Increased lymphatic absorption might contribute to ultrafiltration failure in peritoneal dialysis (PD). Lymphangiogenesis develops during PD, but little is known about the relationship between its morphologic and functional parameters. The relationships between lymph vessel density, the effective lymphatic absorption rate (ELAR), and fibrosis were investigated in a rat model of chronic kidney failure (CKD) with exposure to dialysis solutions.

Wistar rats (n = 44) were allocated to these groups: NKF (normal kidney function), CKD (70% nephrectomy), CKDD [CKD, with daily intraperitoneal (i.p.) Dianeal 3.86% (Baxter Healthcare BV, Utrecht, Netherlands)], CKDP [CKD, with daily i.p. Physioneal 3.86% (Baxter Healthcare BV)]. After 16 weeks, a peritoneal function test was performed, and the ELAR was calculated from the disappearance rate of i.p. dextran 70. The lymph vessel profile density (LVPD) was assessed using STEPanizer image analysis (Java application from Tschanz SA, Bern, Germany) of omental sections after anti-podoplanin immunostaining. Fibrosis was quantified by picro-sirius red staining. The LVPD was significantly increased in CKD rats compared with NKF rats, and no additional effect of dialysis solutions was present. The ELAR was increased compared with NKF rats. For all rats together, the LVPD correlated positively with the ELAR and with the amount of fibrosis.

Chronic kidney disease itself induces lymphangiogenesis and fibrosis and increases the ELAR, independent of exposure to dialysis fluids. The ELAR is related to the LVPD in peritoneal tissue.

Key words
Lymphangiogenesis, lymphatic absorption rate, podoplanin, stereology

Introduction
Ultrafiltration failure is an important reason for discontinuation of treatment with peritoneal dialysis (PD) (1–3). High lymphatic reabsorption can contribute to impaired ultrafiltration. Some PD patients have a high lymphatic absorption rate, but no time trend is evident (4). Lymphangiogenesis is part of normal development during embryogenesis and occurs in several pathogenic conditions (5–9), including peritoneal exposure to dialysis solutions, during which its development is associated with peritoneal fibrosis by the transforming growth factor β–vascular endothelial growth factor C pathway (10,11). Furthermore, compared with uremic patients before the start of PD, PD patients with ultrafiltration failure have been described to show an increase in the number of lymph vessels in peritoneal tissue (11).

To date, little is known about the relationship between the morphologic and functional data of lymphangiogenesis. In the present study, we used a rat model of chronic kidney failure, with exposure to either conventional or biocompatible dialysis solution, to investigate whether the density of lymph vessels in peritoneal tissue and the effective lymphatic absorption rate (ELAR) are related. The
ELAR was calculated based on the rate of dextran 70 disappearance from the peritoneal cavity during a standard peritoneal permeability analysis adapted for rats (SPARa) (12). In addition, we tested whether lymphangiogenesis and the extent of fibrosis of peritoneal tissue were related in this model.

Methods

Study design
Male Wistar rats (n = 44; Harlan, Zeist, Netherlands) with a body weight of 260 – 280 g were randomly assigned to four experimental groups:

• Normal kidney function (NKF), n = 8, no peritoneal exposure
• Chronic kidney failure (CKD), n = 12, 70% nephrectomy, no peritoneal exposure
• CKD exposed to Dianeal (CKDD), n = 12, 70% nephrectomy, with daily peritoneal exposure to Dianeal (Baxter Healthcare BV, Utrecht, Netherlands), a conventional lactate-buffered dialysis solution, 3.86% glucose
• CKD exposed to Physioneal (CKDP), n = 12, 70% nephrectomy, daily peritoneal exposure to Physioneal (Baxter Healthcare BV), a bicarbonate/lactate–buffered dialysis solution, 3.86% glucose

All dialysis solutions were provided by Baxter Healthcare BV. The rats received 6 mL dialysis fluid per 100 g body weight intraperitoneally, and the infused volume was set at 20 mL daily when the body weight of the animal exceeded 420 g. After 16 weeks, at least 2 rats in each group underwent a SPARa, and morphology assay of the peritoneum was performed in all rats. The protocol was approved by the Committee for Animal Experiments of the Academic Medical Center, University of Amsterdam.

Procedures
Housing conditions, catheter implantation, nephrectomy, and SPARa were all performed as previously described (13).

Peritoneal transport parameters
Peritoneal small-solute transport was expressed as dialysate-to-plasma creatinine (D/P Cr), determined by an enzymatic method. The ELAR was calculated based on the rate of dextran 70 disappearance from the peritoneal cavity—that is, the difference between the instilled and recovered dextran mass divided by the geometric mean of the dialysate dextran concentrations. Intraperitoneal dextran 70 was measured by gel permeation chromatography (14).

Renal function
Just before the end of the study, 24-hour urine collected from rats in metabolic cages was used for the calculation of endogenous creatinine clearance.

Immunohistochemistry
Formalin-fixed, paraffin-embedded omental tissue was used for immunohistochemistry. Sections of 4 μm thickness were deparaffinized, and heat-induced epitope retrieval in sodium citrate (pH 6) was then performed. The staining sequence was 5% normal goat serum in phosphate-buffered saline for 30 minutes; the primary antibody [mouse anti-rat podoplanin, 1:10000 (ReliaTech GmbH, Wolfenbuttel, Germany)] was then left for 2 hours at room temperature. The secondary antibody [alkaline phosphatase–conjugated goat anti-mouse immunoglobulin G1, 1:50 (Southern Biotech, Birmingham, AL, U.S.A.)] was followed by washing in Tris-buffered saline with Tween (Sigma–Aldrich, St. Louis, MO, U.S.A.) and visualization using a Vector Red Substrate kit (Vector Laboratories, Burlingame, CA, U.S.A.).

Fibrosis was analyzed on sections of formalin-fixed, paraffin-embedded omental tissue, stained with picro-sirius red (Gurr: BDH Chemicals, Poole, U.K.).

Image acquisition
A BX61VS microscope with 10×/0.40 UPlanSApo objective and an Olympus dotSlide imaging system (Olympus, Zoeterwoude, Netherlands) was used to scan slides for the acquisition of TIFF (tagged image file format) images (1 pixel = 648 nm×648 nm). The TIFF images were exported as 10 MB files for analysis of lymph-vessel profile density (LVPD) and as 20 MB files for quantification of fibrosis. In the latter case, the area of picro-sirius red–positive staining was measured (ImagePro premier 9.1: Media Cybernetics, Rockville, MD, U.S.A.) and expressed as a percentage of the total surface area.

Analysis of LVPD
The stereology tool STEPanizer (version 1: Java application from Tschanz SA, Bern, Germany) was used to
quantify the number of vascular profiles and the surface area (15). We analyzed 8 images from each rat, selected after application of a systematic uniform random sampling of the images. Using a counting frame (area: 924×924 pixels), the vascular profiles were counted if they were located inside the counting frame, but not if they were touching the exclusion line or its extensions. The profile density is reported as a ratio: the number of lymph vessel profiles per tissue surface area.

**Statistics**
Data are presented as means, with standard deviations. Possible differences between the study groups were assessed using analysis of variance and Games–Howell post hoc analysis, or the Mann-Whitney U-test for comparisons between specific groups. Correlations between continuous variables were tested using the Pearson correlation test or the Spearman rho.

**Results**
Because of complications related to nephrectomy, to catheter obstruction, or to suspicion of peritonitis, 5 rats were excluded from the study (2 from CKDD, 3 from CKDP).

**Renal function**
At the end of the study period, endogenous creatinine clearance was highest in the NKF animals (4.3 ± 0.7 mL/min) and decreased in nephrectomized rats (CKD: 1.8 ± 0.5 mL/min; CKDD: 2.3 ± 0.6 mL/min; CKDP: 1.9 ± 0.5 mL/min; p < 0.001 for all groups vs. NKF).

**LVPD**
The LVPD was 0.3 ± 0.2 profiles/mm² in NKF rats and significantly increased in rats with kidney failure (CKD: 0.8 ± 0.4 profiles/mm²; CKDD: 1.0 ± 0.6 profiles/mm²; CKDP: 0.9 ± 0.4 profiles/mm²; p = 0.009 for the differences between groups). No significant differences were evident between the CKDP and CKDD groups (p = 0.9), nor between the CKD group and the groups exposed to dialysis fluids (p = 0.8 for CKDD, p = 0.7 for CKDP). When CKDD and CKDP were analyzed together, the difference between the CKD group and the combined groups remained non-significant (p = 0.4).

**Peritoneal transport parameters**
The ELAR was 12 ± 3 μL/min in NKF rats and was increased in rats with kidney failure (CKD: 19 ± 2 μL/min; CKDD: 29 ± 22 μL/min; CKDP: 23.2 ± 9 μL/min; p = 0.09 for all rats with CKD vs. NKF rats). Although the ELAR was somewhat higher in the groups exposed to dialysis solutions than in the CKD group, the difference was not significant (p = 0.4). The D/P Cr was 0.38 ± 0.06 in NKF rats and somewhat higher in CKD rats (0.45 ± 0.03). Exposure to dialysis fluids induced an additional significant increase (CKDD: 0.59 ± 0.09; CKDP: 0.63 ± 0.05; p = 0.002 for both groups vs. the CKD group). No difference between the CKDD and CKDP groups was evident (p = 0.9 for both parameters). The ELAR was related to the D/P Cr (p = 0.04, r = 0.6).

**Fibrosis of peritoneal tissue**
The surface area positive for picro-sirius red was 1.5% ± 0.8% in NKF rats and was increased in CKD rats (2.9% ± 1.3%, p = 0.05). Exposure to dialysis fluids was associated with a further increase (CKDD: 9.9% ± 6.0%; CKDP: 8.0% ± 4.2%; p = 0.02 vs. the CKD group).

To investigate the relationships between the LVPD, the ELAR, and fibrosis, we analyzed the ELAR as a continuous variable. As Figure 1 shows, the ELAR and LVPD demonstrated a positive relationship (p = 0.04; Spearman rho: 0.63). A strong positive correlation was present between the LVPD and the amount of fibrosis expressed as percentage of positive staining by picro-sirius red (p < 0.001; Spearman rho: 0.7).

**Figure 1** Relationship between the effective lymphatic absorption rate (ELAR) and the lymph vessel profile density (LVPD) in omental tissue (p = 0.04; Spearman rho: 0.63).
Discussion
Analysis of the LVPD in peritoneal tissue shows that lymphangiogenesis develops in CKD and that it is associated with fibrosis. Long-term exposure to dialysis fluids had no significant additional effect. Importantly, the ELAR was also higher in CKD rats than in NKF rats, and it was positively correlated with the LVPD and with small-solute transport. The relationship between the ELAR (which is a functional parameter) and the LVPD has not previously been reported and supports our contention that the ELAR indeed represents lymphatic absorption (16)—a finding hitherto not recognized by some authors (17).

The presence of uremia-induced lymphangiogenesis in rats has previously been reported, and exposure to dialysis fluids for 4 weeks has been claimed to lead to a further increase in the number of lymph vessels (10,18). Also, simple exposure to dialysis fluids induced an increase in the number of lymph vessels in rats with normal kidney function (19). In our study, although the absolute values of the LVPD in the groups exposed to dialysis fluids were higher than the values in the CKD group, we found no additional significant effect of the exposure to dialysis fluids. The methods used for the quantification of lymphangiogenesis in other studies and in ours are different. In addition, we cannot exclude the possibility that a lymphangiogenic effect of exposure to dialysis fluids might be found only early, at initiation of peritoneal dialysis, and that after longer follow-up or in long-term uremia, the effect could become less important.

The ELAR was calculated as peritoneal dextran clearance, which includes both the subdiaphragmatic and interstitial pathways of uptake into the lymphatic system. As previously reported in patients (20), the ELAR and the transport rates of small solutes (determined as D/P Cr) were positively correlated, suggesting that a large lymphatic surface area develops in concert with a large surface area of blood vasculature. The combination of functional and morphologic data, together with the use of computer-assisted stereology for the quantification of LVDPD, are among the strengths of the present study; the small number of rats undergoing the SPARa could be considered a weakness.

Conclusions
The present study shows that lymphangiogenesis is present and is associated with fibrosis in the peritoneal tissue of rats with CKD. An additional significant effect of dialysis solutions could not be established. Importantly, the ELAR, a functional parameter calculated as peritoneal dextran clearance, was related to the LVPD in peritoneal tissue.

Disclosures
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