Patients with advanced chronic kidney disease and vascular calcification have a large hydrodynamic radius of secondary calcioprotein particles

Wei Chen¹, Viktoriya Anokhina², Gregory Dieudonne³, Matthew K. Abramowitz⁴, Randeep Kashyap⁵, Chen Yan¹, Tong Tong Wu⁶, Karen L. de Mesy Bentley⁷, Benjamin L. Miller^{2,8,9} and David A. Bushinsky¹

¹Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA, ²Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA, ³Department of Radiology, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA, ⁴Department of Medicine, Albert Einstein College of Medicine, Bronx, NY, USA, ⁵Department of Surgery, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA, ⁶Department of Biostatistics and Computational Biology, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA, ⁷Department of Pathology and Laboratory Medicine, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA, ⁸Department of Dermatology, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA and ⁹Department of Biomedical Engineering, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA

Correspondence and offprint requests to: Wei Chen; E-mail: Wei_Chen@urmc.rochester.edu

ABSTRACT

Background. The size of secondary calcioprotein particles (CPP2) and the speed of transformation (T_{50}) from primary calcioprotein particles (CPP1) to CPP2 in serum may be associated with vascular calcification (VC) in patients with chronic kidney disease (CKD).

Methods. We developed a high throughput, microplate-based assay using dynamic light scattering (DLS) to measure the transformation of CPP1 to CPP2, hydrodynamic radius (R_h) of CPP1 and CPP2, T_{50} and aggregation of CPP2. We used this DLS assay to test the hypothesis that a large R_h of CPP2 and/or a fast T_{50} are associated with VC in 45 participants with CKD Stages 4–5 (22 without VC and 23 with VC) and 17 healthy volunteers (HV). VC was defined as a Kauppila score >6 or an Adragao score ≥ 3 .

Results. CKD participants with VC had larger cumulants R_h of CPP2 {370 nm [interquartile range (IQR) 272–566]} compared with CKD participants without VC [212 nm (IQR 169–315)] and compared with HV [168 nm (IQR 145–352), $P < 0.01$ for each]. More CPP2 were in aggregates in CKD participants with VC than those without VC (70% versus 36%). The odds of having VC increased by 9% with every 10 nm increase in the R_h of CPP2, after adjusting for age, diabetes, serum calcium and phosphate [odds ratio 1.09, 95% confidence interval (CI) 1.03, 1.16, $P = 0.005$]. The area under the receiver operating characteristic curve for VC of CPP2 size was 0.75 (95% CI 0.60, 0.90). T_{50} was similar in CKD participants with and without VC, although both groups had a lower T_{50} than HV.

Conclusions. R_h of CPP2, but not T_{50} , is independently associated with VC in patients with CKD Stages 4–5.

Keywords: calcification propensity, calcioprotein particle, chronic kidney disease, mineral metabolism, vascular calcification

INTRODUCTION

Vascular calcification (VC) is common and contributes to cardiovascular mortality in patients with chronic kidney disease (CKD) [1–5]. VC is accelerated in CKD because impaired kidney function results in calcium (Ca) and phosphorus (P) redistribution and overload as well as deficiency of calcification inhibitors [1–6]. Recently, an *in vitro* assay (T_{50} test) was developed to determine the calcification propensity in blood [7–9]. This assay is based on the physiochemical properties of calcioprotein particles (CPPs), which are nanoparticles, composed of CaP crystals and calcification inhibitors. When Ca and P concentration exceed the solubility limit, insoluble CaP crystals form and precipitate as hydroxyapatite. *In vivo*, despite having blood that is supersaturated with respect to a CaP solid phase, VC typically does not occur due to the presence of calcification inhibitors, which prevent unwanted calcification by binding to CaP crystals to form CPPs [7, 8, 10, 11]. CPPs are then taken up by the reticuloendothelial system, thus removing excess mineral from the circulation [12]. CPPs are initially amorphous and soluble and are referred to as primary CPPs (CPP1). They then spontaneously convert into secondary CPPs (CPP2), which are more crystalline and less soluble [7]. The process of transformation from CPP1 to CPP2 reflects the intrinsic inhibitory capacity of blood to prevent Ca and P precipitation.

The T_{50} test uses nephelometry to measure the time to transition from CPP1 to CPP2 by quantifying the amount of light scattered in turbid solutions [9]. T_{50} is the time to half maximal turbidity, which is the midpoint of transition from CPP1 to CPP2. In patients with CKD, a lower T_{50} is associated with Ca and P supersaturation [9], aortic stiffness and all-cause mortality [13, 14], but the direct relationship between T_{50} and VC has never been examined. The size of CPP2, which is not measured by the T_{50} test, may also impart additional information on the calcification propensity of the serum [9]. Compared with nephelometry, dynamic light scattering (DLS) directly measures the hydrodynamic radii (R_h) and size distribution of both CPP1 and CPP2 using photon auto-correlation function in addition to T_{50} [15]. DLS has been used to demonstrate the pattern of CPP transformation [7, 9, 11, 16]; however, it has not been used in clinical studies to determine if these measured parameters were associated with VC. We developed a high throughput, microplate-based assay using DLS to measure the transformation of CPP1 to CPP2, R_h of CPP1 and CPP2, and T_{50} and aggregation of CPP2 in serum. We then tested the hypothesis that a large R_h of CPP2 and/or a fast T_{50} were associated with VC in patients with CKD.

MATERIALS AND METHODS

Development of DLS microplate assay

Similar to the T_{50} test [9], serum samples were collected and stored at -80°C . For measurement, samples were thawed at room temperature and centrifuged at 10 000g for 30 min at room temperature to remove cryoprecipitates. After the addition of the Ca (10 mM) and P (6 mM) solutions [9], R_h of CPPs in serum was measured in a 384-well microplate with 40 μL serum-solution mixture in each well (16 μL serum, 4 μL NaCl, 10 μL Ca and 10 μL P solution). We used DynaPro Plate Reader II (Wyatt Technology, Santa Barbara, California), which is an automated, temperature controlled DLS microplate reader allowing us to measure CPP transformation in a high throughput fashion. DLS determines size distribution of nanoparticles in solution using photon autocorrelation function [11, 15, 17]. Studies have shown that CPP transformation detected by DLS mirrored the growth and transformation of CPP detected by transmission electron microscopy (TEM) [7, 9, 11, 16]. Compared with nephelometry that was used in the T_{50} test, DLS provides the R_h and size distribution of CPP2 in addition to T_{50} .

Samples were measured in triplicate at a constant temperature of 37°C for 6 h. Microplates were sealed with tape to ensure even temperature distribution and avoid evaporation. Cumulants analysis was used to estimate cumulants R_h of nanoparticles, whereas regularization analysis was used for size distribution of particles [15, 18]. We observed three distinct phases of CPP transformation and estimated the CPP1 and CPP2 size using the cumulants R_h obtained from Phases 1 and 3, respectively. T_{50} was determined by fitting R_h of CPPs to a sigmoidal equation using Prism 7.02 (GraphPad). The intra-assay coefficients of variation (CVs) for size standards—75 nm and 300 nm in R_h (Alfa Aesar, Heysham, Lancashire, UK) were

0.1–3% and 0.7%, and their inter-assay CVs were 2% and 1%, respectively. The intra-assay CVs for the R_h of CPP2 and T_{50} of the serum of a single individual were 2–7% and 1–6%, whereas the inter-assay CVs were 9% and 6%, respectively.

TEM

To examine the specificity of DLS in detecting CPP transformation, the findings from DLS were compared with TEM. For TEM, 20 μL serum-solution samples (non-centrifuged) from three phases of CPP transformation were applied onto Formvar/carbon-coated grids, which were dried at room temperature. To visualize CPPs, no staining was used. To detect the presence of other nanoparticles such as lipoprotein particles, 2% phosphotungstic acid at a pH of 6.5 was used for staining. The unstained and stained grids were examined using a Hitachi 7650 TEM with attached 11 megapixel Gatan Erlangshen digital camera and DigitalMicrograph software. For ellipsoids, R_h was calculated from long (a) and short (b) axes obtained from TEM using the following equation [11]:

$$R_h = \frac{a\sqrt{1 - \frac{b^2}{a^2}}}{\ln\left(\frac{1 + \sqrt{1 - \frac{b^2}{a^2}}}{\frac{b}{a}}\right)}$$

Study design and population

To determine the relationship between the parameters of CPP transformation and VC, we conducted a cross-sectional study at the University of Rochester Medical Center (URMC), Rochester, NY, from October 2015 through February 2017. Two groups of participants were recruited: participants with predialysis CKD and healthy volunteers (HV). Participants with CKD were recruited from nephrology clinics (Supplementary data, Figure S1). Patients aged between 40 and 85 years with estimated glomerular filtration rate (eGFR) $<30\text{ mL/min/1.73m}^2$, ability to provide informed consent and established care with the URMC nephrology division were included. The exclusion criteria were pregnancy, taking warfarin or chemotherapeutic agents, having a diagnosis of chronic obstructive pulmonary disease (COPD), receiving or had received >3 months of dialysis treatment, or a kidney transplant. We excluded patients with COPD because we were interested in serum bicarbonate levels as a measure of acidosis and acidosis may affect CPP transformation. Seventeen HV without CKD or any traditional risk factors of VC—diabetes mellitus, hypertension, hyperlipidemia or smoking history—were recruited through advertisement via Craigslist. The study was approved by the URMC Research Subjects Review Board and performed under the principles embodied in the Declaration of Helsinki.

Measurement of VC

Calcification was measured in lumbar aorta, iliac, femoral, radial and digital arteries. Lumbar aortic calcification was assessed by lateral abdominal plain radiography and quantified by the Kauppila score [19]. Calcification in the muscular arteries including iliac, femoral, radial and digital arteries was assessed

by plain radiography of hands and pelvis and quantified by the Adragao score [20]. All the films were read by a single radiologist, who was blinded to participants' CKD status, CPP2 size and T_{50} . We defined VC as a Kauppila score > 6 or Adragao score ≥ 3 , as these cutoff points are associated with cardiovascular mortality in patients with CKD [3, 5, 19–21].

Statistical analysis

R_h of CPP2, T_{50} , participants' demographics, medical history, medication use, serum markers of bone-mineral metabolism and VC scores were compared among three groups—HV ($n = 17$), CKD without VC ($n = 22$) and CKD with VC ($n = 23$). A multiple logistic regression was used to evaluate the association between CPP2 and VC in participants with CKD while adjusting for age, diabetes, serum Ca and P. Age and diabetes status were examined as effect modifiers by using first-order interaction terms. To evaluate the diagnostic performance of CPP2, we generated receiver operating characteristic (ROC) curves for VC with CPP2 (univariate model) and compared the area under ROC curves of multiple logistic regressions with and without CPP2 using test equality of ROC areas. Sensitivity analyses were performed by using five other different definitions for VC: (i) Kauppila > 6 and Adragao ≥ 3 , (ii) Kauppila > 6 alone, (iii) Adragao ≥ 3 alone, (iv) Kauppila > 0 alone and (v) Adragao > 0 alone for the comparisons of CPP2 and T_{50} among three groups (HV, CKD without VC and CKD with VC), logistic regressions and ROC curves. Sensitivity analyses were also performed by adding T_{50} or the use of phosphate binders as a covariate. Two-sided P-values < 0.05 were considered statistically significant for all analyses and analyses were conducted in STATA 14.1 (StataCorp, College Station, Texas, USA).

RESULTS

CPP transformation detected by DLS

Using the DLS assay, we found three distinct phases of CPP transformation (Figure 1A), which were also observed on TEM (Figure 1B–D). In Phase 1, CPP1 were spherical particles with an $R_h \sim 50$ nm (Figure 1B). With DLS, Peak 2 (Figure 1E) demonstrates the most amount of light scattered (98%) and represents CPP1 and another far less abundant particle of a similar size (Figure 1B). Peak 1 is the amount of light scattered ($\sim 2\%$) by lipoprotein particles, which were ~ 5 nm in radius (Supplementary data, Figure S2). In Phase 2, CPP1 grew radially and transformed spontaneously into CPP2 (Figure 1C). CPP2 were spindle-shaped and each CPP2 spindle had an $R_h \sim 100$ – 150 nm, which was calculated based on TEM measurements. With DLS, the intensity of Peak 2 decreased as Peak 3 emerged, reflecting the transition of CPP1 to CPP2 (Figure 1F). In Phase 3, CPP2 were present either alone or in aggregates (Figures 1D and 2). As observed with TEM, there was no CPP1 observed in this phase. With DLS, Peak 3 became the major peak ($\sim 90\%$ intensity) representing CPP2 and had a wide size distribution with R_h ranging from 100 nm to 6000 nm corresponding to the difference in the size of CPP2 aggregates seen in TEM (Figures 1D and G and 2). The intensity of Peak 2 decreased to $\sim 9\%$. As CPP1 was not longer present in this phase,

Peak 2 likely represents the other nanoparticles indicated by the solid arrow in Figure 1B and D. Since CPPs contributed to more than 90% of total light scattered, we estimated CPP1 and CPP2 size using the cumulants R_h obtained from Phases 1 and 3, respectively.

Participant characteristics

HV had a mean age of 24 ± 5 years and eGFR of 114 ± 13 mL/min/1.73 m², were all white (100%), and all had Kauppila and Adragao scores of 0 (Table 1). Participants with CKD had a mean age of 65 ± 12 years and eGFR of 19 ± 8 mL/min/1.73 m². Among participants with CKD, 20 (44%) had a Kauppila score > 6 and 18 (40%) had an Adragao score ≥ 3 . Twenty-three participants (51%) had a Kauppila > 6 or Adragao ≥ 3 and were defined as having VC [3, 5, 19–21]. Compared with CKD participants without VC, CKD participants with VC were older, had higher prevalence of diabetes and coronary artery disease, higher serum Ca and lower low-density lipoprotein (LDL). The R_h of CPP1 was similar in all participants.

Association of CPP2 and T_{50} with VC

CKD participants with VC had larger cumulants R_h of CPP2 [370 nm [interquartile range (IQR) 272–566]] compared with CKD participants without VC and to HV ($P < 0.01$ for each), whereas there was no difference in cumulants R_h of CPP2 between HV [168 nm (IQR 145–352)] and CKD participants without VC [212 nm (IQR 169–315), $P = 0.29$, Figure 3A]. The calculated R_h of individual CPP2 spindle based on TEM measurements is ~ 100 – 150 nm. The size distribution of CPP2 is shown by the width of Peak 3 in Phase 3 (Figure 3C–E). A total of 30% ($n = 7$) of CKD participants with VC had CPP2 size < 150 nm, compared with 65% ($n = 11$) of HV and 64% ($n = 14$) of CKD participants without VC indicating that more CPP2 were in aggregates in CKD participants with VC than those without VC.

Among participants with CKD ($n = 45$), R_h of CPP2 remained significantly associated with VC after adjusting for age, diabetes mellitus, serum Ca and P (Table 2). In the multiple logistic regression model of participants with CKD, the odds of having VC increased by 9% with every 10 nm increase in R_h of CPP2 [odds ratio (OR) 1.09, 95% confidence interval (CI) 1.03, 1.16, $P = 0.005$]. Neither age nor diabetes status modified the association between CPP2 and VC. The area under the ROC curve for VC in the univariate model of CPP2 was 0.75 (95% CI 0.60, 0.90; Figure 4). For multiple logistic regressions, the area under the ROC curve was 0.79 without CPP2 as a covariate and was 0.89 with CPP2, but the change was not significant ($P = 0.13$).

Compared with HV ($T_{50} = 221 \pm 31$ min), both CKD participants with and without VC had a lower T_{50} ($P = 0.02$ for each; Figure 3B). There was no difference in T_{50} between CKD participants with and without VC (191 ± 39 versus 190 ± 43 min, $P = 0.89$). CPP2 remained significantly associated with VC after adding T_{50} as a covariate in the logistic model (Supplementary data, Table S1). Sensitivity analyses performed using five other definitions of VC yielded generally similar results with one exception, that is, CPP2 was not

FIGURE 1: CPP transformation and size distribution of CCPs. (A) CPP transformation measured by DLS in 10 representative participants with CKD (5 with VC, solid squares; 5 without VC, hollow circles); (B–D) TEM of serum (magnification: 150 000 $\times$) showing (B) CPP1 and a few other nanoparticles (solid arrow indicates the other nanoparticle) in Phase 1; (C) radial growth of CPP1 into crystalline CPP2 (solid arrow indicates CPP1 in transition; hollow arrow indicates the center of radial growth) in Phase 2 and (D) CPP2 in Phase 3 (solid arrow indicates the other nanoparticle seen in B); (E–G) size distribution of nanoparticles in serum measured by DLS with the percentage of total light scattered in the parenthesis (X-axis is in log scale) in each phase.

FIGURE 2: TEM of CPP2 aggregates in Phase 3 (magnification: 50 000 $\times$).

significantly associated with VC, when VC was defined as Kauppila > 0 alone (OR 1.06, 95% CI 0.996, 1.13, $P = 0.07$, [Supplementary data, Table S2](#)). As shown in [Table 2](#), the serum phosphate level was inversely associated with the odds of having VC; however, after adjusting for the use of phosphate binders in participants with CKD, there was no longer significant association ([Supplementary data, Table S3](#)).

DISCUSSION

We developed a high throughput, microplate-based assay using DLS to measure the transformation of CPP1 to CPP2, R_h of CPP1 and CPP2, T_{50} , and aggregation of CPP2 in serum. We demonstrated that our DLS assay successfully characterized the transformation of CPP1 to CPP2 and found that most CPP2 were present in aggregates. Using this assay, we found for the first time that the R_h of CPP2, but not T_{50} , was significantly associated with VC in participants with CKD Stages 4–5 after

Table 1. Participant characteristics by CKD and VC status

	HV (<i>n</i> = 17)	CKD-No VC (<i>n</i> = 22)	CKD-VC (<i>n</i> = 23)
Age (years), mean (SD)	24 (5)	61 (13)*	69 (9)* [#]
Male, <i>n</i> (%)	5 (29)	16 (73)*	16 (70)*
White, <i>n</i> (%)	17 (100)	15 (68)*	18 (78)*
Diabetes mellitus, <i>n</i> (%)	0 (0)	11 (50)*	18 (78)* [#]
Hypertension, <i>n</i> (%)	0 (0)	22 (100)*	23 (100)*
Coronary artery disease, <i>n</i> (%)	0 (0)	1 (5)	12 (52)* [#]
Hyperlipidemia, <i>n</i> (%)	0 (0)	17 (77)*	20 (87)*
Diuretics use, <i>n</i> (%)	0 (0)	13 (59)*	15 (65)*
Vitamin D use, <i>n</i> (%)	0 (0)	16 (73)*	19 (83)*
Lipid-lowering medication use, <i>n</i> (%)	0 (0)	16 (73)*	19 (83)*
Phosphate binder use, <i>n</i> (%)	0 (0)	3 (14)*	8 (35)*
eGFR (mL/min/1.73 m ²), mean (SD)	114 (13)	19 (8)*	18 (7)*
Serum bicarbonate (mEq/L), mean (SD)	24 (2)	20 (3)*	22 (4)
Serum calcium (mg/dL), mean (SD)	9.1 (0.3)	8.8 (0.6)	9.2 (0.5) [#]
Serum phosphate (mg/dL), mean (SD)	3.6 (0.5)	4.9 (1.6)*	4.3 (0.9)*
25-hydroxyvitamin D (pg/mL), median (IQR)	30 (27–37)	37 (23–43)	35 (28–43)
Parathyroid hormone (pg/mL), median (IQR)	33 (28–39)	155 (84–246)*	139 (69–186)*
Alkaline phosphatase (unit/L), median (IQR)	76 (60–82)	80 (63–96)	81 (60–98)
HDL (mg/dL), median (IQR)	62 (53–72)	49 (43–58)*	46 (37–58)*
LDL (mg/dL), median (IQR)	80 (73–91)	86 (78–102)	71 (51–82)* [#]
Albumin (g/dL), mean (SD)	4.5 (0.2)	4.2 (0.3)*	4.1 (0.2)*
CPP1 (nm), mean (SD)	54 (9)	55 (10)	60 (10)
Kauppila score, median (IQR)	0	0 (0–3)*	8 (7–13)* [#]
Kauppila >6, <i>n</i> (%)	0 (0)	0 (0)	20 (87)* [#]
Adragao score, median (IQR)	0 (0)	0 (0–0)*	4 (4–7)* [#]
Adragao ≥3, <i>n</i> (%)	0 (0)	0 (0)	18 (78)* [#]

If normally distributed, values for continuous variables with normal distribution are provided as mean (SD). Otherwise, they are provided as median (IQR). Categorical variables are presented as numbers with percentage. For continuous variables, comparisons were made using two-sample t-tests if test assumptions were met, Mann–Whitney U tests if not. For categorical variables, comparisons were made using chi-square tests.

**P* < 0.05, versus HV.

[#]*P* < 0.05, versus participants with CKD, but no VC.

HDL, high-density lipoprotein.

adjusting for age, diabetes mellitus, serum Ca and P (Table 2). Our findings suggest that R_h of CPP2 has the potential to be a biomarker for VC in patients with CKD.

To our knowledge, we are the first to characterize CPP2 aggregation in serum without centrifugation. Prior studies using DLS have described CPP2 from either the ascites of a patient with calcifying peritonitis [11] or from pellets obtained after centrifugation of serum [22]. After the addition of supersaturated Ca and P solutions, amorphous CPP1 spontaneously transformed into spindle-shaped CPP2, which were present mostly in aggregates (Figure 1). Despite the presence of other nanoparticles such as lipoprotein, CPP1 and CPP2 contributed to more than 90% of total light scattered in all three phases of the transformation indicating that this technique can be utilized to detect the CPP transformation. The R_h and transformation pattern observed using DLS are consistent with our TEM findings and similar to what was previously described [7, 9]. The calculated R_h of each CPP2 spindle was ~100–150 nm based on TEM measurements (Figure 1D) [11], whereas the DLS measured R_h of CPP2 was larger (Figures 1A and 3C–E), indicating the presence of CPP2 in aggregates (Figure 2). The polydispersity of Peak 3 (i.e. the width of the peak) indicates the heterogeneity in the size of aggregates.

Compared with participants without VC, CKD participants with VC had larger cumulants R_h of CPP2 (Figure 3A) and had

more CPP2 in aggregates (Figure 3C–E). The difference in the R_h and the percentage of CPP2 present in aggregates may reflect a difference in the concentration and/or physical structure of CPPs. That CPP2 aggregation may indicate a high concentration of CPPs in the serum is consistent with prior studies which demonstrate that the amount of CPPs is associated with VC [23, 24]. In these studies, the amount of CPP was measured using fetuin-A sediment assay [23]. Fetuin-A or α -2-Heremans-Schmid glycoprotein is a potent circulating inhibitor of apatite formation [25]. The fetuin-A sediment assay indirectly measures the amount of fetuin-A-containing CPP using differential centrifugation and enzyme-linked immunosorbent assay to quantify fetuin-A bound as fetuin-A mineral complex and total fetuin-A [23]. The amount of fetuin-A-containing CPPs is associated with the extent of coronary artery calcification and aortic stiffness in patients with CKD [23, 24]. The fetuin-A sediment assay only measures fetuin-A containing CPPs and does not discriminate CPP1 from CPP2 [26]. Also, fetuin-A may be undetectable in patients with fetuin-A deficiency such as those with end-stage renal disease [27]. Compared with the fetuin-A sediment assay, DLS assay can discriminate CPP1 from CPP2 and measure the R_h of CPPs. Recently, there were two more assays developed to quantify CPP. One used a gel filtration method [28] and the other used fluorescent probe-based flow cytometry [26]. The gel filtration method developed by Miura

FIGURE 3: Boxplots of (A) cumulants R_h of CPP2 and (B) T_{50} in HV (white), CKD participants without VC (light gray) and CKD participants with VC (dark gray); (C–E) size distribution curves of CPP2 in HV and CKD participants with and without VC, as indicated by Peak 3 in Phase 3. Vertical dash line is the reference line at R_h of 150 nm, which is the calculated size of a CPP2 spindle based on TEM measurements. T_{50} , time for half-maximal transformation of CCPs. * $P < 0.05$ versus HV. # $P < 0.05$ versus CKD participants without VC.

et al. identifies a new class of CPP in the plasma, named low-density CPP, which are thought to be the major form of CPP *in vivo* [28], whereas the flow cytometric assay is able to discriminate CPP1 from CPP2 [26]. However, the relationship between CPP levels measured by these assays and VC is yet to be elucidated.

We observed that CPP2 spindles aggregated almost immediately after transforming from CPP1 and the aggregates have a specific pattern on TEM (Figure 2), suggesting the possibility of oriented aggregation, which is special case of aggregation in which nanocrystals self-assemble and form new secondary single crystals [29]. The various sizes and patterns of CPP2 aggregates may reflect a difference in the physical structure of CPPs between participants with and without VC. CKD participants

with VC might have an either inadequate quantity or activity of fetuin-A [30], which binds to Ca and P to prevent aggregation [7, 31]. When fetuin-A is deficient other serum proteins, such as albumin, act as inhibitors. However, albumin is a weaker inhibitor than fetuin-A [31], so a higher concentration of albumin is required to slow the transformation of CPP1 to CPP2 resulting in larger aggregates [11]. Further studies incorporating the measurement of fetuin-A will be necessary to further examine the relationship between CPP2 and VC.

Similar to prior studies [9], we found that CKD participants had lower T_{50} compared with HV; however, we did not find an association between T_{50} and VC in participants with CKD (Figure 3B). Prior studies using the T_{50} test found that low T_{50} was associated with arterial stiffness, cardiac endpoints and

FIGURE 4: ROC curves for VC in participants with CKD ($n = 45$). Multiple logistic regression models for VC were adjusted for age, diabetes, serum calcium, phosphate, with and without CPP2. AUC, area under the curve.

Table 2. Multiple logistic regression of VC in patients with CKD ($n = 45$)

	Odds ratio	Standard error	P-value	95% CI
R_h of CPP2 (per 10 nm)	1.09	0.03	0.005	1.03, 1.16
Age (years)	1.08	0.05	0.08	0.99, 1.17
Diabetes mellitus (yes versus no)	9.83	10.3	0.03	1.25, 77.0
Serum calcium (per 0.1 mg/dL)	1.19	0.1	0.06	0.99, 1.42
Serum phosphate (per 0.1 mg/dL)	0.91	0.04	0.04	0.84, 0.99

all-cause mortality in patients with CKD [13, 14], but did not examine the direct relationship between T_{50} and VC. While VC results in arterial stiffness, stiff arteries may also result from other mechanisms such as atherosclerosis and accumulation of advanced glycation end-products [32]. In our study, the lack of association between T_{50} and VC can also be due to small sample size. However, the R_h of CPP2 remained significantly associated with VC after adjusting for age, diabetes, serum Ca and P (Table 2). The addition of T_{50} as a covariate in the model did not alter this relationship (Supplementary data, Table S1). Compared with T_{50} , which requires a kinetic test with hours of continuous monitoring, R_h of CPP2 alone is easier to implement clinically as it only needs to be measured once after incubation of the serum-solution mixtures. Thus the R_h of CPP2 may be a useful clinical biomarker of VC.

Our study has several strengths. The major strength is our new DLS microplate assay, which allows us to determine R_h of CPP2 in addition to T_{50} in a high throughput fashion. We demonstrated that DLS was specific in detecting the transformation of CPP1 to CPP2. We measured calcification of both large and medium-size arteries and quantified VC using validated scoring systems (Kauppila and Adragao scores, respectively) [3, 5, 19, 21]. Large elastic arteries such as aorta receive blood directly from the heart and are more prone to intimal calcification [33], medium-size arteries such as iliac and

femoral arteries have more vascular smooth muscle cells and are more susceptible to medial calcification [20]. While both intimal and medial calcification predict mortality [19, 20], medial calcification is a more characteristic feature of CKD and the amount of medial calcification may have a greater prognostic power for mortality than intimal calcification in patients with CKD [5]. To investigate whether the relationships of T_{50} and CPP2 with VC differ with these two types of VC, we defined VC as Kauppila >6 or Adragao ≥ 3 in the main analysis and used five other definitions of VC in sensitivity analyses, which yielded generally similar results as the main findings.

Our study has limitations. Because of the cross-sectional nature of our study, we could not evaluate the role of CPP2 or T_{50} in predicting the risk of VC. CPP2 size and T_{50} were measured from a single serum sample. Studying the variability of these parameters within an individual would be informative and could be a focus of future studies. The small sample size may not have allowed us to detect an association between T_{50} and VC; however, despite having a small sample size, we observed a significant association between CPP2 and VC. While the HV were younger than CKD participants, the main study objective was to compare CPP2 and T_{50} in CKD participants with and without VC, who had a smaller age difference. Finally, we did not measure coronary artery calcification, another predictor of cardiovascular events, to avoid the additional radiation [34].

Using our DLS microplate assay, we measured both the R_h of CPP2 and T_{50} in serum and found that the R_h of CPP2, but not T_{50} , was independently associated with VC in patients with CKD Stages 4–5. Our findings suggest that the R_h of CPP2 has the potential to be a biomarker for VC in patients with CKD and support a larger and prospective study to further evaluate the relationship of CPP2 with VC, cardiovascular endpoints and mortality.

SUPPLEMENTARY DATA

Supplementary data are available at ndt.oxfordjournals.org.

FUNDING

This research was supported by the University of Rochester Clinical and Translational Science award (KL2 TR001999) from the National Center for Advancing Translational Sciences of the National Institutes of Health (W.C.), American Society of Nephrology Carl W. Gottschalk Research Grant (W.C.) and the Renal Research Institute (W.C. and D.A.B.); and the National Institute of Diabetes and Digestive and Kidney Diseases (R01 DK075462) (D.A.B.). The Dynamic Light Scattering DynaPro Plate Reader II was supported by National Center for Research Resources grants 1S10 RR026501 and 1S10 RR027241, as well as National Institute of Allergy and Infectious Diseases P30 AI078495 and the School of Medicine and Dentistry, University of Rochester. Transmission electron microscopy was supported by the University of Rochester Medical Center electron microscopic shared resource.

AUTHORS' CONTRIBUTIONS

W.C., V.A., B.L.M. and D.A.B. were responsible for the research idea and study design. W.C., V.A., K.L.d.M.B. and G.D. were responsible for the data measurement. W.C., V.A., B.L.M., M.K.A., R.K., C.Y., T.W. and D.A.B. were responsible for the data analysis/interpretation. Mentorship was provided by B.L.M. and D.A.B. Each author contributed important intellectual content during manuscript drafting or revision 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.

CONFLICT OF INTEREST STATEMENT

D.A.B. is a consultant for Relypsa, Amgen, Sanofi/Genzyme, Vifor and Tricida, and has an equity interest in Amgen and Tricida. B.L.M. has an equity interest in Adarza BioSystems, and is a consultant for Dodo Omnidata. M.K.A. has consulted for Tricida. Results presented in this paper have not been published previously in whole or part, except in abstract format.

REFERENCES

- Blacher J, Guerin AP, Pannier B *et al.* Impact of aortic stiffness on survival in end-stage renal disease. *Circulation* 1999; 99: 2434–2439
- Blacher J, Pannier B, Guerin AP *et al.* Carotid arterial stiffness as a predictor of cardiovascular and all-cause mortality in end-stage renal disease. *Hypertension* 1998; 32: 570–574
- Wilson PW, Kaupila LI, O'Donnell CJ *et al.* Abdominal aortic calcific deposits are an important predictor of vascular morbidity and mortality. *Circulation* 2001; 103: 1529–1534
- London GM, Guerin AP, Marchais SJ *et al.* Arterial media calcification in end-stage renal disease: impact on all-cause and cardiovascular mortality. *Nephrol Dial Transplant* 2003; 18: 1731–1740
- Gorritz JL, Molina P, Cerveron MJ *et al.* Vascular calcification in patients with nondialysis CKD over 3 years. *Clin J Am Soc Nephrol* 2015; 10: 654–666
- London GM. Mechanisms of arterial calcifications and consequences for cardiovascular function. *Kidney Int Suppl* (2011) 2013; 3: 442–445
- Heiss A, DuChesne A, Denecke B *et al.* Structural basis of calcification inhibition by alpha 2-HS glycoprotein/fetuin-A. Formation of colloidal calciprotein particles. *J Biol Chem* 2003; 278: 13333–13341
- Wald J, Wiese S, Eckert T *et al.* Formation and stability kinetics of calcium phosphate-fetuin-A colloidal particles probed by time-resolved dynamic light scattering. *Soft Matter* 2011; 7: 2869–2874
- Pasch A, Farese S, Graber S *et al.* Nanoparticle-based test measures overall propensity for calcification in serum. *J Am Soc Nephrol* 2012; 23: 1744–1752
- Schafer C, Heiss A, Schwarz A *et al.* The serum protein alpha 2-Heremans-Schmid glycoprotein/fetuin-A is a systemically acting inhibitor of ectopic calcification. *J Clin Invest* 2003; 112: 357–366
- Heiss A, Eckert T, Aretz A *et al.* Hierarchical role of fetuin-A and acidic serum proteins in the formation and stabilization of calcium phosphate particles. *J Biol Chem* 2008; 283: 14815–14825
- Herrmann M, Schafer C, Heiss A *et al.* Clearance of fetuin-A-containing calciprotein particles is mediated by scavenger receptor-A. *Circ Res* 2012; 111: 575–584
- Smith ER, Ford ML, Tomlinson LA *et al.* Serum calcification propensity predicts all-cause mortality in predialysis CKD. *J Am Soc Nephrol* 2014; 25: 339–348
- Pasch A, Block GA, Bachtler M *et al.* Blood calcification propensity, cardiovascular events, and survival in patients receiving hemodialysis in the EVOLVE trial. *Clin J Am Soc Nephrol* 2017; 12: 315–322
- Bernes B, Pecora R. *Dynamic Light Scattering: with Applications to Chemistry, Biology and Physics*. Toronto, Ontario: General Publishing Company, Ltd, 2000
- Pasch A. Novel assessments of systemic calcification propensity. *Curr Opin Nephrol Hypertens* 2016; 25: 278–284
- Rausch K, Reuter A, Fischer K *et al.* Evaluation of nanoparticle aggregation in human blood serum. *Biomacromolecules* 2010; 11: 2836–2839
- Wyatt Technology Corporation. Dynamics User's Guide; Version 7.1 (M1400 Rev. K)
- Kaupila LI, Polak JF, Cupples LA *et al.* New indices to classify location, severity and progression of calcific lesions in the abdominal aorta: a 25-year follow-up study. *Atherosclerosis* 1997; 132: 245–250
- Adragao T, Pires A, Lucas C *et al.* A simple vascular calcification score predicts cardiovascular risk in haemodialysis patients. *Nephrol Dial Transplant* 2004; 19: 1480–1488
- Honkanen E, Kaupila L, Wikstrom B *et al.* Abdominal aortic calcification in dialysis patients: results of the CORD study. *Nephrol Dial Transplant* 2008; 23: 4009–4015
- Young JD, Martel J, Young D *et al.* Characterization of granulations of calcium and apatite in serum as pleomorphic mineralo-protein complexes and as precursors of putative nanobacteria. *PLoS One* 2009; 4: e5421
- Hamano T, Matsui I, Mikami S *et al.* Fetuin-mineral complex reflects extra-osseous calcification stress in CKD. *J Am Soc Nephrol* 2010; 21: 1998–2007
- Smith ER, Ford ML, Tomlinson LA *et al.* Phosphorylated fetuin-A-containing calciprotein particles are associated with aortic stiffness and a procalcific milieu in patients with pre-dialysis CKD. *Nephrol Dial Transplant* 2012; 27: 1957–1966
- 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
- Smith ER, Hewitson TD, Cai MMX *et al.* A novel fluorescent probe-based flow cytometric assay for mineral-containing nanoparticles in serum. *Sci Rep* 2017; 7: 5686

27. Westenfeld R, Schafer C, Kruger T *et al.* Fetuin-A protects against atherosclerotic calcification in CKD. *J Am Soc Nephrol* 2009; 20: 1264–1274
28. Miura Y, Iwazu Y, Shiizaki K *et al.* Identification and quantification of plasma calciprotein particles with distinct physical properties in patients with chronic kidney disease. *Sci Rep* 2018; 8: 1256
29. Yuwono VM, Burrows ND, Soltis JA *et al.* Oriented aggregation: formation and transformation of mesocrystal intermediates revealed. *J Am Chem Soc* 2010; 132: 2163
30. Westenfeld R, Jahnke-Dechent W, Ketteler M. Vascular calcification and fetuin-A deficiency in chronic kidney disease. *Trends Cardiovasc Med* 2007; 17: 124–128
31. Garnett J, Dieppe P. The effects of serum and human albumin on calcium hydroxyapatite crystal growth. *Biochem J* 1990; 266: 863–868
32. van Popele NM, Grobbee DE, Bots ML *et al.* Association between arterial stiffness and atherosclerosis: the Rotterdam Study. *Stroke* 2001; 32: 454–460
33. Coll B, Betriu A, Martinez-Alonso M *et al.* Large artery calcification on dialysis patients is located in the intima and related to atherosclerosis. *Clin J Am Soc Nephrol* 2011; 6: 303–310
34. Wade AN, Reilly MP. Coronary calcification in chronic kidney disease: morphology, mechanisms and mortality. *Clin J Am Soc Nephrol* 2009; 4: 1883–1885

Received: 16.2.2018; Editorial decision: 28.3.2018

Nephrol Dial Transplant (2019) 34: 1000–1008
doi: 10.1093/ndt/gfy132
Advance Access publication 19 July 2018

Effect of everolimus on renal function in patients with tuberous sclerosis complex: evidence from EXIST-1 and EXIST-2

John J. Bissler¹, Klemens Budde², Matthias Sauter³, David N. Franz⁴, Bernard A. Zonnenberg⁵, Michael D. Frost⁶, Elena Belousova⁷, Noah Berkowitz⁸, Antonia Ridolfi⁹ and J. Christopher Kingswood¹⁰

¹St. Jude Children's Research Hospital and Le Bonheur Children's Hospital, Memphis, TN, USA, ²Charité Universitätsmedizin, Berlin, Germany, ³Medizinische Klinik und Poliklinik IV, Klinikum der Universität München, Munich, Germany, ⁴Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA, ⁵Universitair Medisch Centrum Utrecht, The Netherlands, ⁶Minnesota Epilepsy Group, St. Paul, MN, USA, ⁷Moscow Research and Clinical Institute of Pediatrics, Moscow, Russian Federation, ⁸Novartis Pharmaceuticals, East Hanover, NJ, USA, ⁹Novartis Pharmaceuticals S.A.S., Rueil-Malmaison, France and ¹⁰Royal Sussex County Hospital, Brighton, UK

Correspondence and offprint requests to: John J. Bissler; E-mail: jbissler@uthsc.edu

ABSTRACT

Background. A reduction in renal angiomyolipoma volume observed with everolimus (EVE) treatment in patients with tuberous sclerosis complex (TSC) has been postulated to translate to clinical benefit by reducing the risk of renal hemorrhage and chronic renal failure.

Methods. The long-term effects of EVE on renal function (~4 years of treatment) were examined in patients treated with EVE in the Phase 3 EXIST-1 and EXIST-2 studies. Patients in EXIST-1 had TSC and subependymal giant cell astrocytoma (SEGA), and patients in EXIST-2 had renal angiomyolipoma and a definite diagnosis of TSC or sporadic lymphangioleiomyomatosis. EVE was administered at 4.5 mg/m²/day, with adjustment to achieve target trough levels of 5–15 ng/mL in EXIST-1 and at 10 mg/day in EXIST-2. Estimated glomerular filtration rate (eGFR) and creatinine levels were assessed at baseline, at Weeks 2, 4, 6, 8, 12 and 18, then every 3 months thereafter. Proteinuria was graded according to National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0.

Results. A total of 111 patients from EXIST-1 and 112 patients from EXIST-2 were included in this analysis. Respective mean ages at EVE initiation were 10.5 [standard deviation (SD) 6.45] and 33.2 (SD 10.29) years, and 3.6% and 37.5% of patients had undergone prior renal intervention. Mean baseline eGFR was 115 and 88 mL/min/1.73 m² in EXIST-1 and EXIST-2, respectively. Overall, mean eGFR remained stable over time in both studies, with a decline in renal function mostly confined to some patients with severely compromised renal function before treatment. Patients with prior renal intervention exhibited low eGFR values throughout the study. The incidence of proteinuria increased after initiating treatment with EVE and was mostly Grade 1/2 in severity, with Grade 3 proteinuria reported in only two patients. Measurements of proteinuria were limited by the use of urine dipstick tests.

Conclusions. The use of EVE does not appear to be nephrotoxic in patients with SEGA or renal angiomyolipoma associated with TSC and may preserve renal function in most patients.