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Low-GDP peritoneal dialysis fluid ('balance') has less impact *in vitro* and *ex vivo* on epithelial-to-mesenchymal transition (EMT) of mesothelial cells than a standard fluid

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Abstract

Background. Peritoneal membrane deterioration during peritoneal dialysis (PD) is associated with epithelial-to-mesenchymal transition (EMT) of mesothelial cells (MC), which is believed to be mainly due to glucose degradation products (GDPs) present in PD solutions. Here we investigate the impact of GDPs in PD solutions on the EMT of MC *in vitro* and *ex vivo*.

Methods. For *in vitro* studies, omentum-derived MC were incubated with standard PD fluid or low-GDP solution diluted 1:1 with culture medium. For *ex vivo* studies, 33 patients, who were distributed at random to either the 'standard' or the 'low GDP' groups, were followed over 24 months. Effluents were collected every 6 months to determine EMT markers in effluent MC.

Results. Exposure of MC to standard fluid *in vitro* resulted in morphological change into a non-epitheloid shape, down-regulation of E-cadherin, indicative of EMT, and in a strong induction of vascular endothelial growth factor (VEGF) expression. In contrast, *in vitro* exposure of MC to low-GDP solution did not lead to these phenotype changes. This could be confirmed *ex vivo*, as the prevalence of non-epitheloid

phenotype of MC in the standard group was significantly higher with increasing PD duration and MC isolated from this group showed significantly higher levels of EMT-associated molecules including fibronectin, collagen I, VEGF, IL-8 and TGF-β levels when compared with the low-GDP group. Over time, the expression of E-cadherin also decreased in the standard but increased in the low-GDP group. In addition, the levels of EMT-associated molecules (fibronectin, VEGF and IL-8) increased in the standard but decreased in the low-GDP group. A similar trend was also observed for collagen I and for TGF-β (for the first year), but did not reach global statistical significance. Accordingly, effluent MC with non-epitheloid morphology showed significantly lower levels of E-cadherin and greater levels of fibronectin, collagen I, VEGF and IL 8 when compared with MC with epitheloid phenotype. The incidence of peritonitis did not significantly influence these results. Drop-out due to technique failure was less in the 'balance' group. The functional, renal and peritoneal evaluation of patients being treated with either standard or 'balance' fluid did not show any significant difference over time.

Conclusions. MC from PD effluent of patients treated with a PD fluid containing low GDP levels show fewer signs of EMT and the respective molecules than MC from patients treated with standard fluid, indicating a better preservation of the peritoneal membrane structure and a favourable outcome in patients using low-GDP fluid. It also confirms the hypothesis that the protection of EMT by GDP-reduced fluids is also present *in vivo*.

Keywords: epithelial-to-mesenchymal transition; low-GDP peritoneal fluid; mesothelial cells; peritoneal dialysis; peritoneal membrane

Introduction

Chronic exposure of the peritoneum to peritoneal dialysis (PD) fluids leads to peritoneal dysfunction and membrane failure [1,2]. Its non-physiological nature is considered to be one of the factors leading to alteration of the peritoneal membrane (PM) [2]. This persistent strain of chronic peritoneal inflammation, exacerbated by acute periodic episodes of peritonitis, contributes to structural abnormalities of the PM. Both processes result in loss of the mesothelial cell (MC) monolayer, submesothelial fibrosis, angiogenesis and hyalinizing vasculopathy [3–7]. Such alterations are considered to be the major cause of loss of functional membrane capacity, resulting in ultrafiltration failure. The characterization of this response to PD is based on functional, histological and effluent cytological studies [5–8]. Peritoneal biopsy is the gold standard to investigate PM alterations, but invasiveness precludes its regular use. Based on histological data, we could show that epithelial-to-mesenchymal transition (EMT) of MC is the mechanism that probably initiates the damage of the membrane [9,10]. Transdifferentiated MC acquire a non-epitheloid phenotype with the loss of E-cadherin and cytokeratin expression and an increased production of vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), fibronectin and collagen I [11], which correlates with high peritoneal transport [12]. We have also demonstrated that standard fluids induce EMT of MC *in vitro* [11].

'Balance' is a PD fluid with a low content of glucose degradation products (GDPs) relative to standard solutions, and first clinical studies have suggested an improved biocompatibility [13]. GDPs promote the transformation of precursors of glycosylation (Amadori products) into advanced glycosylation end products (AGE) [14]. MC express AGE receptors (RAGE), and their activation is able to initiate the EMT process [15]. Two different papers published by the same group 3 years apart have shown rapid remesothelization and less EMT using low-GDP solutions in two series of PD patients at medium term [16,17]. In the same sense, we have hypothesized that peritoneal MC of patients exposed to a GDP-reduced fluid ('balance'; Fresenius Medical Care Deutschland GmbH, Bad Homburg, Germany) should be at lower risk of developing mesothelial cell transition, demonstrated both *in vitro* and *ex vivo*, compared with a GDP-rich standard fluid, and they should be at lower risk of deteriorating peritoneal function. The aim of this study, therefore, was to reveal if the expression of EMT markers in MC from effluents of PD patients at medium term is reduced by treatment with the low-GDP solution 'balance'.

Materials and methods

Patients and study design

This prospective study was performed over a 4-year period in two university hospitals using the same PD protocols. Only incident patients were included, and the only inclusion criterion was ability and willingness to perform continuous ambulatory peritoneal dialysis (CAPD) therapy with no expressed indication for automated PD. Patients were alternately assigned to either 'balance' or standard PD fluid depending on the time point of inclusion.

Twenty patients (11 female and 9 male) were allocated to the standard fluid group (Stay Safe) (age 59 ± 15 years; 15% diabetics) and 13 (3 female and 10 male) to the 'balance' group (age 62 ± 11; 38% diabetics) (both solutions from Fresenius Medical Care, Bad Homburg, Germany). Balance, containing 1.5, 2.3 and 4.25% glucose respectively is pH neutral, 'low GDP' solution (Fresenius Medical Care). A follow-up period of 24 months was planned for each patient. The study was performed according to the Declaration of Helsinki and was approved by the ethics committees of both hospitals. Written informed consent was given by the patients. Oral informed consent was obtained from omentum donors submitted to elective surgeries. Small solute and water peritoneal transport was determined during a 4-h dwell peritoneal kinetic study performed with 4.25% glucose peritoneal dialysis fluid, using the allocated fluid type of the patient. Mass transfer area coefficients (MTAC) for creatinine, ultrafiltration capacity for the same period and residual renal function were calculated as previously described [18].

We determined EMT markers in MC released into nocturnal peritoneal effluent every 6 months. If peritonitis or haemoperitoneum occurred, the samples were taken after a 4-week symptom-free period.

Culture of MC and treatments

Human peritoneal MC from PD patients were isolated from PD effluent by using the method previously described [19]. MC were cultured in Earle's M199 medium (Biological Industries, Ashrat, Israel) supplemented with 20% fetal calf serum (Gibco BRL; Life Technologies, Paisley, UK), 50 IU/mL penicillin, 50 μg/mL streptomycin, 2% HEPES 1 M, 10 μg/mL ciprofloxacin (Bristol-Myers Squibb, Columbus, OH, USA) and 2% Biogro-2 (Biological Industries). EMT markers were determined *ex vivo* in cultured MC obtained from effluent of an overnight dwell with PD fluid containing 2.3% glucose (standard or 'balance', depending on

group) [19]. For *in vitro* experiments, we used omentum-derived MC that were isolated and cultured from omentum samples as previously described [19]. These cells were used and remained stable for one to two passages. In order to exclude fibroblast contamination, the purity of human MC omentum and effluent-derived cultures was determined by the expression of standard mesothelial markers intercellular adhesion molecule (ICAM-1) [10]. These MC cultures were negative for von Willebrand factor, excluding endothelial cell contamination [19].

Omentum-derived MC were incubated with standard (Stay Safe, 2.3% glucose; Fresenius Medical Care) or low-GDP ('balance', 2.3% glucose; Fresenius Medical Care) solutions diluted 1:1 with culture medium for 48–72 h. MC were also treated with recombinant human TGF-β1 (1 ng/mL) (R&D Systems Inc., Minneapolis, MN, USA) to induce EMT *in vitro* [9,11]. Each experiment was carried out in duplicate, and at least five experiments were performed.

Western blot

MC cultures were lysed in RIPA buffer (1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) with inhibitor cocktail (Pierce, Cambridge, MA, USA), and total protein was quantified using a total protein assay kit (Pierce). An equal amount of protein (30–50 μg) was fractionated by 8–10% SDS–PAGE and transferred to nitrocellulose membranes. Membranes were blocked with non-fat milk and incubated with specific antibodies against E-cadherin (Zymed, San Francisco, CA, USA), Pan-Q Cytokeratin (Sigma-Aldrich, St. Louis, MO, USA) and tubulin (Becton & Dickinson, Franklin Lakes, NJ, USA). Peroxidase-labelled goat anti-mouse antibody (Pharmigen, San Diego, CA, USA) was visualized with an enhanced chemiluminescence detection kit (Pierce), and blot images were acquired with a Kodak Image Station 2000 MM (Kodak, New York, NY, USA).

Quantitative real-time PCR (Q-PCR) analysis

For reverse transcription–PCR analysis, MC were lysed in TRI Reagent (Ambion Inc., Austin, TX, USA), and RNA was extracted as fabricant instructions. cDNA was obtained from 2 μg of total RNA by reverse transcription (RNA PCR Core Kit; Applied Biosystems, Foster City, CA, USA; Roche Systems Inc., Branchburg, NJ, USA). Q-PCR was carried out in a Light Cycler 480 (Roche Diagnostics GmbH, Mannheim, Germany) using a SYBR Green Kit (Roche Diagnostics GmbH) and specific primer sets for E-cadherin, fibronectin and collagen I. Histone 3 primers were used for PCR reaction control. These studies were performed in MC from patients who reached 18 months of treatment in both groups.

Enzyme-linked immunoassay

For the detection of VEGF, IL-8 or TGF-β in culture supernatants, media of MC cultured under the indicated conditions were replaced and collected 18 h later and stored at −80°C until their analysis. The VEGF concentration in supernatants was determined by enzyme-linked immunoassay (ELISA) kit (R&D Systems, Minneapolis, MN, USA). The levels of fibronectin, procollagen and ICAM-1 levels in cell lysates were assessed by commercially available ELISAs [Biomedical Technologies Inc. (Stoughton, MA, USA), Takara Bio Inc. (Shiga, Japan) and Diaclone (Besancon, France), respectively] according to the manufacturer's protocol and normalized with total protein of cell lysate.

Statistical analysis

Results are given as means ± SEM. Comparisons between data groups were performed using the non-parametric Mann–Whitney rank sum '*U*' test. A P value <0.05 was considered statistically significant. Wilcoxon's test for comparison between periods (intra-group) and Mann–Whitney's test for the comparison between groups were applied.

For studying the complete outcome of each variable over time, we applied the linear mixed model using unstructured covariance matrix for quantitative variables and generalized estimating equations (GEE) for qualitative variables (phenotype) both in the framework of 'Generalized Mixed Models'. These results should be interpreted as follows: (i) significant model means that the interaction 'fluid–time' is $P<0.01$, (ii) significant 'fluid' means that the effect of both fluids is different but the variation over time is not significantly different (maintain parallelism) and (iii) significant 'time' means that the effect of time affects both fluids to a similar degree.

To remove the interference of peritonitis with the studied variables, we followed three different approaches applied on the linear mixed model analysis: (i) isolated analysis of the outcome of patients who never suffered peritonitis vs those with at least one episode, (ii) comparison of samples collected before and after the first episode and introducing it as a co-variable, and (iii) introducing peritonitis (those having one episode vs none, cumulated episodes and days of peritoneal inflammation) as another co-variable in the GEE.

We used SPSS 14.5 (Chicago, IL, USA), which, among others, contains details on the 'Generalized Mixed Models', their method and meaning, and GraphPad Prism 4.0 (La Jolla, CA, USA).

Results

Exposure of MC to low-GDP fluid ('balance') in vitro has less impact on EMT than standard fluid

To analyse the effect of GDPs on EMT *in vitro*, omentum-derived MC were incubated for 48 or 72 h with standard PD fluid composed of 2.3% glucose and buffered with lactate (Stay Safe; Fresenius Medical Care) or low-GDP solutions composed of 2.3% glucose and buffered with lactate ('balance'; Fresenius Medical Care) diluted one-half with culture medium. As a positive control of EMT, omentum-derived MC were treated for 48 or 72 h with human recombinant transforming growth factor (TGF)-β1 (1 ng/mL), which has been proven to be a good model of EMT *in vitro* [10,11]. Exposure of MC to standard PD fluid, with high content of GDPs, resulted in a marked cell death (floating round-shaped cells) and in morphology change at 48 and 72 h, with the acquisition of a spindle-like shape, similar to cells treated with TGF-β1 (Figure 1A). In contrast, exposure of MC to low-GDP fluid had no effect on cellular viability and on cellular morphology. In addition, treatment of MC with standard PD fluid or TGF-β1 induced the down-regulation of E-cadherin, indicative of EMT (Figure 1B). The effect on E-cadherin was more evident at 72 h in cells exposed to standard fluid, indicating that the accumulation of soluble factor(s) was required to repress the expression of this epithelial marker. Interestingly, when incubating MC with the low-GDP fluid, the expression of E-cadherin was preserved (Figure 1B). These data were confirmed in a more quantitative manner by measuring the expression levels of E-cadherin mRNA by real-time RT–PCR. Exposure of omentum MC for 72 h to standard PD fluid or TGF-β1 significantly repressed the expression of E-cadherin mRNA (Figure 1C). In agreement with the results of E-cadherin protein expression shown above, in MC exposed to low-GDP fluid, the expression of E-cadherin mRNA was preserved (Figure 1C).To further explore the effects of PD fluid exposure on EMT, we analysed the expression of VEGF, which has been shown to be up-regulated during the mesenchymal conversion of MC [9,11]. As shown in Figure 1D, exposure of MC to standard PD fluid or treatment with TGF-β1 significantly induced the secretion of VEGF, whereas MC exposed to low-GDP fluid did not show a significant up-regulation of VEGF.

Clinical outcome of patients

Patients could be followed up for 6 (standard/'balance') (20/13), 12 (18/11), 18 (11/11) and 24 months (3/9), respective-

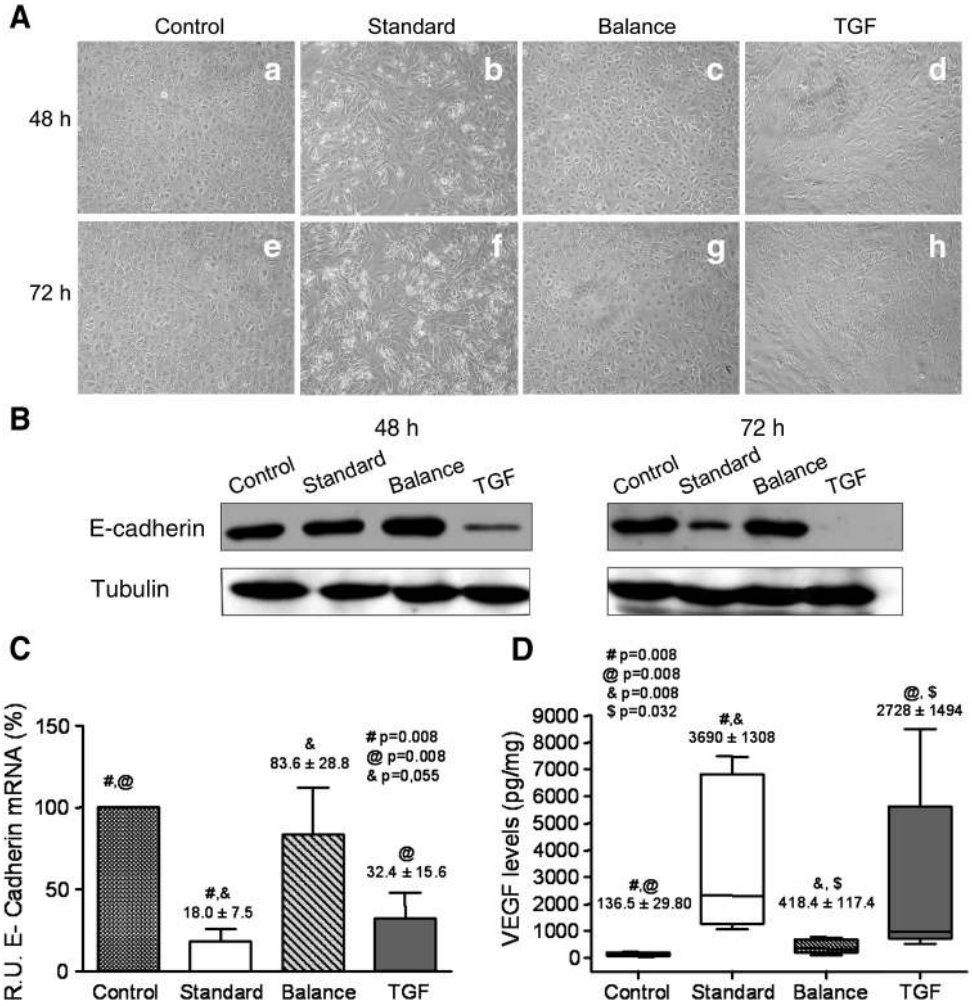


Fig. 1. Effects of PD fluids on MC *in vitro*. (**A**) Effects of PD fluids on MC morphology at 48 and 72 h *in vitro*. Pictures are representative of five independent experiments. (**B**) Western blot showing expression of E-cadherin in MC exposed to PD fluids. Tubulin was used as loading control. Photographs are representative of five independent experiments. (**C**) E-cadherin MRNA levels were analysed by quantitative RT–PCR as described. The results represent the relative expression of E-cadherin mRNA in cells treated with PD fluids or TGF-β1 compared with untreated cells, and data are depicted as mean value±standard error of five experiments. (**D**) VEGF production in supernatant (picograms per milligram of cell protein) in MC from omentum treated with PD fluids or TGF-β1. Box plots represent 75% percentile, 25% percentile, median, maximum and minimum values of five experiments. Symbols show statistical differences between groups.

ly. Patients from the 'balance' group showed significantly higher (P=0.008) technique survival than those from the standard fluid group, specifically after the first year of treatment. The reasons for drop-out were kidney transplantation in 7/0 patients, transfer to haemodialysis in 5/1, transfer to automated peritoneal dialysis (APD) in 3/2 and death in 1/1 patient (standard/'balance'), respectively. Switch to haemodialysis was more frequent in patients treated with standard fluid (P=0.008). The percentage of patients affected by peritonitis was similar in both groups (6 of 20 patients, 30%, for the standard and 5 of 13 patients, 38%, for the 'balance' fluid group). The time to the first episode in

Table 1. Values of peritoneal transport of creatinine (MTAC), ultrafiltration capacity (UF) and residual renal function in both groups (mean ± SD, control indicates standard fluid group)

	Creatinine MTAC (mL/min)		UF (mL/glucose 4.25%, 4 h)		Residual renal function (mL/min)	
	Standard	Balance	Standard	Balance	Standard	Balance
Baseline	7.6 ± 3.5	12.2 ± 4.6	832 ± 199	615 ± 274	5.8 ± 3.9	7.0 ± 4.3
6 months	6.2 ± 2.04	10.6 ± 2.8	915 ± 247	768 ± 154	3.4 ± 2.5*	6.9 ± 4.2
12 months	6.8 ± 3.1	10.1 ± 2.6	883 ± 273	676 ± 121	4.1 ± 3.1	5.5 ± 3.7
18 months	8.3 ± 2.8	11.6 ± 5.6	833 ± 234	581 ± 296	4.0 ± 2.8	5.0 ± 4.2
24 months	6.1 ± 2.6	9.2 ± 3.4	825 ± 106	720 ± 195	4.2 ± 4.0	4.2 ± 2.6

*P=0.016.

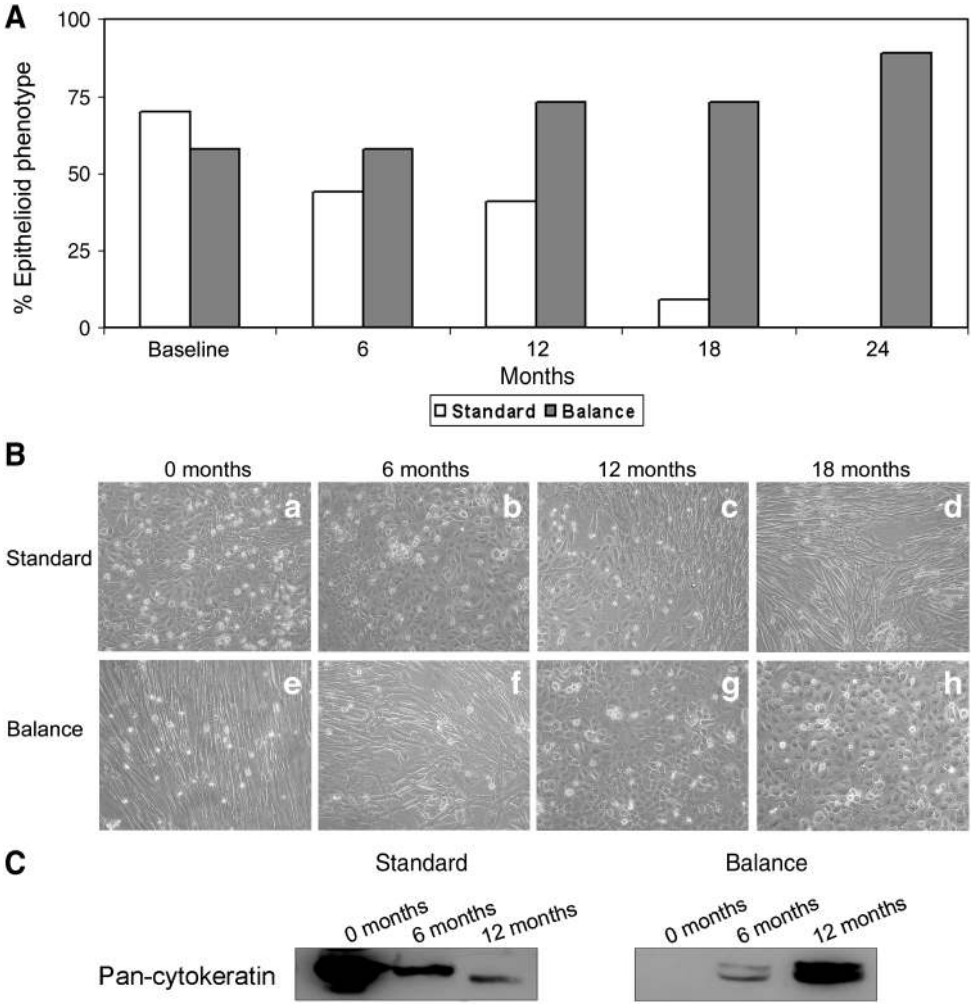


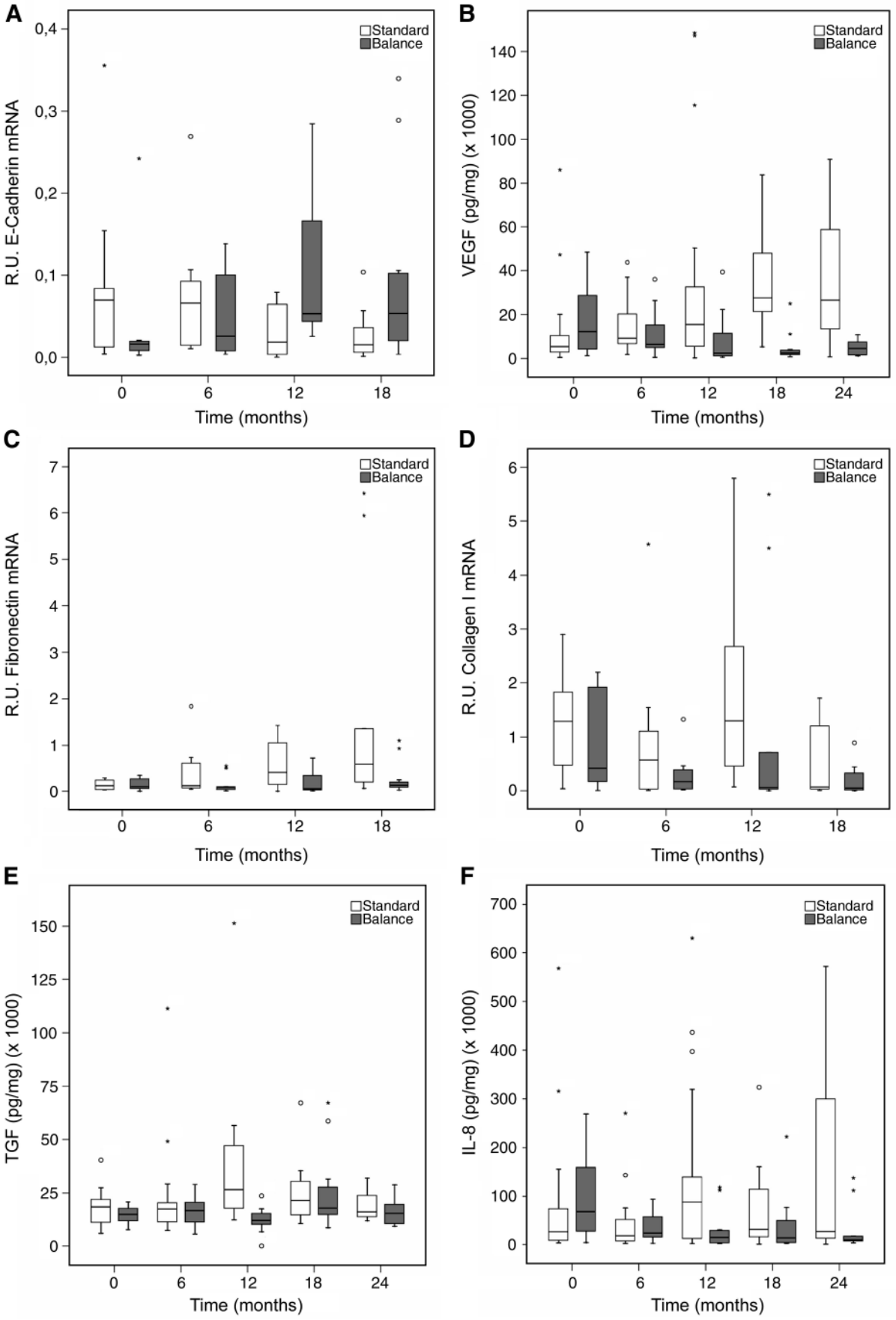
Fig. 2. Epitheloid and non-epitheloid phenotype of MC in standard and 'balance' groups. (**A**) Differences in the percentage of patients showing epitheloid phenotype in MC in each group of fluids over time (mixed models, fluid–time P=0.00001). (**B**) Pictures of MC phenotype from one representative patient from each group of PD fluids (A through D for a standard fluid patient and E through H for a patient treated with 'balance' fluid). (**C**) Western blot of pan-cytokeratin expression in MC from one representative patient of each group of fluids. Red Ponceau staining was used as loading control (data not shown).

each group was also not significantly different (data not shown). The global incidence was slightly higher in the 'balance' fluid group (one episode/16 patient-months vs one episode/27 patient-months in the standard group).

Peritoneal function studies and residual renal function

Creatinine MTAC values were significantly higher in the 'balance' group from the very beginning, but there was no significant change for any group over time. Ultrafiltration capacity was significantly greater in the standard fluid group, but there were no changes for any group over time either. Whereas residual renal function values were slightly higher in the 'balance' group during the first year, the follow-up analysis did not reveal significant differences between groups but a significantly greater 24-h diuresis with 'balance', with no significant modifications for any of the two fluids over time (Table 1).

Fig. 3. Box plots representing the cytokine levels in supernatant or extract from mesothelial cells derived from effluents under 'balance' and standard fluid groups. Statistical comparison is based on the mixed model analysis, which determines the significance of the differences between the two fluids and that from each fluid over time. (**A**) E-cadherin (mRNA levels, expressed in relative units) expression shows an increase in the 'balance' group over time, whereas the contrary is observed in the standard fluid group. This difference for time–fluid is significant (P=0.024). (**B**) VEGF production (picograms per milligram) into supernatant demonstrates lower values in the 'balance' group than in the standard fluid group throughout the study. This difference for time–fluid is significant (P=0.017) as well as for fluids (P=0.00001). (**C**) Fibronectin mRNA (relative units) values in MC from the 'balance' group show lower values than that from the standard fluid group over time. This difference is significant just for fluids (P=0.017) but not over time. (**D**) Collagen I mRNA (relative units) values from MC from the 'balance' group show lower values than that from the standard group in the limit of significance (P=0.056) and no differences over time. (**E**) TGF-β cell supernatant levels (picograms per milligram) were globally and significantly higher in the standard fluid group (fluid, P=0.047), specifically during the first year. (**F**) IL-8 supernatant levels (picograms per milligram) (to be correctly read, IL-8 values should be multiplied by 10) showed significantly higher levels in the standard fluid group with a non-significant trend to grow over time (fluid–time, P=0.041).



Ex vivo cell studies

The percentage distribution of MC with epitheloid phenotype was similar in both groups at baseline. However, <25% of patients from the standard fluid group maintained this phenotype in contrast with >75% of 'balance' patients at medium and long term (Figure 2A). The differences reached statistical significance by linear mixed models in the overall observation both for fluids and over time (fluid–time P=0.00001). Representative examples of patients of the standard PD fluid group showing an epitheloid to non-epitheloid conversion and of the 'balance' group showing a reversion from non-epitheloid to epitheloid phenotypes, as determined by cellular morphology or cytokeratin expression, are depicted in Figures 2B and C.

Figure 3 (A through F) confirms the significant differences between 'balance' and standard groups in terms of EMT-associated molecules in supernatant or cellular extract from effluent-derived MC, along with the observation and with the linear mixed model analysis. E-cadherin expression was progressively lost along the experience in standard fluid patients, whereas it was preserved in 'balance' patients (Figure 3A). On the contrary, and confirming the acquisition of EMT state by MC, VEGF, collagen I (in the limit of significance for fluids, P=0.056), fibronectin, TGF-β and IL-8 levels were significantly (mixed models) higher among standard fluid patients over time (Figure 3B–F).

Evaluating all samples by group of fluid, the standard fluid group showed significantly higher values of VEGF, fibronectin mRNA, collagen I mRNA (P=0.056), IL-8 and TGF-β than the 'balance' group; on the contrary, ICAM-1, procollagen and fibronectin proteins showed no significant differences (Figure 4A). E-cadherin mRNA differences did not reach significance (data not shown).

In general, as a confirmation of the hypothesis (agreement between phenotype and EMT products), we found significantly greater levels of VEGF (4-fold), procollagen and collagen I (2- and 1.2-fold, respectively), fibronectin and fibronectin mRNA (5-fold) and IL-8 (2-fold) in non-epitheloid phenotype cells in all samples. However, TGF-β showed similar values for both phenotypes (Figure 4B). In contrast, E-cadherin showed significantly lower values in non-epitheloid cells (0.037 ± 0.04 vs 0.089 ± 0.09, P=0.02).

Effects of peritonitis episodes in variables related to EMT

Peritonitis incidence did not influence these results. In patients with no peritonitis, the MC phenotype outcome was similar to the overall population. However, in patients who suffered peritonitis and were treated with standard fluid, non-epitheloid phenotype was mostly observed over time. In contrast, only few patients in the 'balance' group who suffered peritonitis showed a loss of the usual epitheloid phenotype over time.

In regard to the levels of cytokines and growth factors in the supernatants or MC extracts, patients without peritonitis showed similar values for all markers when compared with the whole group. As these patients were observed after the first episode of peritonitis, in particular the supernatant VEGF levels were markedly increased (6-fold) afterwards for standard fluid (41 555 ± 47 986 pg/mg, n=12 samples vs 7033 ± 6608 pg/mg, n=24 samples in the 'balance' group). Fibronectin and procollagen levels did not show differences for these subgroups. This difference did not reach statistical significance due to intra-group variability. To confirm this trend, we analysed the outcome of supernatant VEGF levels by linear mixed models including as co-variable the cumulated episodes of peritonitis; the significant differences between both groups of fluids persisted, indicating that dialysis fluid influences the VEGF supernatant levels, independent of suffering or no peritonitis. The days of peritoneal inflammation (leukocyte count >100 cells/mm^3) treated as co-variable neutralized the differences between the two dialysis fluids. This indicates that the severity and duration of inflammation determines a loss of the protection by 'balance' fluid in terms of VEGF production.

Discussion

In the present study, we investigated the effect of a low-GDP solution on EMT of MC *in vitro* and *ex vivo* by defining EMT as acquiring a non-epitheloid phenotype associated with the loss of E-cadherin expression and the production of higher amounts of mesenchymal products. Our data obtained from the analysis of EMT *in vitro* and *ex vivo* showed that exposure of MC to a low-GDP solution is consistently associated with the preservation of an epitheloid phenotype. Factors related to mesenchymal conversion were secreted to a much lower extent after treatment with 'balance' compared with standard PD fluid, which strongly induces these molecules.

MC obtained from omentum *in vitro* cultures preserve their physiological phenotype. But if a standard fluid with high content of GDPs is added to cultures, a non-epitheloid phenotype associated to the loss of E-cadherin expression is acquired. This effect on E-cadherin is evident at later time points (72 h) when compared with cells treated with TGF-β indicating that the accumulation of soluble factor(s) was required to repress the expression of this epithelial marker. In this context, we have previously described that the exposure of omentum-derived MC to standard PD fluids induces the expression of TGF-β [20], which in turn may induce the EMT process. In contrast, the addition of low-GDP solution 'balance' to MC cultures barely affects the epitheloid morphology and E-cadherin expression of these cells, revealing the preservation of the physiological MC identity. These results demonstrate that the content of GDPs in PD fluids has an important role in the induction of EMT of MC.

The *ex vivo* studies performed in effluent-derived MC showed similar results. Changes suffered by MC in their morphology and expression of E-cadherin over time have confirmed that a normal epitheloid phenotype was associated with the use of 'balance' fluid. E-cadherin values were similar for both fluids at baseline and at the sixth month. In contrast, values at the 12th and 18th months were significantly higher for the 'balance' group.

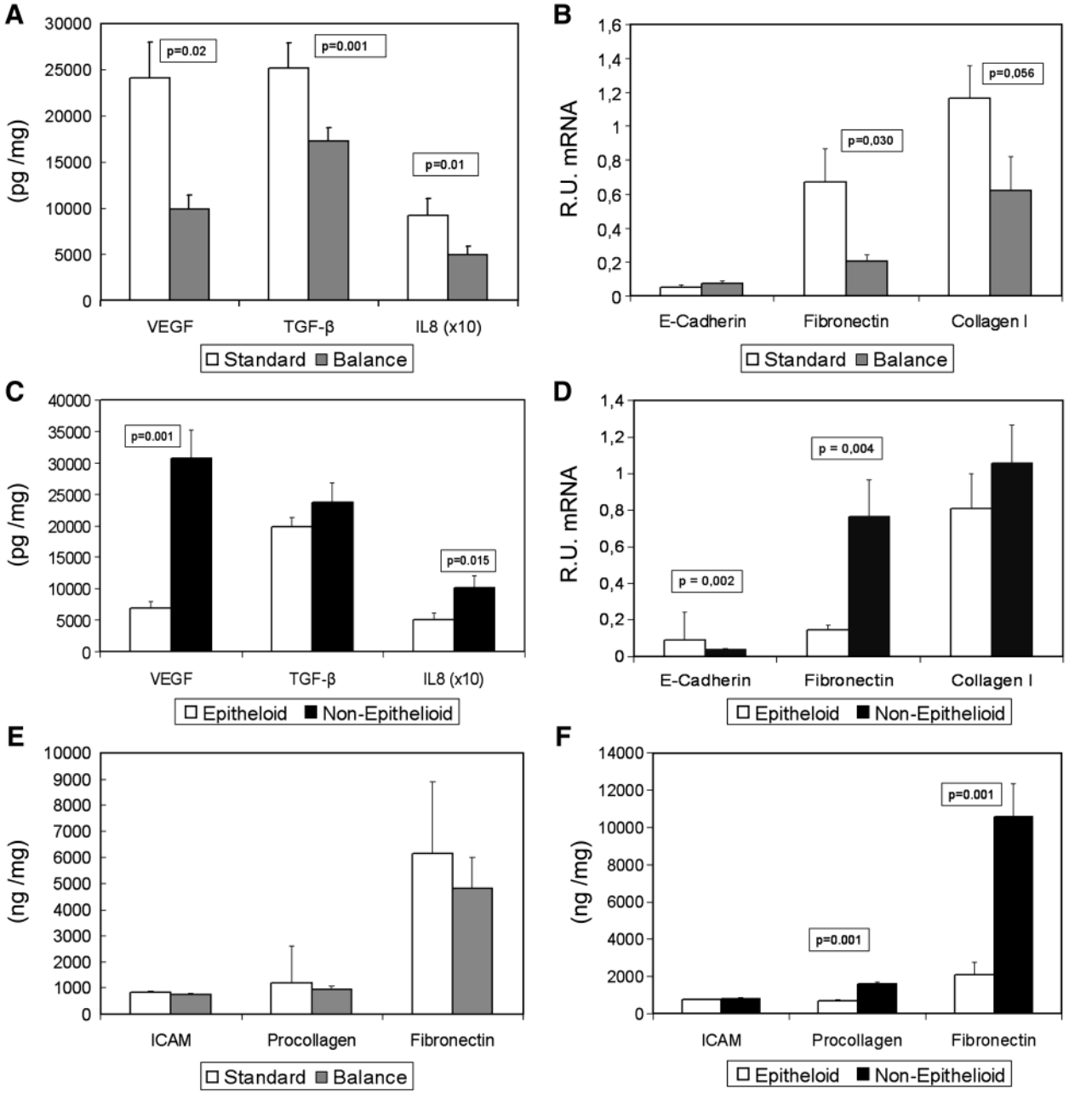


Fig. 4. Mean values of the different mesothelial products studied in culture: evaluation of all samples together. (**A**) Differentiated by group of fluid, the standard fluid group showed significantly higher values of VEGF, TGF-β and IL-8 in supernatant (picograms per milligram). (**B**) Differentiated by group of fluid, the standard fluid group showed mRNA higher values of fibronectin (P=0.03) and collagen I (P=0.05). E-cadherin levels were similar in both groups. (**C**) Differentiated by phenotype of MC, the non-epitheloid phenotype showed higher values of VEGF (P=0.001), TGF-β (NS) and IL-8 (P=0.01) in supernatant (picograms per milligram). (**D**) Differentiated by phenotype of MC, the non-epitheloid phenotype showed lower mRNA levels of E-cadherin (P=0.002) and higher levels of fibronectin (P=0.004) and collagen I (NS). (**E**) Differentiated by group of fluid, the standard fluid group showed no significant differences in cellular lysate (nanograms per milligram) for ICAM-1, procollagen and fibronectin protein levels. (**F**) Differentiated by phenotype of MC, the non-epitheloid phenotype showed higher values of procollagen (P=0.001) and fibronectin (P=0.001) and similar values for ICAM-1 protein levels in cellular lysate (nanograms per milligram).

The assessment of VEGF levels from cultured MC *in vitro* and *ex vivo* demonstrated a marked increment of this growth factor during the EMT process. Globally, this VEGF production was notably increased by standard fluid compared with 'balance' fluid [9,19,21]. This difference accentuated over time. As VEGF is partially responsible for local vasodilatation, and its levels both *ex vivo* in supernatant and in serum of PD patients correlate with high peritoneal transport [1,2,9], we can suggest VEGF as a key molecule for peritoneal function in this context.

Data obtained with other EMT-associated molecules including collagen I, fibronectin, IL-8 and TGF-β have glo-

bally confirmed the differences in MC from peritoneal effluents according to the fluid use, always in the sense that standard fluid induced greater production of these agents.

IL-8 levels were shown to be induced in MC *in vitro* by standard PD fluids [22] and GDPs [23]. Furthermore, there are hints that IL-8 might influence solute transport in PD-related peritonitis and that IL-8 promotes cell migration [24], thereby exacerbating the peritoneal membrane deterioration. Coherently, TGF-β levels did not show any correlation with the phenotype, but comparing MC from the distinctly treated groups, significant differences in TGF-β production for both fluids were only observed when the patients were evaluated by group. The time-dependent course of TGF-β is noteworthy, reaching its maximal levels during the first year but regressing to lower values afterwards. TGF-β has been shown to down-regulate MC proliferation and to accelerate peritoneal fibrosis by inducing the production of extracellular matrix proteins [25–28]. So the down-regulation of several factors associated with membrane deterioration clearly hints towards a benefit of low-GDP solutions in this process.

In agreement with our results, a recent report has shown that different GDPs present in standard PD fluids can induce EMT of MC *in vitro*, in that low-GDP fluid has less impact on peritoneal fibrosis and EMT *in vivo* in a rat model [29].

All these results are in accordance with that obtained in a series of patients (present series and references [16,17]).

Mechanistically, the induction of EMT by standard is related to their high GDP content, which may contribute to AGE accumulation and activation of RAGE, and damage of MC [14,15,30].

The follow-up analysis on EMT markers could be influenced by episodes of peritonitis. Therefore, we compared these markers in MC from patients with and without peritonitis, and we did not find significant influences. However, the variability of the data and the unavoidable reduction of the series precluded a more profound analysis. The most remarkable finding in patients with no peritonitis was that standard fluid maintained its association with non-epitheloid phenotype and high VEGF production. In the same sense, patients treated with 'balance' fluid who suffered peritonitis showed the preservation of epitheloid phenotype with lower production of VEGF, which suggests that 'balance' fluid confers additional MC protection against the effects of inflammation.

In summary, we have shown that MC EMT-associated molecules showed favourable differences and outcome for the low-GDP ('balance') fluid relative to standard fluid at medium term. Although a greater series of patients may be required to confirm these results, the consistency with other studies supports our findings. Moreover, our data extend these results beyond the first year on PD. It is noteworthy that we observed that the differences between fluids markedly increased after this first year.

Limitations of our study

The most important limitations of our study are its non-randomized nature and the low number of patients at the final evaluation. Although a random selection cannot be equal to a randomization procedure, the characteristics of patients included in each group were similar, and the important differences we have found probably are not due to these minor differences. It is important to note that our observation is valid for 18–24 months of PD.

In conclusion, non-epitheloid phenotype, lower expression of E-cadherin and higher fibronectin and VEGF production complete the EMT manifestations by mesothelial cells *in vitro* and *ex vivo*. Based on these parameters, we have demonstrated a significantly favourable outcome in patients using 'balance' fluid relative to those using standard fluid at medium term. These findings confirm the hypothesis that GDP-reduced fluid protects mesothelial cells from the development of EMT, and therefore a better peritoneal membrane preservation can be expected in the long term.

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(See related article by McLoughlin and Topley. Switching on EMT in the peritoneal membrane: considering the evidence. *Nephrol Dial Transplant* 2011; 26: 11–14.)

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The time course of peritoneal transport parameters in peritoneal dialysis patients who develop encapsulating peritoneal sclerosis

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Abstract

Background. Encapsulating peritoneal sclerosis (EPS) is a severe complication of peritoneal dialysis (PD). The first aim was to analyse the risk of EPS in patients who had developed ultrafiltration failure (UFF). The second aim was to identify specific peritoneal transport alterations that distinguish patients with UFF from patients who will develop EPS.

Methods. All patients of this study were treated with PD between July 1995 and December 2008 in the Academic Medical Center, Amsterdam, the Netherlands. *Risk analysis*: all PD patients who developed UFF after at least 2 years of PD. *Peritoneal transport analysis*: all patients who had PD for at least 55 months were included: 12 EPS patients, 21 patients with UFF and 26 patients with normal ultrafiltration (UF). The peritoneal function was measured yearly with a standard peritoneal permeability analysis. UFF was defined as net UF<400 mL after a 4-h dwell with a 3.86% dialysis solution.