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High cut-off dialysis in chronic haemodialysis patients reduces serum procalcific activity

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ABSTRACT

Background. Vascular calcification is enhanced in chronic dialysis patients, possibly due to the insufficient removal of various intermediate molecular weight uraemic toxins such as interleukins with conventional membranes. In this study, we assessed the modulation of *in vitro* vascular calcification with the use of high cut-off (HCO) membranes in chronic dialysis patients.

Methods. In a PERCI trial, 43 chronic dialysis patients were treated with conventional high-flux and HCO filters for 3 weeks in a randomized order following a 2-period crossover design. After each phase, serum predialysis samples were drawn. Calcifying human coronary vascular smooth muscle cells (VSMCs) were incubated with the patient's serum samples. Calcification was assessed with alkaline phosphatase (ALP) and alizarin red staining. In the clinical trial, HCO dialysis reduced the serum levels of the soluble tumour necrosis factor receptor (sTNFR) 1 and 2, vascular cell adhesion molecule 1 (VCAM-1) and soluble interleukin-2 receptor (sIL2R). We therefore investigated the *in vitro* effects of these mediators on vascular calcification.

Results. VSMCs incubated with HCO dialysis serum showed a 26% reduction of ALP with HCO serum compared with high-flux serum. Alizarin was 43% lower after incubation with the HCO serum compared with the high-flux serum. While sIL2R and sTNFR 1 and 2 showed no effects on VSMC calcification, VCAM-1 caused a dose-dependent enhancement of calcification.

Conclusions. The use of HCO dialysis membranes in chronic dialysis patients reduces the procalcific effects of serum on VSMC *in vitro*. The mechanisms of the strong effect of HCO on *in vitro* calcification are not completely understood. One factor may be lower levels of VCAM-1 in HCO serum samples, since VCAM-1 was able to induce vascular calcification in our experiments. Neither sTNFR 1, sTNFR 2 nor sIL2R enhance vascular calcification *in vitro*. Regardless of the mechanisms, our results encourage further studies of highly permeable filters in chronic dialysis patients.

Keywords: atherosclerosis, chronic haemodialysis, coronary artery disease, inflammation, vascular calcification

INTRODUCTION

Cardiovascular mortality is greatly increased in chronic dialysis patients [1, 2]. Arteriosclerosis with vascular calcification is the main underlying pathology, and is in part due to the insufficient removal of pro-inflammatory interleukins with conventional haemodialysis membranes [3]. Uraemic serum is capable of inducing vascular calcification *in vitro* [4, 5].

Vascular calcification in dialysis patients is characterized by extensive calcification of the media of arteries resembling the process of bone formation. Vascular smooth muscle cells (VSMCs) play an active role in this process and differentiate into osteoblast-like cells. They soon express bone-specific genes like *runx2* and *MSX2*, resulting in extraosseous, intraarterial bone formation. VSMCs go into apoptosis and form calcified apoptotic bodies.

The phenomenon of vascular calcification is a highly regulated process with several protective and promoting factors on both sides.

Fetuin-A is a serum protein that inhibits precipitation of calcium and phosphate and protects VSMCs from calcification [6]. The Matrix Gla protein is another important calcification inhibitor that is widely expressed and acts as a potent inhibitor of VSMC calcification. Among other effects, the

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Matrix Gla protein inhibits VSMCs from differentiating into osteoblasts-like cells [7] and inhibits the calcification of arteries [8].

Numerous other calcification inhibitors like Osteopontin [9], Osteoprotegerin [10] and Pyrophosphate [11] have been described with different mechanisms of action, all resulting in the reduced calcification of VSMCs.

On the other hand, there are numerous factors that are known to promote vascular calcification.

Disturbed mineral homeostasis with elevated levels of both phosphate and calcium is a common feature in dialysis patients and correlates with mortality in the chronic kidney disease (CKD) population. Also, phosphate and calcium are capable of enhancing the calcification of VSMCs *in vitro*.

Warfarin effectively inhibits vitamin K-dependent activation of the Matrix Gla protein, and thus enhances calcification [12] and is known to induce calciphylaxis [13].

Furthermore, several proinflammatory interleukins such as tumour necrosis factor (TNF) and interleukin (IL)-6 that are elevated in dialysis patients are known to enhance vascular calcification *in vitro* via direct and indirect mechanisms [14, 15].

Accordingly, the serum levels of these interleukins are predictors of cardiovascular mortality in dialysis patients [16–21].

Since the initiation of dialysis therapy, efforts have been made to reduce the inflammatory levels of CKD patients. The switch from cuprophane dialyzers to more bio-compatible material and the lowering of endotoxin concentration in dialysate are both aimed at a reduction of interleukin production and have made tremendous contributions to the lowering of inflammation in dialysis patients. However, in recent years the enhanced clearance of interleukins with special dialysers has come into the focus of interest.

A new dialysis membrane called a high cut-off (HCO) membrane has been created, allowing the elimination of molecules with a size of up to 45 kDa [22, 23]. These membranes are already used in patients suffering from multiple myeloma in order to remove free light chains [24, 25], and have been shown to remove Beta-2-microglobulin from blood [26]. In the PERCI clinical trial, 43 chronic dialysis patients were treated with HCO membranes. In this trial, the question of whether the use of these HCO membranes modulates inflammation *in vivo* was addressed. While the primary endpoint CD 162 on the monocytes was not altered, markers of inflammation such as vascular cell adhesion molecule 1 (VCAM-1), soluble IL-2-receptor (sIL2R) and soluble TNF receptor (sTNFR) 1 and 2 were lowered significantly after 3 weeks of HCO dialysis [27]. In the current work, we assessed the question of whether the use of HCO membranes in chronic dialysis patients modulates the process of vascular calcification *in vitro*.

MATERIALS AND METHODS

Study design

The clinical trial was conducted as described previously [27]. In brief, 43 chronic dialysis patients were included and dialysed with HCO and regular high-flux membranes in a randomized order following a two-period crossover design. During the trial, every patient was dialysed with each type of membrane (single use) for 3 weeks, with run-in and wash-out phases of 2 weeks in between. Thus, after the trial two samples of every patient who had finished the trial were available: one after 3 weeks of HCO dialysis and one after 3 weeks of high-flux dialysis.

Clinical samples

Serum samples were drawn prior to the first dialysis session after the weekend, centrifuged at 2000 *g* for 15 min and then stored at −80°C. The serum samples of two patients were omitted due to a history of hepatitis. The samples of all other patients who completed the trial were used in cell culture experiments.

Vascular smooth muscle cells

Human VSCMs were purchased from Lifeline Cell Technology (Frederick, MD, USA). All cells used in these experiments were isolated from one deceased donor (male, African American, 18 years old), were characterized as VSMC with Alpha-SMA antibodies but were not immortalized. Cells were used from passages 2–6.

Induction of calcification

For the induction of calcification an ossification medium (OM) was used containing 1.58 mM/L calcium gluconate, 0.1 μM/L dexamethasone, 0.5 mM/L ascorbic acid and 2.5 mM/L beta glycerol phosphate, which were added to a conventional medium (Lifeline Vasculife Medium for VSMCs, Cell Cystems, Troisdorf, Germany). This OM was supplemented with 5% of the serum samples from the clinical trial. For the control medium, 5% of foetal calf serum (FCS; Biowest, Nuaillé, France) was used instead. The medium was changed three times each week.

Determination of calcification

We used two different methods to assess and quantify vascular calcification. Alkaline phosphatase (ALP) activity measurements were performed after an incubation period of 7 days with a commercially purchased kit (Sigma Aldrich, St Louis, MO, USA), according to the manufacturer's protocol. We also used the alizarin red (AZR) staining method (Sigma Aldrich) and performed the measurement according to the protocol after 10 days of incubation [28].

Cell viability

Cell viability was measured indirectly using the mitochondrial activity via water soluble tetrazolium salt (WST-8) assay (PromoCell GmbH, Heidelberg, Germany) as a marker of viable cell number. The WST-8 assay was performed according to the manufacturer's protocol and used to normalize the activity of the calcification markers [29].

Assessment of the calcifying effect of defined substances

The VSMCs were incubated with OM and FCS and the respective protein whose effect we wanted to examine. sIL2R and sTNFR 1 were purchased from R&D Systems (Minneapolis, MN, USA). sTNFR 2 was purchased from Life Technologies (Carlsbad, CA, USA), VCAM-1 was purchased from PeproTech

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GmbH (Rocky Hill, NJ, USA) and TNF-α was purchased from Promocell GmbH (Heidelberg, Germany). The medium was changed three times a week as well. Again, calcification was analysed after 7 days (ALP) and 10 days (AZR).

Statistical analysis was performed using GraphPad PRISM 6.

RESULTS

Incubation of vascular smooth muscle cells with serum samples from the PERCI trial

Serum samples drawn after 3 weeks of HCO dialysis induced 21% less calcification measured via ALP activity compared with the high-flux serum (high-flux 1.01, SEM 0.06625 versus HCO 0.7431, SEM 0.05534; $P < 0.0001$, paired *t*-test; see Figure 1A).

Furthermore, we could show that the HCO sample of every single patient induced less ALP activity compared with its high-flux counterpart (see Figure 1B). Thus, the observed effects were consistent and inter-individual variation was low.

Accordingly, alizarin staining showed significantly less intensity in the cells incubated with HCO serum samples (high-flux 0.3662, SEM 0.01859 versus HCO 0.2057, SEM 0.0105; $P < 0.0001$, paired *t*-test; see Figure 1C). Calcification was 44% less with HCO samples compared with the high-flux samples using AZR staining. This effect was consistent in all patients; the HCO sample of every single patient showed less calcification compared with its high-flux counterpart (see Figure 1D).

These results led us to the question of whether there was a causal relationship between the parameters lowered during the clinical trial and the reduction of vascular calcification *in vitro*.

As published earlier [27], the serum proteins that were significantly reduced by HCO dialysis were sTNFR 1, sTNFR 2, sIL2R and soluble VCAMs. For purposes of clarity, these results from the clinical trial are included in this publication as well (see Table 1).

Therefore, we conducted further experiments to address the question of whether one of these serum parameters in the HCO samples may have induced stronger calcification in the high-flux samples.

Thus, to examine a possible connection between the reduction of these serum proteins and the reduction of *in vitro* calcification we incubated VSMCs with calcification media and added sTNFR 1, sTNFR 2, VCAM-1 and sIL2R, as well as TNF-α, and assessed the calcification. We chose concentrations that were in the broad range or well above the serum concentrations determined in the clinical trial.

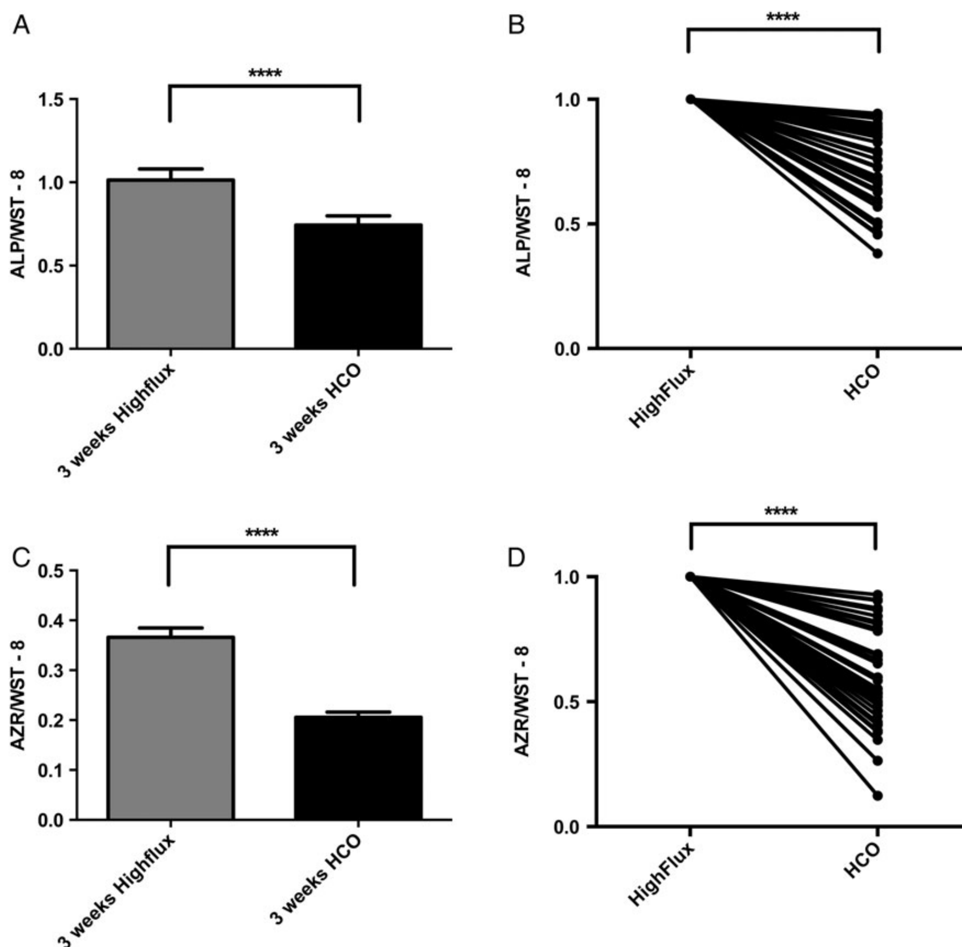

FIGURE 1: (**A**) Ratio of ALP/WST-8 after 7 days; $n = 37$, ****$P < 0.0001$, error bars are SD. (**B**) Ratio of ALP/WST-8 after 7 days; the HCO serum of every individual patient induces less calcification than the respective high-flux serum of the same patient; $n = 37$, ****$P < 0.0001$. (**C**) Ratio of AZR/WST-8 after 10 days; $n = 37$, ****$P < 0.0001$, error bars are SD. (**D**) Ratio of AZR/WST-8 after 10 days; the HCO serum of every individual patient induces less calcification than the respective high-flux serum of the same patient; $n = 37$, ****$P < 0.0001$.

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Table 1. Serum samples from the clinical trial before and after 3 weeks of high-flux or HCO dialysis

	Before HCO	After 3 weeks HCO (SD)	Before high-flux (SD)	After 3 weeks high-flux (SD)	P
sTNFR 1 (ng/mL)	13.25 (±5.3)	10.28 (±3.2)	13.07 (±5.3)	13.13 (±5.1)	<0.001
sTNFR 2 (ng/mL)	16.13 (±5.1)	14.59 (±4.5)	16.05 (±5.6)	16.06 (±5.2)	<0.01
VCAM-1 (ng/mL)	166.46 (±38.1)	147.12 (±50.7)	161.92 (±45.5)	165.55 (±47.8)	<0.05
sIL2R (pg/mL)	145.51 (±120.0)	113.41 (±126.6)	147.42 (±102.9)	145.05 (±93.6)	<0.001
TNF-α (pg/mL)	19.68 (±5.8)	20.11 (±6.7)	20.4 (±5.5)	20.16 (±6.5)	0.56

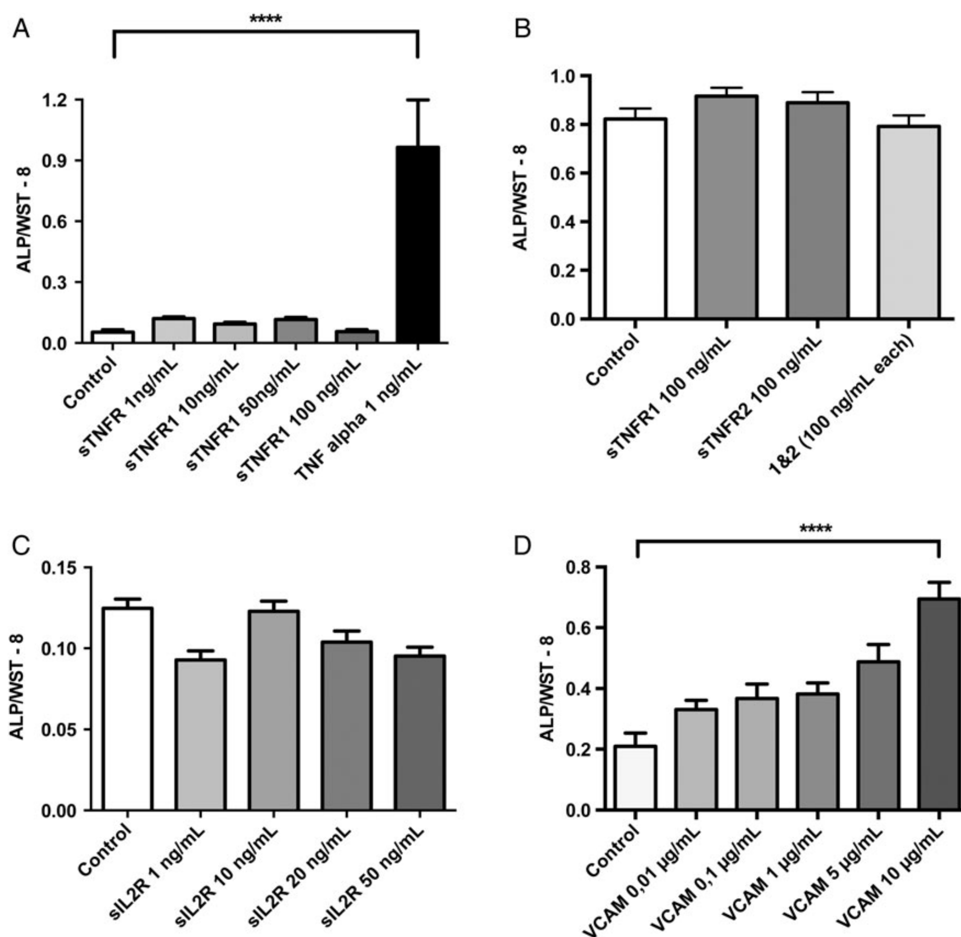

FIGURE 2: (**A**) Ratio of ALP/WST-8 after 7 days; $n = 22$, ****P < 0.0001, error bars are SEM. (**B**) Ratio of ALP/WST-8 after 7 days; $n = 18$, no significant correlation, error bars are SEM. (**C**) Ratio of ALP/WST-8 after 7 days; $n = 18$, no significant correlation, error bars are SEM. (**D**) Ratio of ALP/WST-8 after 7 days; $n = 44$, ****P < 0.0001, error bars are SEM.

TNF-α is known to have a calcifying effect on VSMC [30–33]. We were able to confirm these results in our cell model. The addition of 1 ng/mL TNF-α enhances calcification by a factor of 18 (OM 0.05319, SEM 0.01201; TNF 0.9643, SEM 0.234; see Figure 2A). The sTNFR 1 has been associated with pathways leading to osteogenic differentiation and calcification, and is generally thought to antagonize TNF [34, 35]. However, the observed effects of lowered sTNFR 1 concentrations after HCO dialysis in the clinical trial and lowered *in vitro* calcification with these serum samples led us to the question of whether sTNFR 1 has intrinsic procalcific effects, possibly by acting as a carrier for TNF itself. We therefore analysed whether sTNFR 1 induces vascular calcification *in vitro*. We added sTNFR 1 in ascending doses to the ossification medium. ALP activity was not altered significantly by the addition of sTNFR 1 (1 ng/mL 0.1204, SEM 0.009; 10 ng/mL 0.0932, SEM 0.0095; 50 ng/mL 0.1163, SEM 0.01; 100 ng/mL 0.05, SEM 0.01; see Figure 2A).

Likewise, the incubation of sTNFR 2, a protein involved in inflammation [36, 37], brought no significant change in ALP activity (OM 0.01186, SEM 0.002; 100 mg/mL sTNFR 2 0.009, SEM 0.001; see Figure 2B). Also, the combination of sTNFR 1 and 2 showed no enhancement of ALP activity (see Figure 2B).

Moreover, we analysed the influence of sIL2R, which was also significantly reduced with HCO membranes. This molecule is thought to be a marker of arterial calcification

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[38, 39]. Again, we found no modulation of *in vitro* vascular calcification (OM 0.12, SEM 0.01; OM + FCS + 1 ng sIL2R 0.09, SEM 0.01; OM + FCS + 10 ng/mL sIL2R 0.12, SEM 0.01; OM + FCS + 20 ng/mL sIL2R 0.10, SEM 0.01; OM + FCS + 50 ng/mL sIL2R 0.09, SEM 0.01; see Figure 2C).

Finally, we incubated VSMC with VCAM, which was also reduced significantly after 3 weeks of HCO dialysis during the clinical trial.

VCAM-1 is known to play a role in vascular calcification as a surrogate parameter [40, 41], but there is no evidence as yet for an active role in calcification.

Here, we found a significant and dose-dependent effect on ALP activity reflecting a stronger vascular calcification with VCAM. VCAM-1 10 µg/mL enhanced ALP activity by a factor of 3.3 [OM 0.21, SEM 0.04; OM+VCAM-1 1 µg/mL 0.38, SEM 0.04 ($P = 0.0002$ versus OM); OM+VCAM-1 5 µg/mL 0.48, SEM 0.06 ($P < 0.0001$ versus OM); OM+VCAM-1 10 µg/mL 0.69, SEM 0.05 ($P < 0.0001$ versus OM); see Figure 2D].

DISCUSSION

We demonstrate a significant reduction of *in vitro* calcification by the use of HCO membranes in chronic dialysis patients with regard to ALP and AZR. The observed effects were highly significant and a reduction of calcification was noted with samples from every single patient, underlining the validity of our results. These results alone encourage further investigation to address the question of whether these *in vitro* data can be applied to the process of *in vivo* vascular calcification. Thus, long-term studies using clinical endpoints such as cardiovascular events, changes in the coronary arterium calcium score in cardiac computed tomography scans or pulse wave velocity are justified.

More difficult than the interpretation of these distinct results is the answer to the question of which substances were modulated during dialysis in order to be responsible for such a clear effect on *in vitro* vascular calcification. The mechanism that led to the observed reduction of calcification in our trial is still subject to speculation.

Analysis of numerous inflammatory markers and regulators in the serum samples was performed after the PERCI trial. Several serum parameters were reduced more efficiently with HCO membranes, including substances that are known to induce vascular calcification such as TNF-α. Even though this reduction in TNF-α was not statistically significant compared with the high-flux dialysis, these lower TNF concentrations in the serum samples may provide a possible explanation for the results in our *in vitro* experiments.

Four of the investigated molecules were shown to be significantly lowered with HCO after 3 weeks of HCO dialysis: VCAM-1, sIL2R, sTNFR 1 and 2. However, these four parameters alone are not likely to be responsible for the observed effect of HCO dialysis on *in vitro* calcification. Interpretation of the measured concentrations of serum parameters depends on several factors. Protein-binding, distribution and re-shift from tissue and other factors influence serum concentrations of interleukins, which makes their interpretation difficult. Thus, it is possible that other molecules were eliminated with HCO, which was not detected with the methods used.

To further investigate whether the reduction of these molecules and the *in vitro* vascular calcification are coincidental or linked via a causal relationship, we tested the influence of these molecules on *in vitro* calcification in our cell culture model.

We only analysed the influence of single markers on calcification, and thus can only interpret the results in this context. *In vivo* calcification is a complex and highly regulated process. The combination of different proteins may have a synergistic effect of *in vitro* calcification and needs to be assessed in future experiments.

In ascending concentrations, VCAM-1 induced vascular calcification in a significant manner. Thus, the reduction of VCAM-1 with HCO provides a possible explanation for the lowering of *in vitro* vascular calcification with HCO serum.

VCAM-1 has a size of 96 kDa. Hence, the decline in VCAM-1 concentrations after HCO dialysis cannot be well-explained by the elimination with HCO dialysers. Instead, a reduction of the other inflammatory proteins with secondary down-regulation of VCAM-1 production seems a better explanation of the observed decline in VCAM-1 levels.

In contrast to VCAM, TNF-α is already known to be a potent inducer of vascular calcification, which was confirmed in our model. On the other hand, sTNFR 1 and 2 are generally perceived as TNF antagonists and inhibitors of vascular calcification [42, 43]. However, data on the *in vitro* function of these molecules are sparse.

In our cell culture model, sIL2R, sTNFR 1 and 2 alone had no effect on the process of vascular calcification. Thus, the reduction of the serum concentrations of these molecules alone cannot be used as an explanation for the lowered calcification with HCO.

On the other hand, the significant reduction of sTNFRs may reflect a general lowering of the state of inflammation that was otherwise not measured, possibly due to a lack of statistical power, or perhaps because the critical marker was not measured at all.

Another possible interpretation of these results is that with the use of HCO membranes the degree of chronic inflammation was indeed reduced, but could not be revealed with the chosen markers. Distribution to other compartments of the body besides blood, protein-binding and other factors highly influences free serum interleukin concentrations and makes interpretation difficult.

Possibly, the reduced concentrations of sTNFR 1 and 2 merely reflect a reduced state of chronic inflammation after HCO dialysis that was otherwise not detected with the methods used in this trial. This theory is supported by the fact that HCO serum samples induced markedly reduced TNF mRNA production in monocytes [27].

Another potential explanation for these results provides the speculative theory that with the elimination of sTNFR, TNF-α was also reduced. The ELISA kit used for the analysis of sTNFR concentrations does not to our knowledge reliably discriminate between free sTNFR alone and sTNFR bound to TNF-α. Hence, if the lowered sTNFR concentrations should actually reflect lower sTNFR-TNF compounds, TNF had been cleared and a net reduction of TNF from the body and from the serum samples used in this trial was the result. Since the soluble

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receptor is concentrated 1000-fold higher than TNF itself the free TNF concentrations would be unchanged, and thus even more difficult to interpret.

Finally, the clearance of albumin-bound toxins may serve as a possible yet speculative explanation and shall be mentioned here. Albumin concentrations were significantly lowered with HCO [27, 44], thus albumin-bound toxins may have been cleared more effectively with HCO membranes. Whether albumin-bound uraemic toxins play a role in vascular calcification is broadly unknown.

Limitations

Our study has several limitations. Even though this study was performed in chronic dialysis patients, no clinical endpoint was used to assess *in vivo* vascular calcification. Our data come only from *in vitro* experiments and have to be interpreted as such. Furthermore, we only analysed VSMC calcification in one cell type. In upcoming trials, phenotypic changes of VSMC such as proliferation, migration or apoptosis should be assessed in additional cell lines.

Moreover, the main question of whether the use of highly permeable dialysis membranes leads to a reduction in vascular calcification has to be elucidated in further, longer lasting clinical trials using assessment of clinical endpoints.

In our study, it again became evident that the long-term use of HCO membranes is limited by substantial loss of albumin. As described earlier, albumin loss was indeed significantly higher with the HCO membranes. Thus, before a longer lasting clinical trial with highly permeable dialysis membranes can be conducted, the problem of albumin loss during dialysis needs to be addressed by the development of a membrane restricting albumin filtration.

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CONFLICT OF INTEREST STATEMENT

M.S. is an employee of Gambro Dialysatoren GmbH, Research & Development, Hechingen, Germany. Gambro AB (including all direct and indirect subsidiaries) is part of Baxter International Inc. We declare that the results presented in this paper have not been published previously in whole or part, except in abstract format. Furthermore, we are not aware of any other conflict of interests.

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ORIGINAL ARTICLE

The effects of resistance exercise and oral nutritional supplementation during hemodialysis on indicators of nutritional status and quality of life

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ABSTRACT

Background. Protein-energy wasting (PEW) is common in patients undergoing hemodialysis (HD). Studies have assessed the positive effect of oral nutritional supplementation (ONS) or resistance exercise (RE) on nutritional status (NS) markers in patients undergoing HD.

Methods. The aim of this study was to assess the effect of ONS and RE on NS and the quality of life (QOL) of 36 patients undergoing HD. In a randomized clinical trial, patients were divided into the following two groups: a control group (ONS) that received a can of ONS during their HD sessions and an intervention group (ONS + RE) that received a can of ONS and underwent a 40-min session of RE during their HD sessions. Both interventions lasted 12 weeks. The patients' anthropometric, biochemical, dietetic and bioelectrical impedance measurements as well as their QOL, evaluated using the Kidney Disease Quality of Life Short Form, were recorded.

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