Effluent biomarkers in peritoneal dialysis: A captivating symphony from the peritoneal membrane
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Variability of Effluent Cancer Antigen 125 and Interleukin-6 Determination in Peritoneal Dialysis Patients

Deirisa Lopes Barreto, Annemieke M. Coester, Marlies Noordzij, Watske Smit, Dirk G. Struijk, Susan Rogers, Dirk R. de Waart, Raymond T. Krediet

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ABSTRACT

Background Cancer antigen (CA) 125 is a glycoprotein, which provides data on the state of the peritoneal membrane in peritoneal dialysis (PD). Interleukin-6 (IL-6) acts as a mediator in acute phase responses. The study evaluated the usefulness of CA125 and IL-6 in random effluent samples, by assessing their variability in individual patients during outpatient clinical practice at the department.

Methods This longitudinal prospective study was conducted from 2007 till 2009. Participants included 52 stable PD patients aged 18 years or older. Effluent samples were obtained during regular outpatient visits and appearance rates (AR) were calculated. Inter- and intra-individual variability was determined by the coefficient of variation (CV). A linear mixed model was used to analyze time courses. Furthermore, release patterns of these effluent markers were studied.

Results CA125-AR of short-term patients (≤24 months) ranged from 39.2-766.7 U/min, and IL-6-AR from 15.5-220.0 pg/min. Long-term patients (≥25 months) had a CA125-AR of 7.3-1534.0 U/min, and IL-6-AR of 6.9-956.4 pg/min. Overall CV\textsubscript{intra} was 15% in effluent CA125-AR and 28% in IL-6-AR. Intermediate sampling during a four-hour dwell showed a linear increase of CA125 and IL-6 effluent concentrations. The trend of CA125-AR was different (p=0.001) between short and long-term patients. A negative relationship was found between CA125 (r= -0.44, p=0.02) and PD duration.

Conclusions The clinical relevance of effluent CA125 determinations from an unstandardized dwell during every outpatient visit, as judged from the CV\textsubscript{intra} is reasonable. The inferior CV\textsubscript{intra} values of ARs compared to effluent values indicates that ARs should only be calculated under standardized conditions. A single IL-6 measurement, as a predictor of outcome, should be cautiously interpreted.
INTRODUCTION
Cancer antigen (CA) 125 is a high molecular weight (220 kDa) glycoprotein which is constitutively produced by peritoneal mesothelial cells in vitro.\(^1\) The number of mesothelial cells in peritoneal effluent of stable peritoneal dialysis (PD) patients is proportional to peritoneal dialysate CA125 concentration.\(^2\) Therefore, it can be regarded as a marker for the mesothelial cell mass in stable PD patients and thus provide data on the state of the peritoneal membrane in vivo.\(^3\) Extremely low CA125 concentrations have been found in effluent of patients preceding the diagnosis of peritoneal sclerosis.\(^4\) This is supportive to the contention that a loss of mesothelial cells is implicated in the pathogenesis of peritoneal sclerosis, a serious complication of long-term PD.\(^5\) The small day-to-day variability of CA125, which was found to average 7.7%, supports its reliability as a marker for mesothelial status,\(^6\) at least when measured under strictly standardized conditions.

Inflammatory changes are often seen in the peritoneum, even in the absence of peritonitis, indicating that the peritoneum of a PD patient may be chronically inflamed.\(^7\) Interleukin 6 (IL-6) is a multifunctional protein produced by a wide array of cells such as lymphoid and non-lymphoid cells and by normal and transformed cells, including T cells, monocyte/macrophages, fibroblasts, mesothelial cells and vascular endothelial cells.\(^8\)–\(^10\) Smooth muscle cells in the tunica media of many blood vessels also produce IL-6 as a pro-inflammatory cytokine. IL-6 is one of the most important mediators in the acute phase response, which makes it an interesting protein in the early diagnosis of inflammation. Especially, because an increase is present in effluent IL-6 concentrations shortly before the onset\(^11\) of and during peritonitis,\(^12\) suggesting its local production and reflecting an intraperitoneal inflammatory state. A recent study performed by our group\(^13\) indicates the potential use of effluent IL-6 and CA125 for an early diagnosis of EPS.

At present the CA125 determinations are standardized to be performed at the end of the four-hour standard peritoneal permeability analysis (SPA), while IL-6 has not yet been included in this standard assessment. At our center SPA’s are done annually on a voluntary basis in each PD patient. The variability of effluent CA125 measurements on a more frequent base than once yearly is not known. Knowledge about the variability would be important in analyzing its clinical relevance for decision making in daily practice.
The aim of this study is to evaluate the value of CA125 and IL-6 in random effluent samples by assessing its variation in individual patients during clinical practice at the outpatient department. The time course in effluent CA125 and IL-6 during follow-up was observed. Inter- and intra-individual differences in effluent CA125, IL-6 and possible influencing factors were analyzed in order to study clinical relevance.

PATIENTS AND METHODS
This was a prospective longitudinal, observational open-label study in which patients were included between 2007 and 2008. On average, PD patients visit the outpatient department every six to eight weeks. Therefore, to obtain at least six consecutive effluent CA125 and IL-6 measurements, follow-up duration was at least one year. The patients were divided into a short-term and a long-term PD group, where short-term is defined as patients with a PD duration between 0-24 months and long-term patients with a PD duration of ≥25 months.

Patients
All prevalent stable chronic renal failure patients treated with PD and with a minimum age of 18 years were eligible to participate in this study. Patient characteristics such as primary kidney disease, APD/CAPD treatment at the start of PD and initial regimen were collected. Baseline characteristics of the patients were assessed at the first visit at the outpatient clinic. Patients with a clinically significant diagnosis of peritonitis, malignancy or unstable patients with severe edema were excluded. Furthermore, transfer to hemodialysis, renal transplantation, patient preference or physician’s discretion were reasons for withdrawal from the study. In addition, patients who underwent a four-hour standardized dwell were selected for analysis of CA125 and IL-6 release patterns.

Data collection
During every visit, PD regimen, glucose load, icodextrin use, peritonitis episodes and body weight were registered. The patients were asked to keep their collection bag of the last long day or night dwell, regardless the used dialysis solution. This was done, because in a previous study it was shown that the glucose concentration of the dialysis solution did not influence CA125 concentration. In order to analyze proteins release patterns, intermediate effluent was obtained in a subgroup at timepoint 0’, 10’, 20’, 30’, 60’, 120’, 240’
and 240’ during a four-hour standardized dwell. All samples were centrifuged at 1710 × g for 10 minutes and frozen at -26°C until analysis.

**CA125 and IL-6 assays**

CA125 was determined by using a microparticle enzyme immunoassay in combination with a monoclonal antibody OC125 on an E170 autoanalyzer (Roche Diagnostics, Basel, Switzerland). The total coefficient of variation for this determination is 1.6 to 3.0%. The concentration of IL-6 was measured with an ELISA (RnDSystems, Minneapolis, USA) with a sensitivity of 0.7pg/mL.

**Calculations and statistical analysis**

**Appearance rate dialysate (AR)**

Dwell times and volumes of the bag were recorded in order to calculate the AR of CA125 and IL-6. The AR is the amount of CA125 or IL-6 present in the total drained effluent divided by the duration of the dwell in minutes according to the following equation:

\[
AR = \frac{[\text{Protein}] \times \text{Volume effluent}}{\text{Dwell time}}
\]

**Coefficient of Variation (CV)**

Inter- and intra-individual variability were determined by means of the coefficient of variation (CV). Inter-individual variability (CV\(_{\text{inter}}\)) is identified as the CV within the short-term or long-term study population, and calculated as the standard deviation (SD) within the groups divided by the mean and multiplied by 100%. Intra-individual variability (CV\(_{\text{intra}}\)) is defined as the variability within one patient and was calculated as the overall standard deviation divided by the mean of all experiments and multiplied by 100. The overall SD was calculated as the square root of the mean of the squares of the standard deviations of each patient.

\[
CV = \frac{\text{Overall SD}}{\text{Mean}} \times 100\% \quad \text{and}
\]

\[
\text{Overall SD} = \sqrt{\text{SD}\text{1}^2 + \text{SD}\text{2}^2 + \ldots \text{SD}\text{n}^2} \frac{1}{N}
\]
Statistical analysis
Results are presented as median and ranges, unless stated otherwise. The Mann-Whitney U test was used to compare the baseline characteristics of the short-term and long-term PD patients due to asymmetrically distributed data. A linear mixed model with a repeated statement was used to analyze the time courses of the effluent markers CA125 and IL-6 (dependent variables). This method takes the correlation into account between repeated measurements within the same patient. All available visits were included in the analysis. The multivariate model contained the time (number of visits) as categorical variable and PD duration, both as fixed effects. Several covariance structures were compared using the log likelihood ratio test with Restricted Maximum Likelihood (REML) estimation methods. Based on this comparison, Toeplitz heterogeneous appeared to be the best covariance structure for our data and therefore chosen for the analyses. To evaluate possible relationships, correlation analysis was performed by a Spearman rank correlation analysis. In this analysis only the last measurepoint of every patient was included. All statistical analyses were performed using SPSS version 16.0.

RESULTS
Peritoneal effluent was collected from 52 patients. Included patients had at least two samples available with a maximum interval of six months and were omitted when exclusion criteria were met. Exclusion of patients during the study were due to transfer to HD, transplantation and patients with less than two available samples (Figure 1). Furthermore, measurepoints were lost due to values which were outside of the reference standard curve derived from CA125 and IL-6 assays, peritonitis episodes or missing dwell times and dwell volumes, resulting in the inability of calculating ARs. Therefore, our final study population consisted of 30 PD patients with two or more samples available. Baseline characteristics of the patients are presented in Table 1.

Effluent concentration and appearance rate
Table 2 shows the results of the effluent concentrations and appearance rate of CA125 and IL-6. Long-term patients had significantly lower CA125 AR (p<0.001) when compared with short-term patients. Furthermore, significant higher values of IL-6 AR (p=0.05) between the long-term patients were found as compared to the short-term patients.
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Figure 1. Patient flowchart. The exclusion of patients due to transfer to HD (n=5), transplantation (n=2) and patients with less than two available samples (n=15).

Table 1. Baseline characteristics of the short-term (≤24 months) and long-term (≥25 months) patients.

<table>
<thead>
<tr>
<th></th>
<th>Short term PD (n=17)</th>
<th>Long term PD (n=13)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (female / male)</td>
<td>8 / 9</td>
<td>5 / 8</td>
<td>0.96</td>
</tr>
<tr>
<td>Age (years)</td>
<td>55 (28 - 71)</td>
<td>47 (19 - 75)</td>
<td>0.11</td>
</tr>
<tr>
<td>PD duration at first visit (months)</td>
<td>8.9 ± 5.5</td>
<td>48.4 ± 20.8</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>APD / CAPD</td>
<td>9 / 8</td>
<td>7 / 6</td>
<td>0.74</td>
</tr>
<tr>
<td>Initial regimen</td>
<td></td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>- Dianeeal</td>
<td>-</td>
<td>3 (23%)</td>
<td></td>
</tr>
<tr>
<td>- Physioneal</td>
<td>17 (100%)</td>
<td>10 (77%)</td>
<td></td>
</tr>
<tr>
<td>Primary Kidney Disease</td>
<td></td>
<td></td>
<td>0.16</td>
</tr>
<tr>
<td>- Renovascular</td>
<td>4 (24%)</td>
<td>3 (23%)</td>
<td></td>
</tr>
<tr>
<td>- Diabetic nephropathy</td>
<td>1 (6%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>- Glomerulonephritis</td>
<td>7 (41%)</td>
<td>2 (15%)</td>
<td></td>
</tr>
<tr>
<td>- Other</td>
<td>5 (29%)</td>
<td>8 (62%)</td>
<td></td>
</tr>
<tr>
<td>D/P creatinine</td>
<td>0.81 ± 0.08</td>
<td>0.70 ± 0.14</td>
<td>0.01</td>
</tr>
<tr>
<td>Net UF at 240 min (mL)</td>
<td>463 ± 373</td>
<td>497 ± 291</td>
<td>0.79</td>
</tr>
</tbody>
</table>

Values are presented as median and ranges for age and mean ± standard deviations for transport parameters. APD: automated peritoneal dialysis; CAPD: continuous ambulatory peritoneal dialysis; D/P: dialysate-to-plasma ratio; PD: peritoneal dialysis; UF: ultrafiltration.
Coefficient of Variation (CV)

Results of the CV<sub>inter</sub> in short-term, long-term patients and the total study population, are presented in Table 3. The median interval between the visits was two months. There was no difference in the median interval between the short-term and long-term group. CV<sub>intra</sub> defined as the variability within one patient, of the CA125 AR ranged from 0-37% in short-term PD patients and 2-37% in long-term PD patients (Table 4). Much higher values were found for IL-6 AR. IL-6 CV<sub>intra</sub> ranged from 2-98% in short-term patients and in long-term PD patients from 7-82%. Furthermore, the appearance rates of both markers showed higher CV<sub>intra</sub> values as compared to effluent concentrations.

Table 3. Inter-individual variability expressed by coefficient of variation of CA125 and IL-6 in effluent.

<table>
<thead>
<tr>
<th>Coefficient of inter-individual variation (%)</th>
<th>Effluent concentration CA125</th>
<th>Appearance rate CA125</th>
<th>Effluent concentration IL-6</th>
<th>Appearance rate IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short-term PD</td>
<td>54</td>
<td>56</td>
<td>122</td>
<td>140</td>
</tr>
<tr>
<td>Long-term PD</td>
<td>75</td>
<td>58</td>
<td>139</td>
<td>128</td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>73</td>
<td>141</td>
<td>142</td>
</tr>
</tbody>
</table>

CA125: cancer antigen; IL-6: interleukin-6; PD: peritoneal dialysis.
higher CV term PD patients from 7 presented in Table 3. The median interval between the visits was two months. There was

Table 3. Results

<table>
<thead>
<tr>
<th></th>
<th>Coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Effluent concentration CA125</td>
</tr>
<tr>
<td>Short-term PD</td>
<td>12 (2 - 65)</td>
</tr>
<tr>
<td>Long-term PD</td>
<td>17 (0 - 46)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.63</td>
</tr>
</tbody>
</table>

Values are presented as median and ranges. The p-values represent the difference between short-term and long-term groups. CA125: cancer antigen; IL-6: interleukin-6; PD: peritoneal dialysis.

**CA125 and IL-6 release pattern with the duration of a dwell**

For elucidation of the release patterns, we selected eight stable PD patients who underwent a four-hour SPA. Both CA125 and IL-6 effluent concentrations showed a linear relationship with the duration of the dwell (Figure 2).

Figure 2. Release pattern of CA125 and IL-6 dialysate concentrations. Linear release pattern of CA125 (Panel A) and IL-6 (Panel B) effluent concentrations during a 4-hour standardized dwell in 8 stable PD patients.

**CA125 and IL-6 time courses**

CA125 in the short-term patients was higher than in the long-term patients (p=0.001). During the follow-up period the time course of CA125 AR in short-term patients was also different from long-term patients with a slight decrease at the end of follow-up (Figure 3A). The time course of IL-6 AR was not different (Figure 3B). Furthermore, the time courses of effluent concentrations were relatively similar.
**Figure 3.** Time course of CA125 and IL-6 appearance rates. Appearance rates of CA125 (U/min) and IL-6 (pg/min) in short-term and long-term PD patients, studied by a linear mixed model expressed in means and SD. Panel A shows the time course of CA125 AR, where long-term PD patients have significantly lower time-course (p=0.001). Panel B shows the non-significant time course of IL-6 AR (p=0.12). The number of patients per time-point is presented underneath the graphs.

**Correlation analysis between the effluent markers and PD duration**

To study the relationship between the effluent markers and PD duration, solely the last measurepoint of every patient was included in this analysis. A negative correlation was present between PD duration and CA125 effluent concentrations (Figure 4A), whereas no correlation was present between PD duration and effluent concentrations of IL-6 (Figure 4B). Furthermore, no correlation was found between CA125 and IL-6 (r=0.108, p=0.35).

**Figure 4.** Correlation analysis between effluent CA125 or IL-6 and PD duration. Analysis of relationships was performed by Spearman rank correlation analysis. Panel A presents the negative correlation between CA125 effluent concentrations vs. PD duration (r=-0.44, p=0.02). No relationship between effluent concentrations of IL-6 and PD duration was found (r=0.09, p=0.63), Panel B.
DISCUSSION

Peritoneal dialysis induces peritoneal and morphological alterations and can lead to ultrafiltration failure with time on PD. Therefore, biomarkers in peritoneal effluent may be useful for the detection of abnormalities in peritoneal tissue. Yet, the variability of biomarkers is unknown. The routine measurement of effluent CA125 was included in the standard peritoneal permeability analysis (SPA) in our patient population since 1998, which is performed annually. The present study aimed to evaluate the value of CA125 and IL-6 measurements in prospectively collected random effluent samples at the outpatient department. The use of non-standardized dwell times as was the case in the present study can be overcome by using the appearance rate of a biomarker. However, this can only be done when the increase in effluent concentration is linear in time. This was previously shown for CA125 during a four-hour dwell and beyond, yet no data on IL-6 could be traced. The present study shows that a linear increase in effluent IL-6 was also present during a four-hour dwell.

In a previous study we found a day-to-day coefficient of intra-individual variation for effluent CA125 of 7.7% during a 4-hour SPA. The lack of standardization in the present study led to a doubling of the intra-individual variability, but were similar to the intra-individual coefficient of variation for parameters of fluid and solute transport in CAPD patients found by Imholz et al. No significant difference between inter- and intra-individual variability was present for IL-6 effluent concentrations and appearance rates. This suggests that the linearity of the appearance in effluent may no longer be present for dwell times longer than four hours.

Not all patients reached six visits. Therefore, in the linear mixed model the number of visits was restricted to a maximum of four visits. In IL-6 AR and effluent concentration analyses no difference between short and long-term PD patients was found. This may indicate that the reaction to an inflammatory stimulus was not different. However, only a limited number of patients in the long-term group had more than two samples available for IL-6 determinations. The significant trend difference between short and long-term patients in CA125 AR also showed lower values in the long-term group which confirms the results of previous studies with a longer follow-up. This emphasizes the importance of individual assessment of CA125 with duration of PD to evaluate mesothelial status.

Apart from the higher values of the CA125 AR, also the D/Pcreatinine ratio was higher in the short-term group as compared to the long-term group. Previously, a study by
Parikova et al.\textsuperscript{17} also showed higher initial values for solute transport. A clear relationship was present between small solute transport and effluent CA125 in incident PD patients. These data suggest that fast solute transport rates in incident patients may be caused by vasoactive substances produced by mesothelial cells as described previously.\textsuperscript{18}

During follow-up effluent CA125 was consistently lower in the long-term patients as compared to the short-term patients. When the effluent concentration of CA125 was analyzed with regard to the duration of PD, a negative trend was found. This trend is in accordance with the results of previous studies.\textsuperscript{6} The results of IL-6 suggest that the degree of inflammation may be similar in short- and long-term patients, yet a slight tendency is present to higher values with long-term PD treatment. Our results also support the contention that IL-6 acts, not only as a mediator in the acute phase response, but may also be influenced by angiogenesis and chronic inflammation.\textsuperscript{19} We were unable to find a relationship between effluent CA125 and IL-6. This is probably due to the constitutive synthesis of CA125, whilst IL-6 production also depends on inflammatory stimuli. This absence of correlation is in line with a study performed by Rodrigues et al.\textsuperscript{15} Furthermore, CA125 is released by mesothelial cells only, whilst IL-6 can be produced by various cell types.\textsuperscript{19,20}

The clinical relevance of CA125 and IL-6 determinations from an unstandardized dwell during every outpatient visit is reasonable as judged from the coefficients of variation. The intra-individual variability in the ARs of the effluent markers was much higher as compared to the effluent concentrations. These may have been influenced by possible systematic errors, for instance inaccurate notated dwell times. Moreover, the median dwell time was eleven hours, implicating that this could also be a factor for the cause of large variabilities, which is possibly reflected by the difference between CVs calculations on the average concentration, and AR values of CA125 and IL-6. Therefore, this study illustrates that ARs should only be calculated under standardized conditions. Akman et al.\textsuperscript{21} found that night dwells can be used as a possibility for regular assessment of effluent CA125 in PD patients. However, with duration of the dwell, there was an increase in variation of the results. Our results underline the latter finding. Potentially, the type of dialysis solution may have an influence. This has been investigated for effluent CA125 where the percentage of the glucose based PD solution used, 1.36% versus 3.86%, did not significantly influence the CA125 levels during the dwell. Also the type of osmotic agent had no significant influence on CA125. Currently, no studies have yet been performed to analyze the effect of glucose based PD solutions on IL-6. Differences
between the conventional and more biocompatible PD solution have also not been investigated yet. Therefore, it should be noted that previous studies were performed with the use of the conventional peritoneal dialysis fluid Dianel® and that the present study concerns an almost pure Physioneal® population.

In conclusion, the present study shows that a deviation of the expected effluent CA125 pattern can be of clinical relevance. Especially with the aid of standardization of the dwells. Based on the high coefficients of intra-individual variation, the results of a single measurement of effluent IL-6 have to be interpreted cautiously.

ACKNOWLEDGEMENTS
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