Effects of repetitive diet-induced fluctuations in plasma phosphorus on vascular calcification and inflammation in rats with early-stage chronic kidney disease

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Cardiovascular disease is a major cause of death among hemodialysis patients. Hyperphosphatemia induces cardiovascular disease through vascular endothelial dysfunction and calcification. Repetition of a short-term excessive-phosphorus (P) diet causes transient elevations in plasma P and subsequent vascular endothelial dysfunction in normal rats. The purpose of this study was to investigate the effects of the P fluctuation on vascular calcification and inflammation in rats after unilateral nephrectomy as an early-stage chronic kidney disease (CKD) model. Rats were bred for 36 days; CP group, fed a control P (0.6%) diet; HP group, fed a high-P (1.2%) diet; and P fluctuation group, fed low-P (0.02%) and high-P diets alternately every 2 days. Influences on vascular calcification were analyzed using Von Kossa staining and measurement of vessel Ca content. The influence on inflammation was measured as urinary levels of 8-hydroxy-2'-deoxyguanosine. We demonstrated that the P fluctuation group showed similar vascular calcification and inflammation to the HP group, despite having the same total P intake as the CP group. A diet avoiding P fluctuations may be important for patients with early-stage CKD.

Key Words: chronic kidney disease, vascular calcification, hyperphosphatemia, oxidative stress, phosphorus fluctuation

Cardiovascular disease (CVD) is a major cause of death among patients with chronic kidney disease (CKD) or on hemodialysis.11,12 The presence and progression of vascular endothelial dysfunction and vascular calcification are risk factors for CVD.3–6 Hyperphosphatemia is involved in vascular endothelial dysfunction and vascular calcification.7,8 Hyperphosphatemia is caused by excessive phosphorus (P) intake from the diet. Therefore, as hemodialysis patients are predisposed to develop hyperphosphatemia, dietary P restrictions or pharmacotherapy using P binders are used to manage hyperphosphatemia.10–14 In pre-dialysis patients, high plasma P levels represent a risk factor for higher mortality and declines in renal function.15 In addition, medial calcification has been reported in the aorta from the early stages of CKD.16 For these reasons, management of dietary P is considered necessary from the early stages of CKD.

One of the long-term outcomes of hyperphosphatemia is vascular calcification. Hyperphosphatemia induces P flow into vascular smooth muscle cells (VSMCs) through P transporters, and increases intercellular P.17,18 This, in turn, leads to expression of osteoblast markers such as runt-related transcription factor 2 (Runx2) and bone morphogenetic protein 2 (BMP-2), in VSMCs.19 Increases in these markers cause transformation of VSMCs into osteoblast-like cells, triggering vascular calcification and further progression with high P levels.20–22 Inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and oxidative stressors such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) reportedly play roles in the pathogenesis of vascular calcification in adenine-induced or 5/6 nephrectomy rats, advanced CKD models.23–25 Serum 8-OHdG levels correlate directly with serum levels of BMP-2, an osteoblast marker related to the mechanism of vascular calcification in CKD populations.26 Inflammatory markers such as C-reactive protein (CRP) have also been reported to show significant associations with vascular calcification in hemodialysis patients.27–29 Many of these studies used models of advanced CKD, and details related to early-stage CKD remain unclear.

Recently, not only long-term hyperphosphatemia, but also short-term hyperphosphatemia has been indicated to show effects on vessels. One of the short-term outcomes of hyperphosphatemia is vascular endothelial dysfunction. Transient elevations in P reportedly cause impairment of vascular endothelial function in healthy men.7 Further, repeated transient elevations in plasma P due to intake alternating between low P and high P diet every two days increased concentrations of inflammatory factors and oxidative stress, and caused vascular endothelial dysfunction in normal rats.28 Thus, inflammatory factors and oxidative stress may negatively affect vessel over both the long and short term.

Fluctuations in P are caused by circadian rhythms in healthy men and mice.30,31 Above all, dietary P intake has effects on these P fluctuations in all individuals. P intake from the diet is classified into organic P from natural foods and inorganic P from food additives. In particular, the rate of absorption of inorganic P is high,32 and serum P levels were more elevated after high inorganic P intake than after high organic P intake.33 Further, inorganic P has a stronger influence on vascular endothelial function than organic P in healthy men.33 Fluctuations in P should thus be emphasized in the dietary treatment of CKD patients and hemodialysis patients. However, in the present situation, dietary P recommendations for dialysis patients are set for the day, but not for individual meals.14

For these reasons, transient plasma P fluctuations due to diet may cause adverse effects on the vasculature under conditions of renal dysfunction. However, evidence for the effects of P fluctuations in CKD is lacking and few studies have examined early-stage CKD. We therefore decided to elucidate the effects in CKD, especially early-stage CKD, in which pathogenic vascular

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calcification starts to be seen. Unilateral nephrectomy rats have been shown to mild renal insufficiency in previous studies.\(^{(28,36)}\)

The present study used rats that had undergone unilateral nephrectomy as an early-stage CKD model, to investigate the effects of repetitive P fluctuations on vascular calcification and inflammation.

**Materials and Methods**

**Animals and materials.** All study protocols were approved by the Ethics Committee of the University of Hyogo, School of Human Ethics and Environment. As a model of early-stage CKD, rats that had received unilateral nephrectomy were used. Eleven-week-old male Sprague-Dawley rats receiving unilateral nephrectomies at 10 weeks old were purchased from Japan SLC (Shizuoka, Japan). Rats were maintained on a 12-h light, 12-h dark cycle (09:00–21:00) and allowed free access to extra-pure water. Details of the dietary protocol were shown in Table 1. The CP group (n = 6) was fed the control P diet and the HP group (n = 7) was fed the high-P diet. The P fluctuation group (n = 14) was divided into two groups, an LH group (n = 7) and an HL group (n = 7). These LH and HL groups were alternately fed the low-P diet and the high-P diet every two days.\(^{(28)}\) The LH group started with the low-P diet and finished with the high-P diet, whereas the HL group started with the high-P diet and finished with the low-P diet, to control for any effects of dietary P intake on the last day of the experimental period. During the experimental period, rats were fed each diet under pair-feeding conditions at 09:00–21:00 and allowed free access to extra-pure water.

**Experimental design.** The experimental diets used were a control P diet (0.6% P, 0.6% Ca), a low-P diet (0.02% P, 0.6% Ca) and a high-P diet (1.2% P, 0.6% Ca) based on a commercial diet with casein as the protein source (AIN93-G; Oriental Yeast, Tokyo, Japan).\(^{(28,36)}\) Before grouping, all rats (n = 27) were fed MF (Oriental Yeast) for a week to acclimatize. At 12 weeks old, rats were divided into four groups, each fed a specific diet for 36 days. The CP group (n = 6) was fed the control P diet and the HP group (n = 7) was fed the high-P diet. The P fluctuation group (n = 14) was divided into two groups, an LH group (n = 7) and an HL group (n = 7). These LH and HL groups were alternately fed the low-P diet and the high-P diet every two days.\(^{(28)}\) The LH group started with the low-P diet and finished with the high-P diet, whereas the HL group started with the high-P diet and finished with the low-P diet, to control for any effects of dietary P intake on the last day of the experimental period. During the experimental period, rats were fed each diet under pair-feeding conditions at 11:00 AM, and food intake was recorded every day. Blood samples were taken from the tail vein between 10:00 AM and 11:00 AM every 2 days until 32 days, although collecting blood after that proved difficult due to damaged blood vessels. Urine volume was recorded every 6 days. After 36 days, rats of all groups were administered anesthetic using isoflurane (Wako Pure Chemical Industries, Osaka, Japan) and laparotomized. Blood samples taken from the inferior vena cava, thoracoabdominal aorta, and remaining kidney were collected for analysis.

**Biochemical parameters.** Plasma and urine levels of P, calcium (Ca) and creatinine (Cr) were measured using the Wako Phospha-C test kit, the Wako Calcium-E test kit and the Wako Creatinine test kit, respectively (Wako Pure Chemical Industries). Plasma levels of CRP were determined using the Rat CRP ELISA Kit (Thermo Fisher Scientific, Tokyo, Japan). Plasma levels of TNF-α were determined using the Rat TNF-α Quantikine ELISA Kit (R&D Systems, Minneapolis, MN). Urinary levels of 8-OHdG were determined using the Highly Sensitive 8-OHdG Check ELISA kit (JaIKA, Shizuoka, Japan).

**Analysis of renal fibrosis.** To histologically evaluate renal tissue, kidneys were embedded in paraffin and 4-μm sections were cut. Sections were stained using Masson trichrome stain reagent (Muto Pure Cemicals, Tokyo, Japan) according to the instructions from the manufacturer. The slides were examined using microscope in a blinded fashion. Further, to investigate the influences of P fluctuations on the kidney, renal weight was measured (Fig. 3A). Renal weight was significantly higher in the HP group than in the CP group. Further, to investigate influences of the P fluctuations on renal histology, kidneys in each group were stained and visualized using Masson trichrome staining (Fig. 3B). Renal fibrosis is an indicator of renal tissue associated with decreased renal function. Renal fibrosis was more severe in the HP, LH and HL groups than in the CP group. In the HP, LH, and HL groups, some tubules were observed to be dilated. Influences of P fluctuation diet on the kidney were similar to those of the HP diet, although renal excretion was not clearly different from that on the CP diet. Thus, the HP, LH, and HL groups showed tissue changes compared to the CP group, suggesting that renal function may decline in the near future.

**Statistical analysis.** Data are presented as the mean ± SE. Differences between groups were analyzed using Kruskal-Wallis test followed by a Steel-Dwass method. For all tests, two-tailed p values less than 0.05 were considered statistically significant.

**Results**

**Changes in dietary P intake and plasma P level.** Daily P intakes in the CP and HP groups were nearly constant for 36 days (Fig. 1A). The fluctuation group comprised the LH and HL subgroups to control for the effects of dietary P intake on the last day of the experimental period, because plasma P is strongly influenced most recent diet. As a result, daily P intakes in both P fluctuation subgroups showed great changes (Fig. 1B). Although total P intake in the HP group was approximately double that in the CP and P fluctuation groups (p<0.05), intakes in the CP and P fluctuation groups were the same during the experimental period (Fig. 1C).

To examine the effects of dietary P intake on changes in blood, plasma P levels were measured (Fig. 2). Plasma P levels remained constant in the CP group (Fig. 2A), gradually increased in the HP group, and fluctuated markedly with alternating intakes of P in the P fluctuation subgroups (Fig. 2B).

**Body weight and biochemical parameters on day of sacrifice.** No significant differences in body weight were seen among the four groups (Table 1). To examine the effects of the P fluctuations for 36 days on biochemical parameters, we measured plasma and urinary P, Ca, and Cr on the day of sacrifice. Plasma P, Ca, and Cr levels did not differ significantly between the four groups. Urinary P excretion 24h before sacrifice was significantly higher in the HP and LH groups than in the CP group, and significantly lower in the HL group than in the CP group. The amount of urinary Ca excretion 24h before sacrifice was significantly higher in the HL group than in the CP, HP and LH groups. These results of urinary mineral excretion reflected dietary P intake prior to sacrifice, similar to the results for normal rats excreting minerals in one day.\(^{(28)}\) The amount of urinary Cr excretion 24h before sacrifice did not differ significantly between the four groups.

**Influences of P fluctuations on renal function.** To investigate the influences of P fluctuations on the kidney, renal weight was measured (Fig. 3A). Renal weight was significantly higher in the HP group than in the CP group. Further, to investigate influences of the P fluctuations on renal histology, kidneys in each group were stained and visualized using Masson trichrome staining (Fig. 3B). Renal fibrosis is an indicator of renal tissue associated with decreased renal function. Renal fibrosis was more severe in the HP, LH and HL groups than in the CP group. In the HP, LH, and HL groups, some tubules were observed to be dilated. Influences of P fluctuation diet on the kidney were similar to those of the HP diet, although renal excretion was not clearly different from that on the CP diet. Thus, the HP, LH, and HL groups showed tissue changes compared to the CP group, suggesting that renal function may decline in the near future.

**Effects of P fluctuation on inflammation and oxidative stress.** To determine the effects of P fluctuations on inflammation and oxidative stress, plasma CRP, TNF-α, and urinary 8-OHdG levels were measured (Fig. 4). No significant differences in plasma CRP or TNF-α levels were evident among the four groups (Fig. 4A and B). On the other hand, urinary 8-OHdG levels tended to be higher in the HP group than in the CP group, and levels in the LH group but not the HL group were significantly higher than those in the CP group (Fig. 4C). These results suggested that P fluctuation diets increased oxidative stress, to a similar extent to consuming a high-P diet every day.

**Effects of P fluctuations on vascular calcification.** To determine the effects of P fluctuations on vascular calcification, aortic histology was examined and Ca contents of the aorta were measured. Figure 5A shows the histology of the thoracoabdominal aorta, and remaining kidney were collected for analysis.

**Biochemical parameters.** Plasma and urine levels of P, calcium (Ca) and creatinine (Cr) were measured using the Wako Phospha-C test kit, the Wako Calcium-E test kit and the Wako Creatinine test kit, respectively (Wako Pure Chemical Industries). Plasma levels of CRP were determined using the Rat CRP ELISA Kit (Thermo Fisher Scientific, Tokyo, Japan). Plasma levels of TNF-α were determined using the Rat TNF-α Quantikine ELISA Kit (R&D Systems, Minneapolis, MN). Urinary levels of 8-OHdG were determined using the Highly Sensitive 8-OHdG Check ELISA kit (JaIKA, Shizuoka, Japan).

**Analysis of renal fibrosis.** To histologically evaluate renal tissue, kidneys were embedded in paraffin and 4-μm sections were cut. Sections were stained using Masson trichrome stain reagent (Muto Pure Cemicals, Tokyo, Japan) according to the instructions from the manufacturer. The slides were examined using microscope in a blinded fashion.
Fig. 1. Change in daily P intakes in CP and HP groups (A) and in P fluctuation groups (B). Total P intake for 36 days (C). White circles indicate the CP group, black circles indicate the HP group, white squares indicate the LH group, and black squares indicate the HL group. CP, control P diet group; HP, high-P diet group; LH, alternating low-P and high-P diet group; HL, alternating high-P and low-P diet group. Values are mean ± SE. *p<0.05.

Fig. 2. Change in daily plasma P levels in CP and HP groups (A) and in P fluctuation groups (B). White circles indicate the CP group, black circles indicate the HP group, white squares indicate the LH group, and black squares indicate the HL group. CP, control P diet group; HP, high-P diet group; LH, alternating low-P and high-P diet group; HL, alternating high-P and low-P diet group. Values are mean ± SE.

Table 1. Body weight and Biochemical data at sacrifice day

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<th></th>
<th>CP</th>
<th>HP</th>
<th>LH</th>
<th>HL</th>
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<tr>
<td>Body weight (g)</td>
<td>427.30 ± 9.54</td>
<td>420.54 ± 7.28</td>
<td>437.04 ± 5.78</td>
<td>422.69 ± 10.89</td>
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<tr>
<td>Plasma (mg/dl)</td>
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<tr>
<td>P</td>
<td>5.26 ± 0.34</td>
<td>5.76 ± 0.45</td>
<td>5.52 ± 0.41</td>
<td>4.65 ± 0.46</td>
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<tr>
<td>Ca</td>
<td>10.02 ± 0.50</td>
<td>9.21 ± 0.43</td>
<td>9.55 ± 0.19</td>
<td>10.34 ± 0.45</td>
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<tr>
<td>Cr</td>
<td>0.62 ± 0.03</td>
<td>0.63 ± 0.04</td>
<td>0.64 ± 0.03</td>
<td>0.67 ± 0.04</td>
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<tr>
<td>Urine (mg/day)</td>
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<td></td>
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<tr>
<td>P</td>
<td>34.69 ± 6.41</td>
<td>95.07 ± 11.70**</td>
<td>87.13 ± 9.07**</td>
<td>0.64 ± 0.22**</td>
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<td>Ca</td>
<td>0.47 ± 0.14**</td>
<td>0.84 ± 0.34**</td>
<td>0.52 ± 0.10**</td>
<td>3.42 ± 0.57</td>
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<td>Cr</td>
<td>7.11 ± 1.33</td>
<td>9.76 ± 1.40</td>
<td>8.98 ± 0.80</td>
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CP, control P diet group; HP, high-P diet group; LH, alternating low-P and high-P diet group; HL, alternating high-P and low-P diet group. Values are mean ± SE. *p<0.05 vs CP, **p<0.05 vs HL, ***p<0.01 vs HL.
aorta visualized using Von Kossa staining. Interestingly, vascular calcification was more severely progressed in the LH and HL groups than in the CP group, and was similar to that observed in the HP group. Ca content of the aorta was also significantly higher in the LH group than in the CP group, nearly equal to that in the HP group (Fig. 5B). Although total P intake in the P fluctuation group was the same as in the CP group, the above result indicates that the P fluctuation diets induced vascular calcification, similar to that seen under conditions of consuming the high-P diet every day.

Discussion

We investigated the effects of repetitive P fluctuations on vascular calcification and inflammation in rats that had received unilateral nephrectomy as an early-stage CKD model. In consequence, we demonstrated advanced vascular calcification in the P fluctuation group, similar to the status of animals with chronic consumption of the high-P diet, despite receiving the same total P intake as the CP group. The results suggested that not only chronic hyperphosphatemia, but also repetitive transient plasma P fluctuations, may initiate the mechanisms of vascular calcification in early-stage CKD.

We used unilateral nephrectomy rats as a model for early-stage CKD. Unilateral nephrectomy in rats has been demonstrated to increase glomerular capillary hydraulic pressure, plasma flow rate and glomerular sclerosis, with progression to renal injury. In the study reported that mild renal failure resulted in early cardiac fibrosis with mild diastolic impairment, and the study reported that mild renal insufficiency was associated with relaxation dysfunction of the heart, unilateral nephrectomy rats have been shown to be mild renal impairment. These rats are often used to elucidate the effect of hypertension from a high-sodium diet on the kidney in early-stage CKD. We therefore considered that these rats might be readily affected by a high-P diet.

In this study, high-P and P fluctuation diets caused renal fibrosis and some of the tubule dilatation, although no significant differences in plasma Cr were evident compared with rats receiving the CP diet. This is presumably because renal function was maintained, with renal fibrosis and tubule dilatation being partial. In addition, plasma P levels did not differ significantly among the four groups on the day of sacrifice and urinary P excretion 24 h before sacrifice corresponded to P intake. In early-stage CKD in humans and rats, kidney functions are reportedly maintained by the action of hormones such as fibroblast growth factor 23 (FGF23) and parathyroid hormone (PTH). Kidney function in our rats was thus presumably maintained by these hormones, although this study did not measure hormone levels. The condition of rats on the day of sacrifice for this study model was therefore considered to be comparable to that of mild renal dysfunction. The most important finding seemed to be that P fluctuations caused vascular calcification and inflammation to a similar extent to the high-P diet in this model of early-stage CKD.

Inflammatory cytokines and oxidative stress promote vascular calcification in CKD model rats. Inflammatory cytokines stimulate the generation of reactive oxygen species (ROS) in
Fig. 4. Effects of dietary P on inflammation and oxidative stress. Plasma levels of CRP (A) and TNF-α (B), and urinary 8-OHdG excretion (C). CP, control P diet group; HP, high-P diet group; LH, alternating low-P and high-P diet group; HL, alternating high-P and low-P diet group. Values are mean ± SE. *p<0.05.

Fig. 5. Effects of dietary P on vascular calcification. (A) Thoracoabdominal aorta stained with Von Kossa. Calcium deposition area was stained black. Original magnification: ×40. (B) Quantification of Ca content in the thoracoabdominal aorta. CP, control P diet group; HP, high-P diet group; LH, alternating low-P and high-P diet group; HL, alternating high-P and low-P diet group. Values are mean ± SE. *p<0.05.
response to nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation. ROS production induces osteoblast-like phenotypic changes in VSMCs and may promote vascular calcification. Oxidative stress is reportedly related to BMP-2, a marker of vascular calcification.\textsuperscript{[20]} Also, high P reportedly increases oxidative stress in vascular endothelial cells.\textsuperscript{[4,5]} For these reasons, we measured plasma levels of CRP and TNF-α, as markers of inflammation, and urinary 8-OHdG levels as a marker of oxidative stress. No significant differences in plasma levels of CRP were seen among all groups. This finding may be because CRP is a marker of inflammation in the acute phase.\textsuperscript{[44,45]} Although no significant differences in plasma levels of TNF-α were seen, the HP group tended to show higher levels than the CP group. One possibility is that individual differences affected the statistical results. However, because no differences were seen between the CP and P fluctuation groups, these model rats may be in a state of mild inflammation, and/or P fluctuations may affect the inflammatory system in a different way to the TNF-α system. Despite these results, we found that urinary 8-OHdG levels in the HP group tended to be higher than those in the CP group, as shown in previous studies.\textsuperscript{[7,21]} Levels of 8-OHdG are reportedly related to BMP-2 mRNA expression, which induces vascular calcification.\textsuperscript{[22]} Interestingly, urinary 8-OHdG levels in the LH group were also significantly higher than those in the CP group. Repetitive P fluctuations due to dietary changes are thus inferred to induce increases in oxidative stress, and may lead to the development of vascular calcification, as seen with chronic hyperphosphatemia.

In terms of Ca content in the aorta and urinary 8-OHdG levels, the HL group did not show the significant difference seen with the LH group. One possible reason for such a result was that changes over time in plasma P levels were lower in the HL group than in the LH group (average of plasma P for 32 days: HL 6.28 ± 0.22 mg/dl vs LH 7.13 ± 0.26 mg/dl, p<0.05), although why this difference occurred remains unclear.

This study used an extreme diet, changing from very low P to high P to produce clear fluctuations in plasma P. In humans, intake of such a low-P diet is rare in normal life. Further research is thus needed to clarify the effects of fluctuations from normal P to high P and/or repeated transient hyperphosphatemia on vessels and the ramifications for dietary therapy in CKD patients.

In conclusion, in a rat model of early-stage CKD, vascular calcification and inflammation were exacerbated by repeated P fluctuations, despite the same total P intake as the CP group. Furthermore, the degree of vascular calcification in the group receiving P fluctuation diets was broadly similar to that seen with daily consumption of a high-P diet. These results provide evidence suggesting that a diet avoiding not only chronic hyperphosphatemia, but also repetitive fluctuations in plasma P is important for preventing vascular calcification from early-stage CKD. The present findings suggest that intake of P, especially inorganic P, should be managed on a meal-by-meal basis to prevent calcification in CKD patients.

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Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>BMP-2</td>
<td>bone morphogenetic protein 2</td>
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<tr>
<td>Ca</td>
<td>calcium</td>
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<td>CKD</td>
<td>chronic kidney disease</td>
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<td>Cr</td>
<td>creatinine</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<td>CVD</td>
<td>cardiovascular disease</td>
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<tr>
<td>8-OHdG</td>
<td>8-hydroxy-2'-deoxyguanosine</td>
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<td>P</td>
<td>phosphorus</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
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<td>VSMCs</td>
<td>vascular smooth muscle cells</td>
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Conflict of Interest

No potential conflicts of interest were disclosed.

References

Vascular calcification in chronic kidney disease. 

J Bone Miner Metab 2006; 24: 176–181.


