

Original Article

The circulating calcification inhibitors, fetuin-A and osteoprotegerin, but not Matrix Gla protein, are associated with vascular stiffness and calcification in children on dialysis

Rukshana C. Shroff^{1,2}, Vanita Shah³, Melanie P. Hiorns⁴, Michael Schoppert⁵, Lorenz C. Hofbauer⁶, Gerhard Hawa⁷, Leon J. Schurgers⁸, Atul Singhal⁹, Ian Merryweather⁹, Paul Brogan¹⁰, Catherine Shanahan¹¹, John Deanfield² and Lesley Rees¹

¹Nephrourology, ²Vascular Physiology, ³Infectious Disease and ⁴Radiology Departments, Great Ormond Street Hospital & UCL Institute of Child Health, London, UK, ⁵Department of Internal Medicine and Cardiology, Philipps-University, Marburg, ⁶Division of Endocrinology and Metabolic Bone Disease, Technical University of Dresden, Dresden, Germany, ⁷Biomedica Medizinprodukte, Vienna, Austria, ⁸CARIM and VitaK, University of Maastricht, Maastricht, The Netherlands, ⁹Childhood Nutrition Research Centre, ¹⁰Department of Rheumatology, Great Ormond Street Hospital & UCL Institute of Child Health, London, UK and ¹¹Cardiovascular Division, King's College London, UK

Abstract

Background. Vascular calcification occurs in the majority of patients with chronic kidney disease, but a subset of patients does not develop calcification despite exposure to a similar uraemic environment. Physiological inhibitors of calcification, fetuin-A, osteoprotegerin (OPG) and undercarboxylated-matrix Gla protein (uc-MGP) may play a role in preventing the development and progression of ectopic calcification, but there are scarce and conflicting data from clinical studies.

Methods. We measured fetuin-A, OPG and uc-MGP in 61 children on dialysis and studied their associations with clinical, biochemical and vascular measures.

Results. Fetuin-A and OPG were higher and uc-MGP lower in dialysis patients than controls. In controls, fetuin-A and OPG increased with age. Fetuin-A showed an inverse correlation with dialysis vintage ($P = 0.0013$), time-averaged serum phosphate ($P = 0.03$) and hs-CRP ($P = 0.001$). Aortic pulse wave velocity (PWV) and augmentation index showed a negative correlation with fetuin-A while a positive correlation was seen with PWV and OPG. Patients with calcification had lower fetuin-A and higher OPG than those without calcification. On multiple linear regression analysis Fetuin-A independently predicted aortic PWV ($P = 0.004$, $\beta = -0.45$, model $R^2 = 48\%$) and fetuin-A and OPG predicted cardiac calcification ($P = 0.02$, $\beta = -0.29$ and $P = 0.014$, $\beta = 0.33$, respectively, model $R^2 = 32\%$).

Conclusions. This is the first study to define normal levels of the calcification inhibitors in children and show that fetuin-A and OPG are associated with increased vascular

stiffness and calcification in children on dialysis. Higher levels of fetuin-A in children suggest a possible protective upregulation of fetuin-A in the early stages of exposure to the pro-calcific and pro-inflammatory uraemic environment.

Keywords: dialysis; fetuin-A; matrix Gla protein; osteoprotegerin; vascular calcification

Introduction

Cardiovascular disease is the most common cause of death in patients with chronic kidney disease (CKD) [1]. Structural and functional abnormalities and calcification in the large vessels begin as early as the first decade of life in patients with CKD [2,3] contributing to a 30-fold higher mortality than the age-matched population [1]. *In vitro* studies and animal experiments have shown that ectopic calcification is a highly regulated, cell-mediated process that involves a balance between inducers and inhibitors of calcification [4].

In CKD pro-calcific stimuli such as increased calcium (Ca) and phosphate (PO_4) [2,5–8] and intact parathyroid hormone (iPTH) levels [2], and, potentially, treatment with Ca-based PO_4 binders [2,6–9] and vitamin D [2,8,10] can promote vascular and soft-tissue calcification. Yet, some patients with CKD do not develop calcification despite exposure to the same uraemic milieu [9]. In fact, all extracellular fluid contains Ca and PO_4 in concentrations exceeding their solubility product for spontaneous precipitation [11], suggesting that under normal conditions inhibitors of calcification prevent the development and progression of vascular calcification.

Correspondence and offprint requests to: Rukshana C. Shroff, Research Registrar, Nephrourology Unit, Institute of Child Health, 30, Guilford Street, London WC1N 1EH, UK. Tel: +44-020-7405-9200, ext 0065; Fax: +44-020-7813-8532; E-mail: ShrofR@gosh.nhs.uk

Fetuin-A (α_2 -Heremans-Schmid protein) is the prototypic calcification inhibitor [11,12] that contributes to ~50% of the calcification inhibitory capacity of human plasma [13]. Osteoprotegerin (OPG) is a soluble decoy receptor for the receptor activator of nuclear factor- κ B ligand (RANKL), which stimulates osteoclastic bone resorption [14]. Serum OPG levels are associated with the progression of carotid atherosclerosis [15] and coronary calcification [16]. Matrix Gla (γ -carboxyglutamic acid) protein (MGP) is expressed in the media of arteries where it acts as a local inhibitor of Ca-PO₄ precipitation [17]. Circulating fetuin-A, OPG and MGP levels have been linked with cardiovascular mortality [18–25] in adults with CKD, but there is a complex and poorly understood relationship between these physiological calcification inhibitors at different stages of CKD and also conflicting data on their impact on vascular measures.

Circulating inhibitors of calcification have not been studied in children with CKD, nor have their normal levels been defined in the healthy childhood population. In this study we describe serum fetuin-A, OPG and undercarboxylated-matrix Gla protein (uc-MGP) levels in a cohort of healthy children and dialysis patients. Children provide an ideal opportunity to study uraemic influences on the arterial wall, as they rarely have confounding pro-atherosclerotic risk factors such as diabetes and dyslipidaemia that are prevalent in adults, and also allow us to study the earliest events in the calcification process. We hypothesize that normal levels of the circulating calcification inhibitors protect against vessel stiffness and calcification in children on dialysis.

Methods

Study design

From January 2005 to December 2006, 61 consecutive children (5–18 years) who had been on dialysis for ≥ 3 months were recruited. Serum levels of the calcification inhibitors, fetuin-A, OPG and uc-MGP, as well as hs-CRP were measured and related to carotid artery intima media thickness (cIMT), aortic PWV and calcification score on multi-slice CT scan. Patients were compared with 75 healthy children: 55 children formed part of a larger study investigating nutritional parameters in healthy children and 20 underwent routine corrective surgery for external auricular malformations or squints in our hospital. Controls were age and gender matched with the dialysis cohort and confirmed to have no known medical illnesses, family history of heart disease or active infections at the time of the study. As vascular scans could only be obtained in 18 of the healthy children, the vascular measures in our dialysis cohort were compared with 40 age-matched controls who participated in a previous study from our group [2]. Informed written consent was obtained from all parents or caregivers, and children where appropriate. The study was approved by the local research ethics committee.

Biochemical measures

Blood samples were taken before a mid-week session of haemodialysis (HD) or immediately after a peritoneal dial-

ysis (PD) session. Serum Ca, PO₄ and iPTH levels and the dosage of elemental Ca intake from PO₄ binders and vitamin D therapy were recorded at monthly intervals from the start of CKD stage IV and expressed as mean time-integrated values. In controls, a single blood test at the time of the scans was performed.

1. Serum fetuin-A was measured using a human fetuin-A enzyme-linked immunosorbent assay (ELISA) kit (Epitope Diagnostics Inc., San Diego, CA, USA). The assay utilizes the two-site 'sandwich' technique with two selected polyclonal antibodies that bind to different epitopes of fetuin-A. The intra-assay and inter-assay coefficient of variation were $< 5.5\%$ and $< 6.8\%$, respectively. The reference range for healthy adults quoted by the manufacturer was 0.5–1.0 g/L, and the minimum sensitivity of the assay was 5.0 ng/mL.
2. OPG serum concentrations were analysed using an ELISA system (Biomedica, Vienna, Austria) that detects both free and complexed OPG. In brief, a monoclonal IgG antibody was used as capture antibody and a biotin-labelled polyclonal antihuman OPG antibody as detection antibody. The intra-assay and inter-assay variabilities were 9% and 10%, respectively. RANKL levels were determined by ELISA (Biomedica) based on microtitre plates coated with OPG. Soluble RANKL (sRANKL) from the sample binds to the coated OPG and is detected by a biotin-labelled polyclonal anti-human sRANKL antibody. The intra-assay and inter-assay variations ranged from 3 to 5% and 6 to 9%, respectively.
3. uc-MGP was measured by a novel ELISA technique developed at the vitamin K-research institute (VitaK BV, Maastricht, The Netherlands). In brief, the moAb uc-MGP was used in this competitive ELISA to capture either native MGP in the sample or biotinylated tracer. The uc-MGP concentration was calculated using a calibration curve of synthetic full-length uc-MGP. The intra- and inter-assay variabilities were 7.2% and 11.4%, respectively.
4. hs-CRP levels were measured by an ELISA (Biomedica) according to the manufacturer's instructions.

Vascular measures

1. cIMT was measured by B-mode ultrasound of both common carotid arteries using a 12-MHz linear-array transducer (Vivid 7, GE Medical, Horton, Norway). Longitudinal 2D images of the vessel were acquired on the R wave of the ECG, frozen in diastole and analysed offline. The cIMT was calculated as the distance between the leading edge of the lumen-intima interface and the media-adventitia interface on the far wall of the artery.
2. Applanation tonometry for carotid-femoral PWV and carotid pulse wave analysis was performed with a micromanometer (SPC-301, Millar Instruments, Houston, TX, USA) using proprietary software (SphygmoCor version 7.0, Scanmed, Gloucestershire, UK). PWV was not measured in the controls, as permission was not granted for femoral artery measures. The peripheral pressure-pulse waveform from the right carotid artery was acquired using the same equipment as described above and

the average of waveforms recorded over 10 s was used to calculate a carotid augmentation index.

- The calcification score was measured by 16-slice spiral CT scans (Somatom Sensation 16, Siemens Medical Solutions Inc, Erlangen, Germany) using the standard Ca scoring protocol. Prospective ECG triggering was performed to obtain image acquisition in diastole, using 60% of the RR interval for image reconstruction. The Agatston score [26] for each main epicardial coronary artery, cardiac valves and aorta and an overall score for each patient were determined. In the control group, CT was not undertaken because of radiation concerns.

Statistical analysis

Results are presented as mean \pm SD unless otherwise indicated. Spearman's (non-parametric) correlations were used to test for associations between the calcification inhibitor levels and selected clinical, anthropometric, biochemical and vascular measures. Comparisons between patient and control groups were made using one-way analysis of variance (ANOVA). Two separate stepwise multiple linear regression analyses were performed to test the associations between the calcification inhibitors against the outcome variables PWV and calcification score. Variables with $P < 0.1$ on univariate analyses (age, time in CKD stage IV, dialysis vintage, mean time-integrated $\text{Ca} \times \text{PO}_4$ product PTH and serum albumin and dosage of alphacalcidol) were entered into the multiple regression models. Given the interaction between serum PO_4 and $\text{Ca} \times \text{PO}_4$ ($P = 0.02$), we have excluded serum PO_4 from the regression models despite its significant association with fetuin-A levels on univariate analysis so as to avoid collinearity. All the three calcification inhibitors under study (fetuin-A, OPG and uc-MGP) were entered into both the regression models even though uc-MGP did not show any significant associations with vascular measures on univariate analyses. Also, given the known fluctuations in biochemical measures and calcification inhibitor levels [27–29] following a single HD session, and to address the issue of pooling all dialysis (HD and PD) patients for analyses, the dialysis modality was entered into both regression models despite non-significance on univariate analyses. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA).

Results

The clinical and biochemical characteristics of the patient and control groups are shown in Table 1. Of the 61 patients (37 boys), 39 had renal dysplasia, 9 inherited nephropathies, 5 cystic kidney disease, 4 primary renal tubular disorders, 3 renovascular disorders and 1 Wilm's tumour. None of the patients had diabetes, dyslipidaemias or active infections at the time of the study, and none were on coumadin therapy.

Levels of fetuin-A, OPG and uc-MGP in healthy children

The mean fetuin-A levels in the healthy controls were 0.41 ± 0.13 g/L, and lower than that reported in adults

($0.5\text{--}1.0$ g/L). Fetuin-A showed a linear increase with age ($P < 0.0001$, $r^2 = 0.55$, Figure 1A), but was independent of gender. In children aged 12–18 years, fetuin-A levels were lower in those \geq 50th percentile for age-appropriate height as compared to children below this percentile (0.45 ± 0.1 versus 0.6 ± 0.2 g/L, $P = 0.03$). In this cohort with a normal biochemical profile and no evidence of inflammation, fetuin-A did not show any associations with serum Ca, PO_4 or PTH levels, fasting glucose, triglyceride or hs-CRP. In the 18 controls who underwent vascular scans, no associations were seen with calcification inhibitor levels.

The mean OPG levels in healthy children were 5.2 ± 1.2 pmol/L. OPG levels also showed a linear increase with age ($P = 0.004$, $r^2 = 0.34$, Figure 1B), but no correlation was found between OPG and any anthropometric, biochemical or vascular measure. The mean uc-MGP levels in the healthy controls were 527 ± 185 μM , and were independent of age and unrelated to any other measured parameters.

Fetuin-A levels in dialysis patients (Table 2)

Unlike the levels seen in adult CKD patients, fetuin-A levels in children on dialysis were significantly higher as compared to controls (0.84 ± 0.3 versus 0.41 ± 0.13 g/L, $P < 0.0001$, Figure 2A). The correlation between age and fetuin-A in healthy controls was not seen in the dialysis population ($P = 0.33$). Fetuin-A showed an inverse correlation with time on dialysis ($P = 0.0013$, $r^2 = 0.14$, Figure 2B). Fetuin-A was lower in HD ($n = 18$) as compared with PD patients (0.69 ± 0.4 versus 1.11 ± 0.2 g/L, $P = 0.03$), but this significance was lost after correction for the time on dialysis.

Fetuin-A showed an inverse correlation with the mean time-integrated serum PO_4 levels ($P = 0.03$, $r^2 = 0.19$) and $\text{Ca} \times \text{PO}_4$ product ($P < 0.0001$, $r^2 = 0.24$). Fetuin-A levels showed a strong negative correlation with hs-CRP ($P = 0.001$, $r^2 = 0.42$). Fetuin-A was associated with vessel stiffness: both the aortic PWV and the aortic augmentation index showed an inverse correlation with fetuin-A levels [$P = 0.016$, $r^2 = 0.19$ (Figure 3A) and $P = 0.03$, $r^2 = 0.11$, respectively]. Fetuin-A levels were significantly lower in children with coronary or valvular calcification ($n = 14$) on CT scan than in those without calcification (0.64 ± 0.2 versus 0.89 ± 0.4 g/L, $P = 0.007$, Figure 3B). For every 0.1 g/L increase in serum fetuin-A, there was a 5% decrease in risk of calcification (95% CI 0.84–0.91, $P = 0.013$).

OPG levels in dialysis patients (Table 2)

OPG levels were significantly higher in dialysis patients as compared to healthy controls (6.7 ± 2.2 versus 5.2 ± 1.2 pmol/L, $P < 0.0001$, Figure 4). A linear relationship was seen between OPG and iPTH levels ($P = 0.01$, $r^2 = 0.35$). OPG levels were higher in HD compared to PD patients (8.9 ± 1.6 versus 6.0 ± 0.9 pmol/L, $P = 0.02$), but this reflected the higher PTH levels in the HD patients. OPG levels were associated with vessel stiffness and calcification. The aortic PWV increased with increasing OPG levels ($P = 0.03$, $r^2 = 0.18$, Figure 5A), and children with calcification had significantly higher OPG levels than those without calcification (8.1 ± 1.6 versus

Table 1. Demographic, clinical, anthropometric and biochemical characteristics of patients and controls

	Patients <i>n</i> = 61	Controls <i>n</i> = 75	<i>P</i>
Age (year)	13.4 ± 4.1	12.4 ± 4.1	0.33
Gender (males/females)	37/24	42/33	0.19
Race (Caucasian/Asian/Black/Others)	37/12/9/3	39/19/12/5	–
Estimated GFR (ml/min/1.73 m ²)	8.9 ± 8.0	118 ± 3.4	< 0.0001
Time in CKD stage IV (year)	4.0 ± 2.2	–	–
Time on dialysis (year)	0.9 ± 1.9 [median 1.1 (0.25–8.7)]	–	–
Dialysis modality (PD/HD)	43/18	–	–
Body mass index SDS	–0.5 ± 1.6	0.9 ± 0.6	< 0.0001
Systolic BP index ^a	1.3 ± 0.3	0.9 ± 0.3	0.03
Patients on anti-hypertensive medications	11	0	–
Patients on ACEi or AIIRB	2	0	–
Haemoglobin (g/dL)	11.7 ± 1.5	13.7 ± 2.1	0.08
Albumin (g/L)	38 ± 3.0	41 ± 0.6	0.22
Total cholesterol (mmol/L)	4.0 ± 1.1	3.4 ± 1.0	0.16
Triglycerides (mmol/L)	1.2 ± 1.2	0.8 ± 1.7	0.74
Patients on statins	2	0	–
Diabetes mellitus	0	0	–
Smokers	0	0	–
Serum PO ₄ level (mmol/L) ^b	1.5 ± 0.7	0.9 ± 0.4	0.005
Serum Ca (albumin adjusted) (mmol/L) ^b	2.4 ± 0.1	2.4 ± 0.4	0.22
Ca-PO ₄ product (mmol ² /L ²) ^b	4.2 ± 0.9	3.7 ± 0.2	0.001
Serum iPTH ^b			
pmol/L ^c	10.8 ± 2.9	n/d	–
fold ULN	1.8 ± 1.3	n/d	–
Parathyroidectomy	0	0	–
PO ₄ binders			
Number on Ca-based PO ₄ binders	52 (88%)	–	–
Sevelamer ± Ca-based PO ₄ binders	9 (12%)	–	–
Cumulative intake of elemental Ca from PO ₄ binders (gm/kg) ^b	124 ± 81	–	–
Alphacalcidol (1-α hydroxy Vit D ₃) (μ/kg)	33.1 ± 20.3	–	–

All values expressed as mean ± SD.

^aBP index = measured BP/95th centile BP for age, gender and height.

^bExpressed as mean time-integrated values from the onset of CKD stage IV.

^cFor PTH values in pg/mL multiply by 10.5.

ACEi: angiotensin converting enzyme inhibitor, AIIRB: angiotensin II receptor blocker, SDS: standard deviation score. n/d: not done.

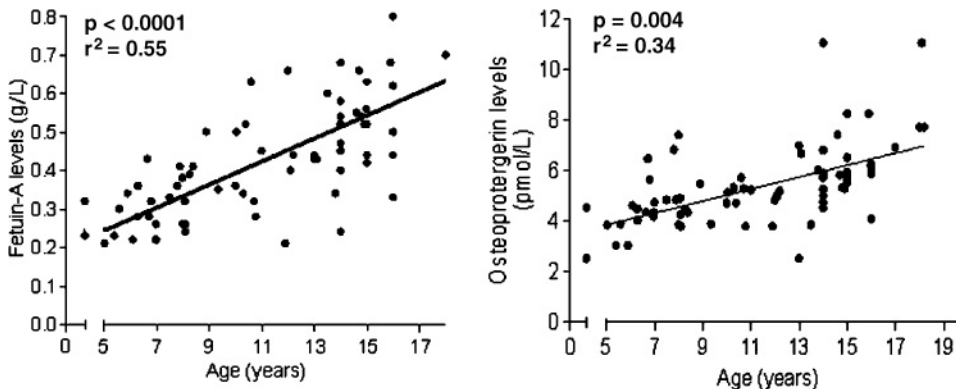


Fig. 1. Circulating serum fetuin-A and osteoprotegerin (OPG) levels showed a linear increase with age in healthy controls, $P < 0.0001$, $r^2 = 0.55$ (A) and $P = 0.004$, $r^2 = 0.34$ (B), respectively.

6.3 ± 2.2 pmol/L, $P = 0.005$, Figure 5B). There was no correlation between RANKL or OPG/RANKL and any clinical, biochemical or vascular measure.

versus 527 ± 185 μM, $P < 0.001$, Figure 6), but no further associations with uc-MGP levels were found.

uc-MGP levels in dialysis patients (Table 2)

uc-MGP levels in children on dialysis were significantly lower as compared to controls (232 ± 116

Correlations between the calcification inhibitors

We were unable to find any correlations between serum levels of fetuin-A, OPG and uc-MGP in the overall cohort,

Table 2. Associations between the calcification inhibitors and clinical, anthropometric, biochemical and vascular measures^a

Variables	Circulation calcification inhibitor levels		
	Fetuin-A	Osteoprotegerin	Undercarboxylated-matrix Gla protein
<i>Clinical measures</i>			
Age	$P = 0.33$	$P = 0.07$	$P = 0.22$
Gender	$P = 0.64$	$P = 0.88$	$P = 0.66$
Time in CKD stage IV	$P = 0.09$	$P = 0.14$	$P = 0.37$
Time on dialysis	$P = 0.0013, r^2 = 0.14$	$P = 0.19$	$P = 0.36$
<i>Anthropometric measures</i>			
Height	$P = 0.28$	$P = 0.08$	$P = 0.78$
Body mass index	$P = 0.16$	$P = 0.15$	$P = 0.56$
<i>Biochemical levels and dosage of medications^b</i>			
Serum Ca	$P = 0.16$	$P = 0.12$	$P = 0.15$
Serum PO ₄	$P = 0.03, r^2 = 0.19$	$P = 0.07$	$P = 0.11$
Serum Ca × PO ₄	$P < 0.0001, r^2 = 0.24$	$P = 0.09$	$P = 0.18$
Serum PTH	$P = 0.08$	$P = 0.01, r^2 = 0.35$	$P = 0.34$
High sensitivity CRP	$P = 0.001, r^2 = 0.42$	$P = 0.24$	$P = 0.56$
Serum albumin	$P = 0.07$	$P = 0.52$	$P = 0.47$
Dosage of elemental Ca intake from PO ₄ binders	$P = 0.11$	$P = 0.62$	$P = 0.51$
Dosage of alphacalcidol	$P = 0.07$	$P = 0.30$	$P = 0.39$
<i>Vascular measures</i>			
Carotid intima-media thickness	$P = 0.08$	$P = 0.14$	$P = 0.09$
Aortic pulse wave velocity	$P = 0.016, r^2 = 0.19$	$P = 0.03, r^2 = 0.18$	$P = 0.22$
Aortic augmentation index	$P = 0.03, r^2 = 0.11$	$P = 0.09$	$P = 0.18$
Coronary calcification score on CT scan	$P = 0.007, r^2 = 0.20$	$P = 0.005, r^2 = 0.23$	$P = 0.61$

^aAll associations have been tested by Spearman’s non-parametric correlations. An r value has only been given for correlations with a p value < 0.05 .

^bExpressed as mean time-integrated values from the onset of CKD stage IV.

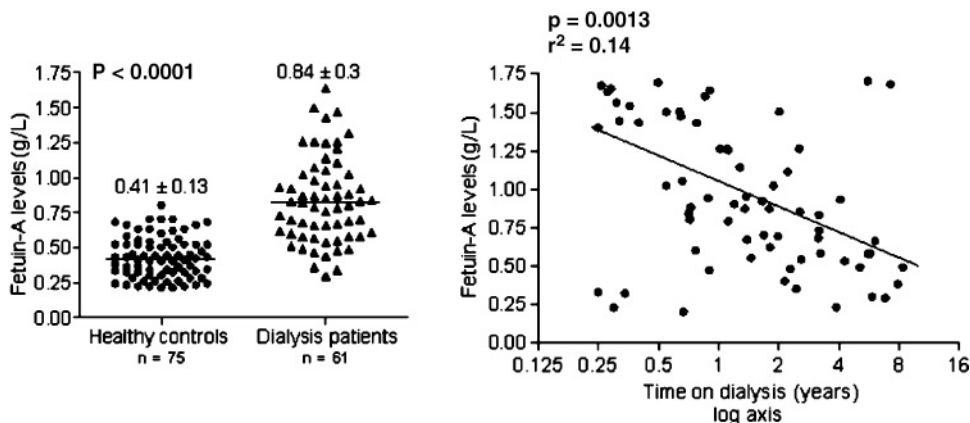


Fig. 2. Serum fetuin-A levels in children on dialysis were significantly higher as compared to controls, 0.84 ± 0.3 versus 0.41 ± 0.13 g/L, $P < 0.0001$ (A), and showed an inverse correlation with time on dialysis, $P = 0.0013, r^2 = 0.14$ (B).

but in the patients with calcification, an association was seen between fetuin-A and OPG levels ($P = 0.04, r = 0.09$). There was no correlation with uc-MGP and fetuin-A or OPG.

Associations between the calcification inhibitor levels and determinants of vascular measures

On stepwise multiple linear regression analyses fetuin-A levels independently predicted aortic PWV ($P = 0.004, \beta = -0.45, \text{model } R^2 = 48\%$) and fetuin-A and OPG predicted cardiac calcification ($P = 0.02, \beta = -0.29$ and $P = 0.014, \beta = 0.33, \text{model } R^2 = 32\%$, respectively).

Discussion

This is the first study to describe circulating levels of the calcification inhibitors fetuin-A, OPG and uc-MGP in a paediatric population and shows that children on dialysis have a significant perturbation of these levels that is associated with increased vascular stiffness and calcification.

In healthy children, both fetuin-A and OPG levels increased with age, presumably because both these proteins are expressed in bone [30] and play a role in skeletal mineralization [31]. Also, in healthy controls, fetuin-A levels were lower in taller children as compared to their age-related peers, suggesting that fetuin-A may be consumed

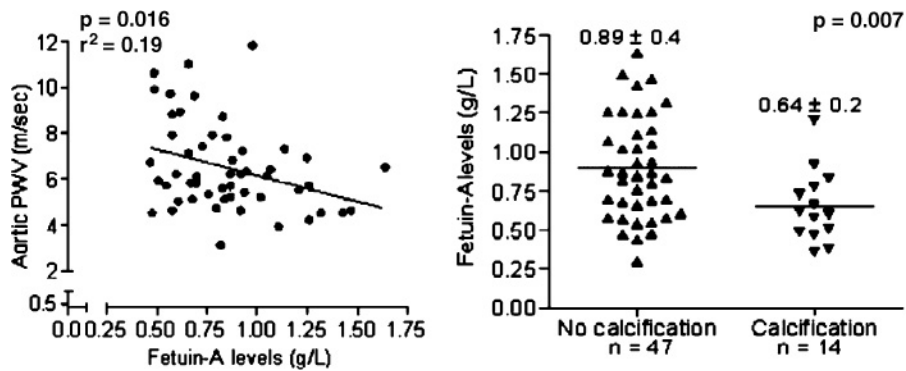


Fig. 3. The aortic pulse wave velocity (PWV) showed an inverse correlation with serum fetuin-A levels, $P = 0.016$, $r^2 = 0.19$ (A). Fetuin-A levels were significantly lower in children with coronary or valvular calcification ($n = 14$) on CT scan than in those without calcification, 0.64 ± 0.2 versus 0.89 ± 0.4 g/L, $P = 0.007$ (B).

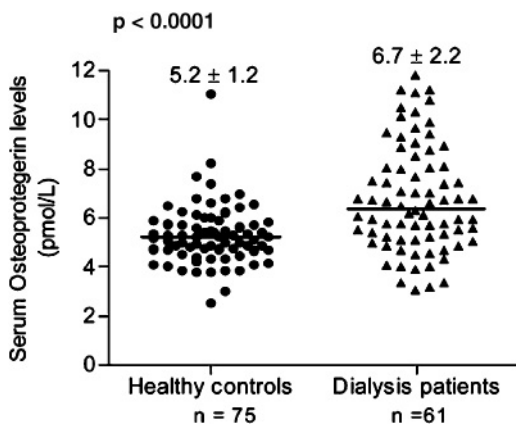


Fig. 4. Serum osteoprotegerin (OPG) levels were significantly higher in dialysis patients as compared to healthy controls, 6.7 ± 2.2 versus 5.2 ± 1.2 pmol/L, $P < 0.0001$.

in the course of active skeletal mineralization, as shown in an animal model [31]. We were unable to find a correlation between fetuin-A and features of bone turnover including height, alkaline phosphatase or PTH levels in dialysis patients; however, as the PTH levels in our cohort were lower than that recommended by the K/DOQI guidelines [32], a high-turnover bone state is unlikely.

Although several studies have reported that adults on dialysis have significantly lower fetuin-A levels than controls [20,22,33–36], we found higher levels of fetuin-A in paediatric dialysis patients as compared to healthy age-matched controls. Only children with evidence of calcification on CT scan had reduced fetuin-A, but even this group had higher levels than the controls. Nevertheless, with increasing dialysis vintage and hs-CRP, fetuin-A levels decreased. Our findings are supported by reports showing that adults with early CKD do not have a reduction in fetuin-A [37] and that there is no change in fetuin-A levels in dialysis patients with low levels of inflammatory activity [38]. Fetuin-A is a negative acute phase reactant [39], and in the pro-inflammatory dialysis environment its production may be reduced. In addition, the pro-calcific uraemic milieu may consume circulating fetuin-A: *in vitro* studies

have shown that fetuin-A contributes to ~50% of the calcification inhibitory capacity of human plasma [13], and by ‘shielding’ mechanisms prevents further crystal growth [12]. Taken together, this suggests that a protective mechanism allows an upregulation of fetuin-A in the early stages of CKD and dialysis, and only severe or prolonged exposure to a pro-inflammatory and/or pro-calcific environment eventually leads to low levels due to reduced production and/or increased consumption.

However, it may also be that patients with calcification have genetically lower fetuin-A levels that predispose them to calcification. Genetic polymorphism studies have given conflicting results [33,40], but the association between reduced fetuin-A levels and cardiovascular [19–22] and even all-cause mortality [19] in four large studies suggests that fetuin-A is likely to have a causal effect on vascular calcification. Moreover, low circulating fetuin-A levels are associated with high serum PO_4 levels even in the general population [40] and have been associated with valvular calcification in patients with normal renal function [41]. In our study, fetuin-A was a significant and independent predictor of vascular stiffness and calcification irrespective of dialysis vintage, $Ca \times PO_4$ levels or hs-CRP, implying that genetic polymorphisms may indeed play a role in an individual patient’s susceptibility to calcify, possibly by modulating the magnitude of change in fetuin-A production in response to a pro-inflammatory or pro-calcific environment.

We found an independent association between fetuin-A levels and aortic PWV and calcification, and there are few and conflicting reports in the literature on this. While a Japanese study has shown that fetuin-A levels predict carotid artery stiffness even in healthy subjects [42], Hermans *et al.* could not find an independent association between PWV or augmentation index and fetuin-A in dialysis patients [38]. The above dialysis cohort in fact had normal fetuin-A levels and a low level of inflammatory activity as compared to controls, and may not be a representative population of dialysis patients. Animal studies have shown that fetuin-A knock-out mice develop widespread soft-tissue and myocardial calcification, whereas their large arteries are spared [43]. In these animals the myocardial Ca content can increase up to 60-fold, initiating a profound

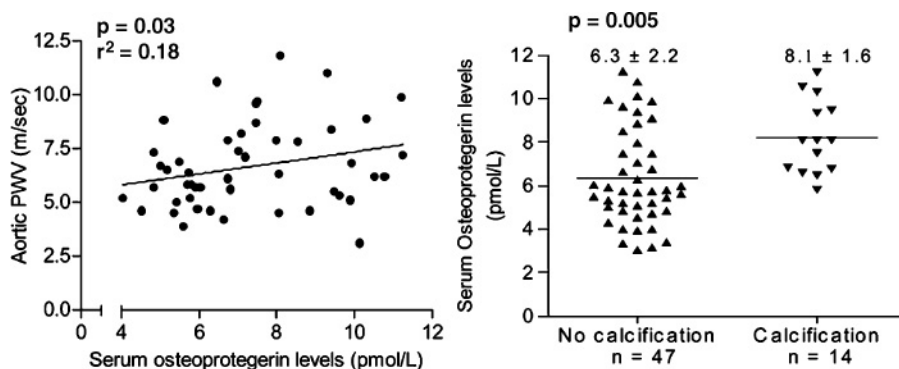


Fig. 5. The aortic pulse wave velocity (PWV) was positively associated with increasing serum osteoprotegerin (OPG) levels, $P = 0.03$, $r^2 = 0.18$ (A). Children with calcification had significantly higher OPG levels than those without calcification, 8.1 ± 1.6 versus 6.3 ± 2.2 pmol/L, $P = 0.0026$ (B).

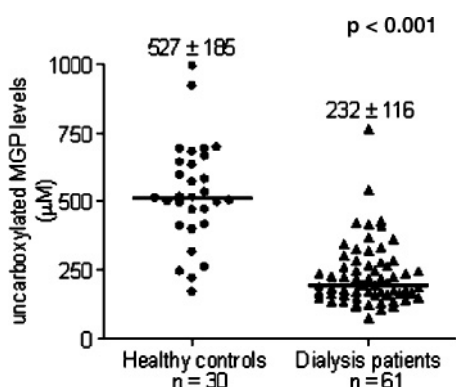


Fig. 6. Serum levels of undercarboxylated-matrix Gla protein (uc-MGP) were lower in children on dialysis as compared to controls, 232 ± 116 versus 527 ± 185 μM , $P < 0.001$.

pro-fibrotic response with the 'myocardial stiffness' leading to cardiac fibrosis, diastolic dysfunction, reduced cardiac output and an impaired tolerance to ischaemia [43]. Indeed, fetuin-A levels are an independent predictor of death in non-renal failure patients following electrocardiogram changes of ST-elevation and acute myocardial infarction [44].

In adults, serum OPG levels are associated with the progression of carotid atherosclerosis [45] and coronary calcification [16], and there are two reports, one in HD patients [46], linking serum OPG levels with vascular stiffness [47]. However, the vessel wall is a site of abundant OPG production, and mouse genetic studies have in fact suggested a vasculo-protective role for OPG [48–50]: targeted deletion of the OPG gene leads to calcification of the aorta and renal arteries [48], transgenic overexpression in OPG-null mice leads to rescue of the phenotype of vascular calcification [49], and finally, vascular calcification induced by warfarin or pharmacological doses of vitamin D can be inhibited by simultaneous application of OPG [50]. Thus, changes in OPG levels may be either cause or consequence of vascular calcification, and high circulating OPG levels in patients with cardiovascular disease could also mean that raised OPG levels detrimentally affect vascular homeostasis.

Although uc-MGP levels were significantly lower in dialysis patients than healthy controls, we were unable to find any correlations with uc-MGP and clinical or vascular measures. *In vitro* work has shown that in vessels with intimal and/or medial calcification, uc-MGP is localized around areas of calcification, whereas in healthy arteries active or γ -carboxylated MGP is present, with no uc-MGP [25,51]. It is possible that uc-MGP levels were so low that correlations with biochemical or vascular parameters is no longer possible. Also, we have not measured total MGP levels, and the low circulating uc-MGP may represent a lack of MGP production or reduced vitamin K-dependent γ -carboxylation of the uc-MGP to its active form. As MGP levels can potentially be modulated by dietary supplementation of vitamin K [52], the role of circulating MGP, if any, needs to be further explored.

There is clearly a complex relationship between the calcification inhibitory proteins and vascular measures in different clinical settings and even at the different stages of uraemic vasculopathy that are impossible to discern given the cross-sectional nature of this study and indeed all the other published work in this field. The results of the present study warrant further investigations of circulating inhibitor levels as well as their genetic polymorphisms to elucidate the potential clinical utility of these biomarkers. As cardiovascular morbidity is known to begin in the early stages of CKD, future studies should assess serial measures of the circulating inhibitors from CKD stages II–III.

In conclusion, we have shown for the first time that fetuin-A and OPG impact on vascular stiffness and calcification in children on dialysis. Paediatric dialysis patients have an upregulation of fetuin-A, possibly as a protective response to the pro-calcific and pro-inflammatory uraemic environment. While further longitudinal studies in both adult and paediatric CKD patients are required to fully characterize these circulating biomarkers, they may prove to be a useful and convenient measure of an individual patient's susceptibility to calcify.

Acknowledgements. This study was funded by the Kidney Research Aid Fund and funding for CT scans was obtained from the Eyck and Strutt scholarship from the British Medical Association. This work was presented

as an oral communication at the American Society of Nephrology meeting in San Francisco in November 2007.

Conflict of interest statement. G. Hawa is full time employee of Biomedica Medizinprodukte, Vienna, Austria. The remainder of the authors do not have any conflict of interest.

References

- Foley RN, Parfrey PS, Sarnak MJ. Clinical epidemiology of cardiovascular disease in chronic renal disease. *Am J Kidney Dis* 1998; 32: S112–S119
- Shroff RC, Donald AE, Hiorns MP *et al.* Mineral metabolism and vascular damage in children on dialysis. *J Am Soc Nephrol* 2007; 18: 2996–3003
- Kari JA, Donald AE, Vallance DT *et al.* Physiology and biochemistry of endothelial function in children with chronic renal failure. *Kidney Int* 1997; 52: 468–472
- Shroff RC, Shanahan CM. The vascular biology of calcification. *Semin Dial* 2007; 20: 103–109
- Block GA, Hulbert-Shearon TE, Levin NW *et al.* Association of serum phosphorus and calcium \times phosphate product with mortality risk in chronic hemodialysis patients: a national study. *Am J Kidney Dis* 1998; 31: 607–617
- Goodman WG, Goldin J, Kuizon BD *et al.* Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis. *N Engl J Med* 2000; 342: 1478–1483
- Goldsmith D, Ritz E, Covic A. Vascular calcification: a stiff challenge for the nephrologist: does preventing bone disease cause arterial disease? *Kidney Int* 2004; 66: 1315–1333
- Litwin M, Wuhl E, Jourdan C *et al.* Altered morphologic properties of large arteries in children with chronic renal failure and after renal transplantation. *J Am Soc Nephrol* 2005; 16: 1494–1500
- Block GA, Spiegel DM, Ehrlich J *et al.* Effects of sevelamer and calcium on coronary artery calcification in patients new to hemodialysis. *Kidney Int* 2005; 68: 1815–1824
- Shroff R, Egerton M, Bridel M *et al.* The bimodal association of vitamin D levels vascular disease in children on dialysis. *J Am Soc Nephrol* 2008; 19: 1239–1246
- Westenfeld R, Jahnen-Dechent W, Ketteler M. Vascular calcification and fetuin-A deficiency in chronic kidney disease. *Trends Cardiovasc Med* 2007; 17: 124–128
- Reynolds JL, Skepper JN, McNair R *et al.* Multifunctional roles for serum protein fetuin-A in inhibition of human vascular smooth muscle cell calcification. *J Am Soc Nephrol* 2005; 16: 2920–2930
- Price PA, Lim JE. The inhibition of calcium phosphate precipitation by fetuin is accompanied by the formation of a fetuin–mineral complex. *J Biol Chem* 2003; 278: 22144–22152
- Simonet WS, Lacey DL, Dunstan CR *et al.* Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997; 89: 309–319
- Kiechl S, Schett G, Wenning G *et al.* Osteoprotegerin is a risk factor for progressive atherosclerosis and cardiovascular disease. *Circulation* 2004; 109: 2175–2180
- Abedin M, Omland T, Ueland T *et al.* Relation of osteoprotegerin to coronary calcium and aortic plaque (from the Dallas Heart Study). *Am J Cardiol* 2007; 99: 513–518
- Shanahan CM, Cary NR, Metcalfe JC *et al.* High expression of genes for calcification-regulating proteins in human atherosclerotic plaques. *J Clin Invest* 1994; 93: 2393–2402
- Kiechl S, Schett G, Wenning G *et al.* Osteoprotegerin is a risk factor for progressive atherosclerosis and cardiovascular disease. *Circulation* 2004; 109: 2175–2180
- Hermans MM, Brandenburg V, Ketteler M *et al.* Association of serum fetuin-A levels with mortality in dialysis patients. *Kidney Int* 2007; 72: 202–207
- Ketteler M, Bongartz P, Westenfeld R *et al.* Association of low fetuin-A (AHSR) concentrations in serum with cardiovascular mortality in patients on dialysis: a cross-sectional study. *Lancet* 2003; 361: 827–833
- Stenvinkel P, Wang K, Qureshi AR *et al.* Low fetuin-A levels are associated with cardiovascular death: impact of variations in the gene encoding fetuin. *Kidney Int* 2005; 67: 2383–2392
- Wang AY, Woo J, Lam CW *et al.* Associations of serum fetuin-A with malnutrition, inflammation, atherosclerosis and valvular calcification syndrome and outcome in peritoneal dialysis patients. *Nephrol Dial Transplant* 2005; 20: 1676–1685
- Kiechl S, Schett G, Wenning G *et al.* Osteoprotegerin is a risk factor for progressive atherosclerosis and cardiovascular disease. *Circulation* 2004; 109: 2175–2180
- O'Donnell CJ, Shea MK, Price PA *et al.* Matrix Gla protein is associated with risk factors for atherosclerosis but not with coronary artery calcification. *Arterioscler Thromb Vasc Biol* 2006; 26: 2769–2774
- Schurgers LJ, Teunissen KJ, Knapen MH *et al.* Novel conformation-specific antibodies against matrix gamma-carboxyglutamic acid (Gla) protein: undercarboxylated matrix Gla protein as marker for vascular calcification. *Arterioscler Thromb Vasc Biol* 2005; 25: 1629–1633
- Agatston AS, Janowitz WR, Hildner FJ *et al.* Quantification of coronary artery calcium using ultrafast computed tomography. *J Am Coll Cardiol* 1990; 15: 827–832
- Ciaccio M, Bivona G, Di SR *et al.* Changes in serum fetuin-A and inflammatory markers levels in end-stage renal disease (ESRD): effect of a single session haemodialysis. *Clin Chem Lab Med* 2008; 46: 212–214
- Cozzolino M, Galassi A, Biondi ML *et al.* Decreased serum fetuin-A levels after a single haemodialysis session. *Nephrol Dial Transplant* 2007; 22: 290–291
- Gonnelli S, Montagnani A, Caffarelli C *et al.* Osteoprotegerin (OPG) and receptor activator of NF- κ B ligand (RANK-L) serum levels in patients on chronic hemodialysis. *J Endocrinol Invest* 2005; 28: 534–539
- Triffitt JT, Gebauer U, Ashton BA *et al.* Origin of plasma alpha2-HS glycoprotein and its accumulation in bone. *Nature* 1976; 262: 226–227
- Schinke T, Amendt C, Trindl A *et al.* The serum protein alpha2-HS glycoprotein/fetuin inhibits apatite formation *in vitro* and in mineralizing calvaria cells. A possible role in mineralization and calcium homeostasis. *J Biol Chem* 1996; 271: 20789–20796
- K/DOQI clinical practice guidelines for bone metabolism and disease in chronic kidney disease. *Am J Kidney Dis* 2003; 42: S1–S201
- Cozzolino M, Biondi ML, Galassi A *et al.* Gene polymorphisms and serum alpha-2-Heremans-Schmid levels in Italian haemodialysis patients. *Am J Nephrol* 2007; 27: 639–642
- Coen G, Manni M, Agnoli A *et al.* Cardiac calcifications: fetuin-A and other risk factors in hemodialysis patients. *ASAIO J* 2006; 52: 150–156
- Moe SM, Reslerova M, Ketteler M *et al.* Role of calcification inhibitors in the pathogenesis of vascular calcification in chronic kidney disease (CKD). *Kidney Int* 2005; 67: 2295–2304
- Stenvinkel P, Ketteler M, Johnson RJ *et al.* IL-10, IL-6, and TNF- α : central factors in the altered cytokine network of uremia—the good, the bad, and the ugly. *Kidney Int* 2005; 67: 1216–1233
- Ix JH, Chertow GM, Shlipak MG *et al.* Fetuin-A and kidney function in persons with coronary artery disease—data from the Heart and Soul Study. *Nephrol Dial Transplant* 2006; 21: 2144–2151
- Hermans MM, Brandenburg V, Ketteler M *et al.* Study on the relationship of serum fetuin-A concentration with aortic stiffness in patients on dialysis. *Nephrol Dial Transplant* 2006; 21: 1293–1299
- Lebreton JP, Joisel F, Raoult JP *et al.* Serum concentration of human alpha2-HS glycoprotein during the inflammatory process: evidence that alpha2-HS glycoprotein is a negative acute-phase reactant. *J Clin Invest* 1979; 64: 1118–1129
- Osawa M, Tian W, Horiuchi H *et al.* Association of alpha2-HS glycoprotein (AHSR, fetuin-A) polymorphism with AHSR and phosphate serum levels. *Hum Genet* 2005; 116: 146–151
- Ix JH, Chertow GM, Shlipak MG *et al.* Association of fetuin-A with mitral annular calcification and aortic stenosis among persons with

- coronary heart disease: data from the Heart and Soul Study. *Circulation* 2007; 115: 2533–2539
42. Mori K, Emoto M, Araki T *et al.* Association of serum fetuin-A with carotid arterial stiffness. *Clin Endocrinol (Oxf)* 2007; 66: 246–250
 43. Merx MW, Schafer C, Westenfeld R *et al.* Myocardial stiffness, cardiac remodeling, and diastolic dysfunction in calcification-prone fetuin-A-deficient mice. *J Am Soc Nephrol* 2005; 16: 3357–3364
 44. Lim P, Collet JP, Moutereau S *et al.* Fetuin-A is an independent predictor of death after ST-elevation myocardial infarction. *Clin Chem* 2007; 53: 1835–1840
 45. Kiechl S, Schett G, Wenning G *et al.* Osteoprotegerin is a risk factor for progressive atherosclerosis and cardiovascular disease. *Circulation* 2004; 109: 2175–2180
 46. Othmane TH, Bakonyi G, Egresits J *et al.* Effect of sevelamer on aortic pulse wave velocity in patients on hemodialysis: a prospective observational study. *Hemodial Int* 2007; 11(Suppl 3): S13–S21
 47. Kim SM, Lee J, Ryu OH *et al.* Serum osteoprotegerin levels are associated with inflammation and pulse wave velocity. *Clin Endocrinol (Oxf)* 2005; 63: 594–598
 48. Bucay N, Sarosi I, Dunstan CR *et al.* osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 1998; 12: 1260–1268
 49. Min H, Morony S, Sarosi I *et al.* Osteoprotegerin reverses osteoporosis by inhibiting endosteal osteoclasts and prevents vascular calcification by blocking a process resembling osteoclastogenesis. *J Exp Med* 2000; 192: 463–474
 50. Price PA, June HH, Buckley JR *et al.* Osteoprotegerin inhibits artery calcification induced by warfarin and by vitamin D. *Arterioscler Thromb Vasc Biol* 2001; 21: 1610–1616
 51. Murshed M, Schinke T, McKee MD *et al.* Extracellular matrix mineralization is regulated locally; different roles of two gla-containing proteins. *J Cell Biol* 2004; 165: 625–630
 52. Schurgers LJ, Dissel PE, Spronk HM *et al.* Role of vitamin K and vitamin K-dependent proteins in vascular calcification. *Z Kardiol* 2001; 90(Suppl 3): 57–63

Received for publication: 23.1.08

Accepted in revised form: 2.4.08