Loss of mesothelial Neutrophil-Gelatinase-Associated-Lipocalin (NGAL) reflects epithelial to mesenchymal transition in experimental peritoneal dialysis

Margot N. Schilte¹, Johanna W.A.M. Celie¹, Pieter M. ter Wee², Eelco D. Keuning¹, Mohammad Zareie¹, Hans van Beek³, Tineke C van der Pouw-Kraan¹, Jacob van den Born¹⁴ and Robert H.J. Beelen¹.

Departments of ¹Molecular Cell Biology and Immunology, ²Nephrology and ³Clinical Genetics, VU University Medical Center, Amsterdam, Netherlands; ⁴Department of Nephrology, University Medical Center, Groningen, Netherlands.

Submitted
Abstract

Introduction: Daily exposure to peritoneal dialysis fluid (PDF) induces mesothelial epithelial to mesenchymal transition (EMT) and secretion of chemokines and cytokines involved in local inflammation, fibrosis and angiogenesis. Over the past years more biocompatible solutions have been developed to improve peritoneal performance. To better understand the influence of PDF on mesothelial damage, we have set up a genome approach to select genes associated with PDF induced damage.

Methods: Rats were daily instilled with conventional or bicarbonate/lactate-buffered PDF (Dianeal® or Physioneal®, 3.86, Baxter, the Netherlands), via an intra peritoneal access port. Untreated animals served as control. After 8 weeks, effluents and liver imprints were collected. RNA was isolated from the parietal peritoneum and RNA micro-array analysis was performed and analyzed using the Panther classification system. Rat mesothelial cells and neutrophils were cultured and analysed for neutrophil-gelatinase-associated-lipocalin (NGAL) expression.

Results: Analysis of the immunity and defence pathway (n=246 genes) showed enhanced gene expression in Physioneal treated animals compared to Dianeal treatment (p=0.01). Single gene analysis of this pathway revealed NGAL to be differentially expressed between the two PDF. Cultured mesothelial cells showed time-dependent production of NGAL, which was significantly impaired upon Dianeal but not after Physioneal exposure (p=0.03). Mesothelial cells on liver imprints stained positive for NGAL, whereas mesothelial cells that underwent EMT lost NGAL expression. A significant higher percentage of EMT was found after Dianeal exposure compared to Physioneal treatment (Dianeal: 3.7% [3.3-4.7] versus Physioneal: 1.5% [1.3-2.6], p<0.001). NGAL concentrations in the effluents significantly increased upon PDF exposure without differences between the Dianeal and Physioneal group, which can be explained by similar numbers of NGAL-producing neutrophils in the effluents of both groups.

Conclusion: Our data shows that NGAL is a possible marker for mesothelial health. Mesothelial cells undergoing EMT in vivo, show loss of NGAL expression suggesting that NGAL may provide a defence mechanism for mesothelial cells to maintain their phenotype during PD.
Introduction

Peritoneal dialysis (PD) is a renal replacement therapy based on the ability of the peritoneal membrane to function as a dialysing membrane. The instillation of PD fluids into the peritoneal cavity allows the peritoneal membrane to exchange water, solutes and waste products between the PD fluid and the circulation. However, chronic PD treatment induces structural and functional changes of the peritoneal membrane, leading to a loss of ultrafiltration capacity.

The different components of the PD fluid, including the buffer, glucose and glucose degradation products (GDPs) generated during heat sterilisation all contribute to peritoneal remodelling. The presence of glucose and especially GDPs enhances the formation of new blood vessels, fibrosis, cell influx and mesothelial regeneration\(^1\);\(^2\).

Over the past years more biocompatible solutions with less glucose, GDPs and a bicarbonate/lactate buffer have been developed leading to less tissue remodelling and improved peritoneal performance\(^3\)-\(^5\).

Mesothelial cells lining the peritoneal membranes are one of the first cells that come into contact with the PD fluid. Chronic exposure to GDP containing PD solutions and the presence of a peritoneal catheter damage the mesothelial monolayer, leading to their partial disappearance from the peritoneal membrane\(^6\). The remaining mesothelial cells become activated\(^7\);\(^8\) and produce chemokines and cytokines involved in angiogenic and fibrotic processes\(^9\).

After PD treatment is initiated, mesothelial cells show a progressive loss of the epithelial phenotype and acquire myofibroblast-like characteristics by an epithelial to mesenchymal transition (EMT)\(^10\). EMT plays an important role in peritoneal fibrosis\(^10\);\(^11\). Moreover, it is suggested that EMT, angiogenesis and fibrosis may be interconnected processes mediated by mesothelial injury and/or response of the mesothelium\(^12\);\(^13\). Exposure of mesothelial cells to more biocompatible solutions have been shown to better preserve mesothelial viability and function and longer retain the typical “cobblestone” morphology\(^14\).

The degree of alteration in mesothelial cell function contributes to ultrafiltration failure and decreased PD efficacy, indicating that the status of the mesothelial monolayer is important for peritoneal outcome.

To better understand the influence of different PD fluids on mesothelial damage we performed mRNA microarray expression analysis to select transcripts associated with inflammation and peritoneal tissue remodelling during PD treatment. Furthermore, we have performed \textit{in vitro} studies to further identify possible new biomarkers.
Material and Methods

Animals and experimental design
Throughout the study, male Wistar rats (Harlan CPB, Horst, the Netherlands), weighing 180–200 gr. at the start of the experiment were used. Rats were housed under conventional laboratory conditions and were allowed one week of acclimatization before the start of the experiment. The experimental design was reviewed and approved by the local ethical committee on the use of laboratory animals. Rats were instilled daily with PD fluid via a subcutaneously implanted peritoneal catheter connected to a mini vascular access port, as described previously\textsuperscript{15}. After operation, during a one week recovery period, rats on PD received daily 2 ml of saline solution containing 1 U/ml heparin to allow wound healing. Thereafter rats were instilled daily with either 10 ml conventional lactate-buffered glucose containing PD fluid (Dianeal\textregistered{} PD4, 3.86% glucose, pH 5.2, high GDP content, Baxter R&D, Utrecht, the Netherlands, n=7) or 10 ml bicarbonate/lactate-buffered glucose containing PD solution (Physioneal \textregistered{}, 3.86% glucose, pH 7.4, low GDP content, Baxter R&D, Utrecht, the Netherlands, n=8). Control animals without peritoneal catheters served as control (n = 8). After 8 weeks of fluid instillation no significant differences were found in body weight and no apparent clinical abnormalities were observed. The morphological changes of the peritoneum and peritoneal function of these animals are described by Zareie \textit{et al}, and are related to the biocompatibility of the PD fluid\textsuperscript{3}.

Analysis of peritoneal cells and effluents
At the end of the experiment effluents were collected and cell number and viability was determined by trypan blue exclusion, as described previously\textsuperscript{3}. Cytocentrifuge preparations were stained by May-Grunwald-Giemsa and cells were differentiated. To determine NGAL positive neutrophils, double stainings for NGAL (Biporto Diagnostics, Gentofte, Denmark) and His 48 (BD Pharmingen San Diego, CA, USA) on cytopsins were performed. As a negative control, conjugate controls (Invitrogen, Carlsbad, USA) without the first antibody were used and proved to be negative. The amount of neutrophil gelatinase associated lipocalin (NGAL) (specific for rat NGAL, Biporto Diagnostics, Gentofte, Denmark) in PET effluents was detected by ELISA.

Mesothelial liver imprints
At the end of the experiment animals were sacrificed and mesothelial imprints of the liver were taken with 3\% gelatine coated slides\textsuperscript{16}. Imprints were fixed in acetone for 10 minutes, blocked with normal goat serum for 15 minutes, and incubated with
primary antibodies cytokeratin (DakoCytomation, Glostrup, Denmark) and vimentin (Serotec, Oxford, UK) for 1 hour. Slides were then exposed for 45 minutes in the dark to fluorescently labelled conjugates (Invitrogen, Carlsbad, CA, USA), 5 minutes to dapi and visualised with a fluorescence microscope. Similarly, imprints were stained for vimentin and NGAL (Bioporto Diagnostics, Gentofte, Denmark).

RNA extraction and microarray analysis
For the micro array analysis pieces of parietal peritoneum were taken. 10-30 μg of total RNA was isolated from the most superficial layer, including mesothelial cells and the underlying connective tissue, of the parietal peritoneum using Trizol reagent (Gibco BRL, Gaithersburg, MD, USA). Small contamination of the underlying muscle layer can not be excluded.
The Compugen Rat OligoLibrary was purchased from Sigma-Aldrich. cDNA probes were generated from total RNA with an oligo (dT) primer (Invitrogen) and SuperScript II Reverse Transcriptase (Invitrogen), with incorporation of aminoallyl-dUTP. Probes were indirectly labelled with Fluorolink Cy3 (sample) or Cy5 (reference pool) monofunctional dyes.
Hybridization protocol was adapted from Snijders et al\textsuperscript{17} with minor modifications. Briefly, Cy3 and Cy5 labelled probes were combined and pre-hybridization was performed in a hybridization mixture containing 2 μg/μl yeast tRNA (ribonucleic acid transfer, Sigma), 0.4 μg/μl polyA (Amersham Biosciences), and 0.8 μg/μl human Cot-a DNA (invitrogen) in 91.2 μl H\textsubscript{2}O, 7.6 μl of 10% SDS, 30.4 μl of glycerol and 250.8 μl of mastermix (1g of dextran sulphate, 6 ml of formamide, 1ml of 20X SSC). The prehybridization mixture was supplemented with 1 μg/μl salmon sperm DNA (Invitrogen). Prehybridization was maintained at 37°C for 45 minutes in the Hybridization Station (Genetac hybridization station). Probe hybridization was initiated by gently removing the prehybridization mix and applying the probe hybridization mix, which is identical to the prehybridization mix but without salmon sperm. The probe mix was denatured at 70°C for 15 minutes and incubated at 37°C for 60 minutes. Hybridization was maintained for 16 h at 30°C on a rocking table (~1 rpm). After hybridization, excess hybridization mixture was rinsed off with PI buffer (0.1M sodium phosphate, 0.1% Igepal CA360, pH 8), and washed for 15 minutes at 35°C in 50% formamide, 2 x SSC, pH7, followed by 15 minutes PI buffer at room temperature. Excess salt was removed by subsequently rinsing in 0.2 x SSC, 0.1 x SSC. Slides were dried by centrifugation at 200 g for 3 minutes.
Data acquisition and analysis
Microarray slides were scanned at a 10μm resolution for Cy3 and Cy5 intensities, using a Perkin-Elmer Multi Laser Microarray Scanner operated by the ScanArray Express v.1.0 software. Array images were processed with Imagine v.5.1. Spots were included in the analysis when in at least 80% of the microarrays a reliable datapoint was obtained for that element in each comparison. Automatically flagged genes (spots with a confidence value <0.11) and manually flagged genes (dirty spots and spots with a confidence value between 0.11-0.15 with a low intensity or bad morphology) were excluded from further analysis.

Data are expressed as normalized log2 ratios of fluorescence intensities of the experimental and the reference sample. The use of a common reference sample allows comparison of the relative expression levels across all samples. Therefore, the expression levels were median centred, i.e., each spotted element was expressed relative to the median expression level of that element across all samples. We calculated for each gene the ratio (fold-change) in expression between the different experimental groups. For an interpretation of the biological processes that are represented by differentially regulated genes we used the fold change expression values of all genes in a Gene Ontology analysis using the PANTHER (Protein ANAlysis THrough Evolutionary Relationships) Classification System at http://www.pantherdb.org/18;19. A Bonferroni correction was applied to adjust for multiple testing. Bonferroni-corrected P-values <0.05 were considered significant.

Cell cultures and treatment
Rat primary mesothelial cells were isolated from 3 different rats by enzymatic digestion of the parietal peritoneal walls of adult rats and subsequently cultured as described20. Briefly, animals were sacrificed and the peritoneal wall was excised under sterile conditions. The peritoneal wall was placed, mesothelial cell-side facing up, in a mesothelial cell isolation device20 and mesothelial cells were removed from the surface of the parietal peritoneal wall by incubation with 0.05% trypsin +0.53 mM EDTA at 37°C for 20 minutes followed by gentle scraping of the mesothelial surface with a cell scraper. The released mesothelial cells were collected and plated on tissue culture dishes. Mesothelial cells showed typical cobblestone appearance at confluence. The cells were maintained in DMEM/F12 (1:1) medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with penicillin, streptomycin and L-glutamine and 10% fetal calf serum. Mesothelial cells were grown to confluence and exposed to either Dianeal or Physioneal diluted 1:4 with culture medium. As a control untreated cells in culture medium were used. After 2, 6, 24, 48 and 96 hours supernatants were collected for NGAL ELISA and cells were collected to determine viability by trypan
blue exclusion. Cytocentrifuge preparations of the mesothelial cells were made and cells were stained for cytokeratin (DakoCytomation, Glostrup, Denmark) and NGAL expression (Bioporto Diagnostics, Gentofte, Denmark), similarly to the liver imprint staining protocol. Mesothelial cells were isolated from three different rats and for each mesothelial cell isolation experiments were performed in triplicate.

Rat neutrophils were isolated from blood of different rats using Lymphoprep (Axis-shield, Dundee, Scotland). Cells were maintained in RPMI medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with penicillin, streptomycin and L-glutamine and 10% fetal calf serum and were left to rest for 2 hours at 37°C. After 2 hours cells were exposed to either Dianeal or Physioneal diluted 1:4 with RPMI medium or to RPMI medium only. After 1, 2, 4 and 20 hours supernatants were collected for NGAL ELISA (Bioporto Diagnostics, Gentofte, Denmark) and cells were collected to determine viability by trypan blue exclusion. All three experiments were performed in triplicate.

**Statistical analysis**

Data is given as median and inter-quartile range and have been analysed using the nonparametric Mann-Whitney U test. Bonferroni correction for multiple comparisons was applied and probability values of $p<0.05$ were considered significant.
NGAL, a marker for mesothelial damage

Results

Differentially expressed pathways between Physioneal and Dianeal treated rats

10-30 μg RNA was isolated of the mesothelial layer of the parietal peritoneum of untreated rats or rats treated for 8 weeks with either the conventional, high glucose and GDP containing fluid (Dianeal), or the more biocompatible, low GDP and bicarbonate/lactate buffered solution (Physioneal). Using these RNA samples, microarray gene expression profiling was performed. Clustered pathway analysis showed that three biological processes were differentially affected by the two PD solutions (Table 1).

PD treatment with either fluid induced significant down regulation of the Nucleoside, nucleotide and nucleic acid metabolism pathway compared to untreated animals, although no differences were found between the two different treatments. The protein biosynthesis pathway was significantly enhanced by PD fluid treatment compared to control animals. Protein biosynthesis was even further enhanced in the Physioneal group, compared to Dianeal treatment. Furthermore, a significant difference between Physioneal and Dianeal treatment was found in the Immunity and Defence pathway, with the Physioneal group showing significantly enhanced gene expression compared to the Dianeal group, suggesting better preservation of immunity and defence in the Physioneal treated rats, whereas Dianeal treated rats have an impaired response.

Other analysed biological processes like carbohydrate metabolism, neuronal activities, proteolysis, oxidative phosphorylation, nerve-nerve synaptic transmission, ion transport and transport showed no differences between the control, Dianeal or Physioneal treated rats (data not shown).

<table>
<thead>
<tr>
<th>Biological Process</th>
<th>Nr of genes</th>
<th>D vs. C</th>
<th>P vs. C</th>
<th>P vs. D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Up/down</td>
<td>p-value</td>
<td>Up/down</td>
</tr>
<tr>
<td>Nucleoside, nucleotide and nucleic acid metabolism</td>
<td>420</td>
<td>-</td>
<td>0.002</td>
<td>-</td>
</tr>
<tr>
<td>Protein biosynthesis</td>
<td>71</td>
<td>+</td>
<td>&lt;0.001</td>
<td>+</td>
</tr>
<tr>
<td>Immunity and defence</td>
<td>246</td>
<td>=</td>
<td>1.00</td>
<td>=</td>
</tr>
</tbody>
</table>

C=Control; D=Dianeal; P=Physioneal.
-: down regulated; +: up regulated; =: no differences
To further investigate the molecular induced changes between the two dialysis solutions, we analysed the single gene expression of the pathways showing significant differences between the two PD fluids, namely the protein biosynthesis and the immunity and defence pathway.

Single gene analysis of Protein biosynthesis pathway

Single gene analysis of the 71 genes in the protein biosynthesis pathway showed that mainly genes of the ribosomal protein family were involved. These proteins are involved in the cellular process of translation, which is the first step in protein biosynthesis. However, all genes in this pathway showed a fold change <1.5 compared to the control group and did not significantly differ between the different treatment groups.

Single gene analysis of the immunity and defence pathway

Similarly, for the immunity and defence pathway a single gene analysis was performed. Table 2 shows the three genes with the highest fold change between Dianeal and Physioneal treated rats. Both PD fluids increased chemokine ligand 2 (CCL2) expression compared to control rats. CCL2 expression was almost two times higher in the Physioneal group compared to the Dianeal treated animals. Secondly, fibrinogen was also found to be differently regulated between the two dialysis fluids. Dianeal treatment slightly reduced fibrinogen expression compared to control animals, whereas Physioneal treatment enhanced fibrinogen expression. Thirdly, neutrophil gelatinase associated protein (NGAL) expression was clearly impaired upon Dianeal treatment, but not after Physioneal exposure.

<table>
<thead>
<tr>
<th>Mapped Ids</th>
<th>Gene name</th>
<th>Fold Change D over C</th>
<th>Fold Change P over C</th>
<th>Fold Change P over D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccl2</td>
<td>Chemokine (C-C motif) ligand 2</td>
<td>1.40</td>
<td>2.77</td>
<td>1.98</td>
</tr>
<tr>
<td>Fgg</td>
<td>Fibrinogen, gamma polypeptide</td>
<td>0.89</td>
<td>1.84</td>
<td>1.69</td>
</tr>
<tr>
<td>Lcn2</td>
<td>Lipocalin 2 (NGAL)</td>
<td>0.66</td>
<td>1.12</td>
<td>1.69</td>
</tr>
</tbody>
</table>

C=Control; D=Dianeal; P=Physioneal.
Both CCL2, also known as monocyte-chemoattractant-protein-1 (MCP-1), and fibrinogen are well-known factors often measured in PD research. During PD treatment, MCP-1 is found in effluents of steady state patients and their levels increase upon peritonitis\textsuperscript{21,22}. From previous rat PD experiments it is known that PD fluid exposure increases MCP-1 levels\textsuperscript{23,24}. Fibrinogen is measured to analyze whether a chronic low-grade activation of the coagulation system is present in PD patients\textsuperscript{25}. Moreover, fibrinogen is seen as a marker for chronic inflammation and activation during PD\textsuperscript{26,27}.

Up to date, there is not much known about the NGAL production of peritoneal cells upon PD treatment with different PD solutions. Interestingly, NGAL has recently been associated with peritonitis during peritoneal dialysis\textsuperscript{28}. We therefore focussed on NGAL as an interesting candidate for further research and a possible new biomarker in PD.

**NGAL production of mesothelial cells *in vitro*\**

To further analyse the role of NGAL during PD treatment, *in vitro* experiments on rat mesothelial cells have been performed. First of all we confirmed that mesothelial cells are involved in the production of NGAL. NGAL was detected in supernatants of unstimulated mesothelial cells and amounts significantly increased over time (Figure 1A). Exposure of mesothelial cells to Dianeal significantly impaired NGAL production (p<0.03), although viability of the cells remained above 95%. Physioneal exposure did not significantly affect NGAL production (Figure 1A). Indeed cytospins of unstimulated as well as of PD fluid stimulated mesothelial cells, stained positive for NGAL expression (Figure 1B).

**NGAL expression by mesothelial cells *in vivo*\**

After the end of the 8 week experiment, mesothelial imprints of the liver were taken of all animals to detect NGAL expression by the mesothelium *in vivo*. Mesothelial cells were first of all stained with cytokeratin and vimentin to differentiate between mesothelial cells and spindle-shaped fibroblast-like cells (Figure 2A). The latter cell type indicates that the mesothelial cells lost their epithelial phenotype and underwent the process of EMT. Figure 2A shows that in control rats all cells retain an epithelial phenotype and no fibroblast-like cells are found. However upon PD treatment, fibroblast like-cells appear with significantly higher numbers in Dianeal treated rats (3.7% [3.3-4.7]) compared to Physioneal treated rats (1.5% [1.3-2.6]) (Figure 2A and 2B, * p=0.001). NGAL and vimentin double staining of the imprints show that the mesothelial cells stain positive in all three groups, although the fibroblast like cells do not (Figure 2C).
Figure 1: NGAL production of rat primary mesothelial cells *in vitro* without stimulation (control) or with Dianeal or Physioneal stimulation (1:4 with medium) for different time points (1A). * p<0.03, Dianeal vs. Control and Physioneal. Cytospins of cultured mesothelial cells stained double positive for cytokeratin (red) and NGAL (green), nuclei are stained with dapi (blue) (1B). N=3 experiments.

Figure 2: Mesothelial cells on liver imprints taken at the end of the study are stained with cytokeratin (red) and vimentin (green), nuclei are stained with dapi (blue) (2A). Cytokeratin negative and vimentin positive cells indicate fibroblast like cells (indicated by arrows). Higher percentages of fibroblast like cells are found between the mesothelial cells on imprints of Dianeal treated animals compared to Physioneal treated rats, * p=0.001 (2B). Mesothelial cells also stain positive for NGAL (red) (2C), whereas vimentin positive fibroblast like cells (green) do not (indicated by arrows). Magnification:40x, scale bar: 50μm.
NGAL measurements in rat dialysates
To see whether we could detect mesothelial damage ex-vivo, we analysed the PET effluents of control animals and rats treated for 8 weeks with PD fluid. NGAL levels in the dialysates where significantly enhanced after PD treatment (Figure 3, p=0.002, control vs. Dianeal and Physioneal). However, no differences between Dianeal and Physioneal treated rats were found. The NGAL levels found in the dialysates differ from the mesothelial cell production measured in vitro and the fold changes given by the micro array. This may indicate that other cellular sources in the effluents are likely to play be involved in NGAL production.

NGAL production by neutrophils
Apart from mesothelial cells, it is known that neutrophils are a potential source of NGAL which may play a role in the NGAL production found in the PET dialysates. Upon PD treatment neutrophil numbers in the PET effluents are significantly increased. Cell differentiation of peritoneal cells in the effluents by May Grunwald Giemsa staining showed a significant increase in neutrophil number after PD fluid exposure from 0.4*10^6 cells [0.40-0.44*10^6] in control animals to 6.8*10^6 cells [4.76-8.40*10^6] and 4.6*10^6 cells [2.35-6.40*10^6] in respectively Dianeal and Physioneal treated animals (p<0.05, control vs. Dianeal and Physioneal). Neutrophil numbers were not statistically different between Dianeal and Physioneal treated rats was found.
Thereafter we stained cytospins of peritoneal cells for NGAL expression. Double stainings for NGAL and His48 showed double positive cells, indicating that in the peritoneum NGAL is not only expressed by mesothelial cells but also by neutrophils (Figure 4A).
To confirm NGAL release of neutrophils *in vitro*, rat blood neutrophils were isolated and stimulated with either Dianely or Physioneal or remained unstimulated for up to twenty hours after isolation. NGAL production of neutrophils significantly increased over time, without significant differences between the different treatments (Figure 4 B).

**Figure 4:** Cytospins of peritoneal cells double stained for neutrophils (his 48, green) and NGAL (red), nuclei are stained with dapi (blue) (4A). Rat blood neutrophils *in vitro* without stimulation (Control) or stimulated with Dianely or Physioneal (1:4 with medium) for different time points. N=3 experiments.
Discussion

Over the past few years, newer more physiologic PD fluids have been developed which lead to less peritoneal changes and better preserve the peritoneum. To gain better insight in the genes and proteins involved in peritoneal tissue remodelling and inflammatory processes, rats were treated with the conventional PD fluid Dianeal or the more biocompatible solution Physioneal or remained untreated for 8 weeks. We thereafter performed a microarray expression analysis on RNA isolated from the parietal peritoneum to detect new biomarkers which may have a predictive value for morphological changes seen during long term PD treatment.

Analysis of our microarray data first of all showed that the Nucleoside, nucleotide and nucleic acid metabolism pathway was down regulated upon PD treatment, although no differences between the two PD fluids were found. Next to having a function as structural units of RNA and DNA, nucleotides play a central role in the cell metabolism in which they can participate in cellular signalling. Down-regulation of this pathway might also indicate lower proliferation /regeneration rates upon PDF treatment.

In contrast to the down regulation of the Nucleoside, nucleotide and nucleic acid metabolism pathway, the Protein biosynthesis pathway showed to be up regulated by PD treatment. It has indeed been shown previously that upon PDF treatment enhanced levels of proteins such as hyaluronic acid (HA), MCP-1, vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGFβ) are found in PD effluents. The combination of lower proliferation and increased protein synthesis might be explained by cellular senescence. Senescent cells do not proliferate anymore, however are metabolically very active and can secrete all kind of proteins and mediators. This has also been described for mesothelial cells exposed to high glucose in vitro.

Thirdly, the microarray showed differences in the immunity and defence pathway between the two PD fluid treatments. Immunity and defence showed to be impaired after Dianeal but not after Physioneal treatment. This indicates a relative preserved peritoneal immunity and a better immunologic defence of the mesothelial cells in Physioneal treated animals. This in accordance to earlier data which describe improved morphology and immune parameters in Physioneal compared to Dianeal treated rats.

Overall, this suggests that the biocompatible solution leads to a better preservation of the mesothelial cells compared to the conventional PD fluid treatment.
Although the single gene analysis of the immunity and defence pathway did not show major fold changes in gene expression, we found NGAL to be a promising new candidate as a marker for mesothelial damage/health. This analysis showed that NGAL gene expression was significantly impaired in Dianeal animals, compared to Physioneal and control animals, which indicates differential effects of the two PD fluids on NGAL production.

NGAL is a small transporter protein of 25kD belonging to the lipocalin superfamily. Lipocalins are generally considered as transporters with several different functions. These functions include the regulation of immune responses, modulation of cell growth and metabolism, transportation of iron and prostaglandin synthesis. It is known that NGAL is stored in specific granules within neutrophils and is rapidly released by neutrophils after exposure to inflammatory stimuli, making it a possible biochemical marker of inflammatory diseases. Recently it has been shown that other types of cells, like epithelial cells in the kidney tubule, produce NGAL in response to various injuries.

Previous studies have indicated that NGAL may represent a novel early urinary biomarker of ischemic renal injury since it is dramatically induced in the kidney early after ischemic injury and acute renal tubular injury. Furthermore, accumulating evidence suggests that NGAL enhances the epithelial phenotype. These findings suggest that NGAL may be expressed by the damaged tubule to induce re-epithelialisation. High levels after tubular injury may play a role in tissue-protective effects and can ameliorate the degree of damage in an ongoing insult. Administration of NGAL seems to limit the morphologic and functional consequences of ischemia-reperfusion injury in a mouse model, by a combination of limiting tubule cell death and enhancing re-epithelialisation.

Recently it has been described that NGAL is upregulated in PD patients undergoing peritonitis and correlates well with the neutrophil count in the effluents. Furthermore they have shown that human peritoneal mesothelial cells are another source of NGAL during peritonitis. Indeed, our study confirmed that mesothelial cells can produce NGAL.

However, damage of mesothelial cells by long term treatment with the conventional PD solution Dianeal, impairs the ability of mesothelial cells to produce NGAL. NGAL staining on liver imprints taken at the end of the study confirmed the expression of NGAL by mesothelial cells in vivo. Little difference in NGAL staining intensity was found between the different groups, which can be explained by the fact that NGAL is stored in granules and directly released under appropriate conditions.
NGAL, a marker for mesothelial damage

Upon PD treatment more vimentin-positive fibroblastic spindle-like shaped cells are found in between the mesothelial monolayer of the liver imprints, indicating EMT of mesothelial cells. We have shown that these cells not only loose cytokeratin, but also the NGAL expression. Also for other cell types loss of NGAL has been related to EMT. These results indicate that NGAL is linked to the mesothelial phenotype and it is therefore speculated that NGAL production in mesothelial cells may exert a protective effect in modulating EMT during PD. Indeed in this and in previous studies, more fibroblast-like cells were found upon Dianonal treatment compared to Physioneal, which we now found to be related to impaired NGAL production by mesothelial cells.

Furthermore, it has been shown that incubation of mesothelial cells with NGAL reverses the TGFβ induced up-regulation of Snail or vimentin and rescues the down-regulation of E-cadherin. Therefore it is thought that NGAL not only protects the epithelial phenotype but may also reverse important events of EMT.

Besides mesothelial cells, neutrophils play an important role during PD. Longterm PD treatment significantly increases neutrophil numbers in the PET effluents, without differences in neutrophil numbers between Physioneal or Dianonal treatment. Our *in vitro* studies with isolated blood neutrophils, showed a time dependent increase of NGAL production in the supernatants of these cells. However, the production of NGAL by neutrophils showed to be unaffected by PD fluid treatment and produced drastically higher amounts of NGAL compared to mesothelial cells. These data indicate that the NGAL levels measured in the PET effluents of the rats are not only produced by the mesothelial cells lining the peritoneum but also by the neutrophils which are attracted to the peritoneum upon PD fluid exposure. The increase in neutrophil numbers after PD treatment makes it difficult to detect the status of the mesothelial cells by measuring NGAL in the dialysate.

In summary, the status of the mesothelial cells and the maintenance of their epithelial phenotype are important for the outcome of PD treatment. As shown by our micro-array data and the additional *in vitro* experiments, the condition of the mesothelial cells is reflected by the amount of NGAL produced by these cells. Mesothelial cells that undergo EMT loose their NGAL expression suggesting that NGAL may provide a defence mechanism for mesothelial cells to maintain their cobblestone phenotype. Unfortunately, the NGAL production of neutrophils present in the effluents overshadows the NGAL production of the mesothelial cells which makes it difficult to detect mesothelial damage by measuring NGAL in PD effluents. However, it is anticipated that in the future NGAL may offer promising diagnostic
and therapeutic possibilities and further research on exogenously administration of NGAL in an attempt to ameliorate EMT \textit{in vivo} during PD is warranted.

\section*{Acknowledgements}

This work was supported by grant #C05-2142 of the Dutch Kidney Foundation.
NGAL, a marker for mesothelial damage

References

21. Tekstra J, Visser CE, Tuk CW et al. Identification of the major chemokines that regulate cell influxes


