




## ORIGINAL ARTICLE

# Pro-calcifying analysis of uraemic serum from patients treated with medium cut-off membrane in a prospective, cross-over study

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## ABSTRACT

**Background.** The retention of a large number of solutes that are normally excreted or metabolized by the kidney is responsible for the symptoms typical in uraemic patients. These molecules are defined as uraemic toxins and can be classified into three groups: small water-soluble molecules, middle molecules and protein-bound toxins. Recently, efforts were put towards developing dialysis membranes that allow the removal of large middle molecules without clinically relevant albumin loss. These membranes are the medium cut-off (MCO) membranes that allow the removal of middle molecules up to ~50 000 Da.

**Methods.** We performed a prospective, open-label, controlled, cross-over pilot study comparing expanded haemodialysis (HDx) (novel MCO membrane Theranova 400) and conventional haemodialysis (HD) in 20 prevalent HD patients. Ten patients used conventional HD high-flux dialyser and 10 patients used HDx for 3 months; later the patients switched and received the other treatment for a further 3 months. We then analysed the pro-calcifying effect of uraemic serum in a model of high phosphate (Pi)-induced calcification in vascular smooth muscle cells (VSMCs).

**Results.** In this study, every patient was the control of himself and, interestingly, we found a tendency of less pro-calcifying potential from HDx-treated patients' serum compared with HD. Studying pathogenetic processes involved in high Pi-induced calcium deposition, we found that uraemic serum of HDx-treated patients induced less VSMC necrosis compared with uraemic serum of HD patients. Nevertheless, no differences were found between the different dialytic treatments in

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the serum potential to induce apoptosis and to modulate the expression of a panel of genes involved in VSMC simil-osteoblastic differentiation such as bone morphogenetic protein 2, runt-related transcription factor 2, osteocalcin, matrix Gla protein, osteopontin, elastin and collagen I  $\alpha 1$ . In an effort to characterize the difference in uraemic toxin profile during the two different dialytic treatments, we measured a panel of 10 uraemic toxins and 3 precursors, finding a significant increased removal during HDx of 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid, tryptophane and some of its metabolites, such as 3-indoxyl sulphate, indole 3-acetic acid and kynurenine.

**Conclusions.** These preliminary data are promising, although larger patients' groups are needed to better understand the effects of HDx on vascular calcification.

**Keywords:** necrosis, protein-bound uraemic toxins, uraemic serum, vascular calcification

## INTRODUCTION

A myriad of symptoms are classic in end-stage renal disease (ESRD), such as functional system impairments and accelerated disease processes related, at least in part, to the retention of a large number of solutes that are normally excreted or metabolized by the kidney. These molecules, characterized by different molecular weights and radii, are defined as uraemic toxins and can be classified into three groups: small water-soluble molecules, middle molecules and protein-bound toxins. The small water-soluble molecules are most efficiently removed by established dialysis methods that remove solutes in the range of 50–15 000 Da, whereas clearance in the molecular weight range up to 50 000 Da of many middle molecules and protein-bound toxins is limited using current dialysis methods. Haemodiafiltration (HDF) at high volumes (>23 L/session) has produced some results on removal of middle molecules and clinical outcomes, although complex hardware and high blood flows are required. Improved removal has been obtained with high cut-off (HCO) membranes, with albumin loss representing a limitation to their clinical daily application [1]. Recently, efforts were put towards developing dialysis membranes that allow the removal of large middle molecules without clinically relevant albumin loss. These membranes are the medium cut-off (MCO) membranes, which allow the removal of middle molecules up to ~50 000 Da, ameliorating the clearance range provided by HDF. The advantage of MCO membranes is the minimal albumin loss compared with HCO membranes. This lack of albumin loss is possible due to a tighter distribution of pores in MCO compared with HCO membranes. Based on the EuroToxin database, we identified 27 middle molecules with a molecular mass >15 kDa. The serum levels of these middle molecules range from <1.5- to >200-fold higher in patients receiving dialysis or with advanced chronic kidney disease (CKD) compared with those of healthy individuals. The 27 middle molecules can be divided into five groups: cytokines, adipokines, immune-related proteins, growth factors and hormones plus other molecules. MCO membranes, besides improving middle molecules removal, cause a marginal loss of albumin that could be beneficial as it partially results in the clearance of protein-bound uraemic toxins [2].

Patients affected by ESRD have an increased morbidity and mortality due to many complications, mainly represented by cardiovascular diseases. In all the causes that induce cardiovascular diseases, an important role is played by vascular calcification (VC), which modifies arterial pulse wave velocity and pressure, leading to hypertension, left ventricular hypertrophy and heart failure [3]. In ESRD patients, VC affects principally the tunica media and vascular smooth muscle cells (VSMCs) and is due to a plethora of different factors, among which the main

ones are high phosphate levels and uraemic toxins. In fact, *in vitro*, the uraemic milieu in patients' sera is responsible for the exacerbation of high phosphate-induced VC, demonstrating a deleterious interplay between these factors [4]. Phosphate stimulates VSMCs to deposit calcium in the extracellular matrix. In this process, VSMCs lose muscular markers and start to express an osteoblastic phenotype, behaving as simil-osteoblasts [5]. Besides the trans-differentiation, high phosphate promotes calcification by causing apoptosis, with the release of apoptotic bodies loaded with calcium that work as VSMC calcification machinery [6]. Another process that induces calcification exacerbation is necrosis, as the massive release of calcium after rupture of the cellular membrane triggers and participates in calcium deposition.

Since high-phosphate VSMC calcification is a well-established *in vitro* model to study calcification, we harvested serum from dialysis patients treated with MCO or conventional bicarbonate haemodialysis (HD) and investigated whether different dialysis treatments could have an impact on calcium deposition in this model. We then tried to characterize uraemic serum composition to find which are the components differentially removed by the dialysis treatment that could play a role in the modulation of serum pro-calcifying effect.

## MATERIALS AND METHODS

### Materials

Dialysis materials for all treatments were provided by Fresenius Medical Care (Bad Homburg, Germany). A novel membrane, Theranova400 (Baxter, Deerfield, IL, USA) was used for patients undergoing expanded HD (HDx), whereas various other membranes (FX8, FX10, FX80, FX100, BK1.6 and BG2.1), based on clinical needs, were used in patients undergoing bicarbonate dialysis. Dulbecco's Modified Eagle Medium

Dulbecco's modified Eagle medium [DMEM; high glucose (4–5 g/L)], sodium chloride (NaCl), foetal bovine serum (FBS) and Pierce bicinchoninic acid (BCA) protein assay kit were purchased from Euroclone (Milan, Italy). Taqman gene assay for bone morphogenetic protein 2 (BMP2), runt-related transcription factor 2 (RUNX2), osteopontin (OPN), osteocalcin (OC), matrix Gla protein (MGP), elastin (Eln), collagen I  $\alpha 1$  (CollI $\alpha 1$ ), microRNAs (miRNAs) and the housekeeping genes  $\beta$ -actin and miRNA 16-5p and all reagents for gene expression assays were from Applied Biosystem (Milan, Italy). The alpha 1 acid glycoprotein enzyme-linked immunosorbent assay (ELISA) kit (ab243675) was from Abcam (Cambridge, UK); the cell death detection ELISA plus kit was from Sigma Aldrich (St. Louis, MO, USA). Unless stated otherwise, all the other reagents were obtained from Sigma Aldrich (St. Louis, MO, USA). Indoxyl

sulphate (IS), indole-3-acetic acid, hippuric acid, kynurenic acid, phenylalanine, trimethylamine-N-oxide (TMAO), kynurenine, indole-3-acetic acid-d5, tyrosine and tryptophan were purchased from Sigma Aldrich (St Quentin Fallavier, France). Kynurenine-d4, phenylacetyl-L-glutamine, phenylacetyl-L-glutamine-d5, *p*-cresyl-sulphate, *p*-cresyl-sulphate-d7, hippuric acid-d5, *p*-cresyl glucuronide, *p*-cresyl glucuronide-d7, TMAO-d9, tyrosine-d4, tryptophan-d5, phenylalanine-d5, kynurenic acid-d5, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), CMPF-d3 and IS-d4 were purchased from Toronto Research Chemicals (North York, ON, Canada). Methanol, water and acetonitrile were liquid chromatography–mass spectrometry (LC-MS) grade and were purchased from Fisher Scientific (Illkirch, France). Sodium dihydrogen phosphate and sodium chloride were purchased from VWR Chemicals (Radnor, PA, USA). Sodium hydrogen phosphate, formic acid LC-MS grade and dimethylsulfoxide were purchased from Sigma Aldrich (St Quentin Fallavier, France).

## Methods

**Study design.** Twenty prevalent HD patients participated in this prospective, open-label, controlled, cross-over pilot study. The study was undertaken in our dialysis unit at the University of Milan, Italy from 1 October 2017 to 31 December 2018. Consecutively unselected male and female adult patients with ESRD on HD were eligible for participation in the study. Participation in the study was voluntary. Patients presenting with cachexia or cancer were excluded. Patients were divided into two groups (A and B) with similar mean age, male:female ratio and dialytic vintage. This was a cross-over design, whereby patients in Group A were treated with Theranova dialyser (HDx) for the first 3 months of the study then switched to conventional bicarbonate dialysis for the remaining 3 months. Patients in Group B were treated with bicarbonate dialysis for the first 3 months of the study then switched to HDx for the remaining 3 months (Figure 1). Patients' characteristics are described in a recent publication from our group [7]. Sera samples from both groups were collected at 1, 2 and 3 months. This study complied with the ethical standards of the 1975 Declaration of Helsinki and the study was approved by the local ethical committee. This study is registered at ClinicalTrials.gov (NCT03169400). All patients provided written informed consent before inclusion in the study, in compliance with Italian law (L. 675/1996).

**Induction of calcification.** Rat VSMCs were obtained by enzymatic digestion, as previously described [5], and were routinely subcultured in growth medium (DMEM containing 10% FBS supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin).

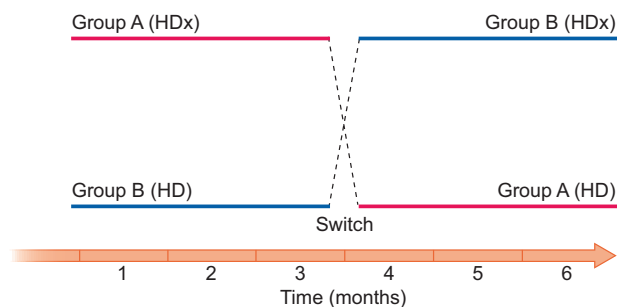


FIGURE 1: Study design.

At 80% confluence, cells were switched to calcification medium (DMEM containing 10% patients' uraemic serum supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 mM sodium pyruvate and 50  $\mu$ g/mL ascorbic acid) and challenged with 3.3 mM trisodium phosphate ( $\text{Na}_3\text{PO}_4$ ; Pi) for up to 4 days. The medium was replaced every 2 days. Cells were used between the sixth and eighth passage.

**Quantification of calcium deposition and alizarin red staining.** Quantification of calcium deposition was performed by Alizarin Red S staining followed by perchloric acid ( $\text{HClO}_4$ ) destaining 3 days after high Pi treatment. Cells were stained with Alizarin Red S solution for 30 min and destained for 24 h with 5%  $\text{HClO}_4$  and calcium content was determined colorimetrically at a wavelength of 450 nm. Protein content was quantified by BCA protein assay kit and calcium deposition was normalized to protein content and expressed as absorbance [optical density (OD)] per milligram of protein.

To visualize calcium deposition, cells were fixed with 70% ethanol (EtOH) and stained with 1 mg/mL Alizarin Red S solution for 30 min.

**Detection of apoptosis and necrosis.** Cytoplasmic histone-associated DNA fragments were detected with a cell death detection ELISA plus kit as a quantitative index of apoptosis 4 days after high Pi treatment. Briefly, after VSMCs were scraped in lysis buffer and sonicated 5  $\times$  20 s at 40% power, samples were centrifuged at 200 g for 10 min at room temperature and 20  $\mu$ L of supernatant was used for the assay. Following the addition of substrate, colorimetric change was determined by the absorbance value measured at 450 nm. Necrosis was detected with the same kit in the medium fraction.

**RNA, miRNA extraction and reverse transcription polymerase chain reaction (PCR).** Total RNA was extracted using the PureLink RNA Mini Kit according to the manufacturer's instructions 4 days after high Pi treatment. After reverse transcription, TaqMan PCR was performed as previously described [5]. Complementary DNA amplification for BMP2 (100 ng), RUNX2 (50 ng), OPN (25 ng), OC (100 ng), MGP (4 ng),  $\text{Coll}\alpha_1$  and Eln (25 ng) was normalized to simultaneous amplification of an internal housekeeping gene,  $\beta$ -actin, and calibrated to a low-expressing normalized target sample.

Exosome isolation was performed as previously described [8]. Briefly, culture medium was centrifuged and exosomes were isolated with Exosome Precipitation Solution (Macherey-Nagel, Bethlehem, PA, USA) following the kit instructions. miRNA was then extracted and reverse transcription was performed with the Taqman Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Waltham, MA, USA). TaqMan quantitative PCR for miRNA 125b, 145, 30b, 30c, 32, 155, 223, 214 and 29b was performed with the TaqMan Advanced miRNA Assay (Applied Biosystem), Taqman Fast Advanced Master Mix and normalized on miRNA 16-5p used as for housekeeping. The relative quantitative evaluation of target genes was performed by comparing threshold cycles using the  $\Delta\Delta$  cycle threshold method.

**Uraemic toxins and precursor detection by ultra-performance liquid chromatography–tandem mass spectrometry (ULPC-MS/MS).** Serum concentrations of uraemic toxins were measured using a ULPC-MS/MS method, which was developed and validated for the simultaneous determination of 10 circulating uraemic toxins (including phenylacetylglutamine, trimethylamine-N-oxide, *p*-cresyl sulphate, IS, kynurenine,

hippuric acid, indole-3-acetic acid, CMPF, kynurenic acid and *p*-cresyl glucuronide) and three precursors (tyrosine, phenylalanine and tryptophan), i.e. relevant uraemic retention solutes derived from both endogenous and colonic microbial metabolism [9]. The limit of quantification was between 1 and 50 ng/mL with linearity up to 10 000 or 50 000 ng/mL according to the compounds. Intra- and interassay variability evaluated at four different concentrations according to the compounds (30, 150, 8000, 40 000 ng/mL) for the 13 compounds were all <13%.

**Alpha-1 acid glycoprotein detection.** Alpha-1 acid glycoprotein was detected by ELISA in patient's serum following the kit protocol. Briefly, serum was thawed and diluted to 1:500 000 with sample diluent and 50  $\mu$ L were used for the assay. Following the addition of substrate, colorimetric change was determined by the absorbance value measured at 450 nm.

### Statistical analysis

Results were expressed as mean  $\pm$  standard error of the mean. Each experiment was performed at least three times and at least in triplicate. Differences between groups were analysed by *t*-test and were considered statistically significant when *P* < 0.05.

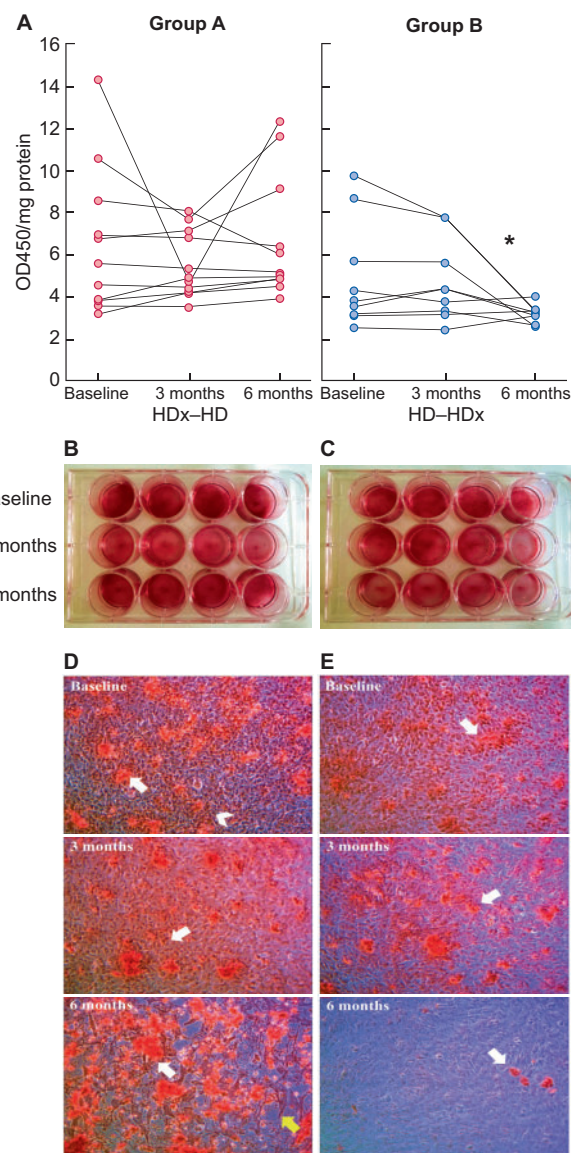
## RESULTS

### Effects of HDx on vascular calcification

VSMCs were treated with uraemic serum at different concentrations but no calcium deposition was detectable at different time points. Thus we added 3.3 mM Pi and 10% uraemic serum for 3 days. Calcium deposition was evaluated for serum collected at three time points for each patient: baseline, after 3 months (before treatment switch) and after 6 months (end of the study). Patients in Group A showed a tendency to a slight reduction of calcium deposition during HDx treatment with a following slight increase after treatment switch [ $6.6 \pm 1.1$ ,  $5.6 \pm 0.6$  and  $6.7 \pm 1.0$  OD/mg protein for baseline, 3 months (HDx) and 6 months (HD), respectively; Figure 2A, left panel]. Patients in Group B showed no modification in calcification in the first 3 months, whereas after treatment switching to HDx we observed a significant reduction in calcification ( $4.7 \pm 0.6$  versus  $3.2 \pm 0.2$  OD/mg protein at 3 versus 6 months, respectively; *P* < 0.05; Figure 2A, right panel). Calcific nodules were visualized by Alizarin Red S staining (Figure 2B and C). Bright red spots are calcium concretion (white arrows), whereas areas of necrosis appeared as darker granular elements (arrowhead) (Figure 2D and E). Figure 2D shows the density of calcific nodules in a patient from Group A, where at 3 months the density and dimension of calcific granules were lower with respect to the baseline and 6 months with an additional evident reduction in necrosis at this time point. Calcification worsened at 6 months and necrosis was so massive that a decrease in cellular density was present (Figure 2D, black arrow). Figure 2E shows the density of calcific nodules in a patient from Group B where there were no appreciable differences between baseline and 3 months, whereas a striking reduction in calcification could be observed after 6 months. These findings are representative of data obtained through quantitative analysis.

### Effects of HDx on VSMC apoptosis and necrosis

We studied whether the dialysis treatment with HDx modulated the toxic effect of uraemic serum during high Pi stimulation.



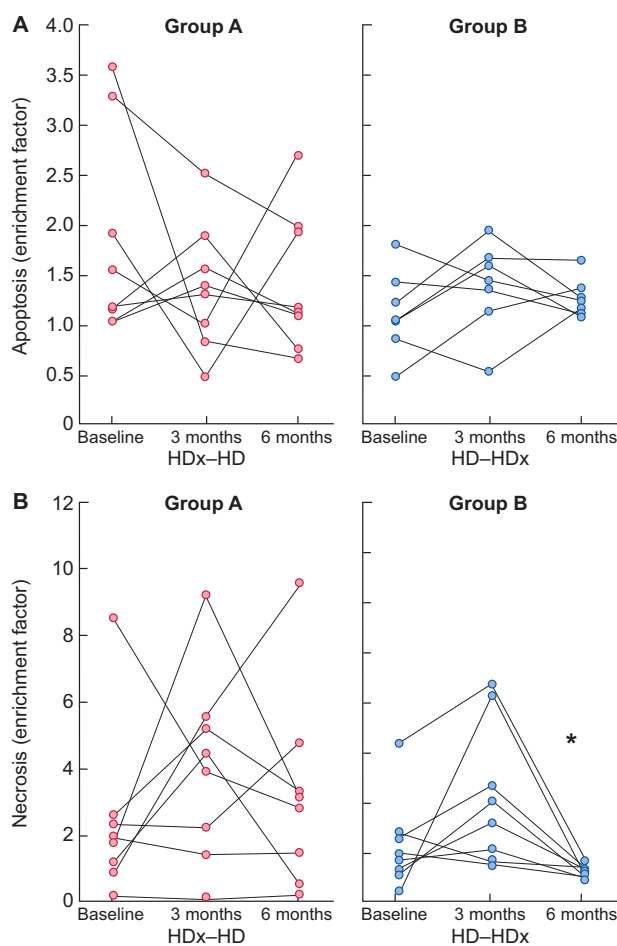
**FIGURE 2:** Effect of HDx on human uraemic serum calcification potential in high Pi-induced VSMC calcification. Rat VSMCs were cultured with 10% human uraemic serum added in the calcification medium, harvested at three different time points (baseline, 3 and 6 months) following either HDx or HD and stimulated with 3.3 mM Pi for 3 days. (A) Calcium deposition was measured by destaining and normalized by cellular protein content. (B–E) Calcium deposition was visualized at the light microscopy level by Alizarin Red S staining. (A) Scatter plot representation of every patient's uraemic serum calcification action in Group A (HDx->HD, left) and in Group B (HD->HDx, right) at three different time points. In Group B, HDx treatment induced a significant reduction of the uraemic sera calcification potential. (B) Group A and (C) Group B calcium deposition in 12-well plate cultured VSMCs stimulated with high Pi (red). Representative images showing the time course of serum calcification potential in a patient from (D) Group A and (E) Group B. The figure shows HDx dialytic treatment reduced the number of red calcific deposits [white arrows (D) 3 months and (E) 6 months]. In contrast, it is possible to detect necrosis as red non-specific staining around calcific red spots (arrowhead) and as a loss of cells during HD [(D) 6 months, black arrow]. Magnification  $\times 200$ . Data are presented as mean  $\pm$  standard error (\**P* < 0.05).

Analysing apoptosis, we found no significant modulation by HDx treatment in Group A [ $1.85 \pm 0.36$  versus  $1.38 \pm 0.22$ , enrichment factor, baseline versus 3 months (HDx); respectively; NS; Figure 3A, left] or in Group B [ $1.38 \pm 0.17$  versus  $1.27 \pm 0.13$ , enrichment factor, 3 months (HD) versus 6 months (HDx),

respectively; Figure 3A, right]. We next studied necrosis, finding a decreasing positive effect of HDx on this process. In Group A there were no statistically significant differences between HDx and HD treatment [ $2.99 \pm 0.99$ ,  $3.69 \pm 0.95$  and  $5.94 \pm 2.85$ , enrichment factor, baseline, 3 months (HDx) and 6 months (HD), respectively; NS; Figure 3B, left]. In contrast, in Group B, HDx induced a significant amelioration of the necrotic process [ $4.47 \pm 1.52$  versus  $1.86 \pm 1.10$ , enrichment factor, 3 months (HD) versus 6 months (HDx); respectively;  $P < 0.05$ ; Figure 3B, right].

### Effects of HDx on VSMC osteochondrogenic shift

We analysed the effect of uraemic serum during HD and HDx treatment periods in high Pi induction of a VSMC shift towards an osteochondrogenic phenotype compared with the absence of high Pi stimulation (controls). We tested seven different molecules, either inducers or inhibitors of calcification, involved in VSMC simil-osteoblast transformation, namely BMP2, RUNX2, OC, OPN, MGP, elastin and Coll1 $\alpha$ 1. All analysed genes showed a strong modulation in the presence of high Pi stimulation, with



**FIGURE 3:** Effect of HDx on human uraemic serum apoptotic and necrotic potential in high Pi-stimulated VSMCs. Rat VSMCs were cultured with 10% human uraemic serum added in the calcification medium, harvested at three different time points (baseline, 3 and 6 months) following either HDx or HD and stimulated with 3.3 mM Pi for 4 days. Apoptosis and necrosis were evaluated by histone-associated DNA fragment detection ELISA in cell lysate or culture medium, respectively. (A) HDx dialytic treatment did not modified high Pi-induced apoptosis that is similar in HDx and HD patients. (B) Sera from HDx patients showed a significant decreased serum necrotic potential in Group B (left panel). Data are presented as mean  $\pm$  standard error (\* $P < 0.05$ ).

the induced targets such as BMP2 increasing  $>20$  times over the control and OC, RUNX2, OPN and MGP increasing more than twice. The downregulated targets such as Eln and Coll1 $\alpha$ 1 were decreased  $\sim 30\%$  in the first and from 60% to 80% in the second (Figure 4). The uraemic serum from the HDx period did not significantly modify the levels of any inducers or inhibitors of calcification compared with the HD period, as shown in Figure 4.

### Uraemic serum characterization following either HD or HDx: uraemic toxins and precursors profile, alpha 1 acid glycoprotein levels and miRNA content

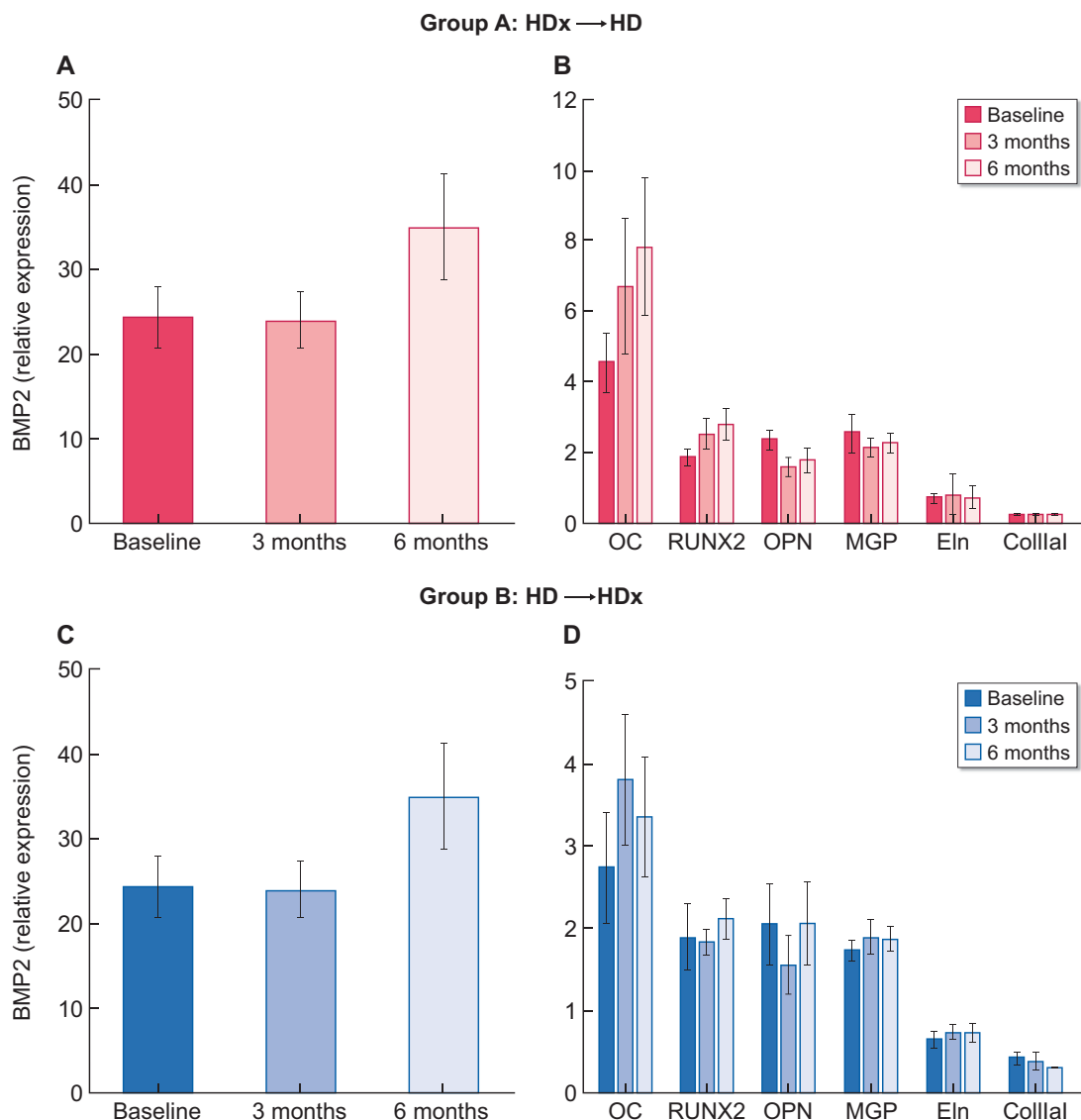
We next measured the levels of 10 uraemic toxins and 3 precursors in patients' serum from either the HDx or the HD period at baseline, 3 months and after the dialysis switch at 6 months. To appreciate the single-patient relative variation with respect to the previous time point, we expressed the data as a percentage of variation. Between the 10 uraemic toxins and 3 precursors tested (see Materials and methods section), we found a significant decrease induced during the HDx period in Group B for tryptophan ( $51.7 \pm 14.2$  versus  $-2.8 \pm 5.8$  for HD versus HDx; Figure 5A;  $P < 0.05$ ), kynurenine ( $44.2 \pm 13.0$  versus  $-0.1 \pm 6.8$  for HD versus HDx; Figure 5B;  $P < 0.05$ ), indole-3-acetic acid ( $43.1 \pm 16.9$  versus  $-2.4 \pm 11.0$  for HD versus HDx; Figure 5C;  $P < 0.05$ ) and CMPF ( $61.8 \pm 27.6$  versus  $-16.1 \pm 9.6$  for HD versus HDx; Figure 5E;  $P < 0.05$ ). For 3-IS the percentage of variation decreased during the HDx period both in Group A, with a rebound effect after the switch to HD ( $-21.1 \pm 13.0$  versus  $54.4 \pm 33.2$  for HDx versus HD; Figure 5D;  $P < 0.05$ ), and in Group B ( $30.6 \pm 13.0$  versus  $-17.1 \pm 10.2$  for HDx versus HD; Figure 5D;  $P < 0.05$ ). We calculated the kynurenine:tryptophan ratio, an index related to intima-media thickness, finding a decrease induced during the HDx period in Group A [ $10.3 \pm 2.1$ ,  $8.4 \pm 1.3$  and  $10.6 \pm 2.1$  nmol/ $\mu$ mol for baseline, 3 months (HDx) and 6 months (HD), respectively; NS] and no variation in Group B [ $11.3 \pm 0.7$  versus  $11.3 \pm 0.5$  nmol/ $\mu$ mol for 3 months (HD) versus 6 months (HDx); NS]. No significant variation was found for the other tested uraemic toxins and precursors.

We next determined alpha 1 acid glycoprotein levels in uraemic serum during both HDx and HD treatments. As shown in Figure 6, in Group A, HDx and HD had almost the same effect, with a dispersion of data that resulted in no difference between the two dialysis treatments ( $-8.7 \pm 7.8$  versus  $2.2 \pm 10.5$  for HDx versus HD; Group A; % variation; NS; Figure 6A). In Group B, excluding the two outliers, there was a tendency towards a decrease from HD to HDx ( $-1.8 \pm 7.8$  versus  $-11.4 \pm 5.1$  for HD versus HDx; Group B; % variation; NS; Figure 6A).

In the attempt to characterize uraemic serum, we evaluated a panel of miRNA, measuring the content in serum-isolated exosomes. Between the nine targets analysed (see the Materials and methods section), only miRNA 125b and 145 showed a detectable and reliable amplification. Due to the high variability between patients, we could not obtain any statistically significant result, we can only note a tendency of miRNA 125b and 145 levels to increase during the HDx period (Figure 6B and C).

## DISCUSSION

VC actively participates in cardiovascular disease, being one of the main causes of arterial stiffness, especially in CKD patients. For the determinant involvement in the alteration of vascular extensibility, it is considered a major independent risk factor for incident cardiovascular diseases and overall mortality in

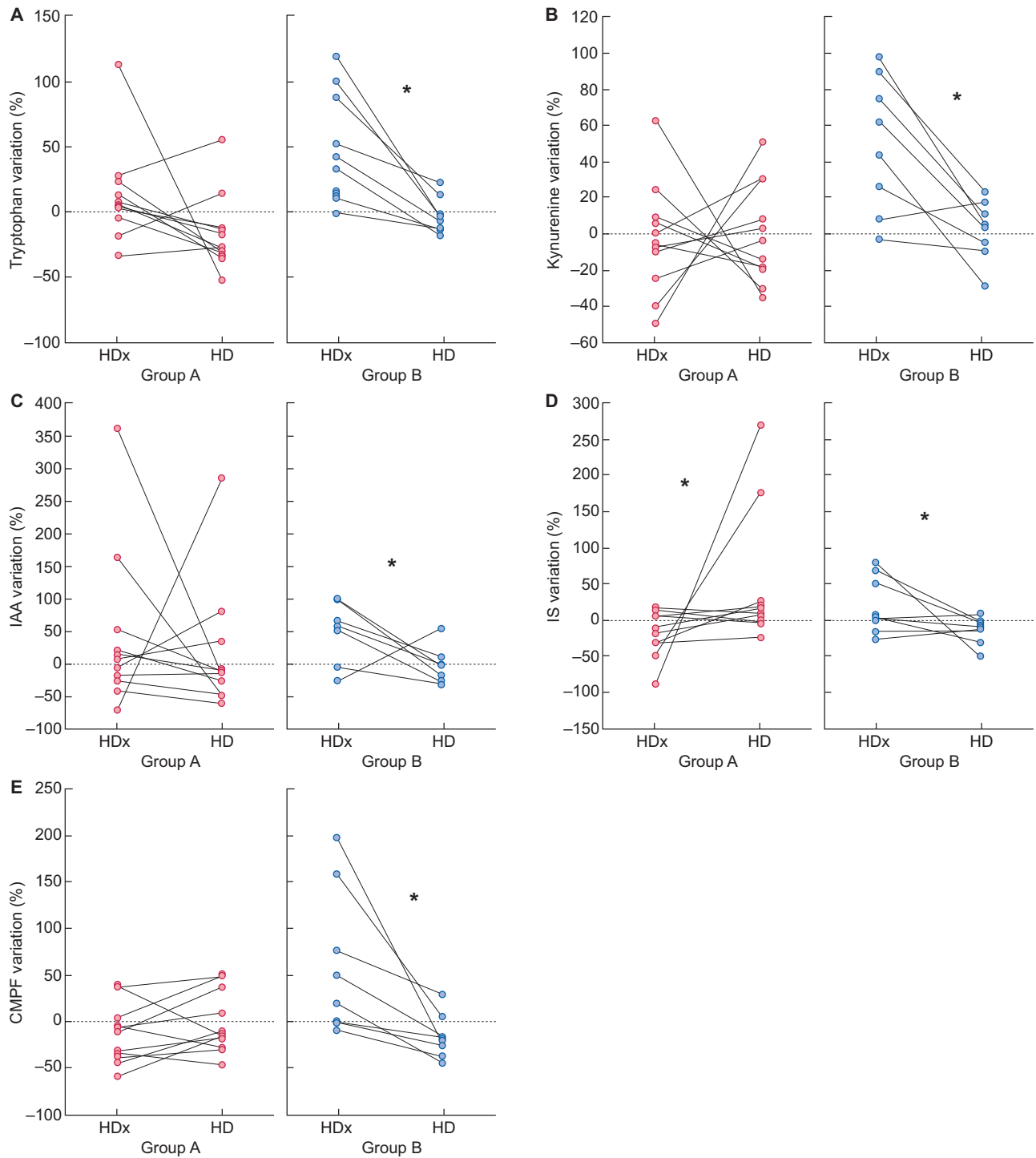


**FIGURE 4:** Effect of HDx on human uraemic serum potential to induce similar-osteoblastic differentiation in high Pi-stimulated VSMCs. Rat VSMCs were cultured with 10% human uraemic serum added in the calcification medium, harvested at three different time points (baseline, 3 and 6 months) following either HDx or HD and stimulated with 3.3 mM Pi for 4 days. mRNA expression was measured by reverse transcription PCR and expressed as relative expression. (A and B) Group A. (C and D) Group B. (A–C) HDx dialytic treatment did not induce any significant variation in the uraemic serum potential to modify BMP2 expression following high Pi stimulation compared with HD. (B and D) HDx dialytic treatment did not induce any significant variation in the uraemic serum potential to modify OC, RUNX2, OPN, MGP, elastin and Coll1a1 expression following high Pi stimulation compared with HD. Data are presented as mean  $\pm$  standard error (\* $P < 0.05$ ).

CKD patients [3]. VC is a tightly cell-regulated pathological process and one of the main causes of calcium deposition in the vessel wall is believed to be the imbalance between inhibitors and promoters of mineralization, even if the precise role of each molecule and their complex interactions are not totally understood [10]. In uraemia, complex miscellaneous factors make an additional contribution to accelerate ageing and VC [4, 11, 12].

One of the strategies to decrease the toxic potential of uraemic toxins is to improve their clearance through the modification of dialysis membrane characteristics. In fact, current dialysis membranes and techniques only remove solutes in the range of 50–15 000 Da and HCO membranes showed an elevated albumin loss that represented the limiting problem in their use in regular dialysis therapy. Nowadays, MCO membranes are used because they are able to remove middle molecules in a

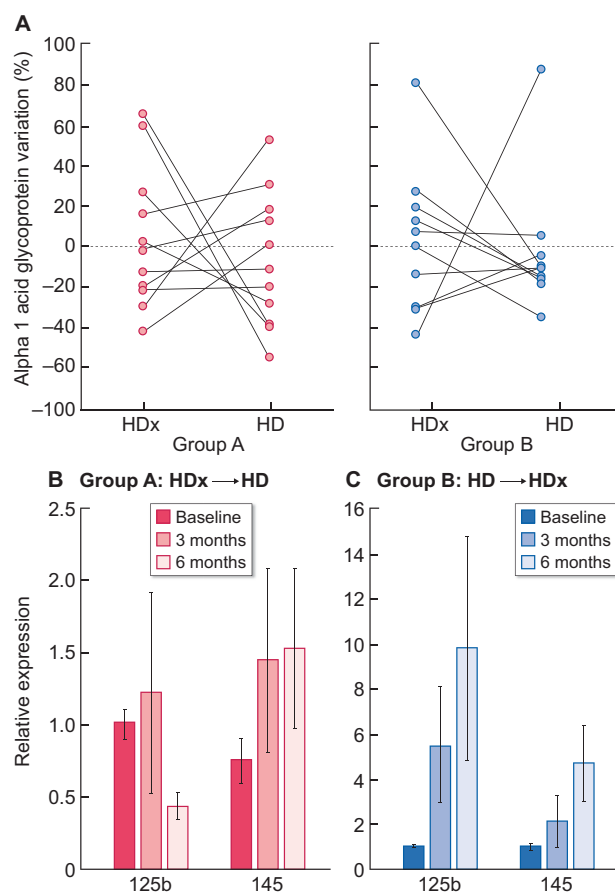
molecular weight range up to 50 000 Da. Recently two clinical studies on MCO membranes were published demonstrating their safety with respect to the excessive albumin loss [13] and an increased reduction rate in myoglobin during the MCO dialysis period [14]. We performed a prospective, open-label, controlled, cross-over pilot study comparing HDx (MCO membrane Theranova VR400) and conventional HD in 20 prevalent HD patients. Ten patients underwent conventional HD with high-flux dialyser and 10 patients underwent HDx for 3 months and the patients then switched and received the other treatment for a further 3 months. In our previous study we found a significant reduction in serum albumin concentration when patients received HDx treatment, but no patients had clinical symptoms of hypoalbuminaemia and needed albumin infusions. Interestingly, we observed a decrease in the number of infections in patients treated with HDx [7].



**FIGURE 5:** Serum content of protein-bound uraemic toxins following either HD or HDx. Protein-bound uraemic toxin levels were measured in serum by UPLC-MS/MS in Groups A and B at baseline, 3 and 6 months and represented as scatter plots of every patient. The percentage of variation is intended as the level variation compared with the level in the precedent time point. (A) Tryptophane, (B) kynurenine, (C) indole-3-acetic acid, (D) 3-IS and (E) CMPF. For all five protein-bound uraemic toxins represented, there was a significant decrease following HDx in Group B. 3-IS was significantly decreased by HDx in Group A. Data are presented as mean  $\pm$  standard error ( $P < 0.05$ ).

In this work we investigated the effect of HDx on the pro-calcifying serum potential in a high Pi calcification model in VSMCs. In general, our research presented a more accentuated sensitivity to the effect of HDx in Group B compared with Group A. In fact, Group B reached the statistical difference for some processes, whereas for the same phenomena, Group A showed

just a tendency. Nevertheless, the major strength of this study is the patient switch between HDx and HD, which allows each patient to be his/her own control. In this context, HDx was able to reduce the pro-calcifying effect of serum in a model of high Pi-induced calcification with a significant inhibition ranging from 15 to 32%. Interestingly, the trend in Group A (HD-HDx-



**FIGURE 6:** Serum content of alpha 1 acid glycoprotein and miRNA following either HD or HDx. In serum, alpha 1 acid glycoprotein was measured by (A) ELISA and miRNA in exosomes by reverse transcription PCR (B and C) in Groups A and B at baseline, 3 and 6 months and represented as scatter plots (alpha 1 acid glycoprotein) and histograms (miRNA) for every patient. (A) No significant effect of HDx on the percentage of variation in serum alpha 1 acid glycoprotein. No significant variation was induced by HDx dialytic treatment in exosome 125b and 145 miRNA levels in (B) Group A and (C) Group B. Data are presented as mean  $\pm$  standard error.

HD) showed an inhibition by HDx in the pro-calcifying potential of serum that was nullified by the return to conventional HD. The decrease in HDx calcifying serum potential was revealed by a reduction in the number and size of calcium-phosphate crystals stained by Alizarin Red S. Moreover, there was also a reduction of the non-specific red staining, the dark red shadow around crystal deposits, the sign of cellular death. This evidence is also supported by clear protection in cell viability in some VSMC samples when treated with patient's serum treated with HDx. We further investigated VC pathogenesis through analysis of the involved processes, either apoptosis or necrosis, finding that HDx protects from necrosis (~58% in Group B) but not from apoptosis.

The finding that HDx decreases the toxic potential of serum is relevant from a clinical point of view. In fact, necrosis is one of the driving mechanisms in calcium deposition in the high Pi-induced *in vitro* calcification model, due to the release from necrotic cells of toxins and calcium in the extracellular space that in turn triggers further calcium deposition in the extracellular matrix [15]. In our study, HDx reduction of uraemic serum toxicity is potentially one of the main mechanisms determining the decrease in calcification potential, since HDx did not alter VSMC simil-osteoblast transformation following high Pi treatment. In fact, we could not detect any difference between serum potential in the HDx compared with the HD patients in high Pi induction of BMP2, RUNX2, the master gene of osteoblastic cascade,

or the downstream genes OC, OPN and the VC inhibitor MGP. In addition, other targets regarding extracellular matrix such as Coll1 $\alpha$ 1 and elastin were not modulated during the HDx treatment. Our data are in line with another study that found a decreased pro-calcifying effect of serum following dialysis with HRO, an MCO membrane, in high Pi-stimulated VSMCs [16]. As in our study, the authors found a decrease in uraemic serum toxic effect when an MCO membrane was utilized, with less apoptosis induction. We did not detect any modulation in the HDx period in the pro-apoptotic potential of serum, probably due to both different cell species (rat versus human VSMCs) and experimental setting. Nevertheless, we found less pro-necrotic potential, whereas necrosis was not investigated in previous studies.

To better clarify the effect of HDx on the pro-calcifying potential of patients' serum we tried to characterize the composition of serum after the two different dialysis treatments. We measured the middle molecule alpha 1 acid glycoprotein (41–43 kDa), a pro-inflammatory glycoprotein. As already found previously for interleukin (IL)-1 $\beta$  and IL-6, we found a tendency towards a decrease of alpha 1 acid glycoprotein after the HDx treatment. Nevertheless, this is an interesting result considering the strict link between inflammation and VC [17] and our previous observation of a decrease in the number of infections when patients were treated with HDx [7]. Of course, larger studies are necessary to better clarify these preliminary data.



The evaluation of a panel of uraemic toxins and precursors in serum showed significant removal during the HDx treatment of tryptophan, together with some of its metabolites and CMPF. Interestingly, uraemic toxins derived from tryptophan metabolism have been associated with cardiovascular diseases in CKD patients. In particular, serum IS levels are associated with overall and cardiovascular mortality [18] and left ventricular diastolic dysfunction in CKD patients [19]. IS and other tryptophan metabolites are endogenous ligands of the transcription factor aryl hydrocarbon receptor (AhR), the activation of which has been associated with an increase in cardiovascular diseases in humans, considering its role as a possible mediator of uraemic toxins adverse effects [20]. We found a decrease in IS serum levels during the HDx period and this can contribute to explaining the reduced calcium deposition in our model, as a pro-calcifying effect of IS has been demonstrated in hypertensive rats [21]. Moreover, the involvement of IS in calcification is supported by its ability to increase oxidative stress and osteoblastic transformation in VSMCs [22]. Uraemic toxins from the kynurenine pathway may also be involved in cardiovascular diseases. We calculated the kynurenine:tryptophan ratio, a parameter that reflects IDO activity, one of the rate-limiting steps of the kynurenine pathway, and that correlates to intima-media thickness disease in CKD patients [23], finding a tendency to be reduced in serum with HDx treatment. The relationship between CMPF and VC has not been studied yet. Moreover, the calcifying action and the relationship with cardiovascular diseases of other tryptophan metabolites through AhR needs to be clarified.

In our study we found decreased necrotic activity of serum during the HDx treatment and this effect was probably related to a reduction of circulating uraemic toxins. In fact, IS has a well-known toxic effect in different cells and organs, such as cardiomyocytes [24], resident tubular and glomerular cells, leading to renal toxicity [25], and it affects the vascular system, damaging vessel function and morphology [26]. Alterations in tryptophan metabolism and in the kynurenine pathway are also involved in different human diseases, such as CKD, brain injury, cancer, neurodegenerative and autoimmune disorders. In fact, uraemic toxins produced by tryptophan metabolism have oxidant, pro-apoptotic and pro-inflammatory action in cells of the cardiovascular system. CMPF exerts its toxic activity, increasing reactive oxygen species production in endothelial cells [27], and accumulates in renal cells, inducing a reduction in cell viability that inversely correlates with reactive oxygen species formation in proximal tubular cells [28].

In our *in vitro* model of VSMC calcification, we could not detect any effect in the HDx period on serum simil-osteoblastic differentiating properties. To investigate this aspect *in vivo*, we measured a panel of miRNAs related to calcification. We analysed selected miRNAs in serum exosomes that are not filtered and cannot be influenced by dialysis. We aimed to evaluate some parameters that can reflect whether 3 months of HDx treatment induced a modification in the patient's calcification progression compared with HD. Recently, efforts were made to identify miRNAs as reliable diagnostic and/or prognostic biomarkers to assess calcification in CKD patients. Circulating miRNA-125b and miRNA-145 have been demonstrated to be valid candidates in *in vitro* models of calcification and, in addition, their circulating levels are associated with VC severity in uraemic patients [29–30]. Due to the high variability between patients' response and the small size of our pilot study we could not find a clear indication of miRNA level variation. Nevertheless, larger studies are advisable to clarify the effect of HDx on miRNA circulating levels.

In conclusion, this pilot study indicates that HDx can decrease the pro-calcifying effect of uraemic serum through a decrease in its necrotic activity. This beneficial effect is due in part to a partial removal of tryptophan, some of its metabolites, such as IS, and CMPF. The effect of MCO on the removal of protein-bound uraemic toxins is probably related to albumin loss, the main carrier of these toxins. Thus we hypothesize that the partial albumin loss during HDx might be beneficial, allowing a decrease in protein-bound uraemic toxin levels that have been difficult to eliminate with extracorporeal strategies until now. MCO might thus decrease uraemic serum pro-calcifying and necrotic effects through albumin loss without any clinical symptoms of hypoalbuminaemia. We think that this hypothesis deserves deep investigation in larger clinical trials.

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## AUTHORS' CONTRIBUTIONS

P.C. and M.C. were responsible for the research idea and study design. Data acquisition was performed by P.C., G.T., L.M. and N.F. Data analysis/interpretation was carried out by P.C., A.G., J.C.A, Z.M., P.M. and M.C. Statistical analysis was performed by P.C. Supervision or mentorship was provided by M.C. Each author contributed important intellectual content during manuscript drafting and accepts accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved. M.C. takes responsibility that this study has been reported honestly, accurately and transparently; that no important aspects of the study have been omitted and that any discrepancies from the study as planned have been explained.

## CONFLICT OF INTEREST STATEMENT

Z.M. and M.C. report having received speaker fees from Baxter. The other authors declare no conflicts of interest. The results presented in this article have not been published previously in whole or part, except in abstract format.

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