Osteopontin levels in the drained dialysate reflect the peritoneal solute transport rate

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Abstract
Background and objectives: Long-term peritoneal dialysis (PD) is accompanied by low-grade intraperitoneal inflammation, may eventually lead to peritoneal membrane injury with high solute transport rate and ultrafiltration failure. Osteopontin (OPN) is highly expressed with the pro-inflammatory cytokines stimulation in many cell types, and evolves in the process of tissue fibrosis. This study aimed to investigate the potential of OPN as a new indicator of peritoneal injury.

Methods: We analyzed a total of 125 PD patients with end-stage renal disease, including 16 patients with continuous ambulatory PD (CAPD)-related peritonitis and 109 patients without peritonitis in a single renal center. The OPN levels in the overnight peritoneal effluents or in serum were analyzed using ELISA. In HMrSV5 cells, The OPN and fibronectin (FN) protein expression were identified using western blot analysis.

Results: The OPN levels in overnight drained dialysate were significantly correlated with D/P Cr (P < 0.0001, R = 0.54) and D/D0 glucose (P < 0.0001 R = -0.39). Logistical regression analysis showed that the OPN levels in peritoneal effluents was an independent predictive factor for the increased peritoneal solute transport rate (PSTR) (p < 0.001). The area under the receiver operating characteristic (ROC) curve of the OPN-PSTR model for identifying PSTR was 0.88, with 95% confidence interval (CI): 0.81-0.95. The OPN was more abundant in peritoneal effluents of the CAPD-related peritonitis group compared with the patients without peritonitis (18.64±13.04 vs. 2.23±1.63 ng/ml, p<0.001). In the in vitro experiment, lipopolysaccharides (LPS) increased the OPN expression in HMrSV5 cells, whereas downregulation of OPN suppressed FN induction with transforming growth factor-β1 (TGF-β1) stimulation.

Conclusions: The OPN levels in drained dialysate were independently correlated with peritoneal transport status in accordance with the PET results. OPN was highly expressed in effluents in patients with CAPD-related peritonitis. Peritoneal mesothelial cells displayed a high expression of OPN under inflammatory stimuli and OPN was likely to be implicated in the progression of peritoneal fibrosis. Thus, OPN may be a useful indicator of peritoneal injury in patients with PD. Keywords: peritoneal dialysis, osteopontin, peritoneal injury, peritoneal solute transport rate
Introduction
Peritoneal dialysis (PD) is a vital replacement therapy for patients with end-stage kidney disease. Approximately 11% of the dialysis population uses PD worldwide. During PD, the peritoneal membrane (PM) naturally removes waste products and excess fluid from the blood and transports them to the dialysis solution. However, long-term exposure to hyperglycemia, hyperosmotic, and acidic dialysis solutions often cause low-grade chronic inflammation and PM injury. The PM presents as progressive fibrosis, angiogenesis, and vasculopathy, which lead to increased solute transport and ultrafiltration failure. Among these conditions, progressing peritoneum fibrosis plays a significant role in peritoneal transport dysfunction, which is characterized by the accumulation of myofibroblasts in the peritoneum with fibronectin (FN) and collagen expression[1–4]. The peritoneal equilibration test (PET) is widely employed to assess PM transport function[5].

Osteopontin (OPN) is a highly phosphorylated glycoprophosphate, that can be secreted from many cell types, including epithelial cells, macrophages, osteoclasts, and fibroblasts in different tissues under inflammatory milieu. OPN participates in diversified important biological functions, including inflammation, biomineralization, cell viability, tissue epithelial–mesenchymal transition (EMT), and fibrosis[6–10]. Different disease models using OPN knockout mice illustrate the role of OPN in fibrogenesis. For instance, OPN-null (OPN-/-) attenuate AngII or unilateral ureteral obstruction induced kidney interstitial fibrosis[11]. Moreover, OPN deficiency results in impaired wound healing and decreased collagen deposition after myocardial infarction[12].

Taken together, we suggest that OPN may be secreted from mesothelial cells, macrophages, or fibroblasts in the PM through long-term and low-grade chronic inflammation stimulation during PD treatment. OPN is also likely to be involved in the development and progression of peritoneal fibrosis, which eventually affects the solute transport and ultrafiltration. We investigated whether OPN may be a new indicator of PM injury characterized by high solute transport via analyzing the association between the OPN concentration in drained dialysate and peritoneal solute transport rate (PSTR) obtained with PET.

Patients And Methods
Patients
From February 2018 to December 2018, 231 PD patients with end-stage renal disease at the PD unit of the First Hospital Affiliated of Soochow University were followed-up regularly. Sixteen patients who suffered from CAPD-related peritonitis were analyzed. Among the remaining 215 patients, we selected the patients who were less than 75 years old and being treated with a CAPD prescription (four times exchanges per day). Other exclusion criteria included acute inflammatory processes, diagnosis peritonitis or abdominal trauma within the past six months prior to the study, active autoimmune diseases, and tumors, with incomplete clinical characteristics. Eventually, 109 CAPD patients without peritonitis were analyzed. Table 1 summarized the clinical characteristics of these patients. All the patients in this study were dialyzed with glucose-based PD fluid (Dianeal®, Baxter).

PET
Semiquantitative assessment of peritoneal membrane transport function was assessed with the PET. The overnight Intrabdominal fluid was drained, and PD fluid containing 2.5% dextrose solution was injected intraperitoneally. 4-hour dialysate creatinine concentration (D) was divided by that of plasma creatinine (P) concentration to obtain the D/P Cr; 4-hour dialysate glucose concentration (D) was divided by that obtained immediately after the injection (D0) to obtain the D/D0 glucose; and ultrafiltration volume. Grouping standard of PET results: High transport (D/P Cr = 0.82~1.03), High-average transport (D/P Cr = 0.65~0.81), Low-average transport (D/P Cr = 0.50~0.64), Low transport (D/P Cr = 0.34~0.49).

ELISA Osteopontin
All participants in the experiment were asked to perform a dialysis exchange according to the usual overnight dialysis regimen prior to PD center visit. The overnight effluent was fully drained next morning in the PD center. We collected a 10ml sample from each patient, which were stored at -80°C immediately. OPN levels were quantified in peritoneal effluents and serum with the ELISA kit (CUSABIO China), according to the manufacturer’s instructions.

Cell culture and treatment
Human peritoneal mesothelial cell line (HMrSV5) was kindly provided by Dr. Zhu nan (Department of Nephrology, Shanghai First People’s Hospital, Shanghai, China). Cells were cultured in Dulbecco ’s modified Eagle’s medium/F12 medium supplemented with 10% fetal bovine serum (Invitrogen,
Grand Island, NY) in a 37°C incubator with 5% CO2. Experiments were performed at approximately 60% to 70% confluence cultures after 16 hours of serum deprivation. Recombinant human TGFβ1 (cat.: 100-B-010-CF, R&D Systems, Minneapolis, MN) was added to the serum-free medium for various periods of time. OPN or Scramble siRNA (Integrated Biotech Solutions, Shanghai, China) was transfected into HMrSV5 cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction.

Western blotting analysis
Cultural HMrSV5 cells were lysed in 1 × sodium dodecyl sulfate sample buffer. An equal amount of protein was loaded into 10 or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The primary antibodies were as follows: anti-OPN (cat.: ab8448, Abcam), anti-GAPDH (cat.: FL-335, Santa Cruz Biotechnology), anti-Fibronectin (cat.: F3648, Sigma-Aldrich).

Statistical analysis
Clinical data were presented as means ± Standard deviation (SD). Baseline characteristics of the study population were compared using one-way analysis of variance (ANOVA) for continuous variables and χ² testing for categorical variables. Relationships between clinical variable and D/P Cr levels were analyzed with Spearman’s correlation coefficient test. Independent factors affecting PSTR were analyzed by Logistic regression analysis, the model with prespecified adjustments for: gender, age, duration, blood albumin, Serum phosphate. In addition, to identify the possible predictors of increased PSTR, a logistic regression model was constructed for the probability of increased PSTR, and the equation as follows: probability = exp(c)/(1+exp(c)), where c is 1.23×OPN levels in peritoneal effluents–0.13×blood albumin+0.944. The optimal cut-off point was identified based on the maximum Youden index (sensitivity + specificity–1). In vitro data were presented as mean±S. E. Comparison between groups was made using one-way analysis of variance, followed by the Mann–Whitney U test. Western blot analysis was completed by scanning and analyzing the intensity of hybridization signals by using NIH ImageJ software package. P-value < 0.05 (two-tailed) was considered statistically significant. These statistical analyses were conducted using SPSS version 25.0 software (IBM Corp.,
Results

Patient characteristics

A total of 109 CAPD patients without peritonitis were enrolled in this study. Among these patients, 55.3% were males with a mean age of 49.14 ±13.25 years and a median PD duration of 37.32 ± 35.01 months. Table 1 summarized the clinical characteristics of the study.

Correlation between OPN and peritoneal transport characteristics

The PSTR determined using PET was correlated with the OPN concentrations in overnight peritoneal effluents. Significant correlations were found between the OPN levels and D/P Cr (p < 0.0001, R = 0.54), D/D0 glucose (P < 0.0001 R = −0.39) through Spearman’s correlation coefficient test (Figure 1). In the blood samples, no firm correlation was observed between the OPN levels and PET results (Table 2). The patients were divided into either low and low-average transport (L/A) group, or high and high average transport (H/A) group based on the PET results. The OPN concentrations in peritoneal effluents were considerably higher in the H/A group than in the L/A group (3.05±1.94 vs. 1.25 ±1.03 ng/ml, P < 0.0001). The H/A group was more likely to be older (p = 0.007), with lower serum albumin (p<0.0001), and higher serum phosphate levels (p = 0.004) (Table 3). Logistic regression analysis showed that OPN in effluents was an independent predictive factor for the PSTR after adjusting for age, PD duration, gender, serum albumin, and serum phosphate (Table 4). To future examine the diagnostic accuracy of the OPN measured for identifying increased PSTR, the OPN-PSTR model was constructed, using two variables (OPN levels in the effluents and serum albumin). As described in Figure 2, the OPN-PSTR model accurately identified the increased PSTR (AUROC = 0.88, 95%CI:0.81–0.95). With the cutoff point of 0.20 (as calculated by Youden’s index), the sensitivity and specificity of the test in discriminating the increased PSTR reached 78% and 92%, respectively.

Patients with CAPD-related peritonitis showed high effluent levels of OPN

We detected the OPN concentrations in peritoneal effluents from 16 patients who suffered from CAPD-related peritonitis. The OPN in peritoneal effluents of these patients had a higher expression level compared with the patients without peritonitis (18.64±13.04 vs. 2.23±1.63 ng/ml, P<0.001) (Figure 3). However, no significant difference was found between the two groups while detecting OPN
levels in the blood sample (data not shown).

**OPN was induced by LPS stimulation and contributed to TGFβ1-induced EMT in HMrSV5 cells**

HMrSV5 cells, a human peritoneal mesothelial cell line, were treated with LPS (500ng/ml) for different time durations. As shown in Figure 4, OPN expression was induced 3–24h after treatment. To explore the role of OPN in peritoneal fibrosis, HMrSV5 cells were transfected with scramble or OPN siRNA, 24 h after transfection, OPN protein expression was downregulated to about 40% of cells transfected with OPN siRNA compared with those transfected with scramble siRNA (Figure 5a). As shown in Figures 5b and 5c, the TGFβ1 treatment can largely increase the FN expression in scramble siRNA-transfected HMrSV5 cells. However, the induction of FN expression was markedly reduced in OPN siRNA-transfected cells.

**Discussion**

During long-term PD, the PM undergoes functional and structural alterations, eventually leading to PD drop-out and increased mortality[2, 3, 13]. PET is the most significant method for evaluating the transfer rate of solute and water across the peritoneal barrier, and includes three parameters: D/P Cr, D/D0 glucose, and 4h ultrafiltration volume. Rajnish Mehrotra et al. demonstrated that D/P Cr is a robust predictor of all-cause mortality and hospitalization through a study that enrolled a large and diverse cohort of patients undergoing PD in both unadjusted and adjusted models[5, 14, 15]. However, PET is an invasive method that requires blood sampling. In addition, patients need to be hospitalized. It will be of great interest to seek relevant biomarkers in peritoneal effluents, which can represent the transfer rate of the solute. The effluent concentrations of interleukin6(IL6), cancer antigen 125(CA125), vascular endothelial growth factor(VEGF), plasminogen activator inhibitor-1(PAI1), matrix metalloproteinase 2(MMP2), matrix metalloproteinase3(MMP–3), bone morphogenetic protein–7(BMP–7), and tissue inhibitor of metalloproteinases1(TIMP–1) levels in the dialysate obtained using PET were correlated with the D/P Cr ratios[14, 16–18]. The present study showed significant correlations exist between the OPN levels in the dialysate and D/P Cr, D/D0 glucose. The OPN concentrations in peritoneal effluents were remarkably higher in the H/A group than in the L/A group. OPN was an independent predictor factor for increased PSTR, which was evaluated using logistic
regression analysis. In addition, by combining the concentrations of serum albumin and OPN in
drained dialysate, our novel model demonstrated good performance in identifying increased PSTR.
Increasing evidence shows that chronic inflammation might be an initial factor for PM injury. After
prolonged PD treatment, both MCs and peritoneal macrophages produce a wide array of inflammatory
cytokines, such as IL-1β, tumor necrosis factor-α (TNF-α), and IL-6. Moreover, pro-fibrotic cytokines
such as MMP-2, TGF-β, and CTGF, are also secreted, which may eventually lead to PM damage and
functional abnormalities[3, 13, 19]. An enhanced secretion of OPN occurs in inflammatory
microenvironment, whereas TNFα, and IL-1β stimulate the transcription and release of OPN genes[6].
Consistent with the prior research, the OPN levels in peritoneal effluents of the CAPD-related
peritonitis group increased markedly compared with the patients without peritonitis; LPS Induced the
OPN expression in human peritoneal mesothelial cell line in a time-dependent manner, additionally,
no extreme change of serum OPN levels were found as the PSTR increased or even under peritonitis
condition. Overall, the high OPN concentrations in the H/A group may be attributed to the high
number of pro-inflammatory cytokines released in the PM. Furthermore, the peritoneal mesothelial
cells can be a significant source of OPN in effluents.
In addition, OPN increased the recruitment of macrophages and T cells, which are implicated in the
pathogenesis of acute and chronic inflammatory diseases, such as rheumatoid arthritis and bacterial
infections[20]. Additionally, OPN can transform fibroblasts into active myofibroblast phenotype, and
evolve in the EMT programs in several different cell lines, which results in fibrotic phenotypes[7, 8]
[21]. The partial EMT of mesothelial cells is implicated in peritoneal fibrosis and TGFβ1, which is a
potent cytokine, that stimulates EMT and induces peritoneal fibrosis[22]. Thus, we further examined
whether OPN plays an important role in TGFβ1-induced EMT in HMrSV5 cells in vitro. The exposure of
HMrSV5 cells to TGβ1 induced FN expression, a hallmark of EMT, and downregulation of OPN in
HMrSV5 cells can inhibit TGFβ1 induced FN expression. Altogether, OPN may play an important role in
mediating TGFβ1 induced mesothelial cells EMT and peritoneal fibrosis.
This study features several important limitations. First, the cross-sectional nature of the study
excluded the establishment of causal relationships and temporal trends between the OPN levels in
effluents and PSTR. Second, this single-center study involved a small sample size. Third, selection bias might be present in this study as a considerable number of subjects was excluded due to the strict inclusion and exclusion criteria. Finally, this study lacked animal experiments to identify which cell types drive OPN secretion in the peritoneum and the roles of OPN in PD induced PM injury.

In conclusion, we put forward significant associations between the OPN levels in the drained dialysate and D/P Cr, and D/D0 glucose. The OPN level is also an independent predictor factor of the increased PSTR. CAPD-related peritonitis can lead to higher levels of the OPN expression in peritoneal effluents compared with the patients without peritonitis. In addition, in vitro studies showed that OPN is highly expressed in peritoneal mesothelial cells with inflammatory simulation and mediates TGFβ1 induced mesothelial cells EMT. Thus, OPN may be a useful indicator of peritoneal injury in PD patients.

Abbreviations
PD: peritoneal dialysis; OPN: Osteopontin; CAPD: continuous ambulatory PD PET: peritoneal equilibration test; PSTR: peritoneal solute transport rate; ROC: receiver operating characteristic; LPS: lipopolysaccharides; TGF-β1: transforming growth factor-β1; PM: peritoneal membrane; EMT: epithelial–mesenchymal transition; IL6: interleukin 6; CA125: cancer antigen 125; VEGF: vascular endothelial growth factor; PAI1: plasminogen activator inhibitor–1; MMP2: matrix metalloproteinase 2; MMP-3: matrix metalloproteinase 3; BMP-7: bone morphogenetic protein–7; TIMP-1: tissue inhibitor of metalloproteinases 1; FN: fibronectin

Declarations
Acknowledgements
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Ethics approval and consent to participate
This study was performed with the written informed consent of all patients and the procedure was approved by the Ethics Committee of The First Affiliated Hospital Soochow University.

Authors ' contributions
Research idea and study design: LJZ, LGY, and SL; data acquisition: LJZ, LJJ, QQ; data analysis: LJZ and LJJ; statistical analysis: LJZ and LJJ; supervision or mentorship: SL, QQ and LGY. All authors read and approved the final manuscript.
Competing interests
The authors declare that they have no competing interests.

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Table 1: Clinical characteristics of CAPD patients without peritonitis (N=109). Clinical values are expressed as mean ± SD.

| Variable                        | Value         |
|---------------------------------|---------------|
| Gender (Male/Female)            | 57/52         |
| Age (years)                     | 49.14±13.25   |
| PD duration (months)            | 37.32±35.01   |
| D/P Cr                          | 0.69±0.11     |
| D/D0 glucose                    | 0.36±0.07     |
| 4h ultrafiltration volume (ml)  | 270.6±138.4   |
| Total Kt/V urea                 | 1.82±0.40     |
| Peritoneal Kt/V urea            | 1.47±0.35     |
| Renal Kt/V urea                 | 0.35±0.44     |
| Cholesterol [mmol/L]            | 4.31±1.08     |
| Triglyceride [mmol/L]           | 1.78±1.33     |
| Uric acid [μmol/L]              | 393.1±89.18   |
| Creatinine [μmol/L]             | 960.3±292.8   |
| Serum albumin [g/L]             | 34.39±4.91    |
| Hemoglobin [g/L]                | 96.61±16.60   |
| Serum calcium [mmol/L]          | 2.19±0.58     |
| Serum phosphate [mmol/L]        | 1.61±0.49     |
| PTH [pg/mL]                     | 419.9±27.78   |

Table 2. Correlation between the PET results and OPN levels in serum by Spearman's correlation coefficient.

| Parameter                        | R   | P   |
|----------------------------------|-----|-----|
| 4h D/P Cr                        | 0.02| 0.77|
| D/D0                             | -0.13| 0.65|
| 4h ultrafiltration volume        | -0.16| 0.74|

Table 3
| Variable                           | L/A (n=37)       | H/L (n=72)       | P       |
|-----------------------------------|------------------|------------------|---------|
| Male gender (%)                   | 0.54             | 0.51             | 0.794   |
| Serum albumin                     | 36.80±3.17       | 33.16±5.19       | <0.0001 |
| Hemoglobin                        | 100.47±18.62     | 94.62±15.21      | 0.081   |
| PD duration                       | 30.09±24.50      | 41.03±38.96      | 0.123   |
| Age                               | 44.41±12.31      | 51.57±13.14      | 0.007   |
| Serum phosphate                   | 1.79±0.58        | 1.51±0.41        | 0.004   |
| Serum calcium                     | 2.25±0.29        | 2.16±0.67        | 0.454   |
| Triglyceride                      | 2.08±1.37        | 1.62±1.29        | 0.084   |
| Cholesterol                       | 4.37±0.99        | 4.45±1.17        | 0.715   |
| Uric acid                         | 391.31±82.40     | 394.07±92.02     | 0.879   |
| Creatinine                        | 990.78±333.16    | 944±270.94       | 0.438   |
| PTH                               | 470.72±260.12    | 394.44±294.68    | 0.197   |
| Total Kt/V                        | 1.44±0.32        | 1.49±0.37        | 0.44    |
| Peritoneal Kt/V                   | 0.43±0.50        | 0.32±0.40        | 0.174   |
| Renal Kt/V urea                   | 1.25±1.03        | 3.05±1.94        | <0.0001 |

Table 3. Data are presented as means ± standard deviation (SD) or n (%);

Table 4

Association between D/P Cr and OPN levels in the peritoneal effluents.

|                      | Unadjusted Model | Adjusted Model | B   | Wald  | 95% CI       | OR   | P       |
|----------------------|------------------|----------------|-----|-------|--------------|------|---------|
| OPN (peritoneal effluents) | 1.26             | 1.29           | 1.84-6.68   | 3.5 | <0.001       |

Table 4. Model 1: univariable logistic regression model; Model 2: including age, gender, PD duration, Serum albumin, Serum phosphate.

Figures
Correlation between OPN level in the peritoneal effluent and peritoneal transport characteristics. Peritoneal solute transport and effluent OPN values. Peritoneal solute transport was assessed with the PET. The levels of OPN in the overnight peritoneal effluents were also analyzed with ELISA. (a) The D/P Cr ratio versus the OPN level. (b) The D/D0 glucose ratio versus the OPN level. (c) The 4h ultrafiltration volume versus the OPN level.
Figure 2

Area under the receiver operating characteristic curve (AUROC) analyses of the predictor performance of OPN-PSTR model identifying increased PSTR.
Figure 3

The levels of OPN in the peritoneal effluents from patients without or with infectious peritonitis. ***P < 0.001.
Western blot assay showing abundance of OPN in HMrSV5 with LPS treatment for different time as indicated.
HMrSV5 cells were pretreated with scramble or OPN small interfering RNA (siRNA) for 24 h, followed by TGFβ1 stimulation. (A) Western blotting analyses revealing the downregulation of OPN protein in HMrSV5 cells at 24h after OPN siRNA transfection. (B) Western blotting analyses showing that knocking down OPN inhibited FN expression at 48 h after TGF β 1 treatment. (C) The graph showing the relative abundance of FN after TGF β 1 treatment.

*P< 0.05 compared with vehicle control (n = 4), #P <0.05 compared with those cells treated with scramble siRNA transfection and TGFβ1 (n = 4).