Effluent biomarkers in peritoneal dialysis: A captivating symphony from the peritoneal membrane
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CHAPTER 8

Part III | Clinical Application

Early diagnosis of Epifluorin P-2 and P-ai in Epiphysiological Epitonia al's Cell Elerosis
The Early Diagnostic Potential of Effluent MMP-2 and PAI-1 in Encapsulating Peritoneal Sclerosis

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Submitted for publication
ABSTRACT

Background
Recently the use of effluent matrix metalloproteinase-2 (MMP-2) and plasminogen activator inhibitor-1 (PAI-1) as potential biomarkers of peritoneal fibrosis has been demonstrated during longitudinal follow-up of incident PD patients. This study focuses on effluent MMP-2 and PAI-1 as early markers in the preceding years of patients who develop encapsulating peritoneal sclerosis EPS.

Methods
In this nested case-control study, patients who developed EPS were compared to controls with a PD duration of at least 57 months. Effluent MMP-2 and PAI-1 levels were determined by an ELISA and dialysate appearance rates (AR) were calculated. Time courses of MMP-2 and PAI-1 AR were studied by means of a linear repeated-measures model in the years prior to EPS diagnosis, adjusted for age and PD duration. Time-specific ROC analyses were performed to assess the diagnostic validity of the markers in the years prior to EPS diagnosis.

Results
In total 11 patients developed EPS and 34 controls were assembled. No difference in MMP-2 AR was present between controls and EPS patients. In contrast, higher PAI-1 AR were found in EPS patients as compared to the controls (p=0.01). Time-specific ROC analyses indicated discriminative ability for PAI-1 (AUC=0.76, p=0.01), whereas this was absent for MMP-2 AR. At a lag time of one year prior to EPS diagnosis the optimal threshold for PAI-1 AR of 8.5 ng/min corresponded to a sensitivity estimate of 100% and 55% for specificity. The latter increased to 88% when the mean PAI-1 AR value of 32.0 ng/min was used.

Conclusions
It is unlikely that MMP-2 can be used as a biomarker of EPS as no distinction between patients who develop EPS and controls can be made. The time course suggests a persistent contribution of effluent MMP-2 in peritoneal tissue remodeling. Elevated levels of PAI-1 AR are present in patients who develop EPS, pointing to progressive peritoneal fibrosis and sclerosis. PAI-1 AR has a fair discriminative capacity from three years prior to EPS diagnosis. Therefore, effluent PAI-1 may aid in monitoring peritoneal fibrosis and serve as biomarker for EPS.
INTRODUCTION
To date no diagnostic instrument or method is available to identify peritoneal dialysis (PD) patients who will develop encapsulating peritoneal sclerosis (EPS), nor strategies for its prevention. Even though the prevalence of EPS is low, this detrimental complication is associated with high mortality rates\textsuperscript{1-3} and severe morbidity. The diagnosis of EPS is frequently based on gastro-intestinal complaints, especially episodes with bowel obstruction and peritoneal membrane failure, confirmed by laparotomy or radiological assessments and therefore often delayed. Anatomical modifications of the peritoneal membrane that are associated with EPS comprise loss, absence or transdifferentiation of mesothelial cells,\textsuperscript{4,5} microvascular changes and interstitial fibrosis.\textsuperscript{6-8} Besides these generic anatomical abnormalities, which may also occur in some long-term patients, EPS is characterized by a more intense phenotype. This is especially reflected by the inability of re-epithelialization, basement membrane thickening and the presence of abundant interstitial fibrosis.\textsuperscript{9} More recently, the use of podoplanin as a morphological marker for EPS has been proposed.\textsuperscript{9} Functionally, an increase in small solute transport, impairment of the ultrafiltration capacity and a decrease in free water transport are observed.\textsuperscript{10} Even though nephrologists and scientists have proposed various theories, the aetiology and pathogenesis of EPS remain to be elucidated.

The paucity of serial peritoneal biopsies due to its invasive character and accompanied risk factors hampers the insight into progressive morphologic alterations. Furthermore, the assessment of peritoneal function provides limited information to address these morphologic changes. Therefore, discovery of biomarkers present in the continuously accessible peritoneal effluent is necessary. These effluent biomarkers may serve as non-invasive instruments for longitudinal follow-up of the peritoneal membrane integrity and possibly aid in the early detection of EPS.\textsuperscript{11}

Recently, the potential use of effluent matrix metalloproteinase-2 (MMP-2) and plasminogen activator inhibitor-1 (PAI-1) as markers of peritoneal tissue remodeling and fibrosis has been investigated.\textsuperscript{12} The foremost function of MMP-2 is maintenance of homeostasis by degrading components of the extracellular matrix such as the basement membrane and interstitial connective tissue in which collagen IV is one of its main substrates.\textsuperscript{13} PAI-1 is present in various cell types where its biological activity includes inhibition of fibrinolysis as well as interactions with integrins and extracellular matrix components.\textsuperscript{14,15} Up-regulation of PAI-1 by transforming growth factor-\(\beta 1\) (TGF-\(\beta 1\)) has been shown to enhance fibrin deposition and interstitial collagen secretion in human
peritoneal mesothelial cells.\(^{16}\)

MMP-2 and PAI-1 are both locally produced within the peritoneal cavity.\(^{12,17,19}\)

In a previous study we found that effluent MMP-2 remained stable during long-term follow-up of PD patients, but that PAI-1 showed an increase with the duration of PD.\(^{12}\)

We therefore examined these markers with respect to long-term PD patients at risk for EPS. As primary aim, the time courses of effluent MMP-2 and PAI-1 were investigated in the four years preceding the diagnosis of EPS. Additionally, to evaluate the clinical validity of MMP-2 and PAI-1 as early diagnostic markers for EPS, their discriminative capacity was assessed.

**PATIENTS & METHODS**

*Study population and design*

A longitudinal nested case-control design was applied as cost-effective subcohort sampling within our center. The diagnosis of EPS was based on pre-specified criteria\(^{30}\) and reviewed by two experienced nephrologists and a radiologist. Patients who did not develop EPS were randomly selected from the same base population as controls in a 1:3 case-control ratio. In order to avoid spectrum bias a restriction on PD treatment duration was set at a minimum of 57 months for all included controls, which correspond to the minimum PD duration of the EPS cases. Due to the low event rate no further matching or stratification was applied. Demographic characteristics and clinical data such as primary renal disease, PD duration and initial regimen, number of peritonitis episodes and peritoneal transport parameters were documented.

*Specimen collection and biochemical assays*

As part of routine patient care all PD patients in our center undergo a yearly standard peritoneal permeability analysis (SPA).\(^{21}\) After this four-hour peritoneal function test dialysate samples are aliquoted and archived at a minimum temperature of at least -20°C. The dialysate specimens in the present study were assembled retrospectively from this local PD biorepository. The collection and processing of the dialysate was therefore similar for cases and controls. For all patients at least two SPA’s with dialysate specimens were available prior to the diagnosis of EPS or in case of the controls their last available SPA. A potential effect of long-term storage of dialysate samples for the EPS cases versus controls was overcome by the aforementioned restriction criteria.
Dialysate MMP-2 and PAI-1 levels were measured by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, USA). The MMP-2 assay has a mean detection limit of 0.047 ng/mL and PAI-1 0.059 ng/mL. The assays were performed by one laboratory scientist unblinded to the clinical outcome. However, cases and controls were assayed within the same analytical batch and the objectivity of an ELISA does not contribute to potential bias.

Prior to biochemical analysis the dialysate samples were homogenized and centrifuged at 1710 x g for 10 minutes. As indicated by pilot tests, the dialysate samples did not require dilution prior to assaying. Analyses were carried out as recommend by the assay protocol. Plates were read within ten minutes by a microplate reader at a wavelength of 450nm (correction λ=650nm). To assess the degree of analytical imprecision, the standards provided by the kit and dialysate samples were assayed in duplicate. The inter- and intra-assay precision of both markers was less than 10%.

Calculations and statistical analysis
To correct for the drained effluent volume, appearance rates (AR) of MMP-2 and PAI-1 were calculated. In this equation the marker concentration is multiplied by the drained volume and subsequently divided by the total dwell time. Based on right skewed distribution of the markers AR, the values were log transformed prior to analysis. Absent marker levels or the inability to calculate AR of MMP-2 and PAI-1 were due to either absence of dialysate specimens, dwell times or dwell volumes. These missings were at random. Therefore, a linear repeated measures model was used for time course assessment, adjusted for age and PD duration. The lag time, e.g. time from dialysate sampling to EPS diagnosis, was incorporated as a categorical variable from maximal four years prior to the diagnosis of EPS or last available SPA for controls. The model included a repeated statement and a random intercept. Additionally, an interaction term was included for lag time and EPS in order to determine differences in trend between the cases and controls. The covariance structure was based on the most optimal Akaike’s Information Criterion.

To evaluate the discriminative capacity of MMP-2 and PAI-1 AR, time-specific receiver operating characteristic (ROC) curves were computed. In these analyses lag time was restricted from three years prior to the diagnosis of EPS. The area under the ROC curve (AUC) and 95% confidence intervals were calculated. ROC curves were also used to estimate threshold values by the Youden’s Index (optimal balance between sensitivity
and specificity), a minimally acceptable true positive rate of 90 percent and minimally acceptable false positive rate of 10 percent. These pre-specified true and false positive rates were used to indicate a negative or positive test result. Comparisons of demographic data and peritoneal transport parameters between EPS patients and controls were performed by non-parametric tests and presented as medians (ranges) or proportions (%). PASW Statistics 20.0 was used for statistical analyses and p-values below 0.05 were considered to indicate statistical significance.

RESULTS
All diagnosed EPS patients (n=11) from our center between 1995-2008 were included in this study, where to date no novel EPS case has been identified. The source population consisted of 417 PD patients in which 15% met the restriction criteria of a PD treatment duration of more than 57 months. A number of 34 controls were randomly selected from this long-term subpopulation.

Data on the patient characteristics have been described elsewhere.\(^2\) In brief, differences were present with regard to age where the patients who developed EPS initiated PD treatment at a younger age as compared to the controls. Moreover, EPS patients had a longer PD treatment duration and a significantly lower net ultrafiltration and free water transport (Table 1).

Dialysate levels of MMP-2 or PAI-1 below the detection limit represented 22% of all dialysate samples and were replaced by the value zero. Missings due to the absence of dialysate specimens, information on dwell time or dwell volume was less than 1.5%.

Table 1. Patients characteristics.

<table>
<thead>
<tr>
<th></th>
<th>EPS (n=11)</th>
<th>Controls (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>35 (21 - 73)</td>
<td>53 (32 - 87)(^a)</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>64</td>
<td>61</td>
</tr>
<tr>
<td>PD duration (months)</td>
<td>104 (57 - 149)</td>
<td>72 (57 - 112)(^a)</td>
</tr>
<tr>
<td>Net UF (mL/4-hour)</td>
<td>121 (-113 - 308)</td>
<td>494 (73 - 920)(^*)</td>
</tr>
<tr>
<td>FWT at 60 minutes (mL)</td>
<td>21 (-41 - 72)</td>
<td>151 (21 - 371)(^b)</td>
</tr>
</tbody>
</table>

EPS: encapsulating peritoneal sclerosis; FWT: free water transport; PD: peritoneal dialysis; UF: ultrafiltration. Data are presented as median and ranges;\(^a\) p < 0.05,\(^b\) p < 0.001.
Diagnostic accuracy measures

To evaluate the clinical performance of the markers, time-specific ROC curves were established from three years prior to EPS diagnosis (Table 2). The AUC, as overall summary of discriminative capacity, indicated no potential for MMP-2 AR. Only one year prior to the diagnosis of EPS the AUC of MMP-2 AR had improved to 0.70. PAI-1 AR showed a fair discriminative ability from three years preceding EPS with increasing AUCs for those with the shortest lag time. Due to these findings, analyses with regard to potential thresholds were restricted to PAI-1 AR.

Table 3 presents the estimated sensitivity and specificity of PAI-1 AR at one and two years prior to the diagnosis of EPS for a series of threshold values. Based on the youden index, a PAI-1 AR threshold of 8.5 ng/min yielded a sensitivity of 100% and a specificity of 55% for a lag time of one year. With the emphasis to achieve a high sensitivity of 90% one year prior to EPS diagnosis, the corresponding PAI-1 AR threshold of 9.0 ng/min estimated a similar specificity of 55%.
Table 2. Time-specific area under the receiver-operating characteristic curve for MMP-2 and PAI-1 appearance rates at various time points prior to diagnosis of EPS.

<table>
<thead>
<tr>
<th>Lag time* (years)</th>
<th>AUC (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2 AR (ng/min)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.70 (0.51 - 0.90)</td>
</tr>
<tr>
<td>2</td>
<td>0.50 (0.31 - 0.70)</td>
</tr>
<tr>
<td>3</td>
<td>0.59 (0.38 - 0.81)</td>
</tr>
<tr>
<td>PAI-1 AR (ng/min)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.76 (0.61 - 0.91)</td>
</tr>
<tr>
<td>2</td>
<td>0.72 (0.55 - 0.89)</td>
</tr>
<tr>
<td>3</td>
<td>0.71 (0.52 - 0.91)</td>
</tr>
</tbody>
</table>

AUC: area under the curve; CI: confidence interval; MMP-2: matrix metalloproteinase-2; PAI-1: plasminogen activator inhibitor-1.
* Time from dialysate sampling to EPS diagnosis.

At a lag time of two years a PAI-1 AR threshold of 15.5 ng/min resulted in a sensitivity estimate of 80% and 58% for specificity. At both lag times PAI-1 specificity decreased when a high sensitivity estimate was used. We also estimated the corresponding proportion of EPS patients that could be detected when a low false positive rate of 10 percent was employed. From this analysis the sensitivity estimates were 33% and 44% at a lag time of one or two years. The corresponding PAI-1 AR thresholds were 38.5 ng/min and 56.0 ng/min. Using the mean value at one year prior to the diagnosis resulted in a sensitivity of 33% and a specificity of 88%.

Table 3. Diagnostic accuracy measures for different threshold values of PAI-1 appearance rates at one or two years prior to EPS diagnosis.

<table>
<thead>
<tr>
<th>Lag time</th>
<th>Threshold value PAI-1 AR (ng/min)</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 year</td>
<td>9.0*c</td>
<td>0.90</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>8.5*c</td>
<td>1.00</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>38.5*d</td>
<td>0.33</td>
<td>0.91</td>
</tr>
<tr>
<td>2 years</td>
<td>5.5*b</td>
<td>0.90</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>15.5*c</td>
<td>0.80</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>56.0*d</td>
<td>0.40</td>
<td>0.90</td>
</tr>
</tbody>
</table>

AR: appearance rates, PAI-1: plasminogen activator inhibitor-1. * Time from dialysate sampling to EPS diagnosis. b Threshold value based on minimally acceptable true positive rate of 90%.
* Threshold value based on Youden Index. d Threshold value based on minimally acceptable false positive rate of 10%.
DISCUSSION

The objective of this longitudinal study was to evaluate the clinical validity of effluent MMP-2 and PAI-1 in the pre-clinical phases of EPS diagnosis. Primarily this was done by serial determinations of these markers in individual patients for time trend assessments in the years preceding EPS diagnosis; second by evaluating their capacity to discriminate between long-term PD patients and those who develop EPS. To our knowledge no study has reported the clinical performance of MMP-2 and PAI-1 in EPS by providing measures of diagnostic accuracy alongside longitudinal follow-up.

The time course of MMP-2 AR showed a large overlap in values between the EPS patients and the long-term PD patients where no difference was present between the groups and in trends. Due to this distribution no discriminative ability for levels of MMP-2 AR could be established. Only one year prior to the diagnosis the trend was divergent which enhanced the overall discriminatory potential.

In a previous study we also found no obvious trend of MMP-2 AR during longitudinal follow-up of incident PD patients. The in vivo verification by an EPS-like rodent model revealed a relationship between the levels of MMP-2 AR and quantity of peritoneal fibrosis was present. Furthermore, AR of MMP-2 correlated with peritoneal transport parameters. Effluent levels of MMP-2 in PD patients with different degrees of peritoneal injury have been reported, but no relationship with the severity of peritoneal fibrosis was found. Additionally in rats, in vivo up-regulation of the MMP-2 gene and its protein expression by TGF-β1 was associated with progressive basement membrane degradation during epithelial-to-mesenchymal transition. These studies support our findings, suggesting that the function of MMP-2 resides most likely in peritoneal tissue remodeling rather than peritoneal fibrosis. The results from the current study indicate that MMP-2 is unsuitable as a diagnostic instrument for the early detection of EPS. MMP-2 seems to be a steady feature that appears in the peritoneal effluent at a relatively constant level as reflected by the time course in long-term PD patients as well as in patients who developed EPS. Even though, the contribution and importance of effluent MMP-2 in the pathophysiology of the peritoneal membrane requires elucidation as MMP-2 could be a relevant risk factor for some morphological alterations.

PAI-1 is involved in multiple pathophysiological pathways. Elevated PAI-1 levels inhibit plasminogen activators and thereby block the conversion of plasminogen to plasmin. This cascade leads to a reduction in fibrinolytic activity and contributes to the accumulation of collagen and fibrin by a decreased rate of extracellular matrix degradation. This study suggests that PAI-1 may play a significant role in the pathogenesis of EPS.
The precise mechanisms of PAI-1 in peritoneal membrane modification have not yet been fully investigated, nor has histological detection of PAI-1 been performed in PD patients. However, evidence accumulates that PAI-1 could be a pivotal factor in the development of peritoneal fibrosis. Several studies have shown proof of locally produced PAI-1 within the peritoneal cavity.\textsuperscript{12,18,19} Furthermore, a linear appearance of PAI-1 in peritoneal effluent during a four-hour dwell has been described, irrespective of the type and glucose percentage of the dialysis solution.\textsuperscript{26} Moreover, elevated levels of dialysate PAI-1 were not accompanied by a rise in tissue-type plasminogen activator concentrations which suggests fibrin formation.\textsuperscript{26} To the same extent, TGF-β1 was able to increase PAI-1 antigen in primary cultured mesothelial cells as well as in a human peritoneal mesothelial cell line, which subsequently lead to a reduction in serine proteases.\textsuperscript{16} Unlike for MMP-2, comparison of effluent PAI-1 concentrations with the histological abnormalities could not be done in our rat model, because a rodent ELISA for PAI-1 determinations is not available. Nevertheless, a strong negative relationship is present between PAI-1 AR and the degree of free water transport.\textsuperscript{12}

The present study showed a discriminative capacity for PAI-1 with regard to the development of EPS in long-term PD patients with a treatment period of at least 4.8 years. Throughout the years preceding the diagnosis of EPS the time course revealed elevated levels of PAI-1 AR in the EPS cases as compared to controls. At one or two years prior to the diagnosis of EPS, PAI-1 AR threshold values of respectively 9.0 ng/min or 5.5 ng/min were necessary to achieve the pre-specified sensitivity estimate of 90 percent. This resulted in a corresponding specificity of 55% at one year and 42% at two years, accompanied by high FPRs. However, determinations of effluent biomarkers are preferably performed in conjunction with peritoneal function tests, such as the modified peritoneal equilibration test or a 3.86% glucose SPA, because standardized conditions are required for both peritoneal transport parameters and effluent biomarkers. Consequently advanced parameters of peritoneal transport such as free water transport could provide additional information and possibly detect the remaining amount of false positives and thereby improve specificity. This study indicated that the optimal threshold of PAI-1 AR, in long-term PD patients at one year prior to EPS diagnosis, is in the region of 8.5 ng/min.

In conclusion, we have demonstrated that MMP-2 is not an effective biomarker for the early detection of EPS as no distinction could be made between long-term PD patients and those who developed EPS. Contrary, the clinical validity of PAI-1 as an
effluent biomarker that may aid in the early detection of EPS has been shown. The discriminative capacity of PAI-1 from three years before the diagnosis of EPS allows early intervention to prevent the development of EPS or postpone discontinuation of PD treatment. Furthermore, to date no single effluent biomarker representative for peritoneal fibrosis has been identified. In this perspective, effluent PAI-1 could also be used as a screening marker in regular PD patients to monitor the degree of peritoneal fibrosis. Finally, we have presented potential thresholds for PAI-1 AR based on estimates of sensitivity and specificity. We advocate a high sensitivity estimate when solely PAI-1 AR levels are available, as under this circumstance a rule out policy is desired. However, it is unlikely that a single effluent biomarker or peritoneal transport parameter is capable of detecting pre-clinical EPS. We believe that a panel of effluent biomarkers in conjunction with advanced peritoneal transport parameters such as free water transport is more competent in facilitating the early detection of EPS. However, future studies with a higher event rate are necessary to verify the degree of PAI-1 in the peritoneal effluent by morphologic investigations and to validate our findings.
REFERENCES


