

Chronic Mineral Dysregulation Promotes Vascular Smooth Muscle Cell Adaptation and Extracellular Matrix Calcification

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ABSTRACT

In chronic kidney disease (CKD) vascular calcification occurs in response to deranged calcium and phosphate metabolism and is characterized by vascular smooth muscle cell (VSMC) damage and attrition. To gain mechanistic insights into how calcium and phosphate mediate calcification, we used an *ex vivo* model of human vessel culture. Vessel rings from healthy control subjects did not accumulate calcium with long-term exposure to elevated calcium and/or phosphate. In contrast, vessel rings from patients with CKD accumulated calcium; calcium induced calcification more potently than phosphate (at equivalent calcium-phosphate product). Elevated phosphate increased alkaline phosphatase activity in CKD vessels, but inhibition of alkaline phosphatase with levamisole did not block calcification. Instead, calcification in CKD vessels most strongly associated with VSMC death resulting from calcium- and phosphate-induced apoptosis; treatment with a pan-caspase inhibitor ZVAD ameliorated calcification. Calcification in CKD vessels was also associated with increased deposition of VSMC-derived vesicles. Electron microscopy confirmed increased deposition of vesicles containing crystalline calcium and phosphate in the extracellular matrix of dialysis vessel rings. In contrast, vesicle deposition and calcification did not occur in normal vessel rings, but we observed extensive intracellular mitochondrial damage. Taken together, these data provide evidence that VSMCs undergo adaptive changes, including vesicle release, in response to dysregulated mineral metabolism. These adaptations may initially promote survival but ultimately culminate in VSMC apoptosis and overt calcification, especially with continued exposure to elevated calcium.

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Cardiovascular disease accounts for more than half of all deaths in patients with chronic kidney disease (CKD).¹ Vascular calcification is an important contributor to this cardiovascular mortality,^{1,2} beginning in the first decade of life in children who are on dialysis.³ Calcification can occur in the tunica intima or media in patients with CKD but, at least in its early stages in young patients with CKD, is typically medial.⁴ Epidemiologic studies have highlighted the impact of dysregulated mineral metabolism and linked both elevated phosphate (P) and calcium (Ca) to accelerated vascular calcification.^{5,6} *In vitro* studies using hu-

man vascular smooth muscle cells (VSMCs) have provided mechanistic insights into the role of Ca and P in the initiation and progression of calcification and have

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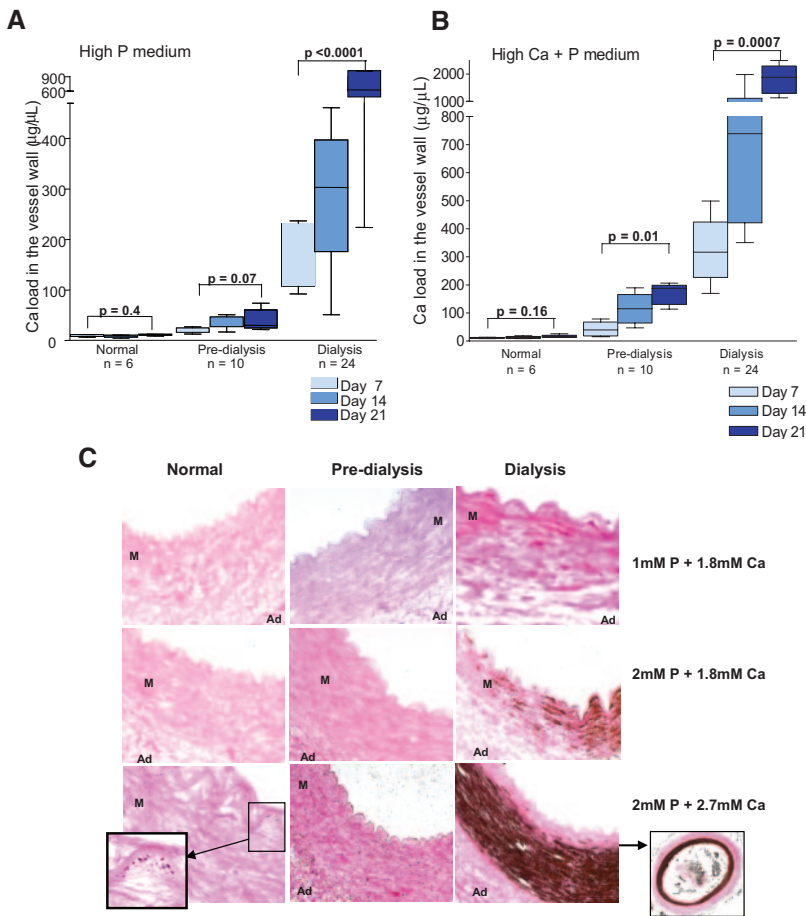


Figure 1. Dialysis vessels show time-dependent Ca accumulation *in vitro*. The Ca load in normal, predialysis, and dialysis vessel rings was quantified after incubation for 7, 14, and 21 d. Normal vessels did not increase Ca loading, whereas dialysis vessels showed maximal Ca load in response to Ca + P. (A) Incubation in high P medium (2.0 mM P + 1.8 mM Ca). The Ca load in normal vessels was 10.0 ± 2.4 versus 8.4 ± 3.6 versus $11.4 \pm 1.6 \mu\text{g}/\mu\text{l}$ ($P = 0.40$), in predialysis vessels was 20.2 ± 5.8 versus 30.1 ± 11.9 versus $41.7 \pm 7.2 \mu\text{g}/\mu\text{l}$ ($P = 0.07$), and in dialysis vessels was 176.3 ± 17.9 versus 298.2 ± 93.7 versus $726.7 \pm 103.9 \mu\text{g}/\mu\text{l}$ ($P < 0.0001$) at 7, 14, and 21 d, respectively. (B) Incubation in high Ca + P medium (2.0 mM P + 2.7 mM Ca). The Ca load in normal vessels was 11.2 ± 1.9 versus 12.9 ± 3.5 versus $17.0 \pm 6.0 \mu\text{g}/\mu\text{l}$ ($P = 0.16$), in predialysis vessels was 43.2 ± 13.8 versus 115.1 ± 49.0 versus $170.8 \pm 35.5 \mu\text{g}/\mu\text{l}$ ($P = 0.01$), and in dialysis vessels was 325.7 ± 137.0 versus 711.0 ± 206.0 versus $1857.0 \pm 90.0 \mu\text{g}/\mu\text{l}$ ($P = 0.0007$) at 7, 14, and 21 d, respectively. (C) Von Kossa staining of vessel rings after 14 d in calcifying media. Normal and predialysis vessels did not show any calcification in high P medium. In high Ca + P medium, some normal and predialysis vessels showed punctate calcification along the internal elastic lamina (inset). Dialysis vessels developed calcification in all *in vitro* conditions with large confluent areas of calcification in the high Ca + P medium (inset). M, medium; Ad, adventitia

shown that in response to raised extracellular Ca and P levels, VSMCs undergo specific phenotypic changes, including osteo/chondrocytic differentiation and vesicle release.^{7,8} Recent work has shown that mineral dysregulation in CKD leads to remarkably similar processes of VSMC phenotypic modulation *in vivo*⁴ beginning before dialysis and culminating in VSMC apoptosis and calcification in dialysis. This suggests that prolonged exposure to worsening mineral dysregulation, as well as other factors specific

the Ca load was significantly higher in dialysis > predialysis > normal vessels in comparable *in vitro* conditions.

Von Kossa staining performed after 14 d of exposure to calcifying conditions revealed that normal and predialysis vessels did not show any calcification in high P medium; however, a small minority (3%) of vessels developed some punctate calcification along the internal elastic lamina in high Ca + P medium (Figure 1C). Strikingly, most dialysis vessels exhibited

to the dialysis milieu, accelerate VSMC damage, reducing their inhibitory capacity and promoting calcification.

To date, studies into the mechanisms that drive calcification in CKD vessels have been hampered by the lack of an appropriate *in vitro* model; explanted VSMCs lack the matrix and architecture of a normal vessel and undergo substantial phenotypic changes so that they may no longer be representative of contractile VSMCs *in vivo*.^{9–11} To address this, we used an *ex vivo* model of intact human arteries from children and tested the response of CKD vessels (from predialysis and dialysis patients) and age-matched healthy subjects to exposure to high Ca and P levels. We show that VSMCs in different vessel types have fundamentally different responses to high Ca and P as a result of phenotypic changes induced during long-term exposure to dysregulated mineral metabolism *in vivo*. We hypothesize that these are adaptive changes that may provide survival benefits in predialysis but are overwhelmed in dialysis, leading to VSMC apoptosis and accelerated calcification.

RESULTS

Dialysis Vessels Undergo Time-Dependent Ca Accumulation in *In Vitro* Calcifying Conditions

Vessel rings were exposed to high Ca and/or P for 7, 14, and 21 d (Figure 1, A and B). Normal vessels did not increase their Ca load in any of the conditions tested. Predialysis vessels showed a small increase in Ca load, only in high Ca + P medium, whereas dialysis vessels showed progressively increasing Ca loads in all of the *in vitro* conditions, with the greatest response in high Ca + P medium. Ca loading in CKD vessels increased with time of exposure, and, at each time point,

von Kossa–positive areas in response to both high P and Ca + P media, with 30% exhibiting dense medial calcification after incubation in Ca + P medium (Figure 1C).

Ca Is a More Potent Inducer of Calcification than P at Equivalent Ca-P Products

To determine the potencies of Ca and P at inducing calcification, we compared media with different Ca and P contents but with an equivalent Ca-P product ($\text{Ca} \times \text{P} = 5.4 \text{ mM}^2$; Figure 2A). As before, vessels from healthy control subjects did not increase their Ca load in any calcifying media. In the presence of high Ca + P, both predialysis and dialysis vessels showed significantly greater calcification than in media with high P alone, highlighting the potency of Ca at inducing calcification.

Dialysis Vessels Are Primed for Rapid Ca Accumulation

We next addressed whether the increased propensity of dialysis vessels to calcify *in vitro* was due to their higher baseline Ca loading (Supplemental Table 1). First, we compared the response to calcifying media of dialysis vessels without and with overt calcification, shown by von Kossa–positive areas at baseline.⁴ This showed greater Ca accumulation in von Kossa–positive vessels (Figure 2B), suggesting that once a nidus for calcification is formed, it can act to accelerate further calcification; however, when we compared von Kossa–negative, predialysis and dialysis vessels with similar baseline Ca loads (25 to 35 $\mu\text{g}/\mu\text{L}$),⁴ dialysis vessels accumulated significantly more Ca than predialysis vessels, under identical *in vitro* conditions (Figure 2C). This suggests that a reduced capacity to inhibit calcification is an intrinsic property of VSMCs in dialysis vessels.

Alkaline Phosphatase Activity Is Differentially Regulated by Extracellular Ca and P

Increased alkaline phosphatase (ALK) activity, an early indicator of VSMC osteogenic conversion, has been shown to enhance calcification. In response to high P medium, normal vessels did not show any significant increase in ALK activity; however, both predialysis and dialysis vessels showed an approximately two- to three-fold increase in ALK. This increase was additional to the already elevated ALK activity observed in most dialysis vessels at baseline⁴ (Supplemental Table 1, Figure 3A). In contrast, incubation in high Ca + P medium significantly decreased ALK activity to below baseline levels in all vessel types. The marked reduction in ALK activity was independent of cell loss, because both normal and predialysis vessels showed no decrease in cell number and trypan blue staining confirmed VSMC viability (data not shown).

To explore the link between ALK activity and calcification, we incubated vessel rings from dialysis patients in high P medium with the addition of levamisole, an inhibitor of ALK activity. Levamisole caused an approximately 50% decrease in ALK levels (Figure 3B), but there was no corresponding reduction in the Ca load (Figure 3C).

ALK is a downstream target of the osteogenic transcription

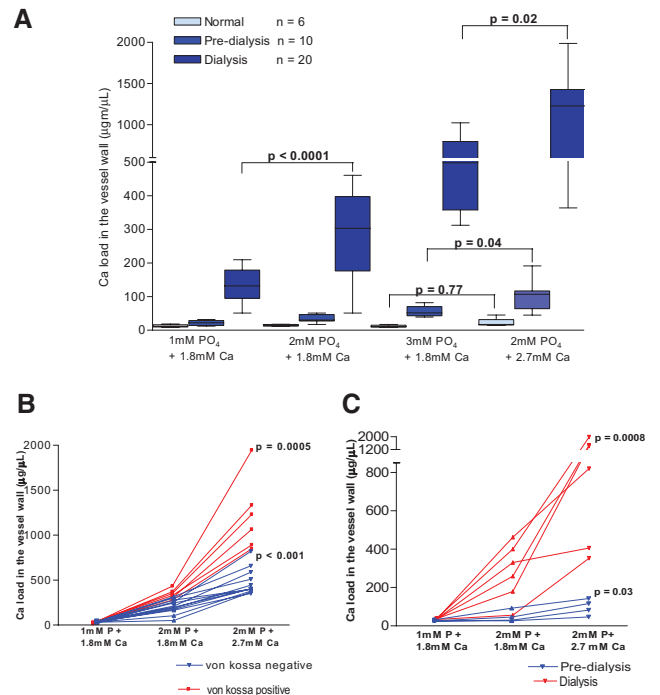


Figure 2. Ca is a more potent inducer of calcification than P. The Ca load in all vessel types was quantified after exposure to calcifying media with the same $\text{Ca} \times \text{P}$ of 5.4 mM^2 ($3.0 \text{ mM P} + 1.8 \text{ mM Ca}$ and $2.0 \text{ mM P} + 2.7 \text{ mM Ca}$) for 14 d. (A) The Ca load in normal vessels was unaffected (6.7 ± 2.4 versus 8.4 ± 3.6 versus 11.8 ± 2.9 versus $12.9 \pm 3.5 \mu\text{g}/\mu\text{L}$; $P = 0.3$), was increased in predialysis (19.7 ± 1.1 versus 30.1 ± 11.9 versus 47.9 ± 8.3 versus $115.1 \pm 49.0 \mu\text{g}/\mu\text{L}$; $P = 0.03$), and was maximum in dialysis vessels (129.0 ± 53.0 versus 298.2 ± 93.7 versus 350.0 ± 147.0 versus $711.0 \pm 206.0 \mu\text{g}/\mu\text{L}$; $P < 0.0001$) in response to in $1.0 \text{ mM P} + 1.8 \text{ mM Ca}$, $2.0 \text{ mM P} + 1.8 \text{ mM Ca}$, $3.0 \text{ mM P} + 1.8 \text{ mM Ca}$, and $2.0 \text{ mM P} + 2.7 \text{ mM Ca}$ media, respectively. On comparing media with similar $\text{Ca} \times \text{P}$, the Ca load was significantly increased in predialysis ($P = 0.04$) and dialysis ($P = 0.02$) vessels but not in normal vessels ($P = 0.77$). (B) Dialysis vessels with baseline von Kossa positivity ($n = 6$) showed greater calcification than baseline von Kossa–negative ($n = 18$) vessels after culture for 14 d in calcifying media. Ca load in von Kossa–positive vessels was 44.2 ± 5.1 , 370.0 ± 25.1 , and $1254.0 \pm 406.2 \mu\text{g}/\mu\text{L}$ ($P = 0.0005$, ANOVA) versus 30.5 ± 5.4 , 208.0 ± 78.6 , and $476.3 \pm 143.7 \mu\text{g}/\mu\text{L}$ ($P < 0.001$, ANOVA) in von Kossa–negative vessels in $1.0 \text{ mM P} + 1.8 \text{ mM Ca}$, $2.0 \text{ mM P} + 1.8 \text{ mM Ca}$, and $2.0 \text{ mM P} + 2.7 \text{ mM Ca}$ media, respectively. (C) Predialysis ($n = 4$) and dialysis ($n = 6$) vessels with similar baseline Ca loads after culture for 14 d in calcifying media showed increased Ca loading in dialysis vessels (31.5 ± 2.4 and $278.2 \pm 105.0 \mu\text{g}/\mu\text{L}$; $P = 0.0008$) compared with predialysis vessels (27.0 ± 3.3 and 48.7 ± 13.2 versus $96.8 \pm 14.4 \mu\text{g}/\mu\text{L}$; $P = 0.03$) in identical calcifying conditions. All probability values calculated by ANOVA.

factor Runx2. Immunohistochemistry showed that normal vessels, under all conditions, showed $<5\%$ Runx2–positive nuclei, and these cells were predominantly at the media–adventitia interface (Figure 3E). Similarly, predialysis vessels showed approximately 5% Runx2–positive nuclei in both control and

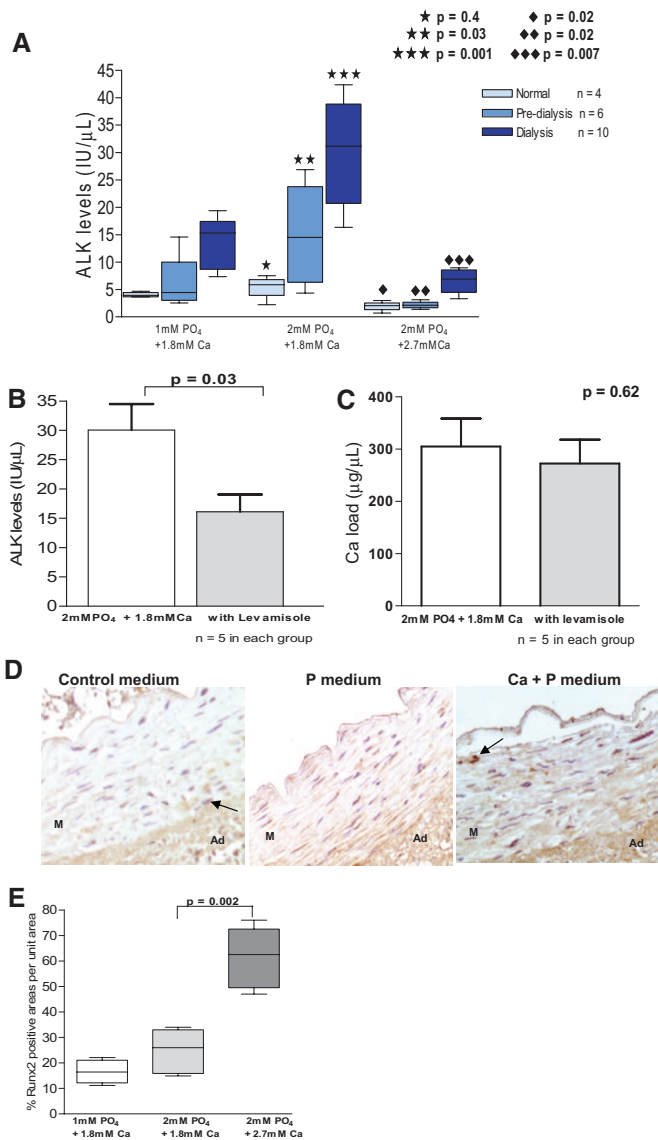


Figure 3. Osteogenic conversion of VSMCs in predialysis and dialysis vessels. (A) ALK levels in normal vessels were 5.7 (2.3 to 7.5) versus 4.2 IU/μL (3.2 to 4.8 IU/μL; $P = 0.4$), in predialysis vessels were 3.9 (2.4 to 15.1) versus 14.4 IU/μL (4.2 to 27.1 IU/μL; $P = 0.03$), and in dialysis vessels were 5.0 (5.7 to 19.3) versus 30.5 IU/μL (16.0 to 42.1 IU/μL; $P = 0.001$) after incubation in 1.0 mM P + 1.8 mM Ca and 2.0 mM P + 1.8 mM Ca media for 14 d, respectively. In 2.0 mM P + 2.7 mM Ca medium, ALK levels were significantly reduced in all vessel types (2.1 [0.9 to 3.1], 2.2 [1.0 to 2.9], and 5.7 IU/μL [3.8 to 8.9 IU/μL] in normal, predialysis, and dialysis vessels, respectively). (B) Dialysis vessels incubated in 2.0 mM P + 1.8 mM Ca medium with the addition of levamisole showed a decrease in ALK levels (31.1 [16.3 to 42.3] versus 17.1 [6.2 to 19.3]; $P = 0.03$). (C) Ca load in vessel rings from the aforementioned experiment remained unchanged (387.2 ± 22.0 versus 356.0 ± 37.0 μg/μL; $P = 0.62$). (D) Immunohistochemistry for Runx2 was performed in dialysis vessels after culture for 14 d in calcifying media. Runx2 positivity increased in high P medium and was significantly greater in high Ca + P medium. Note the cytoplasmic localization of Runx2 in dialysis vessels potentially reflecting alternative isoform usage in these vessels.³⁹ (E) The number of

high P media, and this increased to $10.4 \pm 3.2\%$ after incubation in high Ca + P medium. Dialysis vessels showed extensive Runx2-positive areas with both nuclear and cytoplasmic staining⁴ at baseline that increased approximately 1.5-fold in high P medium and four-fold in high Ca + P medium (Figure 3, D and E).

Dialysis Vessels Undergo VSMC Loss as a Result of Apoptotic Cell Death

Apoptosis has been shown to play a key role in calcification.^{4,7,8} To test whether VSMC loss contributed to calcification, we performed cell counting. In normal vessels, there was no significant change in cell number after exposure to calcifying conditions (Figure 4A). Similarly, both predialysis and dialysis vessels maintained cell numbers in control and high P media; however, both showed a reduction in VSMC number in high Ca + P medium, which was most striking in dialysis vessels (11 versus 30% reduction; predialysis versus dialysis). These findings were confirmed on immunohistochemistry for α-smooth muscle actin that showed prominent cystic areas in the media of dialysis vessels (Figure 4C, top).

To determine whether the increased Ca load in dialysis vessels contributed to greater cell loss, we again examined von Kossa–negative predialysis and dialysis vessels with similar baseline Ca loads.⁴ There was no significant reduction in VSMC number in the predialysis vessels in response to Ca and P (Figure 4B), but dialysis vessels showed a significant reduction in cell number, suggesting VSMCs in dialysis vessels have an increased susceptibility to cell death.

Transferase-mediated dUTP nick-end labeling (TUNEL) staining confirmed that apoptosis was contributing to VSMC loss. Normal and predialysis vessels had <0.5% apoptotic cells, and this remained unchanged in all *in vitro* conditions (Figure 4, C, bottom, and D). Dialysis vessels did not show a significant increase in TUNEL staining in response to P alone, but, in high Ca + P medium, there was a significant increase in TUNEL positivity. Additional indicators of apoptosis, including nuclear fragments and condensed nuclei, were also increased (data not shown; Figure 4C, inset). Apoptosis was detected in both von Kossa–positive and –negative vessels; however, in heavily calcified vessels, TUNEL co-localized with calcification in adjacent sections (Figure 4C).

To confirm experimentally a role for apoptosis in increased calcification, we cultured dialysis vessel rings in the presence of the pan-caspase inhibitor ZVAD.fmk in either high Ca + P medium, which induced apoptosis, or high P medium, which did not. The addition of ZVAD reduced calcification (Figure 4E) in Ca + P treated dialysis vessels but had no effect on

Runx2-positive areas per unit area of tunica media was counted in dialysis vessels after incubation for 14 d. Runx2-positive areas were 16.6 ± 4.1 , 24.9 ± 7.8 , and $61.5 \pm 10.9\%$ ($P = 0.002$) in 1.0 mM P + 1.8 mM Ca, 2.0 mM P + 1.8 mM Ca, and 2.0 mM P + 2.7 mM Ca media, respectively.

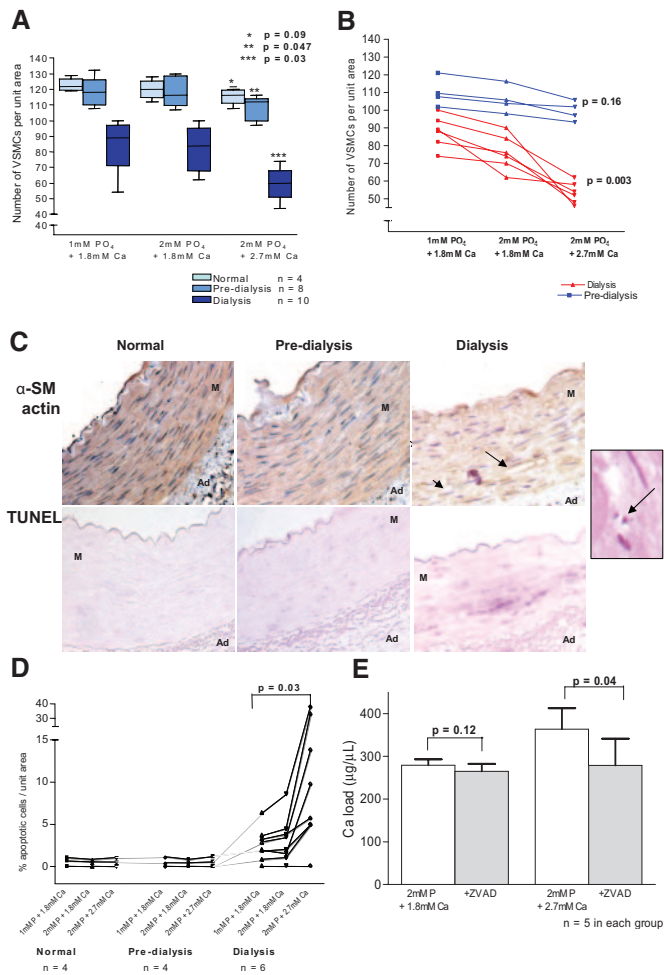


Figure 4. Dialysis vessels undergo VSMC loss as a result of apoptotic cell death. VSMC nuclei per unit area of tunica media were counted on hematoxylin-eosin-stained samples in all vessel types after *in vitro* culture for 14 d in high P and high Ca + P. (A) The VSMC number in normal vessels was 122.0 ± 3.9 versus 120.0 ± 5.8 versus 115.0 ± 4.7 cells per unit area ($P = 0.09$), in predialysis vessels was 118.0 ± 9.1 versus 118.0 ± 9.8 versus 108.0 ± 7.7 cells per unit area ($P = 0.047$) and in dialysis vessels was 85.0 ± 17.9 versus 82.0 ± 14.6 versus 59.0 ± 10.7 cells per unit area ($P = 0.03$) in 1.0 mM P + 1.8 mM Ca, 2.0 mM P + 1.8 mM Ca, and 2.0 mM P + 2.7 mM Ca media, respectively. (B) At similar baseline Ca loads, predialysis vessels had 120.5 ± 8.4 versus 114.3 ± 11.3 versus 107.0 ± 8.6 cells per unit area ($P = 0.16$), and dialysis vessels had 87.8 ± 9.1 versus 76.0 ± 9.9 versus 54.0 ± 6.7 cells per unit area ($P = 0.003$) in 1.0 mM P + 1.8 mM Ca, 2.0 mM P + 1.8 mM Ca, and 2.0 mM P + 2.7 mM Ca media, respectively. (C) Staining for α -smooth muscle actin (α -SM actin) was patchy in dialysis compared with predialysis and normal vessels; arrows show cystic areas denoting VSMC loss. TUNEL-positive areas were observed in dialysis vessels only, and the frequency of nuclear fragments was also increased. Inset shows hematoxylin-eosin staining of nuclear fragments (arrow) associated with a VSMC cyst in a dialysis vessel treated with Ca + P medium. (D) The number of TUNEL-positive cells per unit area of each vessel was counted and expressed as a percentage of total cell number after 14 d of incubation in calcifying media. Normal

calcification in vessels treated in high P medium suggesting Ca acts to enhance apoptosis and calcification in dialysis vessels.

Calcification Is Vesicle Mediated in Dialysis Vessels

Vesicles released from both apoptotic and Ca-stimulated VSMCs have been shown to form the first nidus for calcification^{4,7,12}; therefore, we performed immunohistochemistry for the vesicle marker annexin VI and key vesicle components fetuin-A and matrix Gla protein (MGP). There was no annexin VI positivity in normal vessels in control or high P media, but a few punctate areas of positivity in the tunica media after culture in high Ca + P medium were evident (Figure 5A). In contrast, dialysis vessels showed patchy areas of diffuse staining in control medium, and positive areas increased in high P medium and were maximal in high Ca + P medium, where staining was predominantly along the elastic lamellae. Fetuin-A, an inhibitor of calcification that is taken up by modified VSMCs and released from intracellular stores *via* vesicles, showed minimal deposition in normal vessels in all *in vitro* conditions but was present in dialysis vessels in control medium and increased to maximal levels in high Ca + P medium (Figure 5B). MGP, a locally produced inhibitor of calcification, was deposited in a pattern similar to that of fetuin-A with increased undercarboxylated MGP deposited in calcified dialysis vessels (data not shown).

Electron microscopy (EM) showed no evidence of calcification of the extracellular matrix (ECM) in normal arteries exposed to calcifying conditions. Some vesicle debris was observed in association with rare degenerate VSMCs, but these showed no evidence of calcification (Figure 5C). In contrast, in dialysis vessels, we observed extensive crystalline ECM calcification in association with elastin and collagen. In addition, numerous extracellular membranous vesicles, some containing crystalline apatite, were deposited adjacent to VSMCs, particularly in Ca + P-treated vessels (Figure 5C). An additional striking observation was that the majority of VSMCs in normal vessels, after long-term exposure to Ca + P medium, exhibited extensive intracellular mitochondrial calcification. In contrast, the mitochondria of VSMCs in dialysis vessels seemed intact (Figure 5C).

DISCUSSION

Predialysis and Dialysis Vessels Are Damaged and "Primed" to Calcify

A striking finding in this study was that vessels from healthy control subjects did not calcify even after long-term exposure

and predialysis vessels had <0.5% apoptotic cells in all *in vitro* conditions, whereas dialysis vessels showed a significant increase (2.6 [0.0 to 6.2], 2.9 [0.0 to 8.6], and 7.7% [0.0 to 37.9%] TUNEL-positive cells ($P = 0.03$) in 1.0 mM P + 1.8 mM Ca, 2.0 mM P + 1.8 mM Ca, and 2.0 mM P + 2.7 mM Ca media, respectively). (E) On addition of ZVAD to Ca + P-treated dialysis vessels, the Ca load reduced from 363.7 ± 28.0 versus 278.0 ± 34.0 $\mu\text{g}/\mu\text{L}$ ($P = 0.04$) but remained unchanged in vessels treated in high P medium alone (279.2 ± 24.1 versus 265.3 ± 30.3 $\mu\text{g}/\mu\text{L}$; $P = 0.12$). All probability values were calculated by ANOVA.

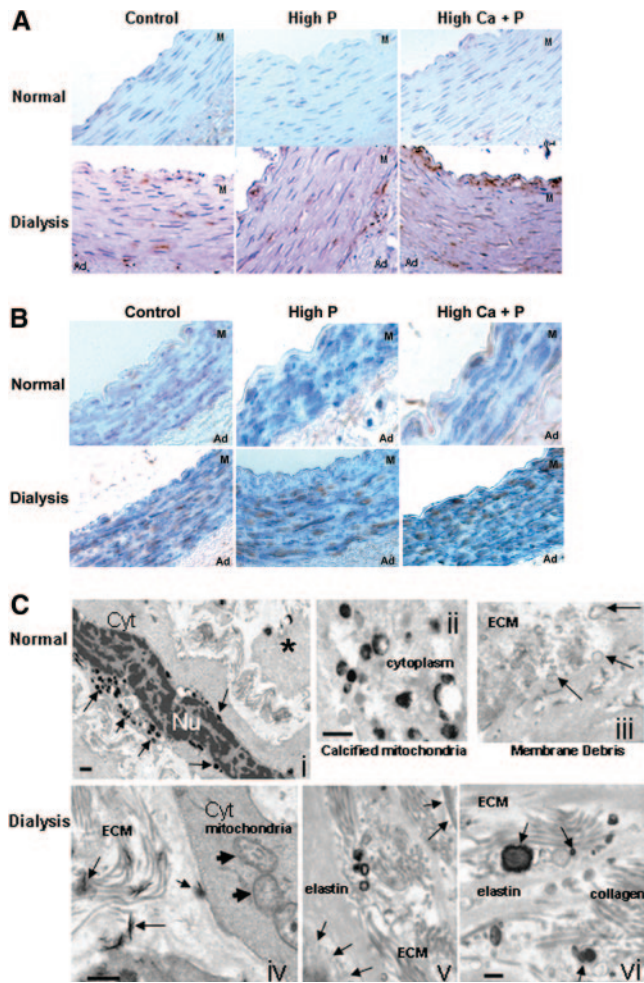


Figure 5. Immunohistochemistry for vesicle markers and EM analysis of vessels. (A) Immunohistochemistry for the vesicle marker annexin VI showed minimal deposition in normal vessels after exposure to calcifying media for 14 d but increased deposition in dialysis vessels. (B) A similar pattern was observed for the vesicle-associated protein fetuin-A, which was absent in normal vessels but heavily deposited in dialysis vessels. Fetuin is present intracellularly as well as deposited in the VSMC matrix. All panels show α -SM actin co-staining (blue) and fetuin-A (brown). (C, i) EM showing calcified mitochondria (arrow) in VSMCs from control vessels exposed to high Ca + P medium *in vitro*. *Degenerate VSMC with remnant calcified mitochondria and membrane debris. (ii) Enlargement of calcified mitochondria. (iii) Enlargement of rare membrane debris found associated with degenerate VSMCs in control vessels. This was never calcified, and no evidence of ECM calcification was observed in normal vessels. (iv) Crystalline ECM calcification (arrow) was observed in calcified dialysis vessels after exposure to Ca + P *in vitro*. These VSMCs exhibited intact mitochondria (arrowheads). (v) Vesicle deposition in the ECM of dialysis vessels exposed to Ca + P occurred in close proximity to healthy intact VSMCs (membranes arrow) and associated with elastin. (vi) Enlargement of vesicles. Note that a large number of these vesicles show crystalline apatite within the lumen (arrow). Bar = 0.5 μ m.

to supraphysiologic levels of Ca and/or P *in vitro*, whereas predialysis and, to a much greater extent, dialysis vessels calcified. This suggests that normal VSMCs possess intact inhibitory pathways that prevent calcification. In contrast, vessels from predialysis and dialysis patients were susceptible to calcification, as a result of their previous exposure *in vivo* to the CKD milieu, which damaged the VSMCs and/or compromised their inhibitory mechanisms, thereby “priming” the vessels for calcification. In support of this notion, a previous study showed that normal rat aortic rings, maintained in high Ca and/or P medium *in vitro*, could be induced to calcify only after direct mechanical injury and ALK activation.¹³ In this study, human dialysis vessels at baseline already exhibited increased ALK activity and VSMC loss, and exposure of the vessels to elevated Ca and/or P *in vitro* increased these indicators of injury and enhanced calcification. The factors that induce VSMC injury in CKD are likely to be multiple; however, consistent with other studies, this study clearly demonstrated that for a fixed Ca \times P, elevated Ca was a more potent stimulus to induce calcification than elevated P, suggesting Ca may be a key mediator of VSMC damage and calcification in CKD.^{4,7}

Ca Acts to Enhance Phenotypic Modulation of VSMCs

In vitro studies have shown that a significant phenotypic adaptation that VSMCs undergo in response to elevated extracellular Ca is vesicle release. Vesicle release is thought to be an adaptive response, because vesicles extrude Ca from the cell, providing protection from intracellular Ca overload¹⁴; however, this adaptive response promotes ECM calcification when the vesicles are not loaded with calcification inhibitors, such as fetuin-A and MGP, that act to block mineral nucleation.^{4,15,16} In this study, annexin VI staining, indicative of vesicle deposition, was increased in CKD vessel rings in response to Ca + P,⁷ and EM showed that many of the vesicles released in dialysis vessels contained crystalline apatite. Release of these calcific vesicles occurred concomitantly with the deposition of dysfunctional undercarboxylated MGP and in the absence of fetuin-A in the serum-free culture conditions; once the intracellular stores of fetuin-A were depleted *via* vesicle release, they could not be replenished by uptake from the media. These observations support the notion that when inhibitory proteins are functional, vesicle release is protective; however, when inhibitors are lacking, vesicles become procalcific.^{4,7}

Importantly, vesicle release occurred only in CKD vessels; it was absent in normal vessels that were resistant to calcification. This suggests that VSMCs in CKD vessels were “preadapted” to release vesicles as a result of their previous exposure to dysregulated mineral metabolism *in vivo* and were phenotypically modified when compared with normal contractile VSMCs. The factors that induce VSMCs to become “vesicle releasing” cells are unknown but may be linked to VSMC osteogenic differentiation. Vesicle release occurs spontaneously in explanted VSMCs and correlates with expression of Runx2¹⁷; consistent with this, we found high baseline expression of Runx2 in dialysis vessels and showed that Ca increased Runx2 expression.¹⁸

The observation that after long-term exposure to high Ca + P *in vitro* VSMCs in normal vessels exhibited intracellular Ca overload and mitochondrial calcification further supports the idea that vesicle release is protective. Whether the mitochondrial Ca overload observed in normal vessels, which is likely to be a prelude to necrotic cell death, also occurs *in vivo* remains unclear^{19,20}; the inability of VSMCs in normal vessel rings to adapt *in vitro* may represent a tissue culture phenomenon, as a result of their acute and prolonged exposure to high Ca and P in suboptimal, serum-free culture conditions.

Although vesicle release may initially be adaptive, we also found that dialysis vessels showed an increased susceptibility to undergo apoptosis in response to elevated Ca. Apoptosis promotes calcification by a number of mechanisms,²¹ and inhibition of apoptosis blocked calcification. Thus, Ca-induced apoptosis may be an important mechanism inducing calcification in CKD. The factors causing increased VSMC susceptibility to apoptosis in dialysis vessels are unknown. They may include increased VSMC damage as a result of oxidative stress and other dialysis-specific stresses as well as depletion of energy stores or other cellular factors as a result of prolonged periods of vesicle release^{10,22}; however, the onset of apoptosis also correlated with the first evidence for the deposition of crystalline apatite in dialysis vessels. It was recently shown that phagocytosis of Ca/P nanocrystals potently induces VSMC apoptosis.²³ Thus, in the earliest stages of mineral deposition, nanocrystals may induce a wave of apoptosis, leading to a vicious cycle of cell death and calcification, and may also account, in part, for why calcification itself can promote further calcification. Our findings are also consistent with a recent study in uremic mice induced to calcify by P feeding. At early stages of calcification, VSMCs upregulated Runx2 and osteopontin, and this phenotypic adaptation was followed by VSMC attrition in highly calcified areas.²⁴

Upregulation of ALK Occurs in Response to High P and Is Uncoupled from Runx2 in Response to Ca

Increased ALK activity in predialysis and dialysis vessels occurred in response to high P. Previous studies have defined a role for ALK in inactivating pyrophosphate, an inhibitor of calcification that binds to nascent hydroxyapatite crystals and prevents further incorporation of inorganic P^{25–27}; however, inhibition of P induced ALK activity in dialysis vessel rings using levamisole failed to inhibit Ca loading, whereas incubation in high Ca + P medium significantly lowered ALK activity in all vessel types but nevertheless induced calcification. This suggests that although pyrophosphate inactivation due to ALK degradation may be an initial priming event, other factors are also required to induce calcification (Figure 6). Importantly, the role of ALK in soft tissues is poorly understood, and its cellular substrates have not been identified. ALK is upregulated in response to injury in multiple tissues¹³ and has roles in phosphorylation/dephosphorylation and cellular transport—factors that are likely to be dysregulated by exposure to elevated P.^{20,28,29} Indeed, in this study and others, ALK upregula-

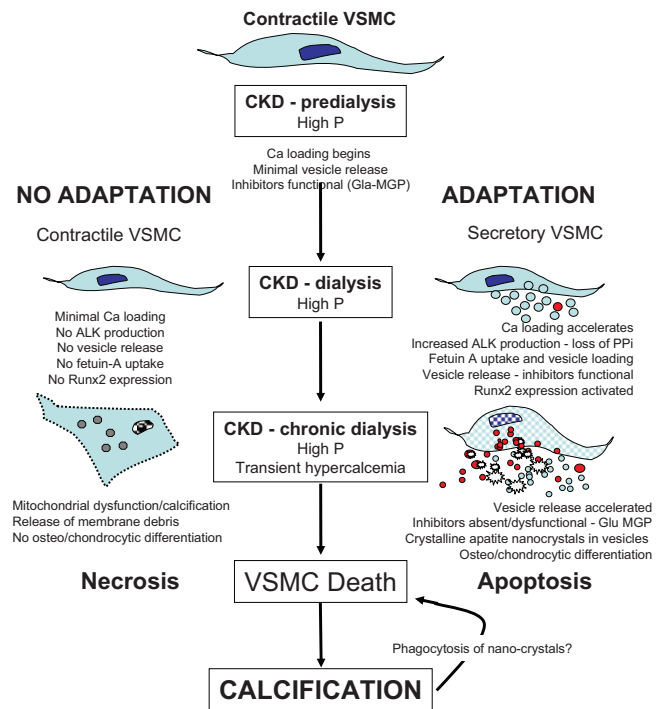


Figure 6. Model shows VSMC phenotypic adaptation in response to mineral dysregulation in CKD. VSMCs that fail to adapt to a synthetic phenotype and release vesicles to protect against Ca overload will eventually undergo necrosis. In contrast, VSMCs that release vesicles do not succumb to intracellular Ca overload but deposit Ca in the ECM, which eventually calcifies. This process eventually results in apoptosis of VSMCs.

tion was uncoupled from Runx2 expression in response to Ca and P,^{13,30} suggesting that it may represent a VSMC adaptation in response to high P, and this idea requires further exploration.

Clinical Implications

These findings may have important clinical implications because they suggest that elevated Ca in the context of high P is a major inducer of VSMC apoptosis, suggesting that transient hypercalcemic episodes, particularly in dialysis patients, may promote vascular calcification. Large observational studies have correlated serum Ca levels with increased mortality in hemodialysis patients.^{6,31} The greatest mortality risk occurs when high Ca and P levels coexist, with the lowest mortality risk seen when Ca levels are in a lower range than previously recommended.³² Although free (ionized) serum Ca levels are tightly regulated, episodic increases, as seen during hemodialysis or with the use of vitamin D analogs or Ca-based P binders, may potentially influence vascular calcification.^{2,33–35} Calcium channel blockers inhibit Ca influx into VSMCs and have been shown in both preclinical studies^{36,37} and clinical trials³⁸ to limit calcification progression, and it may be appropriate to revisit these drugs in the context of CKD.

CONCISE METHODS

Sample Collection

Medium-sized muscular arteries routinely removed and discarded in the course of planned intra-abdominal surgery were collected. Omental arteries (removed during omentectomy at the time of insertion of a peritoneal dialysis catheter) and the inferior epigastric artery (removed at renal transplantation) were compared with mesenteric arteries (removed at major intra-abdominal surgery) from disease-free age-matched control subjects. We studied 24 inferior epigastric arteries (18 from dialysis patients and six from predialysis patients with stage 5 CKD [GFR <15 ml/min per 1.73 m²]), 14 omental arteries (six dialysis patients, four predialysis patient, and four normal control subjects), and two mesenteric arteries (normal control subjects). Clinical and biochemical parameters, baseline Ca load in the vessel wall, and histology have been previously described⁴ and are summarized in Supplemental Table 1. The study was approved by a local research ethics committee.

Media and In Vitro Treatments

Under sterile conditions, vessels were gently stripped of excess adventitia and cut into 1-mm rings. Approximately 12 to 20 rings were typically obtained from a single vessel. The vessel rings were placed in serum-free tissue culture medium and incubated at 37°C in a 5% CO₂ atmosphere with medium changes every 3 d. Using CaCl₂ and NaH₂PO₄, graded concentrations of ionic Ca and P were added to standard culture medium (M199) to give four *in vitro* conditions: Control medium (1.0 mM P + 1.8 mM Ca), high P medium (2.0 mM P + 1.8 mM Ca and 3.0 mM + 1.8 mM Ca), and a high Ca + P medium (2.0 mM P + 2.7 mM Ca). Vessel rings were incubated for 14 d in the these culture media for all experiments except for the time-course studies, which were performed at 7, 14, and 21 d. Baseline refers to vessel measurements made at day 0 (*i.e.*, before *in vitro* culture) and are shown in Supplemental Table 1 and previously described.⁴

Calcification and ALK Assays

Vessels were cut into 2-mm rings immediately after harvest, and the rings were used in assays as described next. The Ca content in the vessel was measured by cresolphthalein complexone chemistry (Sigma): Vessel rings were decalcified in 0.1 M HCl, and the supernatant was removed for colorimetric assay at 540 nm. For the ALK assay, the vessel was lysed with 10% SDS on ice, and the supernatant was used to measure ALK activity colorimetrically at 405 nm using a p-nitrophenyl phosphate substrate (Sigma). The protein content in the vessel rings was measured colorimetrically at 710 nm (Bio-Rad, Hercules, CA). The total Ca and ALK levels per vessel ring were normalized to the protein content in that ring to allow for comparisons. A total of 100 μM of ZVAD.fmk or 10 μM levanisole was added to vessel rings cultured in the aforementioned calcifying conditions, using rings from the same vessel as internal controls.

Vessel Histology

Viability of VSMCs in the vessel rings was confirmed by trypan blue (0.5%) staining as standard. Hematoxylin/eosin (Sigma HHS-32 and

E8017) staining was performed for vessel integrity and cell counting: The number of VSMC nuclei and the percentage of apoptotic cells were counted in a 0.25-m² area and expressed as cells per unit area. Each sample was analyzed twice, and the mean of the readings was used. Vessel rings were stained with von Kossa for calcification (counterstained with 1% Neutral Red Aqueous Solution), and immunohistochemistry was performed for α-smooth muscle cell actin (DAKO M0851, 1:500 dilution) for smooth muscle cells. Apoptosis was examined by immunohistochemistry using TUNEL staining visualized by a rhodamine-labeled anti-digoxigenin antibody. Immunohistochemistry for known vesicle components and osteogenic factors was performed using annexin VI (BD Bioscience 610300, 1:500 dilution) and fetuin-A (AS237 antibodies; a gift from Prof. Willi Jahnen-Dechent, Interdisciplinary Centre for Clinical Research on Biomaterials, Aachen, Germany; 1:200 dilution). The carboxylated and undercarboxylated (Glu and Gla) forms of MGP (from Dr. Leon Schuriger, VitaK, Department of Biochemistry, University of Maastricht; 1:500 dilution) and Runx2 (Santa Cruz SC10758, 1:100 dilution) were examined. Using ImageJ software on the Olympus BX51 microscope, a region of interest was marked around the Von Kossa-positive areas, and this was expressed as a percentage of the total area of the tunica media of the vessel. The percentage of TUNEL-, cbfa-1-, fetuin-A-, and annexin VI -positive areas was expressed in a similar manner, analyzing each sample in duplicate.

Transmission EM

Transmission EM was performed to examine cell morphology, localization of calcification, vesicle release, and mineral deposition. Blood vessels were fixed by immersion in 4% glutaraldehyde containing 2 mmol/L CaCl₂ in 0.1 M PIPES buffer at pH 7.4. A total of 100 μl of 33% H₂O₂ was added to each 10-ml aliquot immediately before use. They were fixed for 4 h at 4°C, washed twice in buffer (0.1 M PIPES), and stored at 4°C. After buffer washes, they were postfixed in 1% osmium ferricyanide for 1 h, rinsed three times in water, and bulk-stained in 2% uranyl acetate for 1 h. They were rinsed in water and dehydrated in an ascending series of ethanol solutions to 100% ethanol, rinsed twice in acetonitrile, and embedded in Quetol epoxy resin (9.0 g of Quetol 651, 11.6 g of nonenylsuccinic anhydride, 5.0 g of methyl nadic anhydride, and 0.5 g of benzyl dimethylamine). Fifty-nanometer sections were cut on a Leica Ultracut UCT, stained with saturated uranyl acetate in 50% ethanol and lead citrate, and viewed in a FEI Philips CM100 operated at 80 kv.

Statistical Analysis

Data are presented as mean ± SD or median (range). The paired or unpaired *t* test was used as appropriate. ANOVA was used for multiple comparisons. Significance was defined as *P* < 0.05. Statistical analyses were performed using SPSS 12.0.1 (SPSS, Chicago, IL).

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DISCLOSURES

None.

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