Artificial Organs

Artificial Organs
35(2):E33–E39, Wiley Periodicals, Inc.

Thoughts and Progress

Molecular Biology-Based Assessment of Vitamin E-Coated Dialyzer Effects on Oxidative Stress, Inflammation, and Vascular Remodeling

**Lorenzo A. Calò, †Agostino Naso, †Angela D'Angelo, *Elisa Pagnin, *Marco Zanardo, *Massimo Puato, †Mirka Rebeschini, ‡Silvano Landini, ‡Mariano Feriani, §Angelo Perego, §Andrea Malagoli, ¶Riccardo Zagatti, ¶Piergianni Calzavara, ¶Carmelo Cascone, and **Paul A. Davis*

**Department of Clinical and Experimental Medicine and †Division of Nephrology, University of Padova-Azienda Ospedaliera di Padova, Padova; Divisions of Nephrology, Hospitals of ‡Mestre (Venezia), §Monselice (Padova), and ¶Treviso, Italy; and **Department of Nutrition, University of California, Davis, CA, USA*

Abstract: Cardiovascular disease represents the most common cause for the excess of morbidity and mortality found in end-stage renal disease (ESRD) and has prompted the exploration of multiple approaches to improve outcomes in these patients. Cardiovascular risk factors such as increased oxidative stress (OxSt) and inflammation are found in ESRD patients. A vitamin E-coated dialyzer using polysulfone membranes has been suggested to have positive effects on these factors. This 1-year study evaluated in 25 ESRD patients under chronic dialysis, the effects of a vitamin E-coated membrane (VitabranE ViE) "ex vivo" on mononuclear cells, OxSt, and inflammation-related biochemical and molecular biology markers using a molecular biology approach. $p22^{phox}$, heme oxygenase (HO)-1, plasminogen activator inhibitor (PAI)-1 protein level, and phosphorylated extracellular signal-regulated kinase (pERK)1/2 status were evaluated at the beginning of the study, after 6 months and after 12 months by Western blot analysis and oxidized low-density lipoprotein (OxLDL) plasma level by enzyme-linked immunosorbent assay, alongside vascular remodeling assessment as measured by carotid intima-media thickness (IMT) in a subgroup of nine randomly selected patients. $p22^{phox}$, PAI-1, OxLDL, and pERK all decreased with VitabranE use, while HO-1 increased. Carotid IMT did not increase. Treatment with VitabranE significantly decreases the expression of proteins and markers relevant to OxSt and inflammation tightly associated with cardiovascular disease, and it appears highly likely that VitabranE use will provide a benefit in terms of cardiovascular protection. **Key Words:** Hemodialysis—Oxidative stress—Inflammation—Cardiovascular remodeling.

doi:10.1111/j.1525-1594.2010.01125.x

Received May 2010; revised July 2010.

Address correspondence and reprint requests to Dr. Lorenzo A. Calò, Department of Clinical and Experimental Medicine, Clinica Medica 4, University of Padova, Via Giustiniani, 2, 35128 Padova, Italy. E-mail: renzcalo@unipd.it

Cardiovascular disease represents the most common cause for the excess of morbidity and mortality found in end-stage renal disease (ESRD) patients (1). Cardiovascular risk factors such as increased oxidative stress (OxSt), inflammation, and endothelial dysfunction are known "nontraditional" risk factors present in ESRD patients. These patients have, in fact, increased levels of inflammation-related proteins, such as interleukin-6 (IL-6) and C-reactive protein (CRP), as well as OxSt-related proteins, such as NAD(P)H oxidase, which lead to reduced nitric oxide availability and endothelial dysfunction (2,3). The inflammatory state present in these patients has a multifactorial origin and also arises directly from dialysis as well as from other nondialysis-related factors (4,5). Mortality rate of ESRD patients on dialysis remains unacceptably high and has prompted the exploration of multiple approaches to improve outcomes (6).

One of these approaches has been the introduction of vitamin E-coated dialyzers in an effort to reduce OxSt (7), lipid peroxidation (8,9), and leukocyte activation (10) as well as scavenge oxygen free radicals. Recently, a vitamin E-coated dialyzer using polysulfone membranes coated with vitamin E has been introduced (VitabranE ViE, Asahi Kasei Kuraray Medical Co., Tokyo, Japan). In this study, the potential beneficial effects associated with this new dialyzer membrane have been evaluated using a molecular biology approach "ex vivo" on mononuclear cells of uremic patients switched from a standard bicarbonate dialysis using a polysulfone dialyzer to a 1-year treatment with a vitamin E-coated polysulfone membrane (VitabranE ViE).

This study examined the levels of proteins relevant to OxSt, such as $p22^{phox}$; subunits of NADPH oxidase, essential for the production of superoxide anions (11,12); plasminogen activator inhibitor (PAI)-1, an

established OxSt-related profibrotic factor (13); and heme oxygenase (HO)-1, the inducible isoform of HO that protects against OxSt (14). We also assessed the state of phosphorylation of extracellular signal-regulated kinases (ERKs), OxSt effector protein for cardiovascular remodeling (15), and a plasma marker of OxSt, oxidized low-density lipoproteins (OxLDLs), considered as crucial in the development of chronic inflammation of the artery wall linked to atherosclerosis (16). In a subgroup of nine unselected patients, in addition to biochemical and molecular markers, vascular remodeling was measured at the beginning and at the end of the study via the evaluation of carotid intima-media thickness (IMT).

PATIENTS AND METHODS

Twenty-five patients, aged between 42 and 64 years, 17 males and 8 females from different dialysis centers (Padova, Mestre, Monselice, and Treviso) undergoing chronic dialysis treatment with 210–240 min, three times a week bicarbonate dialysis for at least 1 year (range 1–5 years), were recruited into a 1-year interventional study. ESRD patients treated with low-flux bicarbonate dialysis with ultrapure dialysate using a 1.8 m^2 polysulfone dialyzer, were switched to 12 months of treatment using VitabranE ViE (Asahi Kasei Kuraray Medical Co.). Vascular access was through the arteriovenous fistula for all the study participants. The etiology of ESRD for the patients was as follows: chronic glomerulonephritis (7 patients), nephroangiosclerosis (17 patients), and undiagnosed (1 patient). Patients were selected based on the following criteria: relatively young age; nonsmokers; lack of comorbidity such as diabetes, chronic obstructive pulmonary diseases, heart failure, and cancer; and lack of hospitalization in the last 6 months.

Blood samples for molecular biology and biochemical determinations were collected at the beginning of the study (baseline), at 6 and 12 months. The patients were also checked at baseline, at 6 months and at the end of the study for biochemical markers of inflammation such as elevated CRP, α2-globulins, monocytes, and lymphocytes as well as for clinical evidence of infectious or inflammatory disease. This was done in particular to minimize the possibility, although not proven, that a different quantitative protein expression between mononuclear cell subtypes due, for example, to a fluctuation of the number of the different mononuclear cell subtypes, could influence the protein expression of the OxSt-related proteins considered in our study.

The patients' blood pressure ranged from 134/84 to 154/92 mm Hg, and antihypertensive treatment included calcium channel blockers, angiotensin converting enzyme inhibitors, and α-blockers. All patients were under erythropoietin (EPO) treatment at the average dose of 8000 U/week at the beginning of the study ranging from 4000 to 16 000 U. During the study, the patients' EPO dose was adjusted to maintain stable hemoglobin levels between 11 and 13 g/dL. Vitamin D, PO_4 binders, and calcium supplements were also present in the therapeutic regimen for some patients. In particular, three patients were under sevelamer HCl (3200–4000 mg/day), five under calcium carbonate (2500–3000 mg/day), and two under lanthanum carbonate (2250 mg/day). None of the patients were under lipid-lowering treatment; all patients were treated with supplements of folic acid (10 mg) after dialysis session with no variation throughout the duration of the study. There was no significant difference in terms of Kt/V values throughout the study (mean Kt/V ratio at the beginning of the study was 1.42 ± 0.08; at 6 months: 1.47 ± 0.07; at 12 months: 1.40 ± 0.08). The study protocol was approved by our institutional authorities, and informed consent was obtained from all the study participants.

MOLECULAR BIOLOGY ASSAYS

Preparation of mononuclear cells

Peripheral blood mononuclear cells were isolated by Ficoll-Paque PLUS gradient (Amersham Biosciences, Uppsala, Sweden) from 35 mL of ethylenediaminetetraacetic acid (EDTA) anticoagulated blood.

Western blot

$p22^{phox}$, HO-1, and PAI-1 protein expression were assessed using Western blot analysis, as previously reported (17,18). In brief, total protein extracts were obtained by cell lysis with an ice-cold buffer (Tris-HCl 20 mM, NaCl 150 mM, EDTA 5.0 mM, Niaproof 1.5%, Na_3VO_4 1.0 mM, sodium dodecyl sulfate 0.1%) added with protease inhibitors (complete protease inhibitor cocktail, Roche Diagnostics, Mannheim, Germany). Protein concentration was evaluated by bichinconinic acid assay (BCA protein assay, Pierce, Rockford, IL, USA). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences), and blocked overnight with no-fat milk (5% in Tween-PBS). Membranes were probed with primary polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz,

CA, USA) and then horse radish peroxidase (HRP) conjugated secondary antibodies (Amersham Biosciences) were added, and immunoreactive proteins were visualized with chemiluminescence using SuperSignal WestPico Chemiluminescent Substrate (Pierce). Protein expression on Western blots was quantified using a PC-based densitometric semiquantitative analysis using NIH image software (Research Services Branch, National Institutes of Health, Bethesda, MD, USA), as previously reported (17,18), and were normalized to GADPH, a housekeeping gene.

Analysis of ERK1/2 phosphorylation

ERK1/2 phosphorylation was performed using Western blot analysis.

Total protein extract was obtained by lysis of mononuclear cells as previously reported (17,18). Total protein extract was obtained, electrophoresed, and blotted as previously described (19,20). The membranes were incubated overnight with antiphospho-ERK1/2 (Cell Signaling, Danvers, MA, USA) and anti-GAPDH (Chemicon International, Temecula, CA, USA). Specific secondary antibodies were HRP-conjugated (Amersham Biosciences), and immunoreactive proteins were visualized with chemiluminescence using SuperSignal WestPico Chemiluminescent Substrate (Pierce).

ERK1/2 phosphorylation was quantified using a densitometric semiquantitative analysis using NIH image software. The ratios between phospho-ERK1/2 and GAPDH were used as indexes of ERK1/2 activation.

OxLDL measurement

Blood OxLDL levels were determined using a commercially available enzyme-linked immunosorbent assay-based kit (Immundiagnostik AG, Bensheim, Germany). Intra-assay and inter-assay variations of the assay were 5 and 8%, respectively.

Carotid artery IMT determination

Carotid ultrasound examinations were performed using the Aspen Advanced Ultrasound System (Acuson, Malvern, PA, USA) equipped with a linear probe (7–10 MHz). The procedure was carried out according to the Mannheim IMT consensus (21). All subjects were examined in the same room in dim light, lying comfortably in a supine position. The right and left carotid arteries of each subject were examined by the same sonographer. Once an optimal longitudinal image was obtained, it was stored on 1/2-inch super VHS videotape. Images were analyzed using a high-resolution video recorder, coupled with a mouse-driven image analysis system. IMT, defined as the distance between the lumen–intima and the media–adventitia interfaces, was measured at end diastole in the far wall of the right and left sides of the common carotid artery, the bulb, and the internal carotid artery (22). IMT measurements were expressed as cumulative mean of mean IMT recorded in each vascular segment. To rule out potential interference of arterial enlargement with IMT measurements, the intraluminal diameter of common carotid artery 1 cm proximal to the dilatation of the bulb was measured at end diastole in lateral projection.

Statistical analysis

Data were evaluated on a Power Macintosh G5 computer (Apple Computer, Cupertino, CA, USA) using the Statview II statistical package (BrainPower, Inc., Calabasas, CA, USA). Data are expressed as mean ± SD and analyzed using analysis of variance. Values at a 5% level or less ($P < 0.05$) were considered significant.

RESULTS

Two patients enrolled in the study dropped out due to the development of bacterial pneumonitis and vascular access thrombosis, respectively.

CRP and α2-globulin levels did not show any significant difference at 6 and 12 months compared with baseline (CRP: baseline 3.5 ± 1.7 mg/L, 6 months 3.8 ± 1.9, 12 months 3.6 ± 1.8, P = not significant [ns]; α2-globulin: baseline 9.3 ± 0.8%, 6 months 9.1 ± 0.6, 12 months 9.2 ± 0.7, P = ns).

Monocytes and lymphocytes number also did not change throughout the study (monocytes: baseline 6.8 ± 2.0%, 6 months 6.8 ± 2.5, 12 months 6.6 ± 2.3, P = ns; lymphocytes: baseline 22.0 ± 5.6%, 6 months 21.6 ± 6.0, 12 months 21.2 ± 6.0, P =ns). None of the study participants had clinical evidence of infectious or inflammatory disease throughout the study. There was no need for significant variations in the patients' EPO dose throughout the study, and hemoglobin levels of our patients remained stable ranging between 11 and 13 g/dL throughout the study.

Figure 1 shows the responses of OxSt-related proteins $p22^{phox}$ (panel A) and HO-1 (panel B) to dialysis treatment with the vitamin E-coated membrane.

Treatment with the vitamin E-coated membrane significantly reduced $p22^{phox}$ levels (baseline: 1.4 ± 0.27, 6 months 0.86 ± 0.57, and 12 months 0.75 ± 0.65, $P = 0.038$), while HO-1 increased (baseline: 0.35 ± 0.28, 6 months 0.57 ± 0.3, and 12 months 0.94 ± 0.65, $P = 0.023$).

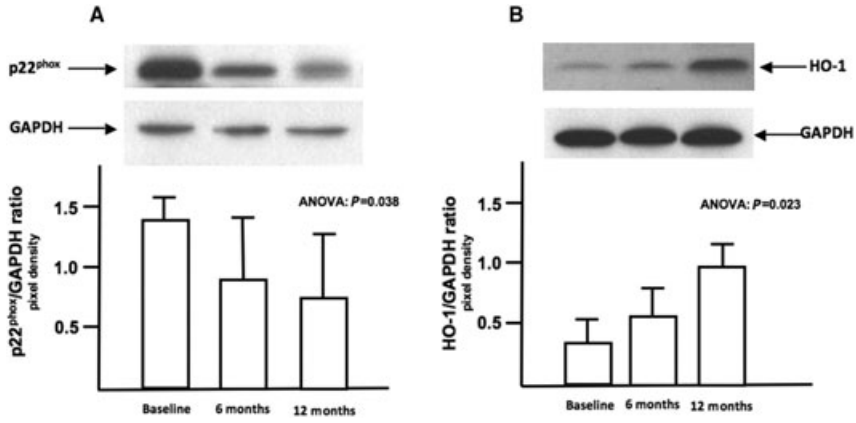

FIG. 1. Densitometric analysis of $p22^{phox}$ (panel A) and HO-1 (panel B) protein expression in mononuclear cells of dialysis patients treated for 1 year with vitamin E-coated dialyzer. The top part of each panel shows representative $p22^{phox}$ (panel A) and HO-1 (panel B) Western blots. ANOVA, analysis of variance.

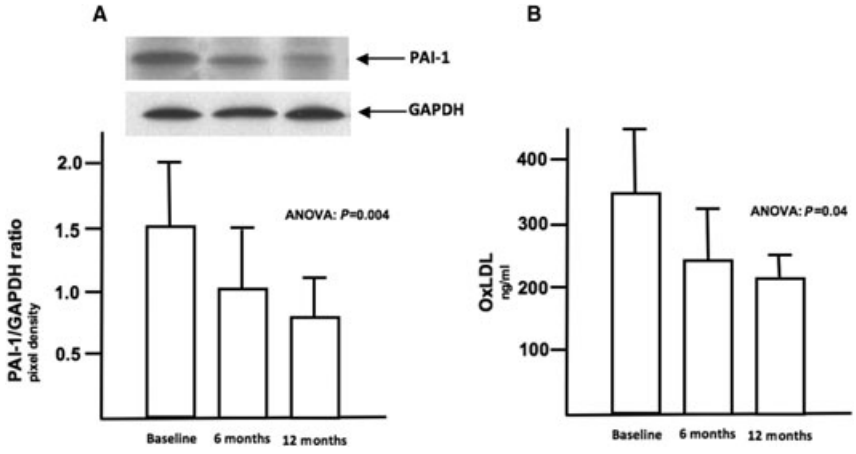

FIG. 2. Densitometric analysis of PAI-1 protein expression in mononuclear cells (panel A) and plasma level of OxLDL (panel B) of dialysis patients treated for 1 year with vitamin E-coated dialyzer. The top part of panel A shows representative PAI-1 Western blots. ANOVA, analysis of variance.

Figure 2 shows the responses of vitamin E-coated membrane treatment on PAI-1 (panel A) and OxLDL (panel B) protein expression.

PAI-1 protein expression (baseline: 1.54 ± 0.55, 6 months 1.14 ± 0.46, and 12 months 0.81 ± 0.30, $P = 0.004$) and OxLDL level (baseline: 373.7 ± 101.3, 6 months 250.7 ± 95.0, 12 months 222.1 ± 45.2 ng/mL, $P = 0.04$) were significantly reduced by the dialysis treatment using vitamin E-coated membrane.

Figure 3 shows the responses in terms of OxSt- and inflammation-related signaling via ERK of dialysis treatment using the vitamin E-coated membrane.

Dialysis treatment with the vitamin E-coated membrane significantly reduced phosphorylated extracellular signal-regulated kinase (pERK)/GADPH ratio (baseline: 4.6 ± 0.98, 6 months 3.72 ± 0.75, 12 months 2.72 ± 1.03, $P = 0.031$).

Evaluation of IMT in the subgroup of randomly selected patients showed, although not reaching statistical significance, a trend toward the reduction, as an effect of treatment with the vitamin E-coated membrane between baseline and 12-month time

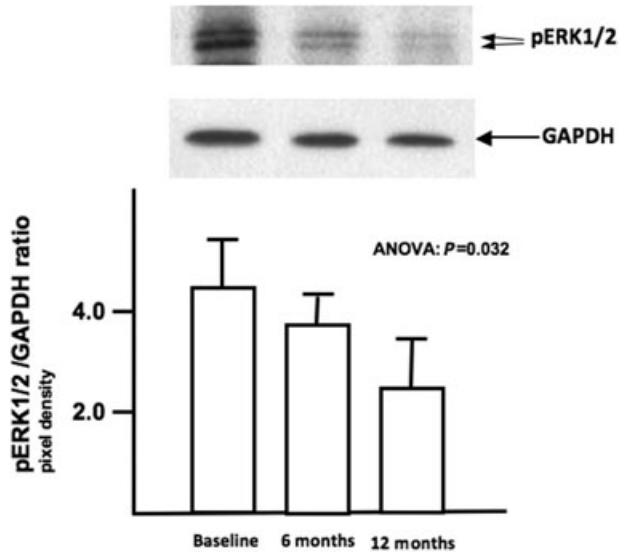

FIG. 3. Densitometric analysis of the ratio of pERK to GAPDH in mononuclear cells of patients treated for 1 year with vitamin E-coated dialyzer. The top of the figure shows a representative pERK1/2 Western blots.

periods (1.32 ± 0.56 mm vs. 1.26 ± 0.52 mm, respectively; $P = 0.064$, ns).

DISCUSSION

In recent years, the quality and technology employed in dialysis have undoubtedly improved regarding the biocompatibility of materials used and the ability to remove uremic toxins (17,18,23). Walker and collaborators (24) showed that switching from a cellulose acetate membrane to a polysulfone membrane reduces the level of protein oxidation. Another recent development is the use of vitamin E as a coating for membrane dialyzers. Vitamin E has antioxidant activity against free radicals and suppresses platelet adhesion and aggregation. The use of vitamin E as a surface layer thereby provides increased antioxidant properties to these dialysis filters as previously demonstrated by studies with vitamin E originally coated on acetate membrane that have reported reduction of OxSt (7), lipid peroxidation (8,9), and leukocyte activation (10).

VitabranE ViE combines the antioxidant and antithrombotic activities of α-tocopherol with the biocompatibility of polysulfone membranes, which have excellent properties for clearance and permeability (25).

This study examined the levels of proteins relevant to OxSt such as $p22^{phox}$, PAI-1, and HO-1, and assessed the state of phosphorylation of ERK, an OxSt-induced effector protein for cardiovascular remodeling, and a plasma marker of OxSt, the level of OxLDL, considered as crucial in the development of chronic inflammation of the artery wall. In addition to biochemical and molecular markers, vascular remodeling was measured in a subgroup of patients by determining carotid IMT using ultrasound at the beginning and at the end of the study.

The $p22^{phox}$ is a 22-kDa subunit of cytochrome b558 of the NADH/NADPH oxidase present both in leukocytes and in the vascular wall that functions as an integral subunit of the final electron transport from NAD(P)H to heme and molecular oxygen in generating O_2 (11). In hemodialyzed (HD) patients treated with VitabranE, the reduction of $p22^{phox}$ protein level not only suggests reduced OxSt, but also, given its presence in leukocytes, an inhibition of leukocyte activation, a very known cause of OxSt in ESRD. As a consequence, this reduction of $p22^{phox}$ protein level therefore suggests an inhibition of OxSt-mediated signaling mechanisms known as responsible for vascular remodeling and atherogenesis induced by ESRD (2,16,26).

While originally studied as part of the regulation of fibrinolysis, PAI-1 is now widely recognized as part of the OxSt-related response (13). Reactive oxygen species (ROS) induces PAI-1 gene expression, and this can be blocked by antioxidants and ROS-scavenging enzymes (13). The ROS-mediated signaling pathway leading to PAI-1 induction involves MAPK/PKB (13), RhoA/Rho kinase (27), and at least three transcription factors such as NF-kB, AP-1, and SP1 (13). Moreover, PAI-1 production has been linked to inflammatory cytokines such as interleukin-1 and tumor necrosis factor-α, which promote vascular inflammation and atherosclerosis (28). In addition, OxLDL, the first step of the atherogenetic process, has been shown to induce PAI-1 expression (29). Therefore, in our patients treated with VitabranE, the reduction of PAI-1 protein levels suggests reduced OxSt and reduced atherothrombogenesis. The results showing that VitabranE-treated patients had lower levels of OxLDL, another indicator of OxSt and cardiovascular risk factor (30), further strengthen the argument that VitabranE reduces OxSt in these HD patients. The concomitant reductions seen in both PAI-1 and OxLDL, both keys for the induction of atherosclerotic cardiovascular disease, upon VitabranE treatment, provide strong evidence for its beneficial effect on OxSt and cardiovascular risk.

HO-1 acts on heme, producing CO and biliverdin (14), which is further metabolized to bilirubin, a potent antioxidant itself (31). There are three different isoforms of HO: HO-1, HO-2, and HO-3. HO-1 has a very low basal expression, but it increases rapidly upon OxSt, while HO-2 and the recently identified isoenzyme HO-3 are constitutively expressed. HO-1-mediated production of the vasodilator CO may contribute to the regulation of vascular tone and thereby blood pressure and endothelial function (14). Finally, HO-1 has been shown to have long-term anti-inflammatory and antiproliferative effect (32). Therefore, the increase in HO-1 noted upon VitabranE treatment further strengthens the evidence for its beneficial effect on OxSt and cardiovascular risk.

The intracellular signaling mediated by ERK is closely linked to OxSt. It represents an important effector for the processes that ultimately lead to the cardiovascular remodeling and atherosclerosis. ERK1/2, a member of the MAPK family, elicits a hypertrophic response via phosphorylation of nuclear targets (e.g., c-myc, c-jun, and ATF-2), leading to transcriptional reprogramming and the altered gene expression associated with hypertrophy (33). The intracellular signaling through members of the superfamily of MAPK including ERK has been strongly linked to cardiovascular hypertrophic response (33). ERK has also been linked to the

induction of PAI-1 mediated by ROS (33). The reduction in the level of phosphorylation of ERK, as demonstrated in patients treated with VitabranE, further underlines the pleiotropic nature of the beneficial effect of this particular dialysis filter on OxSt and inflammation.

The carotid IMT is used as an indicator of atherosclerosis and coronary atherosclerosis in general in patients on dialysis. It has been shown that carotid IMT is an independent predictor of cardiovascular death in dialysis patients, suggesting that the average thickness measurement of carotid IMT may be useful for predicting the mortality of long-term dialysis (34–36). In addition, a 0.1-mm increase in IMT was shown to predict a 24% higher risk for cardiovascular death in dialysis patients (33), and Ekart and coworkers (36) showed that during a 42.4 ± 19.5 month follow-up of 99 nondiabetic hemodialysis patients, 33.3% died, most of them from cardiovascular causes, and the IMT values of the common carotid arteries were significantly higher in patients who died than in those who survived. Kaplan–Meier survival analysis of these patients showed that those in the first tertile of IMT had significantly better survival than HD patients in the third tertile of IMT.

Our study found in a subgroup of randomly selected patients that treatment with VitabranE after 1 year showed a trend, although not statistically significant, toward reduction of carotid IMT from baseline. Nevertheless, the lack in our study of the evaluation of IMT in a control group of dialysis patients treated with standard polysulfone membrane does not directly allow the conclusion that the trend toward the reduction of or at least the lack of IMT increase we found upon VitabranE treatment is indeed an improvement. However, the lack of IMT increase after 1 year of treatment with VitabranE could at least be considered as positive as it would be expected in consideration of the reductions in the markers of OxSt and inflammation-related proteins observed in our study. Relevant with this issue is, in particular, the reduced pERK, which is an important OxSt-related effector for the induction of proliferative processes leading to cardiovascular remodeling and atherosclerosis.

CONCLUSION

Taken together the results of this study strongly indicate that treatment with VitabranE significantly decreases the expression of proteins and markers relevant to OxSt and inflammation, which are tightly associated with cardiovascular disease. In dialysis patients, therefore, a benefit in terms of cardiovascular protection upon treatment with VitabranE appears highly likely.

REFERENCES

1. Luke RG. Chronic renal failure—a vasculopathic state. *N Engl J Med* 1998;339:841–3.
2. Locatelli F, Canaud B, Eckardt K, Stenvinkel P, Wanner C, Zoccali C. Oxidative stress in end-stage renal disease: an emerging threat to patient outcome. *Nephrol Dial Transplant* 2003;18:1272–80.
3. Massy ZA, Stenvinkel P, Drueke TB. The role of oxidative stress in chronic kidney disease. *Semin Dial* 2009;22:405–8.
4. Stenvinkel P, Heinburger O, Paultre F, et al. Strong associations between malnutrition, inflammation and atherosclerosis in chronic renal failure. *Kidney Int* 1999;55:1899–911.
5. Zimmermann J, Herrlinger S, Pruy A, Metzger T, Wanner C. Inflammation enhances cardiovascular risk and mortality in hemodialysis patients. *Kidney Int* 1999;55:648–58.
6. Foley RN. Clinical epidemiology of cardiac disease in dialysis patients: left ventricular hypertrophy, ischemic heart disease, and cardiac failure. *Semin Dial* 2003;16:111–7.
7. Calò LA, Naso A, Pagnin E, et al. Vitamin E-coated dialyzers reduce oxidative stress related proteins and markers in hemodialysis—a molecular biological approach. *Clin Nephrol* 2004;62:355–61.
8. Westhuyzen J, Saltissi D, Stanbury V. Oxidative stress and erythrocyte integrity in end-stage renal failure patients hemodialysed using a vitamin E-modified membrane. *Ann Clin Lab Sci* 2003;33:3–10.
9. Sommerburg O, Sostmann K, Grune T, Ehrich JHH. Oxidative stress in hemodialysis patients treated with a dialysis membrane which has alpha-tocopherol bonded to its surface. *Biofactors* 1999;10:121–4.
10. Pertosa G, Grandaliano G, Soccio M, Martino C, Gesualdo L, Schena FP. Vitamin E-modified filters modulate Jun N-terminal kinase activation in peripheral blood mononuclear cells. *Kidney Int* 2002;62:602–10.
11. Griendling KK, Soresen D, Ushio-Fukai M. NAD(P)H oxidase. Role in cardiovascular biology and disease. *Circ Res* 2000;86:494–501.
12. Babior BM. NADPH oxidase: an update. *Blood* 1999;93:1464–76.
13. Dimova EY, Samoylenko A, Kietzmann T. Oxidative stress and hypoxia: implications for plasminogen activator inhibitor-1 expression. *Antioxid Redox Signal* 2004;6:777–91.
14. Maines MD. The heme oxygenase system: a regulator of second messenger gases. *Annu Rev Pharmacol Toxicol* 1997; 37:517–54.
15. McCubrey JA, Lahair MM, Franklin RA. Reactive oxygen species-induced activation of the MAP kinase signaling pathways. *Antioxid Redox Signal* 2006;8:1775–89.
16. Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. *Am J Cardiol* 2003;91:7–11.
17. Calò LA, Naso A, Carraro G, et al. Effect of haemodiafiltration with online regeneration of ultrafiltrate on oxidative stress in dialysis patients. *Nephrol Dial Transplant* 2007;22: 1413–9.
18. Calò LA, Naso A, Davis PA, et al. Hemodiafiltration with on-line regeneration of ultrafiltrate: effect on HO-1 and iNOS and implication for oxidative stress and inflammation. *Artif Organs* 2011;35:183–7.
19. Calò LA, Pagnin E, Ceolotto G, et al. Silencing regulator of G protein signaling-2 (RGS-2) increases angiotensin II signaling: insights into hypertension from findings in Bartter's/ Gitelman's syndromes. *J Hypertens* 2008;26:938–45.

20. Calò LA, Schiavo S, Davis PA, et al. Angiotensin II signaling via type 2 receptors in a human model of vascular hyporeactivity: implications for hypertension. *J Hypertens* 2010;28: 111–8.
21. Touboul PJ, Hennerici MG, Meairs S, et al. Mannheim intima-media thickness consensus. *Cerebrovasc Dis* 2004;18:346–9.
22. Pauletto P, Palatini P, Da Ros S, et al. Factors underlying the increase in carotid intima-media thickness in borderline hypertensives. *Arterioscler Thromb Vasc Biol* 1999;19:1231–7.
23. Filiopoulos V, Hadjiyannakos D, Metaxaki P, et al. Inflammation and oxidative stress in patients on hemodiafiltration. *Am J Nephrol* 2008;28:949–57.
24. Walker RJ, Sutherland WH, De Jong SA. Effect of changing from a cellulose acetate to a polysulfone dialysis membrane on protein oxidation and inflammation markers. *Clin Nephrol* 2004;61:198–206.
25. Sasaki M. Development of vitamin E-modified polysulfone membrane dialyzers. *J Artif Organs* 2006;9:50–60.
26. Dhalla NS, Temsha RM, Netticadan T. Role of oxidative stress in cardiovascular diseases. *J Hypertens* 2000;18:655–73.
27. Takeda K, Ichiki T, Tokunou T, et al. Critical role of Rho-kinase and MEK-ERK pathways for angiotensin II-induced plasminogen activator inhibitor type-1 gene expression. *Arterioscler Thromb Vasc Biol* 2001;21:868–73.
28. Vaughan DE. PAI-1 and atherothrombosis. *J Thromb Haemost* 2005;3:1879–83.
29. Dichtl W, Stiko A, Eriksson P, et al. Oxidized LDL and lysophosphatidylcholine stimulate plasminogen activator inhibitor-1 expression in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 1999;19:3025–32.
30. Meisinger C, Baumert J, Khuseyinova N, Loewel H, Koenig W. Plasma oxidized low-density lipoprotein, a strong predictor for acute coronary heart disease events in apparently healthy, middle aged men from the general population. *Circulation* 2005;112:651–7.
31. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987;235:1043–6.
32. Immenschuh S, Ramadori G. Gene regulation of heme oxygenase-1 as a therapeutic target. *Biochem Pharmacol* 2000;60:1121–8.
33. Kim S, Iwao H. Molecular and cellular mechanisms of Ang II-mediated cardiovascular and renal diseases. *Pharmacol Rev* 2000;52:11–34.
34. Benedetto FA, Mallamaci F, Tripepi G, Zoccali C. Prognostic value of ultrasonographic measurement of carotid intima media thickness in dialysis patients. *J Am Soc Nephrol* 2001;12:2458–64.
35. Kato A, Takita T, Maruyama Y, Kumagai H, Hishida A. Impact of carotid atherosclerosis on long-term mortality in chronic hemodialysis patients. *Kidney Int* 2003;64:1472–9.
36. Ekart R, Hojs R, Hojs-Fabjan T, Balon BP. Predictive value of carotid intima media thickness in hemodialysis patients. *Artif Organs* 2005;29:615–9.