R-568 reduces ectopic calcification in a rat model of chronic kidney disease-mineral bone disorder (CKD-MBD)

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Abstract

Background. Chronic kidney disease-mineral bone disorder (CKD-MBD), a newly defined disorder in patients with CKD, describes the interacting triad of (1) biochemical abnormalities of calcium, phosphorus and parathyroid hormone (PTH), (2) extraskeletal calcification and (3) abnormal bone.

Methods. We studied the effects of the calcimimetic R-568, R-568 with calcium (R-568 + Ca) or calcium (Ca) alone compared with control CKD rats on this triad in the Cy/+ male rat, a model of progressive CKD that spontaneously develops CKD-MBD on a normal phosphorus diet. Animals were treated for either 14 or 18 weeks beginning at 20 weeks of age (34-week and 38-week animals, respectively).

Results. The results demonstrated similar efficacy of R-568, R-568 + Ca and Ca in lowering PTH levels. R-568 alone lowered plasma calcium compared to control over time, but increased phosphorus compared to control early in the course of the disease, but not at 38 weeks. Animals treated with Ca alone or R-568 + Ca had lower phosphorus levels; the Ca alone group had elevated Ca levels. Bone volume improved in the calcium-treated groups. In contrast, arterial and cardiac calcification worsened by most assessments in the R-568 + Ca and Ca alone treated animals compared with R-568 alone whereas R-568 alone treatment showed beneficial effects on most sites of extraskeletal calcification.

Conclusion. Thus, R-568, with or without Ca, improved the biochemical abnormalities of hyperparathyroidism but with higher and lower calcium levels, respectively, compared with controls. However, R-568 + Ca had more dramatic improvement in bone volume, but more extraskeletal calcification than R-568 alone. This complexity demonstrates that treatment of one component of CKD-MBD may lead to undesirable effects on other components.

Keywords: kidney; mineral bone disorder; parathyroid hormone; renal osteodystrophy; vascular calcification

Introduction

Chronic kidney disease-mineral bone disorder (CKD-MBD) is a newly named clinical disorder defined as a systemic syndrome in patients with chronic kidney disease (CKD) stages 3–5D that manifests as biochemical abnormalities in parathyroid hormone, calcium, phosphorus and vitamin D; bone abnormalities and extraskeletal calcification [1]. The biochemical abnormalities are common in patients with CKD 3–5D, with nearly 60% and 80% of patients with CKD stages 3 and 4, respectively, found to have elevated PTH in a recent North American cohort [2]. The bone abnormalities of CKD-MBD include renal osteodystrophy, the histologic abnormalities of turnover, mineralization and volume, and increased propensity to fracture [1]. These abnormalities are very common, since predialysis patients typically exhibit abnormal bone histology [3]. The extraskeletal calcification affects multiple organs, including the myocardium and arteries. Arterial calcification is similarly common in patients with CKD, appearing in 30–65% of patients with stage 3–5 CKD and 50–80% of patients with stage 5D CKD, and is associated with increased cardiovascular morbidity and mortality [4,5]. In the coronary arteries, this calcification is typically intimal, within atherosclerotic plaques or as circumferential intimal lesions, whereas in the aorta, calcification occurs in both the intimal and medial layers of the vessel wall (atherosclerosis and Mönkeberg’s medial calcific sclerosis). Thus, CKD-MBD is responsible for significant morbidity and mortality in CKD.

This interrelationship of vascular calcification with abnormal serum biochemistries and abnormal bone remodeling is complex and the pathogenesis is multifactorial. The natural history of CKD-MBD is further complicated, and perhaps accelerated, from therapies that may treat one aspect of CKD-MBD but worsen another. For example, calcitriol will lower PTH but raise calcium and phosphorus levels [6,7], and high dose calcium salts such as phosphate binders may increase overall calcium load leading to arterial calcification [8]. Calcimimetics, of which cinacalcet HCl is commercially available, are allosteric activators of the calcium-sensing receptor and act to lower PTH secretion.
They are effective in decreasing PTH while also lowering calcium and phosphorus levels in patients with CKD stage 5D [9], and ongoing studies are evaluating their effect on coronary artery calcification and mortality [10]. In CKD stages 3–4, cinacalcet HCl lowers PTH and calcium levels but, in contrast to results observed in CKD 5D, raises serum phosphorus [11]. The latter is likely due to the removal of the compensatory phosphaturic effects of PTH. The resultant rise in phosphorus would then be offset by improved PTH levels, and it is not known if this biochemical profile has different effects on extraskeletal calcification and bone than lower phosphorus and higher PTH levels.

The complexity of this interrelationship makes studies in humans difficult as one cannot easily control one variable without impacting another. Thus, there is a clear need for an appropriate animal model in which to understand the progressive pathophysiology of CKD-MBD, and to test interventions. We have recently characterized a novel model of CKD-MBD using the Cy/+ rat model of cystic kidney disease, which develops progressive CKD with all of the features of CKD-MBD spontaneously [12]. We utilized this model to aggressively treat secondary hyperparathyroidism with the calcimimetic R-568, with and without calcium, of CKD-MBD using the Cy/+ rat model of cystic kidney disease (ADPKD) [13]. The spontaneous genetic mutation (Cy) that leads to cystic kidney disease and progressive CKD encodes for a protein of unknown function [14]. This rat colony at Indiana University School of Medicine has been maintained as a sub-colony for more than a decade through successive breeding of heterozygous Cy/+ rats. This colony exhibits a consistent pattern of disease severity. This is an autosomal dominant condition, such that at birth, 1/4 of the animals are normal (+/+), 1/2 are heterozygotes (Cy/+), and 1/4 are affected homozygotes (Cy/Cy). Homozygous Cy/Cy rats develop massively enlarged kidneys and severe azotaemia, and normally die by 4 weeks of age. The male Cy/+ rat develops a persistent azotaemia starting at ∼10 weeks of age, which progresses to terminal uraemia by ∼40 weeks with spontaneous and slow development of all three manifestations of CKD-MBD: biochemical abnormalities, extraskeletal calcification and abnormal bone [12]. For the present study, male heterozygotes were utilized and all procedures were reviewed and approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Experimental design
Weaned rats were housed in open top, shoebox cages and had free access to tap water and standard chow until they were 20 weeks old. At that time, animals were separated into four treatment groups:

**Group 1:** Cy/+ (cystic/CKD) male rats fed control grain-based pellet diet (Purina 5002 diet: 20% protein, 0.6% phosphate, 0.8% calcium, 4.5% fat) (control).

**Group 2:** Cy/+ (cystic/CKD) rats fed the same diet as in group 1 with R-568 mixed into the pellets to deliver an estimated 50 mg/kg/day (R-568).

**Group 3:** Cy/+ (cystic/CKD) rats fed the same diet as in group 2, with the addition of 2% calcium gluconate to the drinking water (R-568 + Ca).

**Group 4:** Cy/+ (cystic/CKD) rats fed the same diet as in group 1, with the addition of 2% calcium gluconate to the drinking water (Ca).

The dose of the R-568 was determined by dose finding studies with oral gavage to assess the peak effect given limited oral bioavailability (data not shown). Within each treatment group, there were 14–16 animals assigned to the four groups with two different sacrifice endpoints: all animals had baseline blood draw at 20 weeks at the start of the diet and at the time of sacrifice. In addition, each animal had an intravital blood draw that was done by tail bleed at the time of tetracycline labelling. The animals had IP injections of tetracycline at 5 weeks and 1 week prior to sacrifice for labelling of bone actively undergoing mineralization. Thus, there were measurements for biochemistries at baseline (20 weeks), 29 weeks, 34 weeks (with half of the animals sacrificed) and 38 weeks (the other half of the animals sacrificed). At sacrifice, rats were anaesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and blood was collected by cardiac puncture. At the time of sacrifice, half of the animals from each study duration group were perfusion fixed with 4% paraformaldehyde in a phosphate buffer prior to harvesting bone and heart tissue. The remaining animals from each study duration group were saline-perfused prior to harvesting the thoracic aorta to determine its calcium and phosphate content. Thus, for each endpoint (34 and 38 weeks), blood from all of the animals was used for biochemical determinations, but only half of the animals (6–8 per group) were used for tissue calcium measurements or bone histomorphometry.

**Methods**

**Animal model**
We utilized the Han:SPRD CY/+ rat with autosomal dominant polycystic kidney disease (ADPKD) [13]. The spontaneous genetic mutation (Cy) that leads to cystic kidney disease and progressive CKD encodes for a protein of unknown function [14]. This rat colony at Indiana University School of Medicine has been maintained as a sub-colony for more than a decade through successive breeding of heterozygous Cy/+ rats. This colony exhibits a consistent pattern of disease severity. This is an autosomal dominant condition, such that at birth, 1/4 of the animals are normal (+/+), 1/2 are heterozygotes (Cy/+), and 1/4 are affected homozygotes (Cy/Cy). Homozygous Cy/Cy rats develop massively enlarged kidneys and severe azotaemia, and normally die by 4 weeks of age. The male Cy/+ rat develops a persistent azotaemia starting at ∼10 weeks of age, which progresses to terminal uraemia by ∼40 weeks with spontaneous and slow development of all three manifestations of CKD-MBD: biochemical abnormalities, extraskeletal calcification and abnormal bone [12]. For the present study, male heterozygotes were utilized and all procedures were reviewed and approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

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**Plasma biochemistries.** Blood urea nitrogen (BUN), calcium, phosphorus and creatinine were determined using colorimetric assays (Point Scientific, Canton, MA, USA or Sigma Labs, St. Louis, MO, USA). Intact PTH was determined by ELISA (Alpco, Salem, NH, USA). The kidney function (BUN) for the saline-perfused animals has been previously reported [15].

**Extraskeletal calcification.** To quantify extraskeletal calcification, proximal segments of thoracic aortas and aortic arches from the saline-perfused euthanized animals were snap frozen. The hearts were collected from the paraformaldehyde-perfused animals, weighed and the upper atrium, aortic valve area and the left ventricle dissected. Each section was then weighed and incubated in 0.6 N HCl for 48 h. The samples (n = 6–8 per group) were then homogenized, centrifuged and the supernatant analysed for calcium using the o-cresolphthalein complex 1 method (Calcium kit; Pointe Scientific). The aortic arch and thoracic aorta were similarly analysed for phosphorus content (Point Scientific), but because the heart was only collected from the paraformaldehyde-perfused animals, only calcium could be determined.

**Bone histomorphometry.** Tibiae were removed, cleaned of soft tissue and cut into proximal and distal segments. Segments were fixed in 10% neutral buffered formalin (NBF), dehydrated in ethanol and processed undecalcified into methyl methacrylate. Frontal sections (4 μm thick) of the proximal segments were cut using a Leica RM2165 microtome and stained with von Kossa-tetrachrome/toluidine blue (MacNeal’s stain) [12] for measurement of static bone parameters at 250× magnification 1.0 mm distal to the chondro-osseous junction using a semiautomatic image analysis system (Osteo measure Histomorphometry System, Osteometrics, Inc., Atlanta, GA, USA) [16]. Results are presented using standardized histomorphometry measures [17]. To examine cortical bone geometry and measure dynamic bone parameters, cross-sections of the distal segments containing the tibial shaft immediately proximal to the tibiofibrular junction were taken using a wire saw (Histo-saw, DDM P216), ground and mounted unstained onto slides. Total cross-sectional area was measured from these sections using a Bioquant image analysis system. Periosteal-labelled surface (as a percent) was determined by dividing the sum of the lengths of tetracycline double labels and one half of the lengths of single labels by the periosteal perimeter length. Periosteal bone formation (BFR) was calculated by multiplying the mineral apposition rate (determined by dividing the mean double label widths by the interlabel period of 28 days) by the periosteal-labelled surface and expressed in microns/day.

**Statistical analyses**
Comparisons between the four different treatment arms were done by two-way ANOVA, with treatment and time as dependent variables (Systat, San Jose, CA, USA). Individual comparisons were done with adjustment for multiple comparisons for the subgroup analyses. For the outcomes measured once (bone histomorphometry and aorta/heart calcium content), we used one-way ANOVA with adjustment for multiple comparisons. The results are presented as mean ± SEM.
**Results**

**Effect of R-568 on biochemical parameters of CKD-MBD**

C57BL/6J rats were treated with diet only (control = CTL CKD), R-568, R-568 + Ca or calcium alone for 14 or 18 weeks (34 or 38 week endpoint animals, respectively). The observed changes in serum biochemistries are shown in Figure 1. The PTH increased in the control group over time ($P = 0.01$) with a significant treatment-time interaction ($P < 0.001$). All treatment groups suppressed PTH compared with control ($P < 0.001$), with no differences between the three treatment groups (Figure 1A). There was a significant treatment-time interaction ($P < 0.001$) for the calcium levels (Figure 1B). The animals treated with calcium alone had increased calcium compared to the other groups at all time points ($P < 0.001$). In contrast, the animals treated with R-568 alone had lower calcium levels than the other groups ($P < 0.001$). There was no difference between the control and R-568 + Ca treated animals (Figure 1B). There was also significant treatment-time interaction for phosphorus ($P < 0.001$). The phosphorus levels showed the most dramatic changes during the last 4 weeks, late in the course of CKD.

In the control animals, the phosphorus level increased from 34 to 38 weeks. The calcium alone and R-568 + calcium treated animals had continued phosphorus lowering over time. In contrast, the R-568-treated animals had a higher serum phosphorus than all groups at both 29 and 34 weeks, but similar to control at 38 weeks (Figure 1C). There was a progressive increase in BUN over time from a 20-week mean baseline BUN of 41–43 mg/ml, with no difference at 34 weeks. However, at 38 weeks, all three treatments had lower BUN compared with control animals (mean ± SEM; control = 88.7 ± 5.6 mg/dl; R-568 = 79.1 ± 5.3 mg/dl; R-568 + Ca = 68.2 ± 2.2 mg/dl; Ca = 68.6 ± 4.7 mg/dl; $P = 0.004$). At 38 weeks, both calcium-treated groups had lower mean ± SEM body weights than the control or R-568-treated animals (control = 532 ± 7 g, R-568 = 508 ± 12 g, R-568 + Ca = 486 ± 7 g, Ca = 490 ± 11 g, $P = 0.01$).

**Effect of R-568 on extraskeletal calcification**

The thoracic aorta was analysed biochemically for calcium and phosphorus, which were expressed as µg/g of tissue ($n = 6–8$ per group). At 34 weeks, there was no difference in calcium among the groups (Figure 2A), but the Ca alone group had increased phosphorus content compared with the other groups (Figure 2B). At 38 weeks, the calcium-treated animals (Ca alone) had greater aortic arch calcium content ($P = 0.04$) compared with the other groups ($P = 0.03$, Figure 2A) and both calcium-treated groups (R-568 + Ca and Ca alone) had greater thoracic aorta phosphorus content compared with the R-568-treated animals and controls ($P = 0.002$, Figure 2B). There was no difference in the aortic arch calcium content at either time point (Figure 3A) or in phosphorus content at 34 weeks (Figure 3B). In contrast, there was increased phosphorus content in the aortic arch in the calcium-treated animals at 38 weeks compared with all of the other groups ($P = 0.004$; Figure 3B). For the aortic valve (Figure 4A), there was a trend towards less...
Thoracic aorta calcification. At the time of sacrifice, the thoracic aorta was removed and its calcium and phosphorus content determined biochemically. At 34 weeks, there was no difference in the calcium content (A), but the phosphorus content (B) was greater in the calcium-treated animals than all of the other groups \((P = 0.002)\). At 38 weeks, the calcium-treated animals had greater calcium \((P = 0.04; \text{A})\) and phosphorus content \((P = 0.03; \text{B})\) compared with the R-568-treated animals. At 38 weeks, animals treated with R-568 + Ca had higher calcium content than R-568 alone. Solid black bars = CKD animals without treatment (control); open white bars = CKD given R-568; hatched bars = CKD animals given R-568 plus 2% calcium gluconate in the drinking water; grey bars = CKD animals given only 2% calcium gluconate in the drinking water.

Aortic arch calcification. The aortic arch was dissected from above the aortic valve to the ligamentum arteriosum from either 34- or 38-week animals and its calcium (A) and phosphorus (B) content analysed biochemically \((n = 6–8 \text{ per group})\). The results are expressed as \(\mu\text{mol/g of tissue}\). At 34 and 38 weeks (A), there was a trend for a reduction in calcium content in R-568-treated animals compared with calcium alone, but this did not reach statistical significance. At 34 weeks (B), there was no difference in the phosphorus content. In contrast, at 38 weeks, there was increased phosphorus content in the rats treated with calcium alone compared with the other treatment groups \((P = 0.004)\). Solid black bars = CKD animals without treatment (control); open white bars = CKD given R-568; hatched bars = CKD animals given R-568 plus 2% calcium gluconate in the drinking water; grey bars = CKD animals given only 2% calcium gluconate in the drinking water.

calcification \((P = 0.053)\) in the R-568-treated animals at 34 weeks, and by 38 weeks, both calcium-treated groups had more calcium content than the R-568-treated animals or controls \((P = 0.035)\). The left ventricle (Figure 4B) had less calcium at 34 weeks \((P = 0.008)\) and 38 weeks \((P = 0.005)\) in the R-568-treated animals than in the other treatment groups. The total heart weight was not different in the groups at either time point (data not shown). Taken together, the data demonstrate that R-568 alone had a beneficial effect on vascular and cardiac calcification whereas treatment with calcium alone, and in most assessments, R-568 + Ca, led to increased extraskeletal calcification compared with control and R-568 alone treated animals.

**Effect of R-568 on renal osteodystrophy**

The bone changes in CKD animals were indicative of mild secondary hyperparathyroidism with increased osteoblast number and surface and increased osteoid (Table 1) when compared to normal littermates as we have previously published [12]. No marrow fibrosis was observed in any of the groups. The R-568 + Ca and Ca alone treated animals had reduced osteoblast and osteoclast number and activity compared with control. A trend towards reduced cell number was also observed in the R-568-treated animals but this did not reach significance. There was also a trend towards reduced periosteal bone formation rate in all three treatment groups \((P = 0.09)\). We could not quantify cancellous bone turnover as double tetracycline labels were not found in cancellous bone in the majority of specimens. Mineralization parameters demonstrated that calcium-treated groups (R-568 + Ca or Ca alone) had reduced osteoid volume and surface, whereas all three treatment groups showed reduced periosteal mineralizing surface (MS/BS) but the calcium alone group did not reach significance. Bone volume and trabecular thickness increased and trabecular separation decreased in both calcium-treated groups suggesting improved trabecular volume with calcium. There was no
similar volume to control animals. 

had improved volume parameters whereas the R-568 had 

than the R-568-treated animals and controls (P = 0.035). The left ventricle (B) had less calcium at 34 weeks (P = 0.008) and 38 weeks (P = 0.005) in the R-568 alone treated animals than in the other treatment groups. Solid black bars = CKD animals without treatment (control); open white bars = CKD given R-568; hatched bars = CKD animals given R-568 plus 2% calcium gluconate in the drinking water; grey bars = CKD animals given only 2% calcium gluconate in the drinking water.

Table 1. Renal osteodystrophy results at 38 weeks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CKD control</th>
<th>CKD R-568</th>
<th>CKD R-568 + Ca</th>
<th>CKD Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turnover</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ob.S/BS (%)</td>
<td>3.4 ± 1.0</td>
<td>0.6 ± 0.2</td>
<td>0.3 ± 0.19*</td>
<td>0.2 ± 0.1*</td>
</tr>
<tr>
<td>N.Ob/B.Pm (μm/mm)</td>
<td>2.6 ± 0.7</td>
<td>0.5 ± 0.1</td>
<td>0.3 ± 0.1*</td>
<td>0.2 ± 0.1*</td>
</tr>
<tr>
<td>Oc.S/BS (%)</td>
<td>6.5 ± 1.0</td>
<td>5.6 ± 1.5</td>
<td>2.3 ± 0.6+</td>
<td>1.5 ± 0.3+</td>
</tr>
<tr>
<td>N.Oc/B.Pm (μm/mm)</td>
<td>1.8 ± 0.2</td>
<td>1.5 ± 0.3</td>
<td>0.7 ± 0.2+</td>
<td>0.5 ± 0.1+</td>
</tr>
<tr>
<td>Periosteal BFR (μm³/μm²/day)</td>
<td>89.8 ± 16.8</td>
<td>40.2 ± 14.4</td>
<td>53.3 ± 17.2</td>
<td>51.0 ± 8.4</td>
</tr>
<tr>
<td>Mineralization</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OV/BV (%)</td>
<td>3.2 ± 1.1</td>
<td>2.2 ± 0.5</td>
<td>0.5 ± 0.2+</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>OS/BS (%)</td>
<td>19.6 ± 3.4</td>
<td>16.1 ± 3.1</td>
<td>3.4 ± 1.0+</td>
<td>10.1 ± 2.6</td>
</tr>
<tr>
<td>O.Wi (μm)</td>
<td>3.4 ± 0.4</td>
<td>3.4 ± 0.1</td>
<td>3.5 ± 0.4</td>
<td>4.2 ± 0.2</td>
</tr>
<tr>
<td>Periosteal MS/BS (%)</td>
<td>66.6 ± 3.5</td>
<td>40.9 ± 9.4*</td>
<td>42.4 ± 7.5*</td>
<td>54.0 ± 3.3</td>
</tr>
<tr>
<td>Volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>12.6 ± 1.6</td>
<td>10.2 ± 0.9</td>
<td>31.2 ± 1.7+*+</td>
<td>26.1 ± 1.3+*+</td>
</tr>
<tr>
<td>Tb.Th (μm)</td>
<td>43.3 ± 1.9</td>
<td>41.4 ± 3.2</td>
<td>53.9 ± 2.5+*</td>
<td>51.5 ± 1.7+*</td>
</tr>
<tr>
<td>Tb.Sp (μm)</td>
<td>324 ±27.8</td>
<td>369 ± 13.7</td>
<td>120 ± 6.7+</td>
<td>148 ± 9*+</td>
</tr>
<tr>
<td>Cross-sectional area (mm²)</td>
<td>6.6 ± 0.2</td>
<td>6.4 ± 0.2</td>
<td>6.4 ± 0.1</td>
<td>6.6 ± 0.1</td>
</tr>
</tbody>
</table>

Data presented as mean ± SEM.
* P < 0.05 compared with control CKD.
+ P < 0.05 compared with R-568.
$ P < 0.05$ compared with R-568 + Ca.

Static parameters: trabecular bone volume (BV/TV), osteoblast and osteoclast surface (Ob.S/BS and Oc.S/BS), osteoblast and osteoclast number per millimeter bone perimeter (N.Ob/B.Pm and N.Oc/B.Pm), osteoid volume (OV/BV), osteoid width (O.Wi), osteoid surface (OS/BS), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular number (Tb.N). Dynamic parameters: periosteal-labelled or mineralizing surface (MS/BS) and bone formation rate (μm³/μm²/day).

Discussion

CKD-MBD is characterized by a complex interplay of abnormalities of serum biochemistries, bone and extraskeletal calcification. In the present study, we evaluated the effect of the calcimimetic R-568 on all three parameters of CKD-MBD. In CKD stages 3–4, there is a progressive increase in PTH levels in an attempt to increase phosphaturia and maintain normal serum phosphorus. Initially, this secondary hyperparathyroidism is an appropriate homeostatic response, but at some point the response is no longer beneficial and instead induces renal osteodystrophy. In patients with CKD
3 and 4, the use of cinacalcet HCl not only lower PTH but also lowered serum calcium and increased serum phosphorus [11]. In our rat model of CKD-MBD, we similarly found lower calcium in the R-568-treated animals compared to controls. The phosphorus levels in the 34-week animals treated with R-568 were higher than controls, but by 38 weeks, the phosphorus was similar to control animals whose disease had progressed. The hypocalcaemia and early hyperphosphataemia could be prevented by concomitant administration of oral calcium. In the present study, we evaluated the effect of these different biochemical profiles on bone and cardiovascular calcification.

There is very little data on the effect of calcimimetics on bone. Parathyroid hormone affects both the rapidly exchangeable ion compartment of bone and cellular remodelling, the latter mediated through osteoblasts and the OPG-RANKL system [18]. There is controversy as to whether or not a calcium-sensing receptor exists in bone and, if present, whether this is the same receptor as in the parathyroid gland [19]. Thus, the bone effects of calcimimetics may be due to both indirect effects on lowering PTH and direct effects. Wada et al. found improvement in the 5/6th nephrectomy uraemic rat model in bone histology and, importantly, in bone strength [20]. In the Wada study, the uraemic rats treated with R-568 and a high phosphorus (1.1%) diet had lower PTH, calcium and phosphorus levels compared with controls. In the present study, the bone histology demonstrated that uraemic controls had decreased bone volume and increased osteoblast and osteoclast activity and increased osteoid volume as a percentage of total volume. These changes are consistent with hyperparathyroid bone disease in advanced CKD, especially when compared to normal rats in our previous studies [12]. The administration of R-568 demonstrated a dose-dependent improvement in all of these parameters, but not to the level of normal animals. In the present study, our model represents an earlier stage of CKD and is slowly progressive compared with the sudden acute kidney injury of the 5/6th nephrectomy model. In addition, the secondary hyperparathyroidism and resultant bone histology was less severe than the Wada study and than our previous study in which a casein diet (and thus more bioavailable phosphorus and more severe hyperphosphataemia and hyperparathyroidism) was used [12]. In the present study, the effects of R-568 were only modest and did not reach significance, although there was a trend towards improved indices of turnover (which we were only able to assess by cellular activity and periosteal turnover) and mineralization; without an effect on bone volume compared with CKD control animals. When calcium was added to the R-568, or calcium alone was given, turnover, mineralization and volume all improved significantly compared with CKD control and R-568 alone treated animals for most indices. For unclear reasons, calcium alone had more osteoid than R-568 + Ca.

Dynamic studies found a trend towards improved periosteal bone formation in all three treatment groups ($P = 0.09$). We were unable to quantify turnover in cancellous bone. While nearly all samples had two tetracycline labels present, they were not present together in the cancellous bone. The reason for this is not clear but may be due to remodelling of the first label because of the relatively long interval between our two labels (4 weeks). Overall, our data found that osteoclast number and activity, osteoid volume and surface, and bone volume improved with the combination of R-568 and calcium or calcium alone compared with CKD controls and R-568 alone. This finding suggests that the hypocalcaemia induced by R-568 treatment may have had an adverse consequence on bone, or alternatively, that calcium supplementation had an added benefit. Without trabecular labelling, we were unable to determine if the suppression in the bone cell number was to the level of adynamic bone observed in humans. We did not do tests of bone strength (i.e. three-point bending, a measure of overall bone strength and susceptibility to fractures), but bone strength was improved in the study by Wada in 5/6th nephrectomy animals treated with R-568 [20]. In humans, the only data that exist on the bone effects of cinacalcet are in dialysis patients [21]. That study was limited due to the small sample size and the heterogeneity in baseline bone biopsies. However, these results demonstrated a general improvement in indices of secondary hyperparathyroid bone disease in most patients [21]. Clearly, more bone biopsy studies in humans, including pre-dialysis populations, are needed to fully understand the effects of calcimimetic compounds on bone.

Extraskeletal calcification is a third component of CKD-MBD. In dialysis patients, the presence of significant coroary artery calcification is associated with increased mortality [22] as it is in the general population [23]. In addition, there is an association of increased arterial calcification with suppressed bone formation or indices of adynamic bone disease [24]. The use of calcium-based phosphate binders, and subsequent increased calcium load and episodic hypercalcaemia, increases coronary artery and aortic calcification compared with the non-calcium-based binder, sevelamer [4,25]. However, the effect of cinacalcet on arterial calcification in humans is unknown, although studies are in progress. In uraemic rats, R-568 does not induce arterial calcification and reduces calcification observed in calcitriol and vitamin D analog-treated animals [26,27]. The mechanism of this protective effect was presumed to be less hypercalcaemia and hyperphosphataemia. In our study, R-568 treatment led to reduced aortic, aortic valve and myocardial calcification compared with R-568 + Ca in a slowly progressive rat model. These data confirm an important role of avoiding hypercalcaemia and positive calcium load. In addition, it appears that the hyperphosphataemia induced by R-568 early in the course of CKD does not have negative consequences on arterial calcification, perhaps due to the relative hypocalcaemia, and/or the protective effects of improved PTH. In parathyroidectomized uraemic rats, PTH infusion was shown to have adverse effects on both arterial calcification and bone at all levels of dietary phosphorus intake demonstrating a direct effect of PTH in this process [28].

In summary, CKD-MBD is complex but can be effectively treated with the calcimimetic R-568. The treatment with R-568 in our slowly progressive model of CKD-MBD showed beneficial effects on biochemical parameters and less extraskeletal calcification, but only a trend towards improved bone histology. The addition of calcium to R-568 similarly lowered PTH with normalized calcium and
lower phosphorus, improved bone histology but increased extraskeletal calcification. Thus, the addition of calcium to R-568 in order to avoid hypocalcemia should be done with caution in humans in order to balance biochemical and bone benefits versus worsened extraskeletal calcification. Importantly, the animals were not receiving calcitriol or vitamin D analogs, a therapy also commonly given to humans. Such concomitant administration may have altered our findings. Overall, these data suggest that all therapeutic approaches that improve biochemical parameters of CKD-MBD should similarly evaluate the consequence of the therapies on the bone and extraskeletal calcification components of CKD-MBD.

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