (LMW) oligosaccharide metabolites, detectable in blood mainly as maltose (G2), maltotriose (G3), and maltotetraose (G4) (Mistry et al., 1994; Posthuma et al., 1997). Further metabolism of G2 is limited by the absence of maltase activity in the human circulation (Silver et al., 2014).

Compared with glucose, peritoneal UF with icodextrin is smoother with increased net UF during long dwells, particularly in fast transporters, contributing to better control of fluid balance. In addition, icodextrin may improve glycemic control, lipid profiles, phosphate removal, and cardiac function (Silver et al., 2014) and preserves residual renal function (RRF) better than glucose solutions (Chang et al., 2016).

New applications of icodextrin include initiation of PD in ESKD patients with preserved RRF using one or two icodextrin exchanges; bimodal solutions combining icodextrin with glucose in one bag for the long dwell; and using single daily long-term exchanges with icodextrin in congestive heart failure patients for treatment of overhydration and azotemia (Freida et al., 2007, 2009; Wankowicz et al., 2011; Silver et al., 2014; Kazory, 2017; Dousdampanis et al., 2018; Savenkoff et al., 2018).

Potential side-effects of icodextrin include hypotension from increased UF; loss of RRF; maltose accumulation; hypoglycemia; amylase assay interference; alkaline phosphatase increase, hyponatremia; as well as idiopathic side effects – rash, sterile peritonitis, and antibiotic compatibility (Silver et al., 2014).

The peritoneal UF can be assessed by weighing the drainage bag at the end of dialysis exchange (Silver et al., 2014) and with repeated complete drainages of the dialysate followed by reinfusions of the effluent, an intraperitoneal volume curve can be constructed (Freida et al., 2007). Another approach is to use a macromolecular volume marker to follow the kinetics of dialysate fluid volume changes intraperitoneally. This method was applied for icodextrin-based dialysis fluid using dextran 70 as a volume marker (Ho-dac-Pannekeet et al., 1996).

We have used human serum albumin labeled with iodine 125, ^{125}I-HSA, a volume marker, which remains stable in both standard and alternative dialysis solutions (Baczynski et al., 2000; Marciniak et al., 2000) and applied this for detailed assessments of peritoneal transport using a thermodynamic model of solutes and water transport including comparison of glucose-based and amino acid-based solutions (Olszowska et al., 2007).

In the present study, we investigated (1) peritoneal UF during a very long (16 h) peritoneal dwell using ^{125}I-HSA as intraperitoneal volume marker in clinically stable peritoneal dialysis patients and (2) kinetics of icodextrin fractions, especially LMW oligosaccharide metabolites, and the impact of these metabolites on peritoneal UF during the 16-h dwell.

MATERIALS AND METHODS

Patients

Twenty clinically stable peritoneal dialysis patients (8 women; mean age of 54 ± 16 years) were included in the study, which was performed in years 2006–2011. Their mean body weight was 76 ± 14 kg, height 166 ± 9 cm, and residual urine volume 1,332 ± 1,111 ml/day (median 875 ml/day). The duration of preceding PD treatment was 25.7 ± 19.4 months (median 19.5 months). Seventeen patients were on CAPD and three were on APD. The causes of ESRD were chronic glomerulonephritis (11 patients), hypertensive nephropathy (2 patients), lupus nephritis (1 patient), and diabetic nephropathy (6 patients). None of the patients had peritonitis during the 3 months preceding the study. Six patients were high transporters (H), seven high-average transporters (HA), and seven low-average transporters (LA) according to peritoneal equilibration test (PET)

performed 3–4 weeks before the study. In the whole group (n = 20), intraperitoneal dialysate volume, net ultrafiltration, and sodium concentration in dialysate and plasma were measured.

In a subgroup of 11 patients, we also analyzed fractional absorption of total icodextrin from dialysate to plasma, plasma and dialysate concentrations of amylase, plasma and dialysate LMW (Mw < 2 kDa) icodextrin metabolites comprising mainly those ranging from two glucose units (maltose, G2; Mw 360 Da) up to seven glucose units (maltoheptaose, G7; Mw 1,153 Da), and dialysate high (HMW) molecular weight icodextrin fractions. Their mean age was 50.4 ± 18.3 years (median 59 years), mean duration of PD 26.9 ± 22.4 months (median 17 months), mean body weight 73 ± 13 kg, height 164 ± 9 cm, and residual urine volume 1,113 ± 1,164 ml/day (median 700 ml/day). Three patients in this subgroup had used one exchange with icodextrin solution per day for 14.3 ± 5.1 months before the study. These patients were identified as ICO+ group and the other eight patients who did not use icodextrin before the study were identified as ICO− group (ICO-naïve patients).

The Ethics Committee of Military Institute of Medicine, Warsaw approved the study protocol. Written informed consent was obtained from each patient after an explanation of the purpose of the study.

Study Protocol

The protocol of the study is presented in **Figure 1**. Immediately prior to the study, the patients received 1.0 g of vancomycin intravenously as a prophylactic against peritonitis. After the dialysate from the overnight exchange of 1.36% glucose solution had been drained from the peritoneal cavity, the patients underwent 16-h exchange with 2.0 L icodextrin dialysis fluid (Extraneal®, Baxter, Castlebar, Ireland). Fresh dialysis solution was pre-warmed to 37°C, and a priming dose of 0.2 g of human serum albumin (HSA) was introduced into the bag in order to minimize the adhesion of radiolabelled albumin to the surface of the plastic material. Subsequently, a "flush before fill" procedure with the new solution was performed. Radio-isotopically labeled albumin (9 mg, 7.5 μCi, ^{125}I-HSA, Serlab-125; Cis Biointernational, Gif-sur-Yvette, France) was added to the dialysis solution as volume marker. After thorough mixing, the dialysis fluid was infused into the peritoneal cavity. After complete infusion, the bag was disconnected, and a three-way stopcock was placed to a transfer set. Dialysate samples (10 ml) were taken through the stopcock at 0, 3, 15, 30, 60, 90, 120, 180, 240, 480, 720, and 960 min of the exchange (**Figure 1**). At baseline (0 min), a sample of fresh dialysis fluid was taken from the bag when half of its content was infused. Prior to each sampling, 15 ml of the dialysate was flushed back and forth ten times through the stopcock. Immediately before collecting each sample, the patient was asked to move in order to mix the dialysate in the peritoneal cavity. Blood samples (5 ml) were collected at the 0, 15, 60, 120, 240, 480, 720, and 960 min of the exchange (**Figure 1**). After 960 min of peritoneal dwell, the dialysate was drained, and its volume was recorded. The peritoneal cavity was then rinsed for 5 min with 2.0 L fresh 1.36% glucose dialysis fluid without the labeled albumin to provide data for calculation of the residual peritoneal volume at the end of dwell (at 960 min). The volume of the infused fresh dialysis fluid and the volume of the drained dialysate were measured by weighing the bag and subtracting the weight of the empty bag from the full bag.

Analytical Methods

Radioactivity of blood and dialysate samples was measured using a gamma counter (LKB Wallac1272 Clinicgamma Quatro, Turku, Finland). Sodium in plasma and dialysate was analyzed by means of a direct ion-select electrode in the hospital laboratory using Cobas Integra 760 autoanalyzer (Roche, Basel, Switzerland). Dialysate samples were analyzed for the concentration of icodextrin and its fractions as well as amylase concentration for the subgroup of 11 patients. In plasma, only LMW metabolites and amylase concentration were identified. Plasma and dialysate metabolites were measured using gel filtration high-performance liquid chromatography as described before (Garcia-Lopez et al., 2008). Plasma and dialysate α-amylase activity was determined by a fully automated routine method

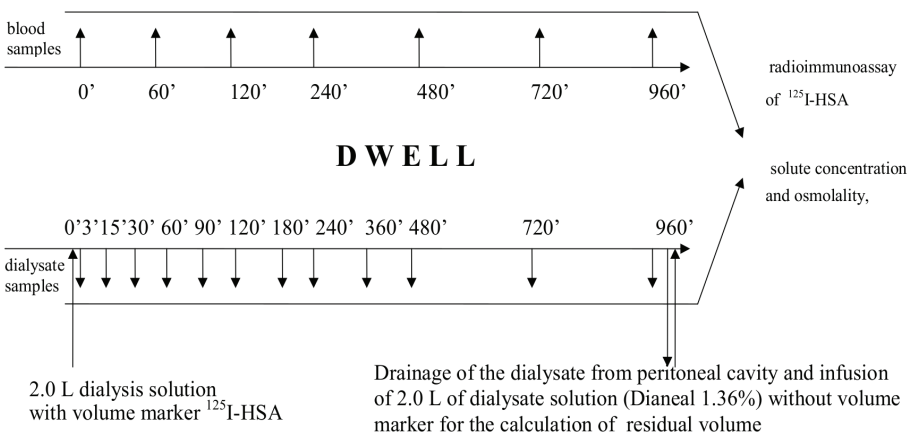

FIGURE 1 | Protocol of the study.

from Konelab 20XT routine biochemical analyzer (Thermo Clinical Labsystems Oy, Finland), using p-nitrophenol maltoheptaoside as a substrate (Garcia-Lopez et al., 2008).

Calculations

Intraperitoneal fluid volume was estimated from the dilution of the volume marker with corrections applied for the elimination of ^{125}I-HSA from the peritoneal cavity (K_E, ml/min) and sample volumes. K_E was calculated as the amount of the marker absorbed from the peritoneal cavity divided by the dwell time and the average marker concentration in the dialysate during the whole dwell (Heimburger et al., 1992). Cumulative transcapillary ultrafiltration at dwell time t was calculated as the difference between dialysis fluid volume at time t and 3 min, plus cumulative fluid absorption during the same period. Net ultrafiltration at dwell time t was calculated as the difference between dialysis fluid volume at time t and 3 min.

The icodextrin concentration was considered as the sum of all high molecular weight (HMW) and low molecular (LMW) icodextrin molecules. HMW molecules of icodextrin were defined as those with molecular weight higher than that of maltoheptaose (G7, Mw 1,153 Da). Low molecular weight (LMW) metabolites of icodextrin were assessed as G2–G7 (Garcia-Lopez et al., 2008).

Peritoneal membrane transport characteristics were determined by PET as described by (Twardowski et al., 1987) and performed 3–4 weeks before the study. The data were evaluated by Student's test. Statistical significance was accepted if p was less than 0.05. Results are expressed as mean ± SD.

RESULTS

We did not observe any clinical complications associated with the use of icodextrin solution in the 16-h dwell study and during 8 weeks of the follow-up.

Fluid Transport

In the whole group of 20 patients, the mean intraperitoneal volume increased gradually throughout the dwell to a mean value of 2,866 ± 402 ml (range 2,372–3,621 ml) at 16 h, corresponding to a significant ($p < 0.001$) mean increase of 810 ml compared with initial values. The individual intraperitoneal volume curves (**Figure 2A**) showed relatively large inter-individual variations; however, in most of the patients, intraperitoneal volume increased steadily, following a linear pattern of volume increase that in 15 out of the 20 patients included the interval between 12 and 16 h. At 4, 8, 12, and 16 h of the dwell, the average net cumulative UF was 360, 546, 570, and 729 ml, respectively (**Figure 2B**). The mean transcapillary UF rate was 1.40 ± 0.36 ml/min. At the end of dialysis exchange, mean cumulative transcapillary UF was 1,393 ± 411 ml, and the mean cumulative net UF was 729 ± 337 ml (range from −18 to 1,360 ml). Only one patient had negative UF of −18 ml at the end of exchange. The mean peritoneal fluid absorption rate (K_E) was 0.68 ± 0.38 ml/min. There was no difference in net ultrafiltration among the PET transport groups. For H transporters, net ultrafiltration reached 736 ± 230 ml, for HA transporters 724 ± 352, and for LA transporters 728 ± 439 ml ($p = 1.0$ for H vs. LA and HA vs. LA; $p = 0.9$ for H vs. HA). The statistical analysis of the correlation between D/P (rate of dialysate to plasma concentration) for creatinine at 4 h of the dwell with water transport (cumulative net UF as well as fluid absorption and transcapillary UF for each interval between sampling points) showed correlation only with cumulative transcapillary UF for time intervals from 15 up to 480 min and to 720 min with $r = 0.53$ ($p = 0.016$) and $r = 0.60$ ($p = 0.006$), respectively. However, the number of patients may be insufficient to draw any conclusion about the potential (and expected) impact of peritoneal solute transport rate.

The mean values of plasma concentrations of albumin and total protein (data not shown) did not correlate with the cumulative values of neither net UF, nor transcapillary UF calculated for the whole dwell period (all $p > 0.25$). Thus, the colloid osmotic pressure effect exerted by plasma albumin could not be demonstrated in the current study.

Sodium Concentration

The concentration of sodium in dialysate decreased from 135.2 ± 2.1 mmol/L at time 0 min to 133.5 ± 2.7 mmol/L ($p < 0.05$) at the end of dwell. It declined immediately after the infusion of fresh dialysis fluid and then gradually increased up to 120 min of the dwell and thereafter remained unchanged. Plasma sodium concentration decreased from 138.7 ± 2.4 mmol/L at the start of the dwell to 136.5 ± 3.0 mmol/L at the end of the dwell ($p < 0.05$), **Figure 2C**.

Kinetics of Icodextrin and Its Metabolites in ICO+ and ICO− Patients

In a subgroup of 11 patients, comprising eight icodextrin naïve (ICO− group) and three icodextrin-exposed (ICO+ group) patients, the kinetics of icodextrin and its metabolites were evaluated. Net cumulative UF at the end of dwell was 706.9 ± 384.7 ml, 624.8 ± 42.3 ml in ICO+ group, and 737.7 ± 454.9 ml in ICO− group (**Figure 3A**).

Dialysate Concentrations of Icodextrin Fractions and Metabolites

Icodextrin was absorbed from the dialysate to blood throughout the dwell among the eight icodextrin naïve (ICO− group) patients while – during the initial 120 min of the dwell – icodextrin was transferred in the other direction, from blood to the dialysate, in the three patients in the ICO+ group (**Figure 3B**). On average, about 41% of the initial intraperitoneal mass of icodextrin was absorbed during the 960 min of dwell (**Table 1**).

The pattern of changes of dialysate icodextrin concentrations differed between the different icodextrin fractions during the 16-h dwell (**Figure 4**). During the dwell, there was a gradual decrease of concentration of all HMW fractions in dialysate starting from 0 min of the dwell (**Figure 4A**). However, after a gradual decline

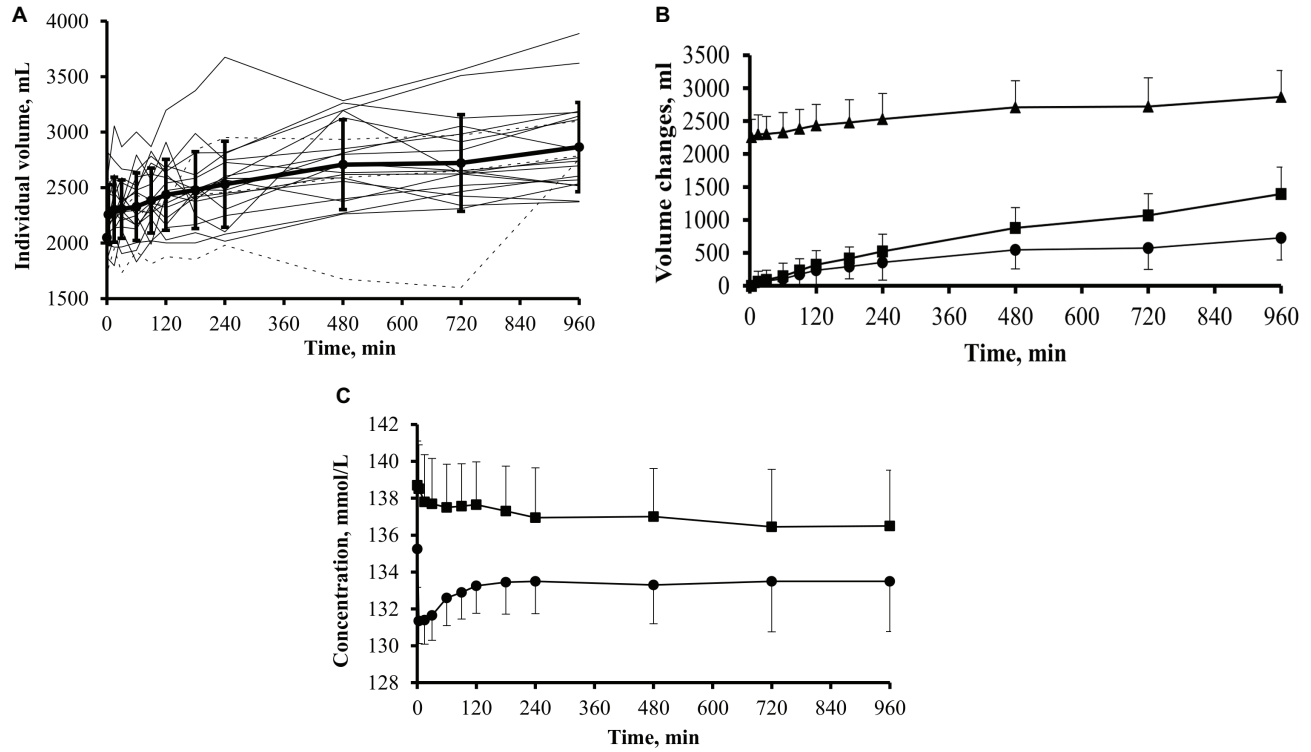

FIGURE 2 | Intraperitoneal dialysate volume, net cumulative ultrafiltration, and dialysate and plasma sodium during 16-h dwells with icodextrin in 20 patients. **(A)** Individual intraperitoneal volume curves in patients previously exposed (dotted line; group ICO+; *n* = 3) and not previously exposed (solid line; group ICO–; *n* = 17) to icodextrin. **(B)** Intraperitoneal dialysate volume (▲; mean + SD), net cumulative ultrafiltration (•; mean – SD), and cumulative transcapillary ultrafiltration (■; mean + SD). **(C)** Sodium concentration in dialysate (•; mean – SD) and in plasma (■; mean + SD).

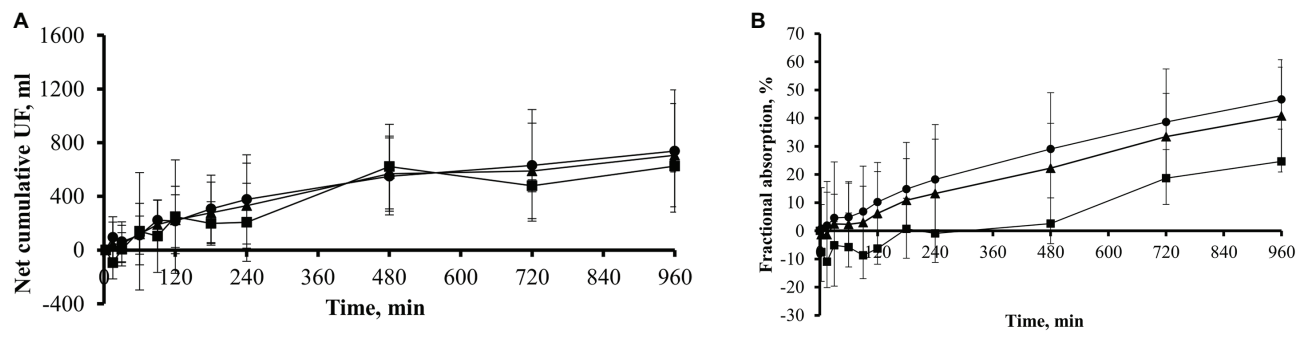

FIGURE 3 | Net cumulative ultrafiltration and fractional absorbed amount of icodextrin during 16-h dwell with icodextrin in a subgroup of 11 patients. **(A)** Net cumulative ultrafiltration (mean ± SD) in subgroup of 11 patients (*n* = 11, ▲) including those previously exposed (group ICO+; *n* = 3; ■) and those not exposed (group ICO–; *n* = 8; •) to icodextrin. **(B)** Fractional (% of initial amount; mean ± SD) absorbed amount of icodextrin in subgroup of 11 patients (*n* = 11, ▲) including those previously exposed (group ICO+; *n* = 3; ■) and not previously exposed (group ICO–; *n* = 8, •) to icodextrin.

lasting up to 240 min, the concentration of oligomers with molecular weight < 1.1 kDa increased from 480 min of the dwell and this increase continued until the end of the dwell.

The total concentration of G2–G7 oligomers in the dialysate increased significantly from 2.34 ± 1.16 mOsm/L at 0 min to 5.16 ± 2.12 mOsm/L (p = 0.01) at the end of the dwell (**Figure 4B**). From 3 min of the dwell, the concentration of G2 to G4 metabolites increased in the dialysate during the entire 16-h dwell while the concentrations of G6 and G7 metabolites decreased in dialysate throughout the whole dwell.

Plasma Concentrations of Icodextrin Metabolites

The analysis of oligomer concentration in plasma was performed only for the G2–G3 metabolites since concentrations of the G4–G7 oligomers were too low to be measured in most plasma

TABLE 1 | Absorption of icodextrin from dialysate to plasma (mean ± SD) during 16-h dwell in subgroup of 11 patients.

Time points during the dwell (min)	Mass of absorbed icodextrin (g)	Mass of absorbed icodextrin as percentage of initial amount (%)
0	0	0
240	20.5 ± 25.8	13.3 ± 16.8
480	34.4 ± 25.8	22.3 ± 16.9
720	51.7 ± 20.7	33.4 ± 13.0
960	63.1 ± 22.9	40.8 ± 14.6

The infused mass of icodextrin was 154.6 ± 16.6 g (mean ± SD).

samples. In the three icodextrin-exposed patients of ICO+ group, the plasma concentrations of G2 (maltose) increased from an already high level of 1.38 ± 0.19 to 1.74 ± 0.23 mg/ml, and the concentration of G3 (maltotriose) increased from 1.12 ± 0.18 to 1.55 ± 0.19 mg/ml. In the eight icodextrin naïve patients of ICO− group, the concentration of G2 increased from 0.24 ± 0.26 to 0.87 ± 0.24 mg/ml and G3 increased from 0.11 ± 0.03 to 0.75 ± 0.12 mg/ml (**Figure 5A**).

Dialysate Concentrations of Icodextrin Metabolites

In the ICO+ group (n = 3), the concentration of G2 increased from 0.02 ± 0.01 to 1.40 ± 0.27 mg/ml, and the concentration of G3 increased from 0.04 ± 0.02 to 1.42 ± 0.28 mg/ml (**Figure 5B**). In the ICO− group (n = 8), G2 increased from 0.01 ± 0.01 to 0.82 ± 0.55 mg/ml and G3 increased from 0.03 ± 0.02 to 0.88 ± 0.20 mg/ml.

Amylase Concentration in Dialysate and Plasma

Amylase concentration in dialysate increased in the whole group of 11 patients from 0.61 ± 0.31 to 2.08 ± 1.40 U/L at the end of dwell ($p < 0.01$). There was a statistically significant decrease of plasma amylase concentration in the whole group during the whole dwell, from initial values of 56.43 ± 46.13 to 22.39 ± 20.57 U/L ($p < 0.05$), but the pattern of changes differed dependent on previous exposure to icodextrin (**Figure 5C**). In the ICO− group (n = 8), amylase concentration decreased from 72.14 ± 44.76 to 23.58 ± 24.20 U/L, while it increased slightly in the ICO+ group from 14.53 ± 2.72 to 19.2 ± 6.80 U/L. Plasma amylase concentration was positively correlated (at sampling points up to 480 min) with cumulative transcapillary UF calculated for initial time interval (up to 15 min) and with cumulative net UF for initial time interval (up to 15 min) and for dwell time up to 180 min: however, correlations had relatively high p (from p = 0.02 up to p = 0.049). The average amylase concentration in plasma correlated with cumulative net UF up to 180 min (r = 0.7, p = 0.017), and with net UF (up to 240 min, r = 0.63, p = 0.036) and cumulative transcapillary UF (up to 180 min, r = 0.66, p = 0.027). The average concentration of amylase in dialysate did not correlate with UF. The concentration of amylase in dialysate at each sampling time showed positive correlation at 15 min with cumulative UF (transcapillary UF and net UF) up to 180 and 240 min (with r = 0.67, p = 0.023 and r = 0.69, p = 0.018 for transcapillary UF and r = 0.73, p = 0.012 and r = 0.76, p = 0.006 for net UF, respectively). There was also positive correlation with $p \leq 0.03$ of amylase level in dialysate at 60 min with net UF up to 120 min (r = 0.73, p = 0.011) and amylase level in dialysate at 90 min with net UF up to 240 min (r = 0.65, p = 0.03).

DISCUSSION

In our study, using a macromolecular volume marker (^{125}I-HSA) to determine the intraperitoneal volume and its changes throughout the dwell, icodextrin resulted in a linear increase of net UF volume lasting up to 16 h in most of the patients. This is in contrast to the short transitory period of effective UF when glucose-based solutions are used with decline of net UF volume, starting already after 120–240 min dependent on glucose concentrations used (Heimburger et al., 1992). While the period of effective net UF is even shorter in patients with fast/high transport status when using glucose-based solutions (Heimburger et al., 1990; Wang et al., 1998b), UF patterns in the present study did not differ between patients using icodextrin solution with different peritoneal small solute transport rates, i.e., between fast/high, fast/high-average, and slow/low-average transporters.

Our findings reflect the fundamentally different molecular mechanisms and the different pathways mediating water flow between colloid osmosis induced by icodextrin versus the crystalloid aquaporin-1 dependent osmosis across the peritoneal membrane induced by glucose, as demonstrated in mice during dwells lasting 120 min (Morelle et al., 2018). Our observation that net UF with icodextrin increases linearly up to at least 16 h agrees with predictions based on computer simulations by Rippe and Levin (2000).

Although there are no previous studies that analyzed intraperitoneal dialysate volumes in patients receiving icodextrin for as long as 16 h using RISA volume marker, our results are in general agreement with some previous studies. Thus, as reported by Davies (2006), most randomized controlled clinical trials show that when using icodextrin solution, average net UF increases after 8 h of long day dwell in APD and up to 16 h of long night dwell in CAPD and this was confirmed in several recent studies (Jeloka et al., 2006; Lin et al., 2009; Takatori et al., 2011; Wang et al., 2015; Chang et al., 2016). However, results are not entirely consistent and great inter-individual variability of net UF has been reported. Jeloka et al. (2006) reported that there was no increase in net UF in patients treated by APD when their dwell time with icodextrin solution was extended from 10 h to up to 14 h; however, re-analysis of the data showed that 16 (44%) of the patients had steadily increasing UF after 10 h (Venturoli et al., 2009). Furthermore, one study did not find any statistically significant differences in UF when comparing 49 patients on ICO with 51 patients on glucose solutions for 12 months follow-up (Chang et al., 2016). Higher UF with CAPD as compared to APD has been reported in patients using icodextrin, possibly due to the increase of intraperitoneal pressure resulting

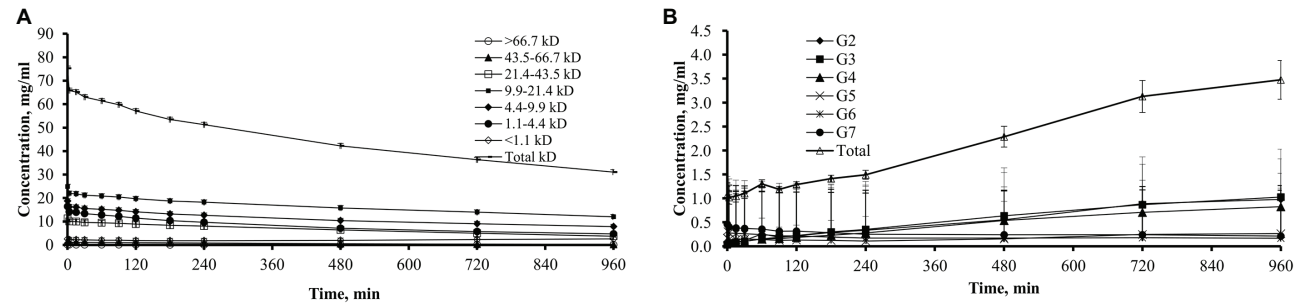

FIGURE 4 | Dialysate concentrations of total icodextrin and icodextrin fractions in 11 patients. **(A)** Dialysate concentrations (mean ± SD) of total icodextrin and separate icodextrin fractions. **(B)** Dialysate concentrations (mean ± SD) of low molecular weight (LMW) icodextrin metabolites (G2–G7) and total sum of LMW icodextrin metabolites.

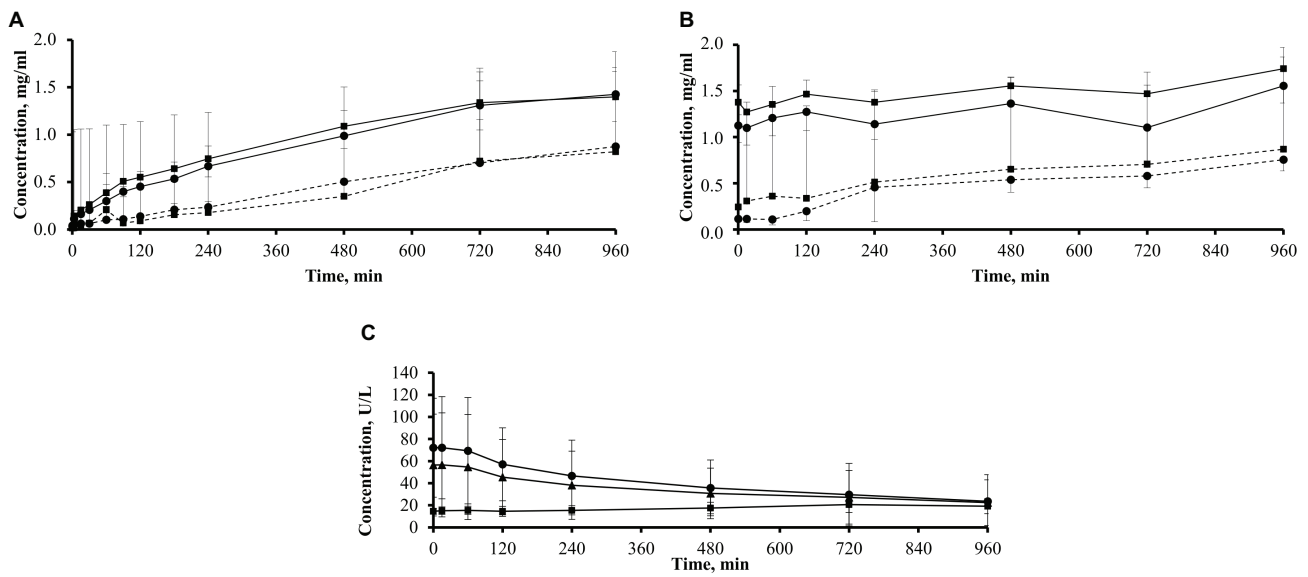

FIGURE 5 | Plasma and dialysate concentrations of icodextrin metabolites G2–G3 and plasma amylase in 11 patients. **(A)** Plasma concentrations of icodextrin metabolites G2 (■; mean + SD) and G3 (•; mean − SD) in patients previously exposed (solid line; group ICO+; *n* = 3) and not previously exposed (dotted line; group ICO−; *n* = 8) to icodextrin. **(B)** Dialysate concentrations of icodextrin metabolites G2 (■; mean + SD) and G3 (•; mean − SD) in patients previously exposed (solid line; group ICO+; *n* = 3) and not previously exposed (dotted line; group ICO−; *n* = 8) to icodextrin. **(C)** Amylase concentration in plasma (mean ± SD) in a subgroup of 11 patients (*n* = 11; ▲) including those previously exposed (group ICO+; *n* = 3; ■) and not previously exposed (group ICO−; *n* = 8, •) to icodextrin during 16-h dwell with icodextrin.

from physical activity and changes in body position during the daily dwell through increase of hydrostatic capillary pressure and increase of lymphatic absorption that consequently result in lower UF (Mistry et al., 1994; Posthuma et al., 1997; Neri et al., 2000). The fact that most of the patients in our study were treated by CAPD prior to the investigation – and that most were icodextrin naïve – may have contributed to higher net UF during the dwell.

Plasma and Dialysate Sodium in Patients Receiving Icodextrin

Icodextrin induces sustained UF even though dialysate osmolality is similar or lower than serum osmolality.

It is often assumed that the absence of free water removal when using icodextrin results in an increased convective contribution to total small solute clearance including also sodium, thereby eliminating the gap between UF and sodium removal that is seen with crystalloid osmotic agents such as glucose. We observed a statistically significant decrease in serum sodium concentration during the dialysis exchange with icodextrin; however, the concentration of sodium remained within the normal range. The decrease in serum sodium concentration is conceivably explained by dilution resulting from the increased osmotic gradient, caused by the generation of metabolites of icodextrin that induces a water flow from cells into the blood compartment. The presence of metabolites, mainly maltose (G2) and maltotriose (G3), in the vascular bed and interstitial fluid appears to have been sufficient to cause the displacement of water from cells into the extracellular space, thus resulting in hyponatremia from dilution, a phenomenon also referred to as hypertonic hyponatremia

(Mistry et al., 1994; Posthuma et al., 1997; Silver et al., 2014). In patients with inadequately controlled diabetes who are treated with PD using icodextrin solution, this phenomenon could potentially add to the risk of severe symptomatic hyponatremia, resulting from inadequately controlled hyperglycemia (Silver et al., 2014).

Our results are consistent with other observations of sodium kinetics during peritoneal dwells with icodextrin based dialysis fluid. In a clinical study on 12-h peritoneal dwell with icodextrin fluid sodium concentration in serum decreased by 3 mmol/L (Moberly et al., 2002), considerably more than reported here by us. Sodium concentration in dialysate in that study increased from 131 to 134 mmol/L in 2 h and was stable throughout the following dwell time (Moberly et al., 2002), and the same pattern of increase and following stability can be found in our data although with lower amplitude of increase. After 12 h of the peritoneal dwell, the sodium concentration in dialysate was still by 5 mmol/L lower than that in plasma (Moberly et al., 2002). Experimental studies in rats demonstrated that the initial sodium concentration in dialysate (i.e., its concentration at third minute after fluid infusion) is similar for glucose and icodextrin based fluids in the control group and in the group with induced peritonitis separately (Wang et al., 2000), and the initial sodium dialysate to plasma ratio is not different if the mixture of icodextrin and glucose is applied as osmotic agent (Wang et al., 1998a). The initial sodium concentration was in the control group of animals around 131 mmol/L that was lower than the nominal concentration of sodium ions in icodextrin fluid of 133 mEq/L (Wang et al., 2000). The initial sodium concentration measured after infusion and immediate drain of icodextrin dialysis fluid in another clinical study was about 130 mmol/L (Freida et al., 2007; Galach et al., 2009). The sodium concentrations in plasma and dialysate were constant with slight tendency to decrease during 15 h peritoneal dwell (Freida et al., 2007; Galach et al., 2009). No tendency to equilibration between dialysate and plasma sodium up to 900 min of peritoneal dwell was observed (Freida et al., 2007; Galach et al., 2009). In all these studies, the sodium concentration was measured by flame photometry (Wang et al., 1998a, 2000; Freida et al., 2007; Galach et al., 2009), and in animal studies, plasma concentrations were corrected to plasma water (Waniewski et al., 1992; Wang et al., 1998a, 2000). Flame photometry measures the total sodium content in the sample and includes also the not dissociated sodium that is about 4% of total sodium, whereas our measurements performed with direct ion selective electrode report the concentration of diffusible sodium ion; the concentration of diffusible sodium ion in the study by Freida et al. might be even lower than in our study. Unfortunately, the other studies discussed here did not provide measurements in fresh dialysis fluid (Wang et al., 1998a, 2000; Freida et al., 2007; Galach et al., 2009). During dwells with glucose based solutions, sodium in dialysate is also far from equilibration with plasma sodium after 360 min in patients and, furthermore, in rats after 240 min, no sodium equilibration is observed for both glucose- and icodextrin-based fluids (Wang et al., 1997, 2000).

We have no clear explanation for the observed decrease in dialysate sodium concentration and the higher than expected initial dialysate sodium concentration. However, one possible reason for the lower than expected dialysate sodium concentration is that the measurements of sodium are somehow influenced by the presence of icodextrin, as can be observed for measurements of dialysate sodium in case of glucose-based dialysis fluid (Wang et al., 1997; La Milia et al., 2004). The observation that dialysate and plasma sodium concentrations did not fully equilibrate by 960 min is puzzling although confirmed by independent studies (Freida et al., 2007; Galach et al., 2009). One may speculate that this is due to sieving of sodium induced by LMW icodextrin fractions. Sodium concentration gradient was also maintained throughout the 6-h dwell using 3.86% glucose due to sieving of sodium in the ultrafiltrate (Wang et al., 1997).

The three pore model, which well predicted the profiles of dialysate volume change for three different fluids with glucose 3.86%, icodextrin 7.5%, and a mixture of glucose and icodextrin, was not able to correctly describe the observed lack of equilibration for sodium in plasma and dialysate for icodextrin fluid in spite of good fit to the sodium profiles in dialysate for glucose- and glucose-icodextrin-based dialysis fluids for 15-h peritoneal dwell (Galach et al., 2009). In contrast to the observed lack of equilibration in clinical data, confirmed also by our results, the modeled sodium concentration approached plasma concentration at about 4 h and remained equilibrated until 15 h (Galach et al., 2009). This failure challenges the assumptions of the three pore model as a single transport barrier between blood and peritoneal dialysate and may need a theoretical extension for polyglucose simulations as proposed for example by the spatially distributed model that involves separately two transport barriers of the capillary wall and interstitium (Stachowska-Pietka et al., 2006; Waniewski et al., 2009). One may expect higher concentration of amylase in interstitial fluid than in dialysate so the osmolality of interstitial fluid may differ from that of dialysate. According to the distributed model, the sodium dip measured for glucose-based hypertonic fluids occurs also in the interstitial fluid, not only in dialysate, and this observation suggests the importance of the processes inside the tissue in contact with peritoneal dialysate (Stachowska-Pietka et al., 2012, 2019). The conjecture about the role of interstitium in the process of osmotic fluid transport induced by polyglucose is hypothetical and will need further theoretical, experimental, and clinical studies to be confirmed.

Kinetics of Icodextrin in Icodextrin Exposed (ICO+) and Icodextrin Naïve (ICO−) Patients

The concentration of icodextrin fractions in the fresh fluid measured in our study shows a similar pattern to that reported previously by Garcia-Lopez et al. (2005). We observed that net UF and the fractional absorption of ICO depend on the previous use of icodextrin. Among icodextrin naïve (ICO−) patients, UF was slightly higher compared to UF in icodextrin exposed (ICO+) patients (**Figure 3A**). This observation confirms the results of computer simulations by Rippe and Levin (2000) showing that the 7.5% icodextrin solution although slightly hypotonic to plasma, resulted in UF of 600 ml after 12-h dwell, but only in patients who were not previously exposed

to icodextrin. In patients on long-term icodextrin and with steady-state plasma levels of icodextrin and its metabolites, UF after 12 h was on average 400 ml (Rippe and Levin, 2000).

The efficacy of icodextrin as osmotic agent depends on colloid osmosis, which is maintained during the long dwell due to its large size, which hinders fast absorption from the peritoneal cavity. Nevertheless, icodextrin is slowly but steadily absorbed, mainly by convective transport, from the dialysate. Because the absorption rate of icodextrin from the dialysate is almost constant during dwell, the percentage of icodextrin being absorbed is directly dependent on the duration of the exchange (Moberly et al., 2002). In our study, 22% of the initial amount of icodextrin was absorbed after 8 h and 33 and 41% after 12 and 16 h, respectively (**Table 1** and **Figure 3B**). Other authors obtained similar results: Davies (1994) reported that the percentage of icodextrin absorbed from the dialysate was 19.6 and 33% at 8th and 16th hour, respectively. Slightly higher absorption of icodextrin was found by Moberly et al. who reported that about 40% of icodextrin was absorbed during 12-h dialysis exchanges (Moberly et al., 2002).

The evaluation of the kinetics of icodextrin during the 16-h dwell in the subgroup of 11 subjects showed a gradual decrease in the concentration of HMW fractions in the dialysate (**Figure 4A**). This is conceivably due to the combined effects of (1) dilution by water flow from blood, (2) intraperitoneal metabolism of the HMW fractions under the influence of α-amylase resulting in increased generation of LMW icodextrin metabolites, and (3) the absorption of these molecules from the peritoneal cavity to the peritoneal tissues, and from them through the lymphatic vessels to the blood (Moberly et al., 2002).

Intraperitoneal metabolism of icodextrin by α-amylase leads to the formation of LMW oligosaccharides, namely G2–G7 metabolites. Our study showed an increase in total LMW concentration of icodextrin metabolites in dialysate, mainly G2–G5 with a concurrent decrease in G6–G7 metabolites (**Figure 4B**). Moberly et al. observed a gradual decrease in the concentration of G5–G7 metabolites in the dialysate in the 12-h dwell with ICO, which they attributed to absorption of these metabolites from the peritoneal cavity into the bloodstream as well as intraperitoneal metabolism under the influence of α-amylase (Moberly et al., 2002).

The MIDAS trial, a multicenter randomized controlled study of 209 CAPD patients, compared overnight icodextrin and dextrose exchanges over a 6-month period (Mistry et al., 1994). Serum samples for estimation of icodextrin and oligosaccharides were obtained immediately prior to the study and at 1, 3, and 6 months. The mean level of icodextrin and its metabolites in plasma increased from baseline value of 0.35 g/L to a steady-state level of 4.87 g/L. The serum maltose followed an identical pattern and rose from 0.04 g/L to a steady-state level of 1.20 g/L. This increase occurred within 2 weeks of icodextrin administration, and steady-state levels were maintained throughout the 6-month study. These metabolites were not associated with any adverse clinical effects (Mistry et al., 1994). We report that the concentration of G2 and G3 metabolites in plasma and dialysate depends on the previous use of icodextrin (**Figures 5A,B**). The concentration of these metabolites was higher both in plasma and in dialysate in icodextrin users (average time on icodextrin solution was 14 months in the ICO+ group) compared to those not using icodextrin before the study. A similar observation was made by Davies et al. in patients receiving icodextrin for over 2 years (Silver et al., 2014); however, the levels of icodextrin and maltose fell to pretreatment values within 2 weeks after the withdrawal of icodextrin-based fluid in a small group who underwent further study. Upon recommencing icodextrin after 3 weeks period of non-use, the ICO and maltose metabolite levels rose to the initial treatment phase and reached a plateau within 2 weeks (Silver et al., 2014).

Amylase can diffuse from the blood into the dialysate, thus contributing to the metabolism of intraperitoneal icodextrin; however, intraperitoneal metabolism of icodextrin seems to be negligible when compared to the substantial transport of HMW icodextrin fractions, and LMW metabolites, from dialysate to plasma, where these fractions undergo rapid hydrolysis (Garcia-Lopez et al., 2008). In our patients, the dialysate concentration of amylase increased during the dwell but still remained low, similar to results of other studies (Garcia-Lopez et al., 2008). In contrast, the plasma concentration of amylase declined significantly in icodextrin naïve patients (ICO− group), while it remained low in the ICO+ patients conceivably related to the preceding icodextrin use (**Figure 5C**). A low plasma amylase concentration in patients using icodextrin may pose difficulties for the correct diagnosis of pancreatitis; however, in such patients, plasma lipase has been suggested to be of value as an alternative (Rubinstein et al., 2016). The decrease in plasma amylase activity in PD patients changing from glucose-based to icodextrin-based solutions is genuine and not only, as had been suggested by others, decreased due to interference by icodextrin in the substrates of the assay (Garcia-Lopez et al., 2008). In the current study, the baseline concentration of plasma amylase was much lower in those treated by icodextrin as compared to icodextrin naïve patients, 14.53 ± 2.72 vs. 72.14 ± 44.76 U/L.

Based on current knowledge, potential determinants of icodextrin-induced UF include: peritoneal solute transport rate, plasma colloid osmotic pressure, metabolism of icodextrin, and peritoneal clearance of macromolecules (Araujo Teixeira et al., 2002; Wiggins et al., 2005; Jeloka et al., 2006; Venturoli et al., 2009). However, some of these factors that were investigated in our study were not predictors for icodextrin-induced UF. Thus, more studies are needed to provide detailed information on the determinants for UF induced by icodextrin.

Some limitations of the study should be considered when interpreting the result. One limitation is that glucose-based solutions were not used as controls; however, the kinetics of glucose solutions have been extensively studied previously and it is unlikely that inclusion of such a control group would have changed the main conclusions. Another potential limitation is that the number of patients is relatively low; only half of the 20 patients underwent more detailed studies on the kinetics of icodextrin and its metabolites, and sodium, and only three patients had received icodextrin prior to the study. The fact that most of the patients were treated by CAPD and not by

APD, and that most were not previously exposed to icodextrin should be considered when interpreting the results. Intraperitoneal pressure was not assessed in this study. Furthermore, while we estimated fluid absorption based on the elimination rate of ^{125}I-HSA, our protocol did not include assessment of free water transport versus water transport through the small pores; however, it is well established that the latter is the dominating route of fluid transport when using icodextrin. Finally, the study conditions required by the complex study protocol using RISA, with need to take samples of blood and dialysate every 1 h of the dwell, deviate from the conditions of home peritoneal dialysis. On the other hand, the applied kinetic model and assessment of the transperitoneal transport of the macromolecular volume marker used in our study allowed us to determine the dynamics of UF at each time point of the 16-h dialysis exchange.

In summary, we report that the use of an icodextrin-based PD fluid resulted in an almost linear increase of intraperitoneal volume with sustained net UF lasting at least up to 16 h in most of the investigated patients and with no significant difference between peritoneal transport types. During the long dwell, plasma sodium and plasma amylase declined. The dialysate amount of icodextrin declined due to decrease of HMW icodextrin fractions while smaller LMW icodextrin metabolites, especially maltose and maltotriose, increased. Plasma maltose and maltotriose increased significantly whereas larger icodextrin fractions were not detectable in blood. The ability of icodextrin to provide sustained UF during very long dwells – which is usually not possible with glucose-based solutions – is especially important in anuric patients and in patients with fast peritoneal solute transport rates. Whether improved net UF during long-term use of icodextrin improves clinical outcomes such as technique and patient survival of PD patients depends on many other factors including residual renal function and adherence to fluid restrictions as well as other aspects of the clinical management of PD patients and will require additional studies.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethics Committee of Military Institute of Medicine, Warsaw with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Military Institute of Medicine, Warsaw.

AUTHOR CONTRIBUTIONS

AO, ZW, and JW designed the study in consultation with BL. AO performed and ZW supervised the clinical study. EG-L performed measurements of icodextrin metabolites. JS-P developed software for data analysis. AO and JS-P analyzed the kinetic data in consultation with JW. AO, ZW, and JW wrote the first draft. All authors revised and approved the final manuscript.

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