

Microparticle Formation in Peritoneal Dialysis: A Proof of Concept Study

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Abstract

Background: Injury to the mesothelial layer of the peritoneal membrane during peritoneal dialysis (PD) is implicated in loss of ultrafiltration capacity, but there are no validated biomarkers for mesothelial cell injury. Microparticles (MPs) are 0.1 to 1.0 μm membrane vesicles shed from the cell surface following injury and are sensitive markers of tissue damage. Formation of MPs in the peritoneal cavity during PD has not been reported to date.

Methods: We designed a single-center, proof of concept study to assess whether peritoneal solution exposure induces formation of mesothelial MPs suggestive of PD membrane injury. We examined MP levels in PD effluents by electron microscopy, nanoparticle tracking analysis (NTA), flow cytometry, procoagulant activity, and Western blot.

Results: NTA identified particles in the size range of 30 to 900 nm, with a mean of 240 (SE: 10 nm). MP levels increased in a progressive manner during a 4-hour PD dwell. Electron microscopy confirmed size and morphology of vesicles consistent with characteristics of MPs as well as the presence of mesothelin on the surface. Western blot analysis of the MP fraction also identified the presence of mesothelin after 4 hours, suggesting that MPs found in PD effluents may arise from mesothelial cells.

Conclusions: Our results suggest that MPs are formed and accumulate in the peritoneal cavity during PD, possibly as a stress response. Assessing levels of MPs in PD effluents may be useful as a biomarker for peritoneal membrane damage.

Abrégé

Contexte: Les lésions causées à la couche mésothéliale de la membrane péritonéale au cours d'une dialyse péritonéale (DP) sont impliquées dans la perte de capacité d'ultrafiltration. Toutefois, il n'existe aucun biomarqueur validé permettant la détection de ces lésions. Les microparticules (MP) sont des vésicules membranaires de 0,1 à 1,0 μm qui se détachent de la surface des cellules à la suite des lésions. Les microparticules sont sensibles aux marqueurs de dommages tissulaires. À ce jour, la formation de microparticules dans la cavité péritonéale au cours de la DP n'a pas été observée.

Méthodologie: Nous avons conçu une étude de preuve de concept que nous avons menée dans un seul centre. Nous voulions déterminer si l'exposition à la solution de dialyse péritonéale induisait la formation de microparticules mésothéliales, ce qui pourrait indiquer la présence de dommages membranaires provoqués par la DP. Nous avons mesuré les taux de microparticules dans les effluents de la DP par microscopie électronique, par analyse du suivi individuel de particules (*Nanoparticle Tracking Analysis*—NTA), en cytométrie de flux, par la mesure de l'activité pro-coagulante et par *Western Blot*.

Résultats: L'analyse par NTA a identifié des particules allant de 30 à 900 nanomètres, dont le diamètre moyen était de 240 \pm 10 nanomètres. Les taux de MP ont augmenté d'une façon progressive au cours des quatre heures que durait la DP. La microscopie électronique a confirmé la taille et la morphologie de vésicules conformes aux caractéristiques des MP, de même que la présence de mésothéline en surface. L'analyse par Western Blot de fragments de MP a également indiqué la présence de mésothéline après 4 heures, ce qui suggère que les microparticules recueillies dans les effluents de dialyse pourraient provenir de cellules mésothéliales.

Conclusions: Nos résultats suggèrent que des microparticules sont formées au cours de la DP et qu'elles s'accumulent dans la cavité péritonéale, possiblement en réponse au stress. Par conséquent, la mesure des taux de microparticules dans les effluents de DP pourrait s'avérer un bon biomarqueur pour indiquer la présence de lésions dans la membrane péritonéale.



Keywords

biomarker, extracellular vesicle, membrane, microparticles, peritoneal dialysis

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What was known before

- Injury to the mesothelial layer of the peritoneal membrane occurs during peritoneal dialysis (PD) and is implicated in loss of ultrafiltration capacity, but there are no validated biomarkers for mesothelial cell injury.
- Alterations in levels of membrane-derived microparticles in plasma or urine samples have been shown to reflect underlying tissue injury; however, whether microparticles are released into the peritoneal cavity during PD was not known.

What this adds

- Our study provides the first evidence that membrane-derived microparticles are released into the peritoneal cavity during PD.
- The mesothelium is a major source of microparticles in peritoneal effluents.
- Mesothelial microparticle levels increase over a 4-hour exposure to PD solution (Dianeal 4.25%).

Introduction

Progressive loss of ultrafiltration capacity is a major cause of attrition in chronic PD patients.¹ Pathologically, ultrafiltration failure arises from epithelial to mesenchymal transition of mesothelial cells and submesothelial collagen deposition. This leads to thickening and fibrosis, further resulting in diminished osmotic conductance of the membrane.² In addition, there is proliferation of submesothelial capillaries which results in a more rapid decay of the osmotic gradient between the blood and the dialysate, thus further decreasing fluid removal.³ It is believed that this peritoneal remodeling is related to mesothelial cell stress and injury induced by

peritoneal solution exposure. This in turn may lead to chronic peritoneal membrane inflammation and fibrosis. At present, there is no universally accepted clinical biomarker for mesothelial cell stress induced by PD solutions.⁴ Such a biomarker could allow for patient stratification according to their risk of developing peritoneal membrane fibrosis, guide optimal selection of PD solutions, and aid in the development of therapeutic interventions to preserve peritoneal membrane health.

Microparticles (MPs) are a class of extracellular vesicles, 0.1 to 1.0 μm in diameter, which are formed through the outward blebbing of the plasma membrane and are released into the extracellular space.⁵ MPs are formed by all cell types and contain membrane and cytosolic protein, messenger RNA (mRNA) and microRNA (miRNA) but lack nuclear material.⁶ MPs are formed under conditions of cell stress, but cell death is not a requisite for their formation.⁷ Because of this property, and the fact that they retain the surface properties of the cell from which they originate, MPs are seen as ideal biomarkers of cell/tissue injury.^{6,8,9} In this regard, increases in endothelial MPs strongly correlate with measures of vascular damage and independently predict risk of cardiovascular mortality/morbidity.^{8,10} Accordingly, the assessment of MPs in biological samples may be used to identify tissue damage at the earliest stages. To date, no studies have examined the formation of MPs in the peritoneal cavity during PD, and indeed mesothelial MPs have not been previously described in any model. We therefore set out to test the hypothesis that the formation of mesothelial cell MPs is induced by the instillation of PD solution.

Patients and Methods

Study Design

We carried out a single-center study of incident PD patients followed up at The Ottawa Hospital. The inclusion criteria

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were adult patients with end-stage renal disease (as defined by stage 5 chronic kidney disease [CKD]) new to PD and presenting for their first peritoneal equilibration test (PET). Exclusion criteria were patients on PD >6 months or patients with a history of peritonitis preceding the first PET. The Ottawa Health Sciences Research Ethics Board approved the study; all patients gave their informed consent to participate, and experiments were conducted in accordance with the *Declaration of Helsinki*.

Following a 10-hour overnight dwell with 2 L of Dianeal 2.5%, patients were drained and 2 L of Dianeal 4.25% was instilled. Ten milliliters of the effluent was immediately collected to provide a baseline sample. A minimum of 10 mL of PD effluent was subsequently collected at 1 hour, 2 hours, and 4 hours postinstillation. A subset of samples were excluded due to delays in collection of PD effluents. A blood sample was collected at 4 hours postinstillation for assessment of dialysate-to-plasma (D/P) creatinine ratio. The clinical characteristics were retrieved from patient files and de-identified for analysis.

D/P ratio was measured at 4 hours and used to classify patients as low (D/P creatinine ≤ 0.49), low average (D/P creatinine 0.50-0.64), high average (D/P creatinine 0.65-0.80), or high (D/P creatinine ≥ 0.81) transporters according to the criteria defined by Twardowski et al.¹¹ Serum creatinine was determined using an Ortho Vitros 250 Analyzer (Ortho-Clinical Diagnostics Inc, Raritan, New Jersey), and dialysate creatinine concentration was measured using a Beckman Coulter LX20 (Beckman Coulter Inc, Mississauga, Canada).

MP Isolation

PD effluent samples were immediately processed after isolation to remove any potential large contaminants such as cells or apoptotic bodies. Briefly, samples were centrifuged at 2500g for 20 minutes at 20°C, and the supernatant was frozen prior to analysis. MPs were then isolated from cell-free samples by centrifugation at 20 000g for 20 minutes at 20°C, and the MP-containing pellet was collected, while the supernatant, which contains exosomes, smaller vesicles, and soluble factors, was discarded. The MP-containing pellet was resuspended in Annexin V binding buffer for flow cytometric analysis, or 1× phosphate buffered saline (PBS) for nanoparticle tracking analysis (NPA), or electron microscopy.

Flow Cytometric Detection of MPs

MPs were quantified using a MoFlo Aria Fluorescence Activated Cell Sorter as described.¹² Mesothelial cell origin was confirmed by staining for the mesothelial cell surface marker mesothelin using a rabbit polyclonal anti-human mesothelin antibody (1:100, Abcam, Toronto, Ontario, Canada) followed by a Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:2000, Sigma, Oakville, Ontario, Canada). As a negative control,

MPs were incubated with secondary antibody alone. MPs were defined as particles of less than 1.0 μm and greater than 0.1 μm in size that exhibited significantly more fluorescence than their negative controls.

Nanoparticle Tracking Analysis

Sizing and enumeration of MPs was achieved by NTA using a Nanosight LM10 instrument (NanoSight Limited, Amesbury, UK) equipped with NTA 2.3 software.^{13,14} NTA is a light-scattering technique which utilizes video analysis for sizing and enumeration of extracellular vesicles.¹³ Peritoneal effluents were collected and diluted in PBS to a particle concentration within optimal working range of the system. Approximately 300 μL of sample was loaded into the sample chamber, and videos were recorded for 60 seconds for each sample, with a shutter speed of approximately 30 milliseconds and a camera gain between 250 and 650. Settings for software analysis were the following: detection threshold: 30 to 50; blur: 5×5 ; minimum expected particle size: auto. Size distributions are presented as the average and standard error of 3 to 4 video recordings per sample.

Measurement of MP Levels by Procoagulant Activity

Levels of phosphatidylserine (PS)-positive MPs were also assessed using a Zymuphen MP-Activity kit (Aniara, West Chester, Ohio, USA) as described previously with modification.¹⁵ The assay utilizes immobilized Annexin V to capture PS-expressing MPs. MPs are then detected by the addition of coagulation factor Va, factor Xa, Ca^{2+} , and prothrombin. Effluent samples, collected after the initial low-speed (2500g) centrifugation step, were loaded according to the manufacturer's instructions, and the rate of thrombin production (proportional to PS availability and, by extension, MP concentration) was assessed using a chromogenic substrate with absorbance read at 405 nm. Results are expressed in arbitrary fluorescence units (AU).

Electron Microscopy

Negative staining of MPs. Isolated MP fractions were spotted on formvar-coated copper grids (200 mesh; Canemco, Lakefield, Ontario, Canada) for 30 seconds. Samples were negatively stained with 2% uranyl acetate in water for 6 minutes and dried with filter paper. Samples were examined on a transmission electron microscope (TEM, JEOL JEM 1230, Japan).

Immunogold staining of MPs. Isolated MP fractions were fixed with 4% paraformaldehyde/0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), pelleted, and postfixed with reduced osmium. The pellet was then washed in 0.1 M sodium cacodylate buffer, dehydrated in a graded ethanol

Table 1. Patient Characteristics.

	1	2	3	4	5	6	7	8	Mean \pm SD or (frequency)
Age, y	77	73	67	76	72	65	78	41	69 \pm 12
Sex	M	F	M	F	F	M	F	F	
Etiology of CKD	Vascular	Vascular	Postnephrectomy	Myeloma	Diabetes	IgA nephropathy	Diabetes	Reflux	
Weight, kg	79	56	126	68	58	84	74	74	77 \pm 22
Diabetes	No	No	No	No	Yes	No	Yes	No	(25%)
Hypertension	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	(88%)
Prior hemodialysis	No	Yes	Yes	No	No	No	Yes	Yes	(50%)
Prior transplant	No	No	No	No	No	No	No	No	0
Prior abdominal surgery	No	No	yes	No	No	No	No	No	(12%)
Time on PD, wk	8	2	1	7	5	7	12	2	5.5 \pm 3.7
4-h D/P Creatinine	0.86	0.73	0.87	0.76	0.82	0.88	0.76	0.67	0.79 \pm 0.08

Note. Weight refers to weight at time of PET. CKD = chronic kidney disease; PD = peritoneal dialysis; D/P Cr = dialysate to plasma creatinine ratio at 4 hours; PET = peritoneal equilibration test.

series, and embedded in LR White Resin. The thin sections (70 nm) were obtained by ultracut (Leica EM UC 6) and taken on nickel grids covered by formvar film. Sections were blocked with blocking buffer (2% skim milk in 1 \times PBS) for 1 hour, and incubated in a rabbit monoclonal antibody for anti-human mesothelin (1:100, Abcam) for 1 hour at room temperature. The antibody solution was then replaced by the same blocking buffer for 5 minutes for rinsing. After rinsing, grids were postincubated with 12-nm colloidal gold-conjugated goat anti-rabbit IgG antibody (1:50, Jackson, Bar Harbor, Maine, USA) for 2 hours at room temperature. The grids were further washed in distilled water, dried, stained with 5% uranyl acetate in ethanol and Reynold's lead citrate, and examined under the TEM as mentioned earlier.

Western Blot Analysis

Mesothelin protein expression was examined in MP fractions obtained from 4-hour PD effluents. MP fractions were resuspended in radioimmunoprecipitation assay buffer (RIPA buffer; 50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% NP40, 0.1% sodium dodecyl sulfate [SDS], 0.5% deoxycholic acid [DOC], 1 mM phenylmethanesulfonyl fluoride [PMSF], 25 mM MgCl₂). Equivalent amounts of protein were separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking in 5% milk, membranes were incubated with an anti-human mesothelin antibody (1:2000, Abcam) overnight. Protein was visualized by staining with the appropriate secondary horseradish peroxidase-labeled antibody for 1 hour at room temperature and probed for immunoreactive proteins by chemiluminescence. As a negative control, unused 4.25% Dianeal solution was subjected to MP isolation procedures (lane 1). Lysates of HeLa cells (a mesothelin-expressing cervical cancer line) were used as a positive control (lane 3).

Statistical Analysis

Results are expressed as mean \pm standard error of the mean (SEM). All values for MP quantification were normalized to PD drain volumes to ensure that differences in interpatient drain volumes did not influence MP levels. The 4-hour volume was the actual volume of effluent drained, while the volumes at 1 and 2 hours were estimated using the PD ADEQUEST program (Baxter International, Deerfield, Illinois) program. Differences between all groups were assessed by Kruskal-Wallis test with Dunn's multiple comparisons post-test. A $P < .05$ was considered significant. Analysis was conducted using Graphpad Prism version 5.0 (GraphPad Software, La Jolla, California).

Results

Patient Characteristics

To assess whether MPs are formed during PD, we carried out a proof of concept study examining MP levels during a standardized PD dwell. A total of 8 patients were assessed and 10 mL aliquots of effluent were collected at baseline, 1 hour, 2 hours, and 4 hours during a modified PET with 2L Dianeal 4.25% (Baxter International).¹⁶ Patient characteristics are summarized in Table 1.

Characterization of MPs in Peritoneal Dialysis Effluents

Using NTA, a video light-scattering technique for identification of small particles,¹³ we observed the presence of extracellular vesicles in all of the 4-hour effluents with a size range between 30 and 900 nm and mean size of 240 (SE: 10 nm) (Figure 1A). These particles were further confirmed to possess characteristic size and morphology by electron

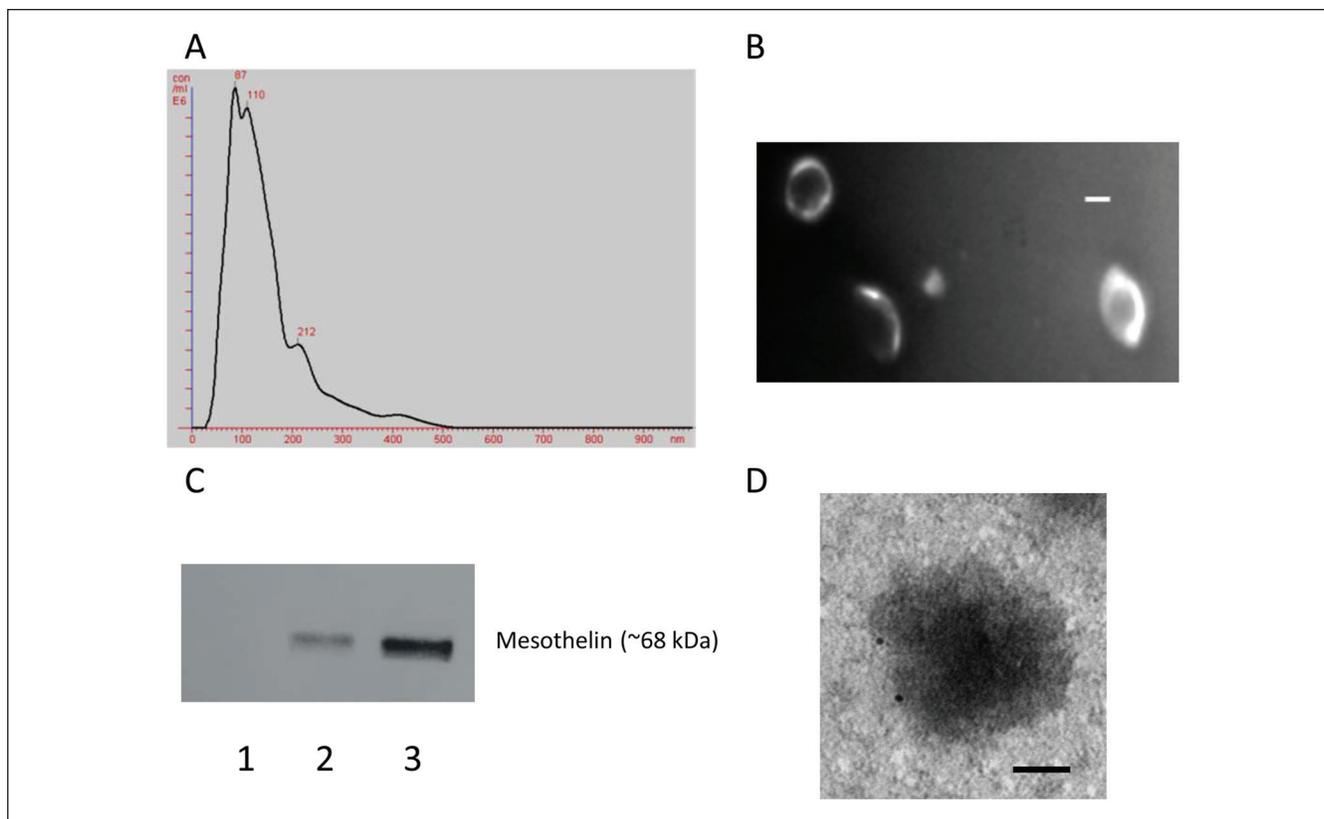


Figure 1. Characterization of MPs in PD effluents.

Note. A, Nanoparticle tracking analysis (NTA) of MPs isolated from peritoneal effluents. PD solutions consisted of Dianeal 4.25% collected after 4 hours PD. B, Transmission electron micrographs of MPs isolated from PD effluents (Dianeal 4.25% collected after 4 hours PD). Scale is 100 nm. C, Detection of mesothelin (surface marker of mesothelial cells) in MPs isolated from peritoneal effluents taken from 4-hour dwell (lane 2). As a negative control, unused 4.25% Dianeal solution was subjected to MP isolation procedures (lane 1). Lysates of HeLa cells (a mesothelin-expressing cervical cancer line) were used as a positive control (lane 3). D, Transmission electron micrographs of MPs isolated from PD effluents and labeled for mesothelin by immunogold electron microscopy. Scale bar is 100 nm. MPs = microparticles; PD = peritoneal dialysis.

microscopy (Figure 1B). Importantly, Western blot analysis of MPs isolated from 4-hour PD effluents also revealed the presence of mesothelin suggesting that at least a portion of MPs in the effluent arise from mesothelial cells which comprise the outer layer of the peritoneal lining (Figure 1C). The presence of mesothelin on the surface of MPs from PD effluents was further confirmed by immunogold labeling and electron microscopy (Figure 1D).

Assessment of MP Release During Peritoneal Dialysis

Peritoneal MP levels were quantified using 3 independent techniques (NTA, flow cytometry, and a MP procoagulant activity assay). Both NTA and the MP activity assay enumerate MPs from all cellular origins, while flow cytometry identified MPs as mesothelial in origin through the use of fluorescent labeling of mesothelin. By NTA, MPs were present at low levels at baseline and accumulated over the 4-hour dialysis period with Dianeal 4.25% (Figure 2A). Similarly, MP levels, determined by assessment of MP procoagulant

activity, increased over the course of PD (Figure 2B). Levels of MPs also increased with an overnight exposure to Dianeal 2.5% (4.6×10^{11} , SE: 1.0×10^{11} vs 1.0×10^{11} , SE: 1.2×10^{11} total MPs in PD cavity by NTA, $P < .01$).

Levels of mesothelial cell-derived MPs, determined by flow cytometry, were also increased at 4 hours (Figure 3). Interestingly, mesothelial MP formation at 4 hours was significantly higher than at 1 and 2 hours, suggesting a progressive increase in MP formation in response to PD fluid instillation.

Discussion

The present study reports for the first time not only that mesothelial-derived MPs are detectable in PD effluents but also that they accumulate over the duration of PD exchange, which suggests that PD solution exposure induces this process. Western blot analysis, flow cytometry, and immunogold electron microscopy suggest that the mesothelial cell layer lining the peritoneal cavity is a major source of effluent MPs. It is noteworthy that levels of mesothelial MPs,

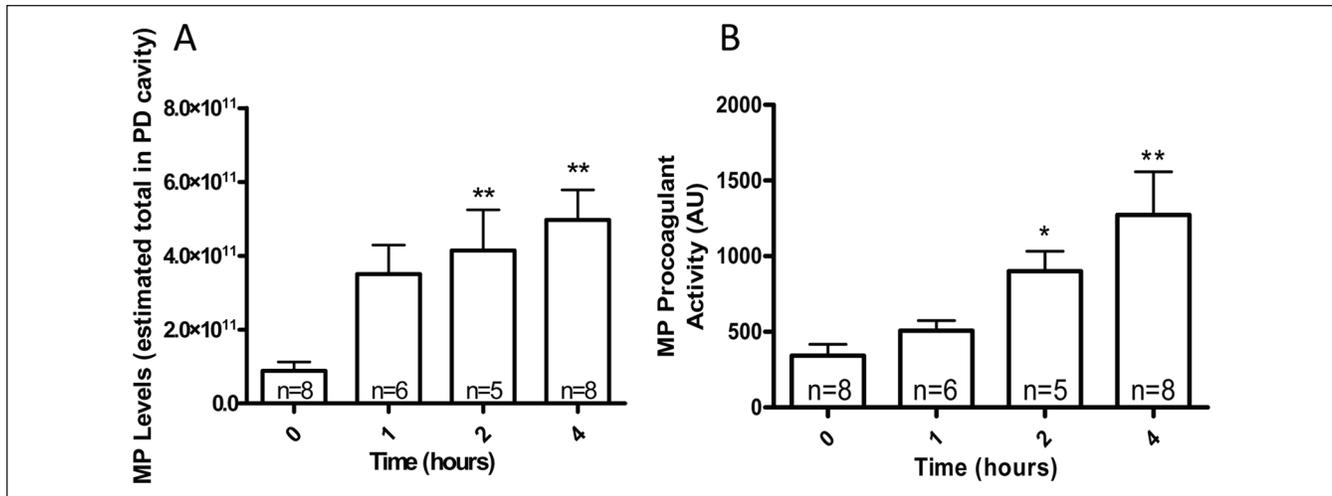


Figure 2. Levels of MPs in PD effluents as determined by nanoparticle tracking analysis (A) and MP activity assay (B). Note. PD solution consisted of Dianeal 4.25%. A, Total MP counts (identified as particles of 0.1-1.0 μm by NTA), normalized to drain volumes are reported as the estimated total number of MPs in the PD cavity. B, MP procoagulant activity, normalized to drain volumes is shown in AU. MPs = microparticles; PD = peritoneal dialysis; NTA = nanoparticle tracking analysis; AU = arbitrary units. * $P < .05$, ** $P < .01$ vs initial MP concentration in effluent collected immediately after PD initiation, $n = 5-8$.

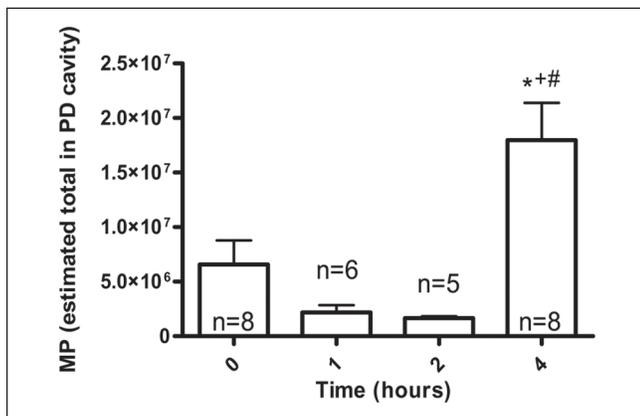


Figure 3. Levels of mesothelin positive MPs in peritoneal dialysates as determined by flow cytometry. Note. PD solution consisted of Dianeal 4.25%. Total mesothelial MP counts (identified as particles of 0.1-1.0 μm with immunostaining for mesothelin) normalized to drain volumes are reported as the estimated total number of mesothelial MPs in the PD cavity. MPs = microparticles; PD = peritoneal dialysis. * $P < .05$ vs initial MP concentration in effluent collected immediately after PD initiation. + $P < .01$ vs 1 hour. # $P < .01$ vs 2 hours, $n = 5-8$.

identified by flow cytometry, did not increase until 4 hours. Based on this, we hypothesize that mesothelial MP formation during PD represents an acute mesothelial cell response to hypertonic dialysate rather than a steady background formation due to a chronic inflammatory state where one expects progressive formation.

We observed a fair degree of interindividual variability in baseline MP levels. The reason for these differences is unclear; however, MP levels are a function of the dynamic

balance between their formation by stressed cells and their clearance through various cellular systems including macrophages.¹⁷ Accordingly, there may be interpatient differences in either the basal rates of MP formation or the rate of clearance from the peritoneal cavity. An alternative explanation could be that certain individuals are more susceptible to this type of injury. There may also be differences in between-patient residual dialysate in the peritoneal cavity after drainage of the overnight dwell. Regardless of the explanation, it is likely that the assessment of dynamic changes in MP formation over a period of time (as was done here) will be far more informative than merely quantifying levels at a single time point as this would account for interindividual variations in rates of MP formation.

Our study has several limitations. First, this was a single-center study. Therefore, it will be important for these data to be verified in other PD populations. Second, our patients' membrane transport characteristics were skewed toward higher transporters with a mean 4-hour D/P creatinine of 0.79. We have previously reported a mean D/P creatinine of 0.71 in a larger cohort from our program, and the variance in our current population falls just over one standard deviation from this mean, in keeping with expected random variation.¹⁸ We have also not fully characterized the origins of all MPs in the PD effluent. NTA and the MP procoagulant activity assay quantify all MPs in the effluent, regardless of cellular origin. Our flow cytometry, Western blot, and electron microscopy data all strongly suggest that mesothelial MPs make up a significant fraction of the MPs formed during PD as each approach indicated the presence of a mesothelial cell surface antigen in a subfraction of MPs. Nevertheless, it is possible that MPs in PD effluents may also originate from

nonmesothelial sources. Importantly, however, the dynamic changes in mesothelial MP levels seen with flow cytometry mirrored the changes seen in total MP levels assessed by NTA and the MP activity assay. Finally, we have focused largely on the effects of Dianeal 4.25% exposure on MP formation. It would therefore be of interest to assess the effects of additional PD solutions that may cause less stress to the peritoneal membrane.

The biological significance of mesothelial MPs in the peritoneal cavity is unclear at this time. We and others have previously reported that MPs serve as intercellular signals capable of inducing a host of responses in target cells.^{12,19-22} Although not examined in the present study, it is possible that mesothelial MPs may exert biological effects directly in the peritoneal cavity. Other MP populations (ie, endothelial, leukocyte) have been reported to exert proinflammatory, proapoptotic, and profibrotic effects on target cells, and it is possible that mesothelial MPs have similar biological activity; however, this remains to be tested.²³⁻²⁵

In summary, we have shown for the first time that MPs are formed during PD exchange. Mesothelial cells represent a major source of MPs in PD effluent and increase in a time-dependent fashion. These increases may reflect acute mesothelial injury in response to PD solutions. This discovery opens a new window into the peritoneal cavity and sets the stage for further study to determine the clinical significance of mesothelial MPs.

Ethics Approval and Consent to Participate

This study was approved by The Ottawa Health Science Network Research Ethics Board. All patients gave their informed consent to participate and experiments were conducted in accordance with the *Declaration of Helsinki*.

Consent for Publication

All authors gave their final approval of this version of the manuscript.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Author's Note

Brendan B. McCormick, Marcel Ruzicka, and Dylan Burger contributed equally as corresponding authors.

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Declaration of Conflicting Interests

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