Chlorthalidone with potassium citrate decreases calcium oxalate stones and increases bone quality in genetic hypercalciuric stone-forming rats

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To study human idiopathic hypercalciuria we developed an animal model, genetic hypercalciuric stone-forming rats, whose pathophysiology parallels that of human idiopathic hypercalciuria. Fed the oxalate precursor, hydroxyproline, every rat in this model develops calcium oxalate stones. Using this rat model, we tested whether chlorthalidone and potassium citrate combined would reduce calcium oxalate stone formation and improve bone quality more than either agent alone. These rats (113 generation) were fed a normal calcium and phosphorus diet with hydroxyproline and divided into four groups: diets plus potassium chloride as control, potassium citrate, chlorthalidone plus potassium chloride, or potassium citrate plus chlorthalidone. Urine was collected at six, 12, and 18 weeks and kidney stone formation and bone parameters were determined. Compared to potassium chloride, potassium citrate reduced urinary calcium, chlorthalidone reduced it further and potassium citrate plus chlorthalidone even further. Potassium citrate plus chlorthalidone decreased urine oxalate compared to all other groups. There were no significant differences in calcium oxalate supersaturation in any group. Neither potassium citrate nor chlorthalidone altered stone formation. However, potassium citrate plus chlorthalidone significantly reduced stone formation. Vertebral trabecular bone increased with chlorthalidone and potassium citrate plus chlorthalidone. Cortical bone area increased with chlorthalidone but not potassium citrate or potassium citrate plus chlorthalidone. Mechanical properties of trabecular bone improved with chlorthalidone, but not with potassium citrate plus chlorthalidone. Thus in genetic hypercalciuric stone-forming rats fed a diet resulting in calcium oxalate stone formation, potassium citrate plus chlorthalidone prevented stone formation better than either agent alone. Chlorthalidone alone improved bone quality, but adding potassium citrate provided no additional benefit.

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Translational Statement

Calcium oxalate stones are the most common type of kidney stone formed by humans, most of whom have idiopathic hypercalciuria and reduced bone density. To model human idiopathic hypercalciuria, we generated the genetic hypercalciuric stone-forming rats, which form solely calcium oxalate stones when fed hydroxyproline. In humans, both potassium citrate and thiazide diuretics individually decrease stone formation; however, there are few data comparing these medications in combination. In genetic hypercalciuric stone-forming rats, the combination of potassium citrate and a thiazide prevented calcium oxalate stone formation better than either agent alone. Chlorthalidone alone improved bone quality, and adding potassium citrate provides no additional benefit for bone.

Calcium oxalate (CaOx) stones are most common in human stone formers,[1] the majority of whom have hypercalciuria (IH).[2] The increase in urine Ca leads to increased supersaturation with respect to Ca-containing solid phases, principally CaOx and Ca hydrogen phosphate (CaP), which increases the probability for nucleation and growth of crystals into clinically significant stones.

To model human IH, we generated a strain of genetic hypercalciuric stone-forming (GHS) rats. Selectively inbred for over 113 generations, GHS rats, compared with their parent Sprague-Dawley rats, are hypercalciuric.[3] When fed a normal Ca diet, all GHS rats form CaP kidney stones.[4] Like patients with IH, these rats have increased intestinal Ca absorption,[4,5] decreased renal Ca reabsorption,[6] and increased bone resorption,[7] leading to increased urine Ca excretion and CaP stone formation,[4] as well as a decrease in bone mineral density (BMD).[8,9] The addition of the Ox precursor, hydroxyproline, to the diet of GHS rats results in universal formation of CaOx stones.[10] Serum 1,25-dihydroxyvitamin D_3 levels are normal.[11] We have shown that hypercalciuria in the GHS rats is polygenic,[12] as it is in humans.[13] Thus the pathophysiology of the hypercalciuria in the GHS rats closely mirrors that of humans with IH.

Two pharmacologic therapies used to decrease recurrent stone formation in humans are potassium citrate (KCit) and thiazide diuretics, alone or in combination.[2,14] In humans, both KCit and thiazides individually have been shown to decrease stone formation[15] and improve bone quality; however, there are few data directly comparing the efficacy of these 2 medications in combination. We have previously shown that giving GHS rats thiazides (specifically chlorthalidone [CTD]) decreases urine Ca, reduces urine CaP supersaturation, and decreases CaP stone formation.[16] We also found that CTD improves BMD and bone quality in GHS rats.[17] In another study, we observed that giving GHS rats KCit also decreases urine Ca, but increases CaP supersaturation and does not decrease stone formation.[18] Most recently, we found that CTD alone was superior to the combination of CTD plus KCit for reducing CaP stone formation and improving BMD and bone quality in the GHS rats.[19] As CaOx stones are the type most commonly found in humans, in the current study we tested the hypothesis that the combination of CTD and KCit would reduce CaOx stone formation and improve bone quality in GHS rats better than either agent alone would.

METHODS

Complete methods information is in the Supplementary Methods.

Study protocol

Three-month-old male 113th generation GHS rats were randomly divided into 4 groups ($n = 10$ in each) and housed individually in metabolic cages. All rats were fed a fixed amount of a normal Ca (1.2% Ca) and P (0.65%) diet containing 5% hydroxyproline, supplemented with one of the following: KCl (4 mmol/d) as control, KCit (4 mmol/d), CTD (4–5 mg/kg/d) + KCl, or KCit+CTD. At weeks 6, 12, and 18, 24-hour urine was collected for analyses as described previously.[19] Each rat received an i.p. injection of 1% calcein green at 10 and 2 days prior to being killed for dynamic histomorphometry. At 18 weeks, rats were killed, blood collected, and organs removed. The University of Rochester Committee for Animal Resources approved all procedures.

Urine and serum chemistries

Urine Ca, P, ammonium (NH_4), K, and Na were measured on a Beckman AU autoanalyzer (Beckman Coulter, Brea, CA) and urine pH using a glass electrode. Urine Cit and sulfate were measured by ion chromatography and Ox was measured enzymatically. All urine solutes were measured at 6, 12, and 18 weeks, and a mean value for each time period as well as an overall mean was calculated. Serum Ca, P, Na, K, Cl, bicarbonate, and creatinine were measured with a Roche 501 clinical chemistry analyzer (Basel, Switzerland), which utilizes ion selective electrodes for determinations of Na, K, and Cl and chemical assays for Ca, P, bicarbonate, and creatinine. All methods have been used previously.[17–23]

Urine supersaturation

Urine supersaturation with respect to CaOx and CaP solid phases were calculated using the computer program EQUIL2,[24] as done previously.[4,10,18,19,23]

Kidney stone formation

Kidneys, ureters, and bladders were imaged to determine extent of kidney stone formation. Three observers blinded to treatment scored all radiographs on a scale ranging from 0 (no stones) to 4 (extensive stones), and the mean score was reported for each rat.

Bone measurements

Dual energy X-ray absorptiometry. Dual energy X-ray absorptiometry was used to determine tissue density and mineral content. The areal BMD, bone mineral content, and bone area were measured.

Micro-computed tomography. Micro-computed tomography was used to measure volumetric BMD and microarchitecture of the mid-diaphysis of right femurs and L6 vertebrae.

Tissue-level remodeling. Tissue-level remodeling was assessed via histomorphometry on both mineralized (undecalcified) bone and unmineralized (decalcified) bone. Stained sections were viewed microscopically and results quantified.

Histomorphometry

Undecalcified histomorphometry differentiates between mineralized and demineralized tissue. Sections were used for static and dynamic histomorphometric analysis. Cross-sections of the left distal tibiae were used for back-scattered electron microscopy. Sections of undecalcified right tibiae were stained and quantified. Trabecular bone was analyzed in the proximal tibia metaphysis. Sections of calcein-labeled undecalcified right tibiae were used for dynamic histomorphometry and quantified. Sections of decalcified left tibiae were stained for tartrate-resistant acid phosphatase for assessment of osteoclasts and bone resorption.

Biomechanical properties

Biomechanics of femurs were assessed to define a load-displacement curve and ultimate load, stiffness, ultimate displacement, and energy to break. Data were normalized for specimen geometry. Three-point bending was performed on the right femurs. Vertebral compression was measured on L6 vertebrae. Vertebral compression does not result in complete fracture; the failure point was determined by an 8% to 10% reduction in load. The proximal end of the femurs was subjected to femoral neck fracture.

Degree of mineralization

Back-scattered electron microscopy on both right tibiae and left distal tibiae cross-section samples was done with a scanning electron microscope, and images were taken using a solid-state back-scattered electron microscopy detector.

Statistical analysis

Urine analytes, serum values, and stone formation, expressed as mean ± SE, were compared among the 4 treatment groups by a one-way analysis of variance with subsequent Bonferroni correction for pairwise comparison among the treatment groups (Statistica;

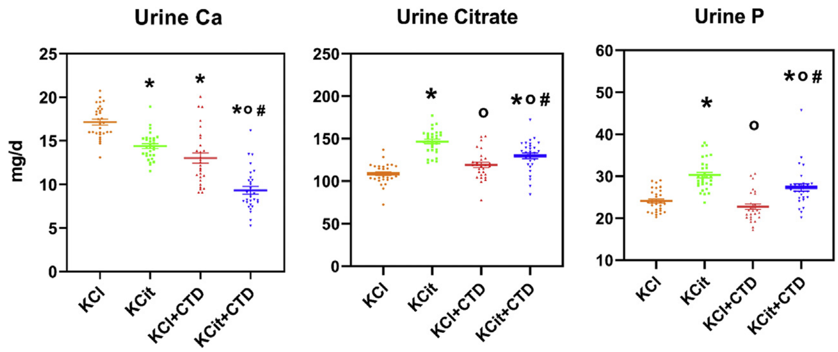

Figure 1 | Urine Ca, citrate (Cit), and P. Rat diets were all supplemented with hydroxyproline and with KCl (4 mmol/d) as a control, KCit (4 mmol/d), chlorthalidone (CTD; 4–5 mg/kg/d) + KCl, or KCit+CTD. Twenty-four–hour urine collections were done at 6, 12, and 18 weeks for analysis of solute levels as described in the methods and an overall mean of all 3 collections was calculated. Results are mean ± SE for 10 rat per /group. *$P < 0.05$ versus KCl; °$P < 0.05$ versus KCit alone; #$P < 0.05$ versus CTD alone.

StatSoft, Tulsa, OK). The interaction effects of KCit and CTD at 18 weeks were tested by comparing to KCl using linear regression models on urine analytes, serum values, and stone formation (SAS 9.4; SAS Institute, Cary, NC), assuming normal distribution. Bone parameters were compared by Student's *t* test using the SPSS Statistics 20 program (IBM Corp, Armonk, MY) and expressed as mean ± SD. $P < 0.05$ was considered significant.

RESULTS

Urine and serum

Mean overall urine Ca for the entire 18-week study was decreased in rats fed KCit and in those fed CTD while the combination of KCit+CTD decreased it further (Figure 1, Supplementary Figure S1, for each collection period). Mean overall urine Cit and urine P were both increased by KCit but not by CTD. KCit+CTD increased both urine Cit and urine P compared with KCl and with CTD; however, the increases were less than that observed with KCit alone. There were no drug interactions between KCit and CTD for any of these parameters.

Mean overall urine Ox was not altered by KCit alone or CTD alone compared with KCl while KCit+CTD decreased urine Ox compared with each of the other groups (Figure 2, Supplementary Figure S2, for each collection period). Mean urine NH_4 was decreased by KCit, but increased by CTD. KCit+CTD decreased urine NH_4 comparably to KCit alone. There was a negative drug interaction for the effects of KCit and CTD on urine NH_4 ($P < 0.0001$). Urine pH was increased by KCit and decreased by CTD. KCit+CTD increased urine pH comparably to KCit alone. There was a positive drug interaction for the effects of KCit and CTD on urine pH ($P < 0.0001$).

Mean overall urine volume for the entire 18-week study was decreased in rats fed CTD while the combination of KCit+CTD decreased it further (Figure 3, Supplementary Figure S3, for each collection period). There were no drug interactions between KCit and CTD on urine volume. Mean overall urine Na was not altered by KCit and KCl+CTD, but fell with KCit+CTD. There were no drug interactions between KCit and CTD on urine Na. Mean urine K was not altered by KCit but increased with CTD and fell with KCit+CTD compared with CTD alone. There was a positive drug interaction between KCit and CTD for urine K ($P = 0.012$).

Mean overall urine supersaturation with respect to CaP was increased by KCit, while CTD, compared with KCl, had

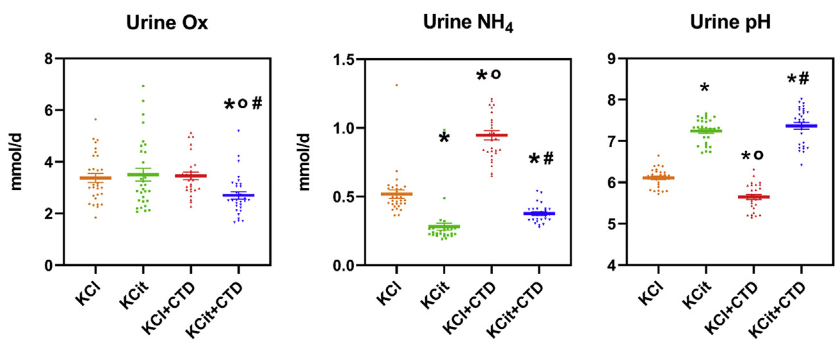

Figure 2 | Urine oxalate (Ox), ammonium (NH_4), and pH. Rat diets were all supplemented with hydroxyproline and with KCl (4 mmol/d) as a control, K citrate (Cit; 4 mmol/d), chlorthalidone (CTD; 4–5 mg/kg/d) + KCl, or KCit+CTD. Twenty-four–hour urine collections were done at 6, 12, and 18 weeks for analysis of solute levels as described in the methods and an overall mean of all 3 collections was calculated. Results are mean ± SE for 10 rats per group. *$P < 0.05$ versus KCl; °$P < 0.05$ versus KCit alone; #$P < 0.05$ versus CTD alone.

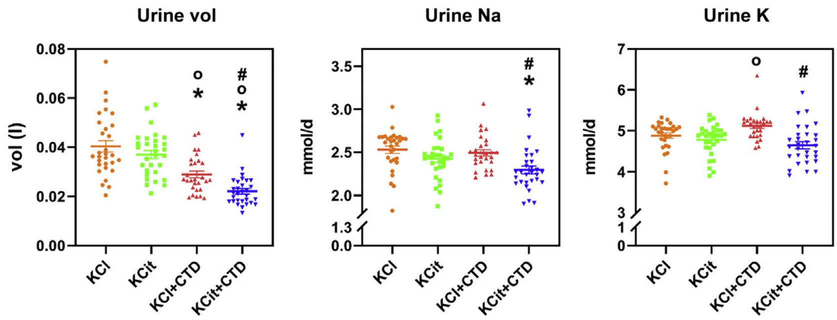

Figure 3 | Urine volume (vol), Na, and K. Rat diets were all supplemented with hydroxyproline and with KCl (4 mmol/d) as a control, K citrate (Cit; 4 mmol/d), chlorthalidone (CTD; 4–5 mg/kg/d) + KCl, or KCit+CTD. Twenty-four–hour urine collections were done at 6, 12, and 18 weeks for analysis of solute levels as described in the methods and an overall mean of all 3 collections was calculated. Results are mean ± SE for 10 rats per group. *$P < 0.05$ versus KCl; °$P < 0.05$ versus KCit alone; #$P < 0.05$ versus CTD alone.

no effect on supersaturation (Figure 4, Supplementary Figure S4, for each collection period). KCit+CTD, compared with KCl and with CTD, increased CaP supersaturation. There was a positive drug interaction for the effects of KCit and CTD on CaP supersaturation ($P = 0.0003$). There was no effect of KCit, CTD, or the combination of KCit+CTD on overall CaOx supersaturation and no drug interaction for the effects of KCit and CTD on CaOx supersaturation.

At 18 weeks on each diet, serum values for Cl, bicarbonate, P, and creatinine were not different between groups (Table 1). Serum Na was decreased by KCit+CTD compared to KCl. Serum K was decreased by CTD and by KCit+CTD compared with KCl. Serum Ca was increased by CTD and by KCit+CTD compared with both KCl and KCit alone.

Stone formation

Representative radiographs of kidneys at 18 weeks on the indicated diets are shown in Figure 5a and quantitation of the radiographs from all the rats in Figure 5b. Significant CaOx calcification was found in all rats fed the KCl control diet. KCit alone and CTD alone had no effect on CaOx stone formation. The combination of KCit+CTD led to less stone formation than did KCl or KCit. There was no drug interaction for the effects of KCit and CTD.

Bone

Micro-computed tomography analysis demonstrated that trabecular (Tb.) bone volume (bone volume/total volume) in the L6 vertebrae was increased with both CTD and KCit+CTD compared with KCl and with CTD compared with KCit (Figure 6a). There was no difference in Tb. thickness in L6 in response to any of the treatments. Tb. number in L6 was increased by CTD compared with KCl and with KCit, and KCit+CTD, compared with KCl, increased Tb. number. Tb. spacing in L6 was decreased by CTD compared with KCl and with KCit while KCit+CTD, compared with KCl, decreased Tb. spacing.

There was no change in volumetric BMD in cortical bone from the femur of rats on any of the treatments (Figure 6b). Bone area and cross-sectional thickness in femurs from rats fed KCit were not different from KCl and were increased with CTD alone compared with both KCl and KCit. KCit+CTD, compared with KCl and with KCit, did not alter bone area or cross-sectional thickness; however, both were decreased compared with use of CTD alone. There was no change in percentage of bone volume to total volume on any of the treatments.

Vertebral compression tests indicate that CTD supported a greater ultimate load, a greater failure load, and a greater energy to fail compared with the KCl control, while KCit and KCit+CTD had no effect on any of these parameters (Table 2). For material properties, there was increased ultimate stress, failure stress, and toughness with CTD compared with KCl and with KCit+CTD. When rats received KCit+CTD, compared with KCl, there was no difference. The results from 3-point bending tests indicate that cortical bone from KCit-fed rats supported a larger failure displacement and larger failure strain compared with bone from KCl-fed

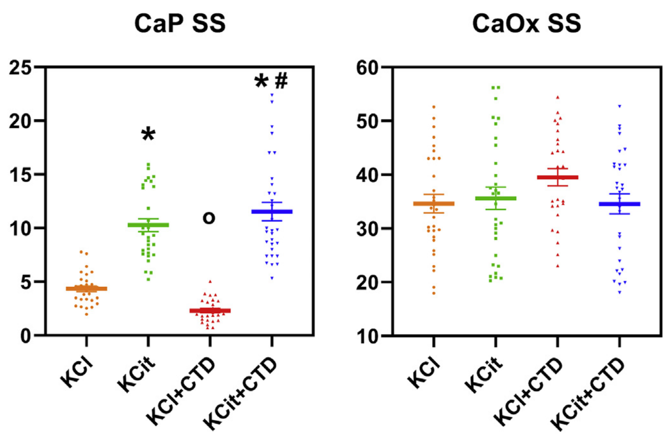

Figure 4 | Urine supersaturation (SS) of CaP and Ca oxalate (Ox). Rat diets were all supplemented with hydroxyproline and with KCl (4 mmol/d) as a control, K citrate (Cit; 4 mmol/d), chlorthalidone (CTD; 4–5 mg/kg/d) + KCl, or KCit+CTD. Twenty-four–hour urine collections were done at 6, 12, and 18 weeks for analysis of solute levels as described in the methods. These values were used to calculate relative supersaturation and an overall mean of all 3 collections was calculated. Values for relative supersaturation are unitless. Results are mean ± SE for 10 rats per group. *$P < 0.05$ versus KCl; °$P < 0.05$ versus KCit alone; #$P < 0.05$ versus CTD alone.

Table 1 | Serum measurements after 18 weeks in GHS rats fed KCl, KCit, KCl+CTD, or KCit+CTD with hydroxyproline

Solute	KCl	KCit	KCl+CTD	KCit+CTD
Na (mmol/l)	144.8 ± 0.7	142.7 ± 0.7	142.7 ± 0.5	141.5 ± 0.9[a]
K (mmol/l)	4.67 ± 0.13	4.44 ± 0.18	3.88 ± 0.17[a]	3.73 ± 0.14[a,b]
Cl (mmol/l)	100.4 ± 0.5	89.4 ± 8.9	95.3 ± 0.6	93.9 ± 0.6
Bicarbonate (mmol/l)	24.6 ± 0.5	24.8 ± 0.7	25.0 ± 0.4	26.4 ± 0.2
Ca (mg/dl)	10.2 ± 0.08	10.2 ± 0.07	10.7 ± .06[a,b]	10.6 ± 0.3[a,b]
P (mg/dl)	5.56 ± 0.20	6.08 ± 0.40	5.97 ± 0.21	5.74 ± 0.22
Creatinine (mg/dl)	0.30 ± 0.01	0.30 ± 0.01	0.32 ± 0.01	0.32 ± 0.02

Cit, citrate; CTD, chlorthalidone; GHS, genetic hypercalciuric stone-forming.
Results are mean ± SE for 9 to 10 rats per group. There were no significant differences comparing KCl+CTD with KCit+ CTD.
[a]$P < 0.05$ vs. KCl alone.
[b]$P < 0.05$ vs. KCit alone.

rats (Table 2), while stiffness and failure strain were increased in CTD-fed rats. KCit+CTD, compared with KCl, did not alter any cortical mechanical properties.

For dynamic undecalcified histomorphometry, the percentage of bone volume to total volume was increased by KCit, CTD, and KCit+CTD when compared with the KCl control (Table 3). Percentage of osteoid volume and percentage of osteoid surface were decreased by KCit, CTD, and KCit+CTD when compared with KCl. There were no changes in dynamic histomorphometry with any of the diets. The number of osteoclasts per total volume was significantly increased by KCit+CTD.

DISCUSSION

CaOx stones are most prevalent in human stone formers,[1] the majority of whom have IH.[1,2] The increased urinary Ca excretion leads to an increase in supersaturation with respect to the Ca containing solid phases, which increases the probability for nucleation and growth of crystals into clinically significant kidney stones. In addition to nonpharmacological therapy, CaOx stones are generally treated with KCit or thiazide diuretics such as CTD or both.[2,14] Each of these pharmacologic therapies has been shown to be effective in reducing stone recurrence; however, there are few data with respect to efficacy of combining them to further reduce stone formation.[15] Patients with IH often have reduced bone density and increased rate of fractures.[25] KCit and thiazide diuretics have also been shown individually to increase bone density,[26] and 1 study of 18 men and 10 women found that the combination of thiazides and KCit reduced stone formation and increased bone density when combined with a low-calcium diet.[27] The study presented here found that in GHS rats fed a diet that results solely in CaOx stone formation, the combination of KCit+CTD prevented stone formation better than either agent alone and that CTD alone improves bone quality, but the addition of KCit provides no additional benefit for bone.

The administration of KCit led to an increase in urinary Cit, a reduction in urinary Ca, and an increase in urinary P, all similar to what we have reported previously.[18,19] After intestinal absorption, Cit leads to systemic alkalization leading to less tubular reabsorption of Cit and an increase in its excretion.[28] Increasing dietary Cit leads to a decrease in urine Ca through several known mechanisms. The systemic alkalization induced by dietary Cit leads to an increase in tubular fluid pH and an increase in renal tubular Ca reabsorption. It also leads to increased bone formation and decreased bone resorption.[29] The increased urinary P with KCit appears secondary to intestinal Cit binding Ca, which decreases intestinal CaP complexation allowing more P to be absorbed and excreted. The administration of CTD also decreased urinary Ca. Thiazide diuretics increase tubular Ca reabsorption in animals and in man. We have shown in rats[16] and in humans[30] that the decrease in urinary Ca persists due to a concomitant reduction in intestinal Ca absorption. It is unclear why urine Ox was not altered by either KCit or KCl+CTD but fell significantly with KCit+CTD. This reduction of urine Ox with the combination therapy, but not

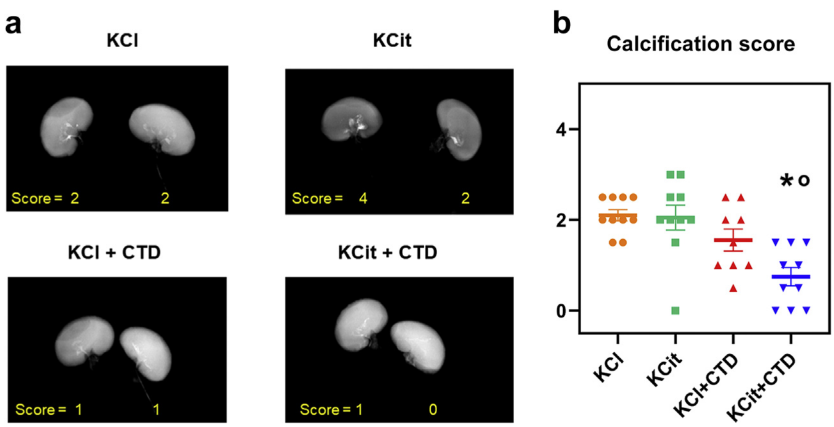

Figure 5 | Kidney stones and calcification. At the conclusion of the 18-week study, the extent of kidney stones and calcification were determined by 3 observers as described in the methods. (**a**) Representative X-rays of kidneys from rats receiving KCl, K citrate (Cit), chlorthalidone (CTD), or KCit+CTD. (**b**) Quantitation of stone formation and calcification in all rats (mean ± SE, $n = 10$ per group). *$P < 0.05$ versus KCl; ○$P < 0.05$ versus KCit alone.

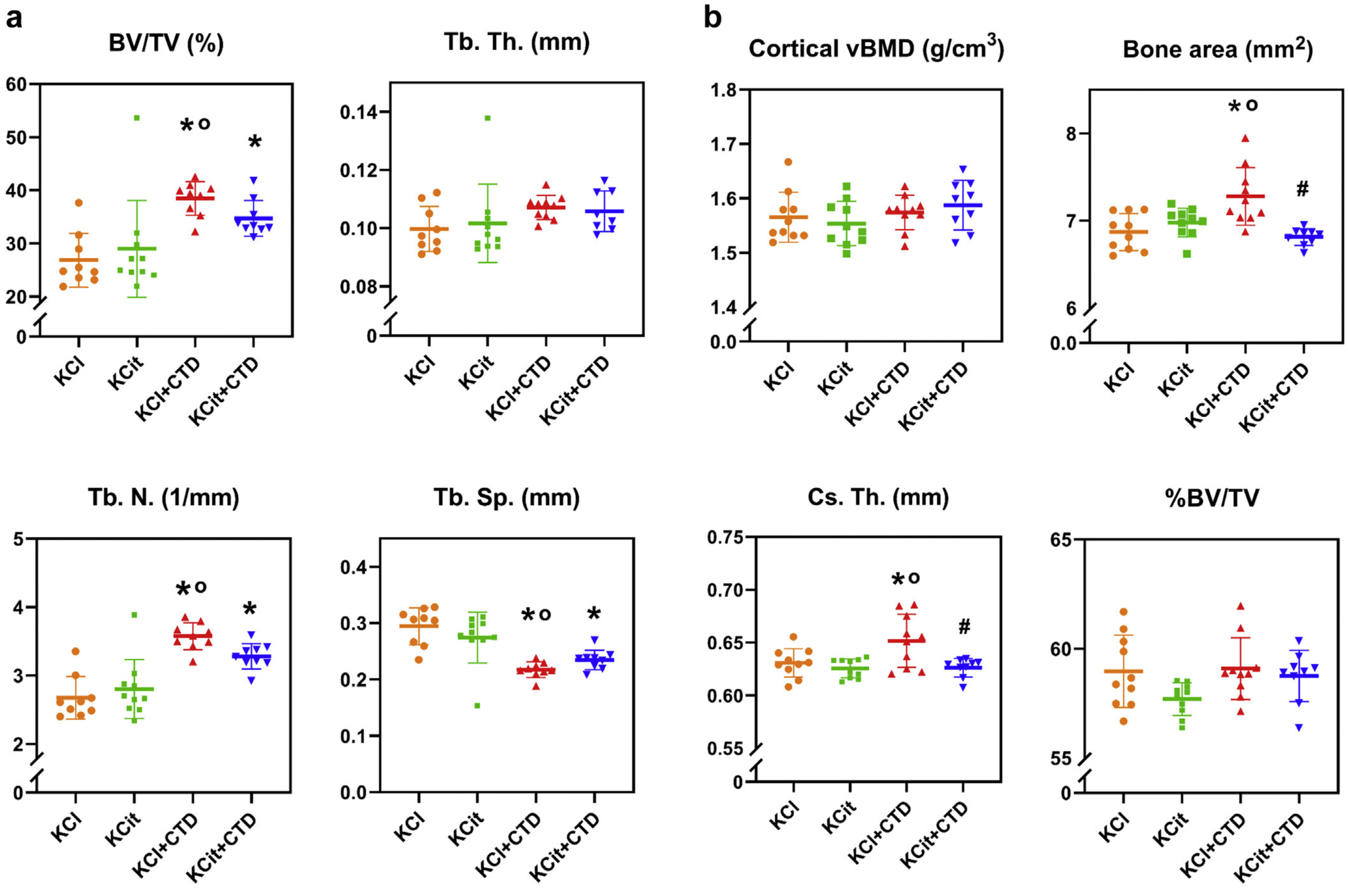

Figure 6 | Changes in bone parameters after 18 weeks. Rat diets were all supplemented with hydroxyproline and with KCl (4 mmol/d) as a control, K citrate (Cit; 4 mmol/d), chlorthalidone (CTD; 4–5 mg/kg/d) + KCl, or KCit+CTD. At the conclusion of the 18-week study, bones were collected from all rats and analyzed as described in the methods. (**a**) Percentage of bone volume per total volume (BV/TV), trabecular thickness (Tb. Th.), trabecular number (Tb. N.), and trabecular separation (Tr. Sp.) of L6 vertebrae are presented. (**b**) Cortical volumetric bone mineral density (vBMD), bone area, cortical thickness (Cs. Th.), and %BV/TV of femoral cortical bone are presented. Results are mean ± SD for $n = 10$ bones per group. $^{*}P < 0.05$ versus KCl; $^{o}P < 0.05$ versus KCit alone; $^{\#}P < 0.05$ versus CTD alone. There were no significant differences comparing KCl+CTD with KCit+CTD.

with either therapy alone, was observed in each of the 3 individual urine collection periods. In our previous publication,[19] urinary Ox also fell with KCit+CTD, providing further validity to the observation. While we do not have a clear explanation as to why Ox excretion fell with the combined therapy, it is possible that thiazides reduce Ca absorption and therefore leave more Ca in the intestine to bind oxalate and lower net absorption.[31] Cit has been shown to reduce intestinal Ca absorption in a rat model,[32] so CTD and Cit together may be additive, or even synergistic, in lowering Ca absorption, providing greater Ca to bind intestinal Ox and therefore reducing renal Ox excretion. Further studies will be required to understand this interesting observation.

Neither KCit nor CTD alone or in combination led to an alteration in CaOx supersaturation yet the combination significantly reduced CaOx kidney stone formation. The reasons that stone formation fell, yet supersaturation was not altered, are not clear from this study. Urine CaOx supersaturation was almost twice as high in each of the 4 groups compared with GHS rats fed a diet without added hydroxyproline;[19] perhaps any changes induced by these medications were not sufficient to alter these high levels of supersaturation. While CaOx supersaturation was numerically lower with KCit+CTD than in any of the other groups, this reduction was not significant. While we present supersaturation data, as it appears to be an important parameter in regulating stone formation,[33,34] we recognize that it is not the sole determinant of stone formation. For example calculated supersaturation dues not account for inhibitors or promoters of stone formation.[24] In addition, no calculated measure of supersaturation has been formally validated for use in rodents.

The increase in urine Ox caused by the addition of the Ox precursor hydroxyproline to the diet of GHS rats results in the formation of only CaOx stones.[10] Indeed in this study, with hydroxyproline added to the diet, urine Ox was approximately twice that of a similar study in which hydroxyproline was not added to diet.[19] Previously we have shown that in GHS rats not given hydroxyproline, which form only CaP stones, CTD alone is superior to KCit or to KCit+CTD in

Table 2 | Mechanical properties of trabecular (vertebral compression) and cortical (3-point bending) bone from GHS rats fed KCl, KCit, KCl + CTD, or KCit + CTD

	KCl	KCit	KCl + CTD	KCit + CTD
Vertebral compression				
Structural properties				
Ultimate load (N)	185.9 ± 34.1	225.6 ± 34.4	269.5 ± 40.6[a]	227.5 ± 43.8
Failure load (N)	169.1 ± 44.2	216.2 ± 43.2	262.9 ± 38.2[a]	219.6 ± 52.3
Energy to fail (mJ)	28.5 ± 16.8	44.5 ± 17.1	62.8 ± 17.7[a]	46.9 ± 18.0
Failure displacement (mm)	0.45 ± 0.21	0.49 ± 0.22	0.57 ± 0.20	0.58 ± 0.26
Stiffness (N/mm)	744.0 ± 417.4	685.6 ± 219.1	686.6 ± 123.3	634.9 ± 129.7
Material properties				
Ultimate stress (MPa)	33.6 ± 6.8	41.9 ± 6.5	51.9 ± 11.9[a]	38.7 ± 8.5[b]
Failure stress (MPa)	30.5 ± 8.4	40.1 ± 7.9	50.7 ± 11.6[a]	37.2 ± 9.6[b]
Failure strain (%)	9.8 ± 4.1	10.0 ± 4.5	11.9 ± 4.2	12.0 ± 4.8
Toughness (mJ/mm^3)	0.7 ± 0.4	1.4 ± 0.6	2.1 ± 0.8[a]	1.2 ± 0.6[b]
Young's modulus (MPa)	614.6 ± 364.6	598.4 ± 140.5	626.0 ± 145.3	512.6 ± 112.4
3-point bending				
Structural properties				
Failure displacement (mm)	0.44 ± 0.07	0.57 ± 0.10[a]	0.57 ± 0.12	0.57 ± 0.14
Failure load (N)	152.1±5.0	144.8±15.2	155.0±14.9	144.5±11.7
Energy to fail (N)	49.2±10.3	45.7±15.2	47.1±14.5	43.4±15.4
Stiffness (N/mm)	450.8±47.3	496.0±49.2	513.9±46.4[a]	480.5±40.8
Material properties				
Failure stress (MPa)	89.1 ± 6.0	85.5 ± 9.6	88.1 ± 16.8	85.4 ± 7.8
Failure strain (%)	4.9 ± 0.7	6.3 ± 1.1[a]	6.4 ± 1.4[a]	6.2 ± 1.5
Toughness (mJ/mm^3)	317.1 ± 67.7	300.0 ± 106.0	303.7 ± 123.7	280.3 ± 101.2
Young's modulus (MPa)	2395.5 ± 244.2	2660.5 ± 216.6	2602.5 ± 430.1	2606.1 ± 291.5

Cit, citrate; CTD, chlorthalidone; GHS, genetic hypercalciuric stone-forming.
Results are mean ± SD for 9 to 10 rats per group.
[a]$P < 0.05$ vs. KCl alone.
[b]$P < 0.05$ vs. KCl+CTD.

reducing CaP stone formation and improving bone quality.[19] In both studies, KCit and CTD, alone and in combination had remarkably similar effects on urine Ca, Cit, P, NH_4, and pH. However, CaP crystal formation is sensitive to pH, the higher the pH, the greater the supersaturation with respect to CaP, while CaOx crystal formation and supersaturation is relatively pH insensitive.[35] Similar to this prior study, CTD, but not KCit, led to an improvement in bone density and quality, and the addition of KCit to CTD did not improve density or quality further.

Thus based on the results of this study and that of our previous study,[19] the approach to preventing stones in hypercalciuric stone formers differs with stone type. With CaOx stones, KCit+CTD is most beneficial in preventing stones, while with CaP stones CTD alone appears to be the optimal treatment.

Table 3 | Histomorphometry analyses of tibiae from GHS rats fed KCl, KCit, KCl + CTD, or KCit + CTD

	KCl	KCit	KCl + CTD	KCit + CTD
Histomorphometry				
Undecalcified static				
BV/TV (%)	0.18 ± 0.05	0.25 ± 0.03[a]	0.31 ± 0.04[a,b]	0.29 ± 0.04[a]
OV/BV (%)	0.002 ± 0.002	0.0005 ± 0.0005[a]	0.0003 ± 0.0003[a]	0.0002 ± 0.0002[a]
OS/BS (%)	0.10 ± 0.18	0.01 ± 0.01[a]	0.006 ± 0.007[a]	0.008 ± 0.008[a]
Osteoid width (μm)	4.4 ± 2.1	2.7 ± 2.0	3.6 ± 5.9	1.4 ± 1.68
Dynamic				
MS/BS (%)	0.27 ± 0.26	0.26 ± 0.08	0.20 ± 0.08	0.18 ± 0.06
MAR (μm/d)	1.51 ± 0.21	1.43 ± 0.24	1.38 ± 0.16	1.38 ± 0.14
BFR/BS ($\mu m^3/\mu m^2/d$)	0.42 ± 0.28	0.38 ± 0.15	0.28 ± 0.11	0.25 ± 0.09
Decalcified				
Oc. S./BS (%)	0.47 ± 0.29	0.32 ± 0.19	0.41 ± 0.17	0.42 ± 0.12
N. Oc./BS (1/mm)	6.7 ± 2.9	7.4 ± 1.4	5.6 ± 2.0	6.8 ± 1.7
N. Oc./TV ($1/mm^2$)	15.4 ± 11.6	23.8 ± 5.8	25.1 ± 10.0	32.6 ± 12.0[a]

BFR, bone formation rate; BS, bone surface; BV, bone volume; Cit, citrate; CTD, chlorthalidone; GHS, genetic hypercalciuric stone-forming; MAR, mineral apposition rate; MS, mineralized surface; N. Oc., number of osteoclasts; Oc. S., osteoclast surface; OS, osteoid surface; OV, osteoid volume; TV, total volume.
Results are mean ± SD for 8 to 10 rats per group. There were no significant differences comparing KCl+CTD with KCit+CTD.
[a]$P < 0.05$ vs. KCl alone.
[b]$P < 0.05$ vs. KCit alone.

In considering a direct interaction between the effects of KCit and the effects of CTD, there were differences in some, but not all, urine parameters that demonstrated a significant interaction effect when rats received both KCit+CTD. There was a lesser effect on urine NH_4 and uric acid supersaturation when both drugs were given than the sum of either alone. There was a greater effect on urine pH and CaP supersaturation when both drugs were given than the sum of either alone. There was no significant interaction on urine Ca, Cit, Ox, CaOx supersaturation, or stone formation when both drugs were given. Because there is no drug interaction with respect to CaOx supersaturation or stone formation, the importance of these drug interactions is not clear.

Hypercalciuric stone formers generally have decreased bone density and increased propensity to fracture.[25] As we found previously, GHS rats given CTD with or without KCit had increased Tb. bone volume, though there was no increase in cortical BMD. Most rat models are more sensitive to changes in Tb. bone than cortical bone because Tb. bone has many more cells than cortical bone and remodels 8 times faster.[36] Rats given CTD alone demonstrated increased vertebral bone strength and material properties, which was not observed when KCit was also given. KCit and CTD increased different structural properties of the bone, though the combination had no effect when compared with control rats fed KCl. An increase in percentage of bone volume and a decrease in unmineralized osteoid was found in rats fed KCit alone, CTD alone, or the combination when compared with bone volume and unmineralized osteoid in control rats. The greater improvements in bone quality in response to CTD compared with KCit are similar to what we have found previously.[17,19]

In human studies, both thiazide diuretics and KCit have been shown to improve BMD.[26,35] In 3 small studies of hypercalciuric stone formers, improvements in bone density or quality or both were found.[25,37,38] Many patients receive thiazide diuretics for hypertension. A meta-analysis of 21 observational studies including almost 400,000 patients concluded that there was a significant 24% reduction in the risk of hip fracture in those receiving thiazide diuretics.[39] KCit therapy for 11 months improved vertebral BMD in patients with Ca nephrolithiasis.[40] KCit also improved distal radius BMD in hypercalciuric patients.[41] BMD as measured by computed tomography scan was also improved by both thiazides and KCit.[42] In this study, we again demonstrate that a thiazide diuretic improves bone density and the mechanical properties of bone, which is consistent with observations in humans.[37,38] Why we did not find that KCit improved bone in GHS rats is unclear. Humans tend to eat an acid-producing diet and KCit has been shown, in at least 1 study, to improve bone density in osteoporotic humans.[43] Perhaps if our GHS rats were fed a diet that results in more endogenous acid production, KCit would lead to an improvement in bone density and quality.

In conclusion, we found that in GHS rats who universally form CaOx stones when fed hydroxyproline, the combination of KCit+CTD led to a greater reduction in stone formation than either agent alone. CTD alone improved bone density and quality while the addition of KCit had no additional benefit. Previously we have shown that for CaP stones, CTD alone is superior to KCit alone or KCit+CTD in preventing stone formation and, similar to the current study, to improve bone density and quality.[19] Thus, in this hypercalciuric model of kidney stones, optimal treatment appears to differ for the 2 most common types of kidney stones found in humans: KCit+CTD for CaOx stones and CTD alone for CaP stones. If these results are confirmed in human hypercalciuric CaOx stone formers, then the combination of these 2 agents, which are often used alone, would be beneficial in prevention of stone formation and at least the thiazide would help improve bone density and quality.

DISCLOSURE

NSK reports grants from National Institutes of Health, during the conduct of the study; owns stock in Tricida and Amgen; and has stock options in Tricida; her spouse is a consultant for Tricida, Sanofi Genzyme, and Relypsa/Vifor/Fresenius; owns stock in Amgen; receives speaking fees from Sanofi Genzyme; and is an adjudicator for adverse events from Novo Nordisk/Covance. DAB reports personal fees and other from Tricida, Amgen, and Sanofi Genzyme; and personal fees from Relypsa/Vifor/Fresenius and Sanofi Genzyme; all outside the submitted work. All other authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods.

Figure S1. Urine calcium, citrate, and phosphate at 6, 12, and 18 weeks.

Figure S2. Urine oxalate, NH_4, and pH at 6, 12, and 18 weeks.

Figure S3. Urine volume, sodium, and potassium at 6, 12, and 18 weeks.

Figure S4. Urine supersaturation of calcium phosphate and calcium oxalate at 6, 12, and 18 weeks.

REFERENCES

1. Bushinsky DA. Kidney stones. In: Melmed S, Auchus RJ, Goldfine AB, et al., eds. *Williams Textbook of Endocrinology, 14th ed*. Philadelphia, PA: Elsevier; 2020:1318–1335.
2. Coe FL, Worcester EM, Evan AP. Idiopathic hypercalciuria and formation of calcium renal stones. *Nat Rev Nephrol*. 2016;12:519–533.
3. Bushinsky DA, Frick KK, Nehrke K. Genetic hypercalciuric stone-forming rats. *Curr Opin Nephrol Hypertens*. 2006;15:403–418.
4. Bushinsky DA, Parker WR, Asplin JR. Calcium phosphate supersaturation regulates stone formation in genetic hypercalciuric stone-forming rats. *Kidney Int*. 2000;57:550–560.
5. Li XQ, Tembe V, Horwitz GM, et al. Increased intestinal vitamin D receptor in genetic hypercalciuric rats: a cause of intestinal calcium hyperabsorption. *J Clin Invest*. 1993;91:661–667.
6. Tsuruoka S, Bushinsky DA, Schwartz GJ. Defective renal calcium reabsorption in genetic hypercalciuric rats. *Kidney Int*. 1997;51:1540–1547.
7. Krieger NS, Stathopoulos VM, Bushinsky DA. Increased sensitivity to 1, 25(OH)2D3 in bone from genetic hypercalciuric rats. *Am J Physiol*. 1996;271:C130–C135.
8. Grynpas M, Waldman S, Holmyard D, et al. Genetic hypercalciuric stone-forming rats have a primary decrease in BMD and strength. *J Bone Miner Res*. 2009;24:1420–1426.

9. Ng AH, Frick KK, Krieger NS, et al. 1,25(OH)2D3-enhanced hypercalciuria in genetic hypercalciuric stone-forming rats. *Calcif Tissue Int*. 2014;94: 531–543.
10. Bushinsky DA, Asplin JR, Grynpas MD, et al. Calcium oxalate stone formation in genetic hypercalciuric stone-forming rats. *Kidney Int*. 2002;61:975–987.
11. Karnauskas AJ, van Leeuwen JP, van den Bemd GJ, et al. Mechanism and function of high vitamin D receptor levels in genetic hypercalciuric stone-forming rats. *J Bone Miner Res*. 2005;20:447–454.
12. Hoopes RR Jr, Middleton FA, Sen S, et al. Isolation and confirmation of a calcium excretion quantitative trait locus on chromosome 1 in genetic hypercalciuric stone-forming congenic rats. *J Am Soc Nephrol*. 2006;17: 1292–1304.
13. Moe OW, Bushinsky DA. Genetic hypercalciuria: a major risk factor in kidney stones. In: Thakker RV, Whyte MP, Eisman JA, Igarashi T, eds. *Genetics of Bone Biology and Skeletal Disease*. London: Elsevier; 2013:585–604.
14. Zisman AL. Effectiveness of treatment modalities on kidney stone recurrence. *Clin J Am Soc Nephrol*. 2017;12:1699–1708.
15. Fink HA, Wilt TJ, Eidman KE, et al. Medical management to prevent recurrent nephrolithiasis in adults: a systematic review for an American College of Physicians clinical guideline. *Ann Intern Med*. 2013;158:535–543.
16. Bushinsky DA, Asplin JR. Thiazides reduce brushite, but not calcium oxalate, supersaturation and stone formation in genetic hypercalciuric stone-forming rats. *J Am Soc Nephrol*. 2005;16:417–424.
17. Bushinsky DA, Willett T, Asplin JR, et al. Chlorthalidone improves vertebral bone quality in genetic hypercalciuric stone-forming rats. *J Bone Miner Res*. 2011;26:1904–1912.
18. Krieger NS, Asplin JR, Frick KK, et al. Effect of potassium citrate on calcium phosphate stones in a model of hypercalciuria. *J Am Soc Nephrol*. 2015;26:3001–3008.
19. Krieger NS, Asplin JR, Granja I, et al. Chlorthalidone is superior to potassium citrate in reducing calcium phosphate stones and increasing bone quality in hypercalciuric stone-forming rats. *J Am Soc Nephrol*. 2019;30:1163–1173.
20. Asplin JR, Bushinsky DA, Singharetnam W, et al. Relationship between supersaturation and crystal inhibition in hypercalciuric rats. *Kidney Int*. 1997;51:640–645.
21. Asplin JR, Donahue SE, Lindeman C, et al. Thiosulfate reduces calcium phosphate nephrolithiasis. *J Am Soc Nephrol*. 2009;20:1246–1253.
22. Frick KK, Asplin JR, Favus MJ, et al. Increased biological response to 1, 25(OH)2D3 in genetic hypercalciuric stone-forming rats. *Am J Physiol Renal Physiol*. 2013;304:F718–F726.
23. Frick KK, Asplin JR, Krieger NS, et al. 1,25(OH)2D3-enhanced hypercalciuria in genetic hypercalciuric stone-forming rats fed a low calcium diet. *Am J Physiol Renal Physiol*. 2013;305:F1132–F1138.
24. Werness PG, Brown CM, Smith LH, et al. Equil2: a BASIC computer program for the calculation of urinary saturation. *J Urol*. 1985;134:1242–1244.
25. Sakhaee K, Maalouf NM, Kumar R, et al. Nephrolithiasis-associated bone disease: pathogenesis and treatment options. *Kidney Int*. 2011;79:393–403.
26. Moe OW, Pearle MS, Sakhaee K. Pharmacotherapy of urolithiasis: evidence from clinical trials. *Kidney Int*. 2011;79:385–392.
27. Pak CY, Heller HJ, Pearle MS, et al. Prevention of stone formation and bone loss in absorptive hypercalciuria by combined dietary and pharmacological interventions. *J Urol*. 2003;169:465–469.
28. Sakhaee K, Williams RH, Oh MS, et al. Alkali absorption and citrate excretion in calcium nephrolithiasis. *J Bone Miner Res*. 1993;8:789–794.
29. Bushinsky DA. Metabolic alkalosis decreases bone calcium efflux by suppressing osteoclasts and stimulating osteoblasts. *Am J Physiol*. 1996;271:F216–F222.
30. Coe FL, Parks JH, Bushinsky DA, et al. Chlorthalidone promotes mineral retention in patients with idiopathic hypercalciuria. *Kidney Int*. 1988;33: 1140–1146.
31. Bushinsky DA, Favus MJ, Coe FL. Mechanism of chronic hypocalciuria with chlorthalidone: reduced calcium absorption. *Am J Physiol*. 1984;247: F746–F752.
32. Rümenapf G, Schwille P. The influence of citrate on the duodenal absorption of calcium in the rat. *Calcif Tissue Int*. 1988;42:326–330.
33. Ferraro PM, Ticinesi A, Meschi T, et al. Short-term changes in urinary relative supersaturation predict recurrence of kidney stones: a tool to guide preventive measures in urolithiasis. *J Urol*. 2018;200:1082–1087.
34. Prochaska M, Taylor E, Ferraro PM, et al. Relative supersaturation of 24-hour urine and likelihood of kidney stones. *J Urol*. 2018;199:1262–1266.
35. Bushinsky DA, Coe FL, Moe OW. Nephrolithiasis. In: Brenner BM, ed. *The Kidney*. 9th ed. 2. Philadelphia, PA: W.B. Saunders; 2012:1455–1507.
36. Parfitt AM. The physiological and clinical significance of bone histomorphometric data. In: Recker R, ed. *Bone Histomorphometry. Techniques and Interpretations*. Boca Raton, FL: CRC Press; 1983:143–223.
37. Adams JS, Song CF, Kantorovich V. Rapid recovery of bone mass in hypercalciuric, osteoporotic men treated with hydrochlorothiazide. *Ann Intern Med*. 1999;130:658–660.
38. Steiniche T, Mosekilde L, Christensen MS, et al. Histomorphometric analysis of bone in idiopathic hypercalciuria before and after treatment with thiazide. *APMIS*. 1989;97:302–308.
39. Aung K, Htay T. Thiazide diuretics and the risk of hip fracture. *Cochrane Database Syst Rev*. 2011;10:CD005185.
40. Pak CYC, Peterson RD, Poindexter J. Prevention of spinal bone loss by potassium citrate in cases of calcium urolithiasis. *J Urol*. 2002;168:31–34.
41. Vescini F, Buffa A, LaManna G, et al. Long-term potassium citrate therapy and bone mineral density in idiopathic calcium stone formers. *J Endocrinol Invest*. 2005;28:218–222.
42. Alshara L, Batagello CA, Armanyous S, et al. The impact of thiazides and potassium citrate on bone mineral density evaluated by CT scan in stone formers. *J Endourol*. 2018;32:559–564.
43. Jehle S, Hulter HN, Krapf R. Effect of potassium citrate on bone density, microarchitecture, and fracture risk in healthy older adults without osteoporosis: a randomized controlled trial. *J Clin Endocrinol Metab*. 2013;98:207–217.