Human uremic serum displays calcific potential *in vitro* that increases with advancing chronic kidney disease.

[1]Ashish Patidar (MSc), [2]Dhruv K. Singh (MD), [3]Peter Winocour (MD), [1,2]Ken Farrington (MD), and [1]Anwar R. Baydoun (PhD)

[1]School of Life and Medical Sciences University of Hertfordshire, College Lane, Hatfield, AL10 9AB, UK

[2]Renal Unit, Lister Hospital, Coreys Mill Lane, Stevenage, SG1 4AB, UK

[3] Department of Diabetes and Endocrinology, QEII Hospital, Howlands, Welwyn Garden City, AL7 4HQ, UK

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Author for correspondence: Professor Anwar R Baydoun
School of Life and Medical Sciences
University of Hertfordshire
College Lane
Hatfield
AL10 9AB

Telephone: 01707285120
Fax: 01707285046
e-mail: a.baydoun@herts.ac.uk

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Abstract

Vascular calcification strongly correlates with declining renal function and contributes to the high morbidity and mortality of patients with chronic kidney disease (CKD). It is closely regulated by circulating factors but little is known about the potential of serum from patients to induce calcification outside the disease setting – the calcific potential of serum. We have therefore examined the ability of serum from age- and sex-matched subjects with and without advancing CKD to induce calcification of cultured smooth muscle cells. Samples from patients with CKD induced significant calcification compared with controls. More importantly, samples from patients on haemodialysis induced significantly higher calcification than those with moderate or advanced CKD. The calcification induced by the latter two but not those on haemodialysis could be enhanced with calcium chloride and β-glycerophosphate. A positive correlation was evident between measured serum creatinine, phosphate, parathyroid hormone, osteoprotegerin and the degree of calcification *in vitro*. Estimated glomerular filtration rate, diastolic blood pressure, haemoglobin and serum albumin correlated negatively. Stepwise multivariate analysis of log-transformed calcific potential data highlighted serum creatinine, albumin and osteoprotegerin as significant predictors, explaining around 50% of the variation. Thus, other regulators either not investigated or as yet unidentified, may contribute to the calcification potential of serum *in vitro*. Furthermore, uremic serum can induce graded calcification outside of the disease milieu that reflects the degree of kidney impairment *in vivo*. These findings could have important clinical relevance in terms of developing novel diagnostic and/or therapeutic strategies for subjects with CKD.

INTRODUCTION

Calcification is a common occurrence in end-stage renal failure and shows a strong correlation with declining renal function [1]. It often results in a diverse range of pathologies including calcific uraemic arteriolopathy, solid as well as extra-osseous soft tissue calcification of which vascular calcification (VC) is the most common and often the most clinically relevant corollary. The existence of VC in chronic kidney disease (CKD) results in increased arterial stiffness, becoming predictive of secondary cardiovascular complications with high morbidity and mortality [2, 3].

The precise mechanisms that lead to VC in CKD subjects are poorly understood but it is accepted to be a delicate and well-controlled biological process that results in smooth muscle cells (SMCs) gaining an osteoblastic phenotype [4]. These complex changes may be regulated *in vivo* by circulating factors in plasma and, interestingly, *in vitro* by uremic serum especially under hyperphosphatemic conditions [5]. The latter demonstrates that serum from pathological settings may have a potential to induce VC even outside the disease milieu and provides a means through which the mechanisms associated with this process can be investigated. More importantly, the ability of serum to induce calcification *in vitro* could be exploited to predict the risk of calcification *in vivo* during routine tests, and applied to the clinical management of patients. To achieve this, a robust biological model is required in which the calcific potential of individual serum samples can be validated, and trends within defined groups of CKD patients identified to allow the establishment of threshold scores which might guide the need for intervention.

We have developed an *in vitro* model of calcification in smooth muscle cells and embarked on a program of studies to establish the calcific potential (i.e. ability to induce calcification) of individual serum samples from cohorts of patients with CKD. More specifically, we have investigated whether serum from patients with stages 3, 4/5 CKD or on haemodialysis (5D) has a greater *in vitro* calcific potential than normal age- and sex-matched controls. We also wished to establish whether *in vitro* calcific potential correlated with the degree of renal impairment. In parallel, we have characterised each patient in terms of disease vintage, extra-renal co-morbidity, and smoking history. Furthermore, we have related the calcific potential of serum in individuals to the levels of biochemical markers and report for the first time that CKD serum induces graded calcification outside of the disease milieu that correlates with the degree of kidney impairment, and with specific biomarkers which may, in part, explain the potential of serum to induce calcification *in vitro*.

METHODS

Ethical approval

Ethical approval was obtained from the Hertfordshire Research Ethics Committee. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. The study subjects gave written informed consent prior to recruitment into the study.

Patient recruitment

All the patients were recruited from the renal clinics in East and North Herts NHS Trust hospitals in Hertfordshire, UK. Inclusion criteria for the patients with CKD were age >18 years, glomerular filtration rate (eGFR) by MDRD-4 formula less than 60 ml/min. Exclusion criteria were immunosuppressive therapy, pregnancy, hormone replacement therapy and malignancy. Twenty age- and sex-matched healthy individuals with normal renal function, without diabetes or *in vivo* calcification as detected with CT scan were recruited as controls. There were 70 subjects in the patient group who, based on eGFR, were classified into a moderate (stage 3 CKD; 21 subjects), advanced (stage 4 and 5 CKD; 18 subjects) or haemodialysis group (5D; 30 subjects). In the 5D group only those patients who had undergone at least 3 months of dialysis were considered.

Data collection

Demographic and clinical variables including age, gender, and blood pressure were recorded. Blood samples in controls and pre-dialysis patients were collected during routine visits and those of haemodialysis patients were collected immediately before dialysis sessions. Samples were taken into Z serum sep clot activator VACUTTE®, centrifuged at 2500 rpm for 15 minutes at 4^{o}C and the serum stored at -80^{o}C until analysed.

Biochemical analysis

Routine biochemistry analyses were conducted for urea, creatinine, and C-reactive protein (CRP). Levels of the latter > 5 mg/l were considered high. Parathyroid hormone (PTH) concentrations were measured on the Beckman Access® 2 immunoassay system (Beckman Coulter, High Wycombe, UK). Assays for osteoprotegerin (OPG), receptor activator of nuclear factor kappa B ligand (RANKL), bone alkaline phosphatase (BAP) were performed on the automated ELISA (enzyme linked immunosorbent assay) analyzer, Triturus® (Grifols, Cambridge, UK). Matrix Gla protein (MGP) was quantified using the BIOMEDICA GRUPEE kit (BI-20062). All analyses were performed in duplicate.

Isolation and culture of rat aortic smooth muscle cells

Cells were isolated as previously described [6] using aortic explants obtained from male Wister rats and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}) and either 10% foetal bovine or human serum.

Induction of calcification in rat cultured aortic smooth muscle cells

Cells in 96-well plates were allowed to grow to 60-70 % confluency over 48 hours before being incubated with 10% serum in the absence (Controls) and presence of a widely used standard calcification buffer consisting of 7mM calcium Chloride ($CaCl_2$) plus 7mM β-glycerophosphate (β-GP) [7]. To ensure that any subsequent mineralisation was regulated and reflective of genuine calcification of cells rather than non-physiological spontaneous precipitation of excess calcium phosphate, parallel experiments were carried out where $CaCl_2$ (7 mM) and β-GP (7 mM) were incubated either in empty wells or with cells fixed with 10% formaldehyde. All incubations were terminated after the designated time period which varied from 1 to 7 days.

Determination of calcification and total cell protein content

Cell monolayers were lysed with buffer consisting of 10 mM TRIS (pH 7.4) and 10 % sodium dodecyl sulphate. Calcification was determined using the DICA-500 Ca^{+2} assay Kit from Bioassay System (Universal Biologicals, UK), according to the manufacturer's instructions. Total protein content was measured using the bicinchoninic acid (BCA) protein assay as described previously [8]. Once determined, calcium levels in lysates were normalised for total cell protein per well and the data presented as nmoles Ca^{+2} protein mg^{-1}.

In parallel experiments, cells were viewed at 10x magnification using a Nikon confocal microscope under normal view to visualise plaques formed as a consequence of calcification.

Cell Viability Assay

Cell viability was determined under the different experimental conditions by assessing the metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan as described previously [8].

Statistical Analysis

Statistical analysis was carried out using SPSS, version 17 and the data presented as the mean ± SD for normally distributed, and as median (interquartile range) if not normally distributed. For continuous variables the significance of differences between two groups was determined by student t test (for normally distributed data) and the Mann-Whitney U-tests (for non-normally distributed data). One Way Analysis of

Variance followed by Bonferroni post-hoc testing or the Kruskal-Wallis test was used to compare multiple groups as appropriate. The Chi-squared test was used to differentiate between the groups with respect to categorical data. Correlations utilised the Pearson and Spearman test as appropriate. For multivariate analysis, values of calcific potential were log-transformed.

Materials

Cell culture reagents and other general biochemical reagents including $CaCl_2$ and β-GP were purchased from Sigma-Aldrich (London, UK). Penicillin and streptomycin was from Fisher Scientific (Loughborough, UK) and foetal bovine serum (FBS) from Gibco (Scotland, UK). The BCA protein quantification Kit was from Thermo Scientific (Hemel Hempstead, UK) and the DICA-500 Ca^{+2} assay Kit was from Universal Biologicals (Cambridge, UK). Reagents for OPG, RANKL, BAP were obtained from Oxford biosystems, Oxford, UK.

RESULTS

Demographics and clinical factors

All subjects were Caucasian. There were no significant differences in age or gender ratios across the groups. Systolic blood pressure (SBP) was significantly lower in the 5D group than in patients with advanced CKD, and diastolic blood pressure (DBP) was significantly lower in the 5D than in both pre-dialysis groups (Table 1).

Baseline biochemistry and hematology

CKD groups differed significantly from controls with respect to haemoglobin, serum albumin, cholesterol, phosphate, PTH, OPG, and RANKL levels and with respect to OPG/RANKL ratio (Table 1). Serum phosphate increased with advancing CKD while serum calcium was unchanged. Haemoglobin levels were higher in moderate CKD than in the 5D group. Serum albumin was higher in both moderate and advanced CKD groups than in 5D patients. Likewise the proportion with elevated CRP levels was greater in the 5D group then in both other CKD groups. Bone alkaline phosphatase levels were similar in all CKD groups. Parathyroid hormone and OPG levels were significantly higher in 5D patients than both pre-dialysis groups, whilst RANKL levels and OPG/RANKL ratios did not differ significantly between CKD stages. Matrix Gla protein has so far been determined only in the 5D group in which levels were significantly lower than in controls (Table 1).

Development of an in vitro model of calcification

In order to be able to determine the effects of serum from human patients on the development of VC, an *in vitro* model of this process was initially established using rat cultured aortic smooth muscle cells (RASMCs) exposed to $CaCl_2$ and β-GP as a phosphate source. Each compound was used at a concentration of 7mM and this was predetermined from pilot studies using 2mM to 20mM of either component. At 2mM there was little or no evidence of calcification, while at higher concentration (≥15mM) Ca^{+2} precipitated with β-GP in the medium (data not shown). A 7mM concentration of each combined was found to give consistent and optimum calcification. As shown in Figure 1A, incubation of cells for 5 days with complete culture medium supplemented with 10 % FBS resulted in marginal calcification. Inclusion of 7mM $CaCl_2$ enhanced calcification while β-GP (7mM) was without significant effect. By comparison, inclusion of both together caused a marked increase in the calcification which was much greater than the additive effects.

Photographs taken of cells following the different treatment conditions (Figure 1B) revealed a normal monolayer under control conditions with little evidence of calcification as indicated by the lack of calcific plaques. Incubations with $CaCl_2$ at 7mM showed moderate plaque formation while cells incubated with β-GP exhibited less marked effects. Consistent with the calcification data (Figure 1A), co-incubations with $CaCl_2$ and

β-GP (both at 7mM) caused marked calcific plaque formation throughout the monolayer (Figure 1B).

In parallel studies, the time course of calcification was determined over 7 days. The data presented in Figure 1C show a time dependent increase which was evident after Day 3, reaching a peak at Day 5 but declining by Day 7. This trend was only evident when cells were incubated with $CaCl_2$ together with β-GP. The basal levels of calcification seen with culture medium alone did not change significantly over time (Figure 1C).

Assessment of cell viability using the MTT assay showed that survival was unaffected at days 1 to 5 but significantly reduced at day 7 as reflected by the marked inhibition of MTT metabolism to formazan (Figure 1D). This decrease in cell viability may account for the decreases in calcification observed at day 7 and as a result all subsequent experiments were carried out over a 5 day period.

3.3. Effects of human serum on calcification of RASMCs

Incubation of cells with serum from patients with CKD induced calcification of RASMCs outside the disease milieu. More importantly, there was a graded response in total cellular calcium content and deposition of calcium phosphate crystals. These changes which we have defined as the calcific potential of serum, correlated with the degree of renal impairment. This is clearly evident in Figure 2 which shows that serum from 5D patients had a greater calcific potential than serum obtained from stage 3 or stage 4/5. Control human serum did not cause any obvious calcification of cell monolayers and the total detected levels of calcium (50.8±9.3) were not significantly different to those in cells incubated with FBS (32.2±8.4 nmoles Ca^{+2} protein mg^{-1}; p=0.30). These levels are consistent with other studies in smooth muscle cells [9, 10]. However the degree of calcification in our studies was less, perhaps due to the difference in the species and incubation period.

In the whole group, the calcific potential of serum correlated positively with blood urea, serum creatinine, phosphate, OPG and PTH, and negatively with DBP, haemoglobin and albumin (Table 2). In stepwise multivariate analysis the significant predictors of log transformed calcific potential were serum creatinine (p<0.001), albumin (p=0.009) and OPG (p=0.010). These factors however only explain around 50% of the variation in calcific potential (r^2=0.506). Other parameters including age, serum cholesterol, phosphate, PTH and high CRP were not significant in this model. The calcific potential of serum could therefore be influenced by other as yet unidentified factors.

In view of the graded response reported above, we were interested in establishing whether the calcific potential of the stages 3, 4/5 or 5D sera could be enhanced under conditions that may be associated with calcification *in vivo* (such as the presence of high calcium and phosphate). We therefore investigated the effects of co-incubating RASMCs with serum samples and both $CaCl_2$ (7mM) and β-GP (7mM). In these studies, the calcific potential of serum from stage 3 and 4/5 CKD groups was

significantly enhanced whilst no significant increment was apparent with control or 5D samples (Figure 3). Interestingly, within the latter (5D group) we observed that half the samples (referred to here as responders) did show an increase in calcification above that seen with serum alone when co-incubated with $CaCl_2$ and β-GP while the other half (non-responders) did not (Figure 4). What was even more striking was that responders had significantly higher levels of MGP ($p=0.003$), high density lipoprotein (HDL; $p=0.004$) and RANKL ($p=0.012$) when compared to the non-responders. Serum phosphate was lower and serum albumin higher in the responders but this did not attain statistical significance. Other levels of other potential modulators of calcification in Table 1 were not significantly different between the two groups.

DISCUSSION

Although plasma constituents are believed to regulate vascular calcification, it is not entirely clear how the various molecules act, nor is the complex interplay amongst the key factors established. Furthermore, the critical role of each biomarker, in terms of which might be the most important molecule(s) remains unresolved. Of particular relevance to the current manuscript is the additional question about whether their presence in serum may be sufficient to regulate calcification outside of disease settings. If established, the latter may be of clinical significance in defining thresholds of calcific potential and predicting risks of calcification *in vivo* which might inform therapeutic strategies.

With the exception of studies by Chen *et al* demonstrating that pooled uremic sera may accelerate calcification in bovine cultured vascular smooth muscle cells, there are no studies that have examined the direct calcific potential of non-pooled serum or its constituents in an *in vitro* setting [5]. More importantly, the key question of whether the calcific potential of serum is dependent on the degree of renal impairment has not been addressed. We have therefore correlated the degree of *in vitro* calcification with the severity of renal impairment *in vivo* and have looked at the association between several biomarkers and the degree of calcification induced *in vitro*.

In developing our model, we examined the effects of Ca^{+2} alone, phosphate in the form of β-GP and a combination of both at pre-determined concentrations. The degree of calcification was less pronounced with $CaCl_2$ and even less so with β-GP alone but clearly evident when both were used in combination. These results suggest that, on their own, neither $CaCl_2$ nor β-GP may be sufficient to induce profound calcification, at least *in vitro*. That this contradicts other studies may be due to differences in the duration of experiments, which in ours were over a shorter period (5 days). Others were prolonged, obtaining calcification after incubations of between 20 to 30 days [5, 11]. It is therefore likely that when used individually, $CaCl_2$ or phosphate may require a longer duration to cause calcification. The advantage of our model however is that calcification was significantly induced and evident within a period during which optimal cells confluency was maintained and viability was not compromised. Moreover, the process was time-dependent supporting the notion that it is regulated and not simply a consequence of aggregation of Ca^{+2} and phosphate to form crystals. One conclusion we have not been able to draw from our studies is whether the changes we observed involved a change in phenotype or indeed differentiation of the cells into bone-like structures and this remains to be established.

The results obtained with the clinical samples confirm that serum from CKD settings can induce calcification of normal healthy smooth muscle cells *in vitro*. Moreover, we have provided the first demonstration of this effect being graded, correlating well with the severity of renal impairment *in vivo*. In the first instance, the 5D sera had the most striking calcific potential which was effectively 2-fold higher than the increases seen with the CKD3 or CKD 4/5 and at least 8-fold higher than the controls samples. With the limited sample size used in our studies, there was no notable difference in the

effects obtained with the CKD3 and the CKD4/5 sera but both were significantly higher than baseline values.

The events and mechanisms underlying the effects described above have not yet been investigated. Studies aimed at addressing these are now planned and will include analysis of fetuin A and FGF-23 which are emerging as critical regulators of calcification [12]. In the interim we have analysed our samples for calcium and phosphate as well a number of other putative biomarkers of *in vivo* calcification and correlated these with the calcific potential of serum in our model.

The first notable observation was that calcium was not significantly elevated above controls nor was it significantly different between the three CKD groups. Phosphate levels on the other hand increased relative to the degree of renal impairment and correlated positively with *in vitro* calcification. However, in our multivariate model phosphate was not independently associated with calcific potential, suggesting that it may not be critical in this process. This would be consistent with our earlier demonstration that phosphate, on its own, is not sufficient to induce calcification *in vitro* over periods not associated with detrimental changes in cell survival and viability. Moe *et al* have also indicated that serum factors other than phosphate may be required for inducing calcification *in vitro* [13]. These observations do not support other published literature [14-16].

Blood urea, serum creatinine, PTH and OPG also increased in parallel with the severity of renal failure and correlated positively with calcific potential. Although serum creatinine was an independent predictor of calcific potential in our multivariate model it is only a marker of renal dysfunction. Parathyroid hormone on the other hand remains controversial, with some studies claiming elevated levels determine the severity and progression of VC [17] while others have suggested that it may inhibit calcification [18]. To further compound the controversy, other groups have failed to establish a link between PTH and VC in patients with CKD [19, 20]. There was also no independent association of PTH with calcific potential in our multivariate model suggesting that it is not directly involved in the process. Whilst OPG has been implicated as a key inhibitor of VC in animal models [21, 22], levels are reported to be elevated in serum from CKD patients on dialysis [23, 24] and have been strongly correlated with the severity of coronary calcification [11, 25]. We found OPG to be independently associated with calcific potential in multivariate modeling, supporting a role *in vitro* and in the genesis of VC in humans.

Albumin decreased with advancing CKD and was negatively correlated with calcific potential with which it was independently associated in our multivariate analysis. This may still be a reflection of the severity of the degree of renal impairment but may also indicate a regulatory role in the pathogenesis of VC perhaps by inhibiting calcification through attenuation of seeding of calcium phosphate crystals [26]. C-reactive protein and cholesterol which may have roles in the development of *in vivo* calcification [27] were associated with serum calcific potential in univariate analyses of our data. We did not

however find evidence of independent association, but this does not preclude a role for inflammation or lipid abnormalities in the *in vitro* model.

In parallel with establishing the direct effects of serum we also explored whether calcific potential could be enhanced by increasing calcium and phosphate concentrations in the incubation medium. Addition of $CaCl_2$ and β-GP increased the calcific potential of the predialysis sera but did not alter that of the control or haemodialysis group significantly. The lack of enhancement of the calcific potential of 5D sera led us to examine individual samples more closely and this identified two distinct groups which we have referred to as responders and non-responders. The former showed decreased calcific potential when compared to the non-responders. However, the calcific potential of serum from responders could be enhanced by $CaCl_2$ and β-GP which was not evident with serum from non-responders. Further analysis of the biochemical parameters revealed that non-responders had significantly lower levels of BAP, HDL, RANKL and MGP. Phosphate levels were higher and albumin levels lower in this group but not significantly so. It may be that in non-responders, the potential to calcify is maximal even before addition of extra calcium and phosphate. In contrast, in responders, perhaps associated with higher ambient levels of inhibitors (e.g. HDL, MGP, RANKL) and lower levels of promoters (ie high albumin and low phosphate), the potential to calcify is sub-maximal, and only fully expressed on addition of extra calcium and phosphate.

In summary, we have developed a model of VC which we have exploited to demonstrate the calcific potential of serum and shown that this correlated with the degree of uraemia in patients. The model appears suitable for screening clinical samples and offers a reproducible but yet simple system that could be employed in predicting risks of calcification *in vivo.* Additionally, the model can now be utilised to identify critical biomarker surrogates and mechanisms responsible for the calcific potential of the serum. This may lead to the development of diagnostic tools and inform therapeutic strategies.

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Table 1: Biochemical parameters of serum from controls and CKD subjects

NS = non-significant, SBP = systolic blood pressure, DBP = diastolic blood pressure, eGFR (ml/min/1.73m^2) = estimated glomerular filtration rate calculated using MDRD 4 formula, CRP = C-reactive protein, BAP = bone specific alkaline phosphatase, PTH = parathyroid hormone, OPG = osteoprotegerin, RANKL = receptor activator of nuclear factor kappa B ligand, MGP = matrix Gla protein. Normally distributed parameters represented as mean ± standard deviation. Non-normally distributed parameters represented as median (minimum, maximum). *$p < 0.05$; **$p < 0.01$; $^{\dagger}p < 0.001$ denote significant difference from controls by ANOVA.

Table 2: Correlation coefficients of serum calcific potential with biomarkers

Serum PTH = serum parathyroid hormone, serum OPG = serum osteoprotegerin and eGFR (ml/min/1.73m^2) = estimated glomerular filtration rate calculated using MDRD 4 formula. Calcification potential values were correlated with particular biochemical variables using Pearson's or Spearman correlation as appropriate.

Figure 1: Induction of calcification and assay for cytotoxicity in RASMCs.

Cells cultured to 60 % confluency were incubated for 5 days with either complete culture medium alone (Control; open bar), media containing 7mM $CaCl_2$, 7mM β-GP (solid bars) or a combination of both (Panel A). In parallel studies cells were incubated with 7mM $CaCl_2$ plus 7mM β-GP for different time points (Panel C). Calcification was quantified and normalised for total cell protein as described in the methods. The formation of calcific plaques was visualised under an inverted microscope at x40 magnification and the photograph (Panel B) is representative of at least 3 independent experiments. Changes in cell viability was determined as described in methods and represented in Panel D. The data is the mean ± SEM from 3 experiments with 5 replicates in each. • denotes $p<0.05$, •• denotes $p<0.01$, ••• denotes $p<0.001$ when compared to controls (C).

Figure 2: Calcification of RASMC by serum from patients with CKD.

Cells cultured to 60 % confluency were incubated for 5 days with complete culture medium and 10% serum from controls (open bar) or from subjects with CKD3, CKD4/5 or on haemodialysis (solid bars). Calcification was quantified and normalised for total cell protein as described in the methods. The data represents the mean ± SEM from 3 experiments with 5 replicates in each. • denotes $p<0.05$, •• denotes $p<0.01$, ••• denotes $p<0.001$ statistical significance.

Figure 3: Effects of $CaCl_2$ and β-GP on serum-induced calcification.

Cells cultured to 60 % confluency were incubated for 5 days with complete culture medium containing 10% control (open bar) or CKD Sera in the absence (solid black bars) and presence of $CaCl_2$ (7mM) and β-GP (7mM) (hatched bars). Calcification was quantified and normalised for total cell protein as described in the methods. The data

represents the mean ± SEM from 3 experiments with 5 replicates in each. •• denotes $p<0.01$ statistical significance.

Figure 4: Effects of $CaCl_2$ and β-GP on serum-induced calcification in responders and non-responders.

Cells cultured to 60 % confluency were incubated with (hatched bars) or without $CaCl_2$ (7mM) and β-GP (7mM) (solid bars) for 5 days in complete culture medium containing 10% Serum from subjects on haemodialysis. Calcification was quantified and normalised for total cell protein as described in the methods. The data represents the mean ± SEM from 3 experiments with 5 replicates in each. • denotes $p<0.05$ statistical significance.

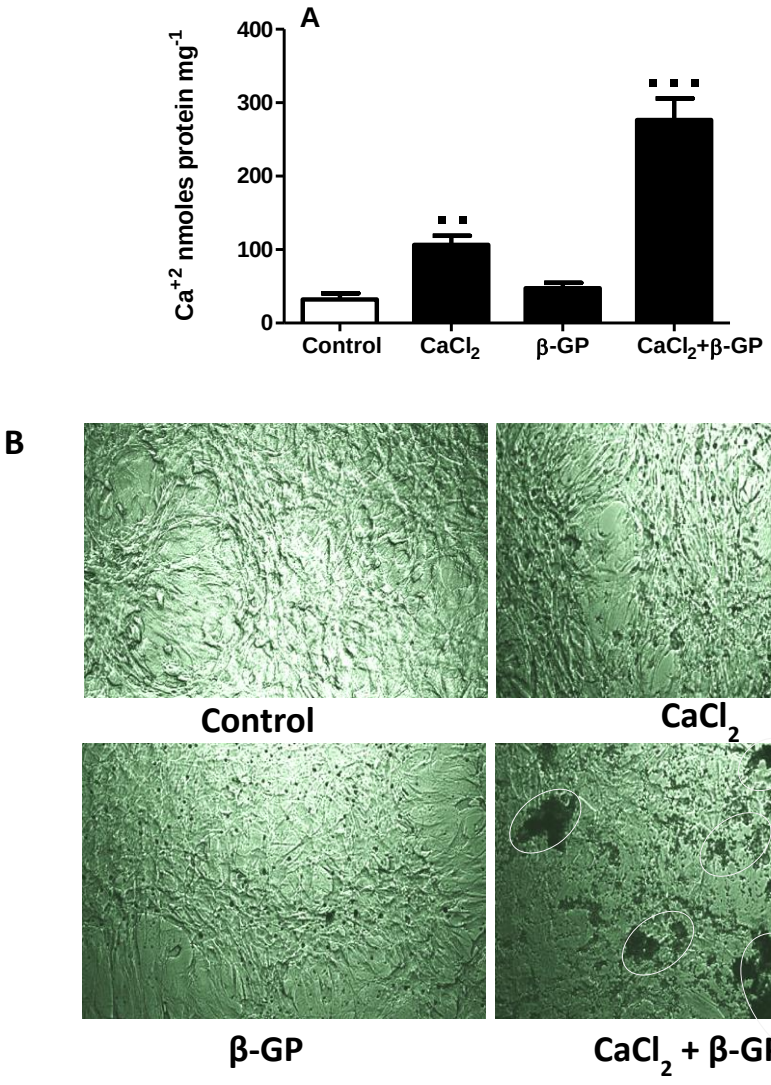
Table 1: Biochemical parameters of serum from controls and CKD subjects

Variables	Groups						
	Control (C)	Moderate CKD (M)	Advanced CKD (A)	Haemodialysis (HD)	M v A p-value	M v HD p-value	A v HD p-value
Number	20	21	18	30	NS	NS	NS
Age (yrs)	63 ± 6.2	64 ± 9.8	66 ± 17	64 ± 14	NS	NS	NS
Gender (M:F)	12/8	15/6	10/8	22/10	NS	NS	NS
SBP (mmHg)	150 ± 24	145 ± 13	152 ± 27	134 ± 17	NS	NS	0.026
DBP (mmHg)	87 ± 9	85 ± 12	89 ± 11	73 ± 11†	NS	0.002	<0.001
Creatinine (umol/l)	72 ± 17	150 ± 29	291 ± 80†	751 ± 235†	0.017	<0.001	<0.001
eGFR (MDRD 4)	94 ± 24	42 ± 8†	19 ± 5†	NA	<0.001	NA	NA
Urea (mmol/l)	5.2 ± 1.2	10.3 ± 2.4**	20.3 ± 6†	20.7 ± 6†	<0.001	<0.001	NS
Haemoglobin (g/dl)	14.0 ± 1.0	12.7 ± 2.4	11.2 ± 2	11.1 ± 1.1**	NS	0.010	NS
High CRP %	15	33	22	50*	NS	NS	0.016
Calcium(mmol/l)	2.49 ± 0.13	2.41 ± 0.12	2.37 ± 0.1	2.37 ± 0.2	NS	NS	NS
Cholesterol (mmol/l)	5.8 ± 1.0	4.8 ± 1.1*	4.6 ± 1.0**	4.1 ± 1.2†	NS	NS	NS
HDL Cholesterol (mmol/l)	1.5 ± 0.3	1.2 ± 0.3	1.4 ± 0.4	1.3 ± 0.6	NS	NS	NS
Phosphate (mmol/l)	1.02 ± 0.15	1.11 ± 0.24	1.34 ± 0.26*	1.66 ± 0.47†	NS	<0.001	0.010
Albumin (g/l)	45.4 ± 2.5	41.1 ± 4.7**	41.8 ± 4.8*	37.4 ± 2.9**	NS	0.005	0.001
BAP (IU/l)	-	26.2 ± 6.4	28.6 ± 6.8	24.1 ± 6.7	NS	NS	NS
PTH (pmol/l)	4.0 (2.3, 6.1)	3.0 (1.2, 22.9)	5.6 (1.9, 25.6)	17.8 (1.0, 43.7)†	NS	<0.001	<0.001
OPG (pmol/l)	4.0 ± 1.5	5.4 ± 1.7	5. 8 ± 2.2	9.8 ± 3.7†	NS	<0.001	<0.001
RANKL (pmol/l)	0.0 (0, 0.44)	0.05 (0, 0.47)†	0 (0, 0.42)†	0.02 (0, 0.37)†	NS	NS	NS
OPG RANKL Ratio	75.7 (7.4, 291)	12.7 (5.3,41.2)†	15.9 (6.1, 71.5)†	16.3 (7.3, 35.4)†	NS	NS	NS
MGP (ng/ml)	15.4 ± 1.4	-	-	1.94 ± 0.8	-	-	-

Table 2: Correlation coefficients of serum calcific potential with biomarkers

Variables	Calcific potential of serum	
	Coefficient	**p-value**
Serum Creatinine	0.725	*<0.001*
Blood urea	0.600	*<0.001*
Serum PTH	0.455	*<0.001*
Serum OPG	0.419	*<0.001*
Serum phosphate	0.539	*<0.001*
MDRD eGFR	-0.551	*<0.001*
Diastolic blood pressure	-0.411	*<0.001*
Haemoglobin	-0.382	*<0.001*
Serum albumin	-0.451	*<0.001*

Figure 1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400
300
200
100
0
Ca+2 nmoles protein mg-1
Control
CaCl2
β-GP
CaCl2+β-GP
B
Control
CaCl2
β-GP
CaCl2 + β-GP
C
300
200
100
0
Ca+2 nmoles protein mg-1
1D
3D
5D
7D
D
150
100
50
0
% Cell Viability
Control
1
3
5
7
Day

Figure 2

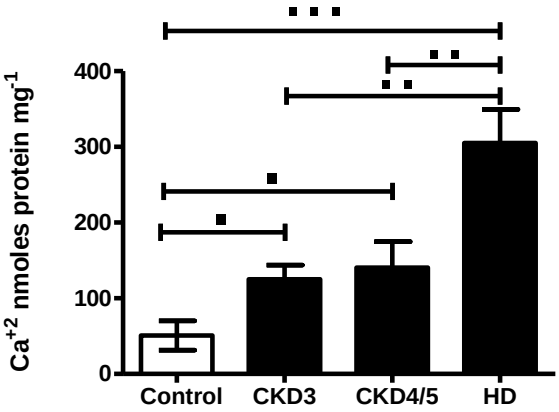

Figure 3

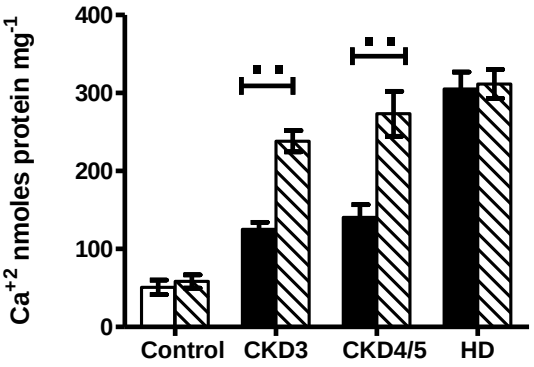

Figure 4