Partial Human Genetic Deficiency in Tissue Kallikrein Activity and Renal Calcium Handling

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A loss-of-function polymorphism of the human tissue kallikrein (TK) gene (R53H) induces a major decrease in enzyme activity. Inactivation of the TK gene in mice causes a defect in tubular calcium (Ca) reabsorption. Therefore, this study investigated the Ca phenotype of carriers of the 53H allele. In a crossover study, 30 R53R homozygous and 10 R53H heterozygous young white male individuals were randomly assigned to two 7-d low-Ca diets (10 mmol/d) associated with either a low-sodium (Na)/high-potassium (K) diet or a high-Na/low-K diet to modulate TK synthesis. On the seventh day of each diet, the participants were studied before and during a 2-h infusion of furosemide that functionally excludes the thick ascending limb and increases Ca delivery to distal tubular segments. Urinary kallikrein activity was 50 to 60% lower in R53H participants than in R53R participants. Adaptation of urinary Ca excretion to the contrasted Na/K diets was unaffected in R53H participants. By contrast, R53H participants after furosemide infusion had significantly lower serum ionized Ca concentrations than did R53R participants (P < 0.0001) and tendency toward nonsignificantly higher urinary Ca excretions than did R53R participants (P = 0.14). These effects were more marked under low-Na/high-K diet. Despite nonsignificant differences in urinary Ca excretions between the two groups, these results suggest in R53H individuals an increase in Ca reabsorption in the thick ascending limb under baseline conditions that counteracts a distal tubular defect that is revealed by furosemide infusion. In humans as in mice, TK thus may act as an intrarenal modulator of Ca reabsorption.


Renal tubular calcium (Ca) transport along the nephron is one of the major determinants of urinary Ca excretion and consequently of serum Ca concentration (1). Although this renal transport is more substantial in the proximal tubule and the cortical thick ascending limb (TAL) of Henle’s loop, renal tubular Ca and magnesium (Mg) transport is finely tuned in the distal and connecting tubules, which actively reabsorb approximately 10% of the filtered load of both cations (2). The renal reabsorption of Ca is controlled physiologically by both nonhormonal (extracellular volume, acid/base status, and serum Mg and Ca concentrations) and hormonal (parathyroid hormone [PTH] and 1,25-dihydroxycholecalciferol) factors. The kidney tissue kallikrein (TK), the main kinin-forming enzyme (3), also may regulate physiologically renal tubular Ca transport. In human and in other mammal kidneys (e.g., mouse, rabbit), TK is distributed mainly in the distal nephron, close to Ca transporters that are involved in the active distal Ca reabsorption (4–6). TK gene expression increases during a low-Ca diet (6). In addition, TK-deficient mice are hypercalcemic, and hypocalcemia can be observed in this model under a low-Ca diet through a non–kinin-mediated mechanism (6). Whether TK might play a role in Ca metabolism in humans remains unknown.

We previously characterized a loss-of-function–coding polymorphism (Arg53His) of the human TK gene that results in a major loss of kallikrein activity in vitro and in vivo (7). Its allelic frequency (approximately 5 to 7%) provides an opportunity to study the physiologic role of TK in humans. We already showed that, like the TK-deficient mice, heterozygous R53H humans with a partial genetic deficiency have arterial dysfunction, highlighting the important role of vascular TK and kinins in the control of arterial function (8,9).

This study aimed to investigate the Ca phenotype of R53H allele carriers. We studied R53H heterozygous and R53R homozygous individuals who were on controlled high- and low-sodium (Na) and -potassium (K) diets, which modulate kallikrein synthesis and excretion (10,11). We also tested the effect of acute administration of furosemide, which blocks electrolyte reabsorption in the TAL and enhances the distal tubule function, the main site of kallikrein synthesis in the kidney (12).

Materials and Methods

Participants

The study design and protocol have been reported previously (8). Briefly, 206 white male individuals who were aged 18 to 35 yr were screened for the R53H mutation and for the following inclusion criteria: Nonsmoker, normotensive (BP <140/90 mmHg in the supine position after 5 min of rest), normal clinical examination, normal plasma creat-
in urine concentration (<100 µmol/L), and no proteinuria. Among the 11 R53H heterozygous individuals (5.3%) identified, all except one smoker were included in the study. Among the R53R homozygous individuals, we included the first 30 consecutive individuals who matched all of the inclusion criteria. All individuals completed the study after giving written informed consent. The protocol was approved by the “Comité Consultatif de Protection des Personnes se prêtant à des Recherches Biomédicales” (Paris-Cochin, France). All procedures were in accordance with institutional guidelines.

Study Design
First, we studied urinary kallikrein activity (UKLKa) for all of the participants, who received a noncontrolled normal Na/K/Ca diet. The participants then were randomly assigned to one of the two experimental diets for 7 d: Diet 1 characterized by a low-Na (<20 mmol NaCl/d) and high-K diet (>140 mmol KCl/d [LowNa/HighK]) and diet 2 with a high-Na (>250 mmol NaCl/d) and low-K diet (<50 mmol KCl/d [HighNa/LowK]). Ca intake was kept at a constant 10 mmol/d (400 mg/d) during the two periods as were calories and protein (70 g/d) contents. The administration order of the diets was randomized according to a crossover study design. Each controlled diet period was separated from the previous diet by a 7-d washout period during which the Na, K, and Ca intakes were unrestricted. All meals were provided by the metabolic kitchen of the Clinical Investigation Center and were taken in the unit. During the noncontrolled diet, 166 ± 68 mmol of Na and 73 ± 23 mmol of K were excreted in the 24-h urine. The control of the Na and K intake allowed us to achieve the desired balances, as shown by the previously reported 24-h urinary Na and K excretion rates (8). Blood and urine were sampled for hormone and electrolyte determination at inclusion on the noncontrolled Na, K, and Ca diets and then on day 7 of each controlled diet period at 9:00 a.m. after an overnight fast and after 1 h of rest in the sitting position.

Studies of Ca Metabolism before and during Furosemide Infusion
For investigation of the interaction between diet-induced and genotype-induced changes in Na, K, Ca, and Mg reabsorption, furosemide was infused at a constant rate (5 mg/h in 0.9% saline solution infusion for normally distributed parameters. The diet-induced variations in 24-h UKLKa were smaller in R53H participants than in R53R participants at baseline (5228 [2084 to 6630] versus 14,534 [6700 to 26,118] DFU/s per 24 h, respectively; P = 0.006). The diet-induced variations in 24-h UKLKa were smaller in R53H participants than in R53R participants (8). On the LowNa/HighK diet, UKLKa was significantly lower in R53H than in R53R participants (20,017 [14,536 to 24,872] versus 51,515 [32,557 to 84,699] DFU/s per 24 h, respectively; P = 0.0045). The difference between genotypes was smaller on the HighNa/LowK diet (1759 [403 to 4950] versus 2560 [222 to 10,773] DFU/s per 24 h, respectively; P = 0.26).

Interaction between Diet and Furosemide and Genotype on Ca Balance
In the whole population, the contrasted diets affected baseline urinary excretion of Na (UNaV/GFR), K (UKV/GFR), Ca (UCaV/GFR) and Mg (UMgV/GFR; Tables 1 and 2). Baseline UNaV/GFR and UKV/GFR reflected the Na and K content of the diet (Table 2). Under the LowNa/HighK diet, baseline UCaV/GFR was significantly lower and was associated with a significantly higher baseline serum total Ca concentration than on the HighNa/LowK diet, with no change in baseline serum ionized Ca concentration (Table 1).

All enzyme and hormone values were measured blind to genotype and order of diet.

Calculations
Variations in filtered loads as a result of variations in GFR can cause variations in urinary Ca and Mg excretions. Therefore, we related urinary excretion values to the GFR to assess the tubular reabsorption of solutes (X) independently of GFR values as follows: UxV/GFR = [X]u × [creat]p × [creat]u−1, where [X]u, [creat]p, and [creat]u are urinary concentration of solute X and the plasma and urinary concentration of creatinine, respectively.

Statistical Analyses
To analyze the effects of the R53H polymorphism on various electrolytes and hormones taking into account all experimental conditions, we used an ANOVA model for a crossover study design. The model included the genotype effect, the experimental factors (diet, furosemide infusion, and diet × furosemide interaction), the period, and the participant interactions. The interactions between genotype and the various experimental factors were tested and kept in the final model when P < 0.10. All analyses were carried out using SAS Statistical Software (version 8.2, Cary, NC), and P < 0.05 was considered to be significant. Data are expressed as medians and interquartile ranges for UKLKa and for the other non-normally distributed parameters and as the mean ± SD for normally distributed parameters.

Results
Consequences of Diet and the R53H Polymorphism on Urinary Kallikrein Activity
As previously reported, both the diet and the R53H polymorphism influenced urinary excretion of kallikrein (8). We found that the 24-h UKLKa level was 50 to 60% lower on average in R53H participants than in R53R participants at baseline (5228 [2084 to 6630] versus 14,534 [6700 to 26,118] DFU/s per 24 h, respectively; P = 0.006). The diet-induced variations in 24-h UKLKa were smaller in R53H participants than in R53R participants (8). On the LowNa/HighK diet, UKLKa was significantly lower in R53H than in R53R participants (20,017 [14,536 to 24,872] versus 51,515 [32,557 to 84,699] DFU/s per 24 h, respectively; P = 0.0045). The difference between genotypes was smaller on the HighNa/LowK diet (1759 [403 to 4950] versus 2560 [222 to 10,773] DFU/s per 24 h, respectively; P = 0.26).

Analytical Methods
All analytical methods for determining ionized Ca (Ca2+), Mg, Na, K, creatinine, PTH, and calcitriol levels were as described previously (13,14). UKLKa was measured as described previously (8). Briefly, UKLKa was quantified using an amidolytic assay with the fluorogenic peptide substrate DPFP-Nmec (gift from Dr. F. Gautier, Tours, France). The results were expressed in Δ fluorescence units (DFU) per second of incubation for the total urine collected (DFU/s per 12- or 24-h urine).
Table 1. Effect of diet, furosemide treatment, and genotype on UCaV/GFR, and serum concentrations of TCa and Ca\(^{2+}\)^

<table>
<thead>
<tr>
<th>Parameter</th>
<th>UCaV/GFR (μmol/L(_{\text{GFR}}))</th>
<th>[TCa] (mM)</th>
<th>[Ca(^{2+})] (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Furosemide</td>
<td>Basal</td>
</tr>
<tr>
<td>HighNa/LowK diet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>all (n = 40)</td>
<td>40.2 ± 12.8</td>
<td>145.5 ± 33.3</td>
<td>2.26 ± 0.06</td>
</tr>
<tr>
<td>R53H (n = 10)</td>
<td>42.2 ± 8.4</td>
<td>148.4 ± 32.5</td>
<td>2.26 ± 0.05</td>
</tr>
<tr>
<td>R53R (n = 30)</td>
<td>39.5 ± 14.0</td>
<td>144.5 ± 34.0</td>
<td>2.26 ± 0.07</td>
</tr>
<tr>
<td>LowNa/HighK diet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>all (n = 40)</td>
<td>24.7 ± 10.3</td>
<td>121.3 ± 24.7</td>
<td>2.29 ± 0.06</td>
</tr>
<tr>
<td>R53H (n = 10)</td>
<td>26.1 ± 10.7</td>
<td>130.9 ± 25.8</td>
<td>2.27 ± 0.05</td>
</tr>
<tr>
<td>R53R (n = 30)</td>
<td>24.2 ± 10.3</td>
<td>118.1 ± 24.0</td>
<td>2.30 ± 0.07</td>
</tr>
</tbody>
</table>

ANOVA model

- genotype: 0.14
- diet: <0.0001
- furosemide: <0.0001
- diet × genotype: 0.17
- genotype × diet: NS
- genotype × furosemide: NS

*NS: interaction \(P \geq 0.10\) not included in the model. Ca\(^{2+}\), ionized calcium; GF, glomerular filtrate; HighNa/LowK, high-sodium/low-potassium; LowNa/HighK, low-sodium/high-potassium; TCa, total calcium; UCaV/GFR, urinary excretion of calcium.

Table 2. Effect of diet, furosemide treatment, and genotype on UNaV/GFR, UKV/GFR, and UMgV/GFR\(^a\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>UNaV/GFR (μmol/L(_{\text{GFR}}))</th>
<th>UKV/GFR (μmol/L(_{\text{GFR}}))</th>
<th>UMgV/GFR (μmol/L(_{\text{GFR}}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Furosemide</td>
<td>Basal</td>
</tr>
<tr>
<td>HighNa/LowK diet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>all (n = 40)</td>
<td>1910 ± 538</td>
<td>13486 ± 2478</td>
<td>198 ± 78</td>
</tr>
<tr>
<td>R53H (n = 10)</td>
<td>1986 ± 570</td>
<td>13680 ± 2928</td>
<td>180 ± 76</td>
</tr>
<tr>
<td>R53R (n = 30)</td>
<td>1885 ± 535</td>
<td>13422 ± 2362</td>
<td>203 ± 79</td>
</tr>
<tr>
<td>LowNa/HighK diet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>all (n = 40)</td>
<td>96 ± 93</td>
<td>6250 ± 1873</td>
<td>618 ± 223</td>
</tr>
<tr>
<td>R53H (n = 10)</td>
<td>67 ± 49</td>
<td>6209 ± 1356</td>
<td>611 ± 186</td>
</tr>
<tr>
<td>R53R (n = 30)</td>
<td>105 ± 102</td>
<td>6263 ± 2036</td>
<td>621 ± 237</td>
</tr>
</tbody>
</table>

ANOVA model

- genotype: 0.80
- diet: <0.0001
- furosemide: <0.0001
- diet × furosemide: <0.0001
- genotype × diet: NS
- genotype × furosemide: NS

*NS: interaction \(P \geq 0.10\) not included in the model. UKV/GFR, urinary excretion of potassium; UMgV/GFR, urinary excretion of magnesium; UNaV/GFR, urinary excretion of sodium.

As expected, furosemide infusion induced a massive increase in Na, K, Mg, and Ca excretions in the whole population. The magnitude of the natriuretic and kaliuretic responses to furosemide was influenced by the Na/K content of the diet (for diet × furosemide interaction: \(P < 0.0001\) and \(P < 0.0001\) for Na and K excretion, respectively; Table 2). The magnesuric response was more marked on the LowNa/HighK diet than on

(120.5 ± 29.6 pmol/L) than on the HighNa/LowK diet (140.8 ± 35.2 pmol/L; \(P = 0.0002\)). None of the baseline ionic or hormonal parameters (PTH, calcitriol) was influenced by the R53H polymorphism (data not shown). Baseline urinary Ca excretion was negatively correlated to 24-h UKLKa that was measured in the individuals who were on each diet (\(r = 0.35, P = 0.002; n = 80; \text{data not shown})\).
the HighNa/LowK diet (for diet × furosemide interaction: \( P = 0.0019 \); Table 2). There was no significant effect of the R53H polymorphism on UNaV/GFR, UKV/GFR, or UMgV/GFR (\( P = 0.80, 0.24, \) and 0.80, respectively; Table 2).

In the whole population, furosemide infusion massively increased UCaV/GFR (\( P < 0.0001 \)), with the increase being of similar magnitude on both diets (for interaction diet × furosemide: \( P = 0.17 \); Table 1). The calciuretic effect of furosemide infusion was associated with a significant decrease from baseline in ionized and total Ca concentrations (for diet × furosemide interaction, \( P < 0.0001 \) and \( P = 0.0003 \), respectively; Table 1 and Figure 1). The decrease in ionized Ca concentration was associated with a significant increase in PTH concentration, which was more marked on the LowNa/HighK than on the HighNa/LowK diet (for diet × furosemide interaction: \( P < 0.001 \); data not shown). The response of Ca metabolism to diet and furosemide differed significantly according to the R53H polymorphism (Table 1 and Figure 1). After furosemide infusion, R53H participants had significantly lower ionized Ca concentrations than did the R53R participants as shown by the significant mean difference between the two groups (−0.027 mmol/L; 95% confidence interval [CI] −0.036 to −0.018; \( P < 0.0001 \)). This contrasted with the very small mean difference in serum ionized Ca concentrations that were observed at baseline between the two groups (−0.002 mmol/L; 95% CI −0.011 to 0.007; \( P = 0.65 \); Table 1 and Figure 1). The R53H genotype influenced significantly the response of serum total Ca concentration to the diet (genotype × diet effect, \( P = 0.0635 \)) with significantly lower serum total Ca concentrations achieved in R53H than in R53R participants, especially on the LowNa/HighK diet (mean difference between participants −0.045 mmol/L; 95% CI −0.070 to −0.020; \( P = 0.0005 \); Table 1). The R53H genotype did not affect significantly the response of serum total Ca concentration to furosemide infusion (genotype × furosemide effect, \( P > 0.10 \)).

Finally, the R53H participants had nonsignificantly higher UCaV/GFR after furosemide infusion than did R53R participants (\( P = 0.14 \)), which was more marked on the LowNa/HighK diet. The postfurosemide PTH concentration did not differ significantly between genotypes (data not shown).

**Discussion**

After the demonstration that homozygous TK-deficient mice display renal hypercalciuria and can become hypocalcaemic under low-Ca diet conditions as a result of defective tubular Ca reabsorption (6), we hypothesized that humans who genetically differ by their TK activity might display an abnormality in the regulation of their Ca balance. We used the opportunity of the presence of a loss-of-function polymorphism in the human hKLK1 gene to investigate blood and urine Ca-dependent parameters under baseline conditions and after a 2-h infusion of furosemide. These investigations were performed twice, each time after a period of a 7-d controlled diet in which Ca intake was kept constant and Na and K intake was manipulated. Ca intake was set at a low value of 400 mg/d (10 mmol/d) to minimize the net intestinal absorption. In these conditions, the fasting urinary Ca excretion at baseline should reflect primarily net bone resorption (15). The protein content of the diet also was kept constant to avoid variations in net acid load that may affect Ca metabolism and renal Ca handling (14). Conversely, we altered the Na and K intake to modify renal kallikrein synthesis. Salt restriction is known to stimulate the excretion of renal kallikrein by an unknown mechanism possibly mediated by aldosterone (10,11). High K intake also upregulates kallikrein synthesis and excretion either directly or by triggering aldosterone secretion (3,10).

The effects of the diet and the R53H genotype on the Ca balance first were analyzed at baseline. As expected, baseline urinary Ca excretion was significantly lower on the LowNa/HighK diet than on the HighNa/LowK diet (16–18). The low-Na diet enhances renal tubular Ca reabsorption through mechanisms that may involve the contraction of extracellular volume and increased concentrations of plasma angiotensin II (18). High K intake also stimulates renal Ca reabsorption through a mechanism that currently is unclear (17,18). A decrease in Ca intestinal absorption on the low-Na diet also

![Figure 1. Effect of furosemide infusion on serum concentration of ionized calcium in R53H (○) and R53R participants (●) on the low-sodium/high-potassium (LowNa/HighK) and the high-sodium/low-potassium (HighNa/LowK) diets. (Top) Mean ± SD. (Bottom) Individual values. There was a significant genotype × furosemide interaction (\( P = 0.0002 \)) in the ANOVA.]
theoretically can contribute to the lower Ca excretion as a result of a reduction in calcitriol synthesis (16), but this effect should be attenuated in this study because participants were studied in fasting condition and under a chronically low-Ca diet. The increase in renal Ca reabsorption on the LowNa/HighK diet occurred with no change in the ionized Ca concentration and was independent of the two main stimulatory Ca reabsorption hormones, which either remained unchanged (PTH) or were decreased (calcitriol).

Because TK is expressed mainly in the distal tubule (12) in which the epithelial Ca channel TRPV5 is expressed (19), we looked for differences in urinary Ca reabsorption between R53H and R53R participants, particularly when both Ca reabsorption (17,18) and UKLKa (10,11) were stimulated by the LowNa/HighK diet. We expected to find physiologic differences in distal Ca reabsorption because R53H participants had on average between 50 and 60% lower UKLKa levels than did R53R participants at baseline. However, we detected no significant difference between the genotypes for urinary Ca excretion and other Ca-related measurements (serum total and ionized Ca, PTH, and calcitriol concentrations) at baseline, even after 7 d of a controlled LowNa/HighK diet. This lack of significant difference between the two genotypes may be due to a low statistical power of the study for urinary Ca excretion. We alternatively suggest that if a defect in distal Ca reabsorption was present in R53H participants, then it may have been compensated for in the TAL of the tubule, which is the second site of tubular Ca reabsorption regulation.

We tested this hypothesis by studying Ca excretion after an infusion of furosemide, which functionally excludes the TAL by binding to the Na-K-Cl type 2 co-transporter (NKCC2). Furosemide thus blocks the Na-dependent Ca and Mg reabsorption at this site and leads to an increase in Ca delivery to distal segments (20). A distal tubular defect in Ca reabsorption therefore could be revealed by furosemide. Indeed, the main result of our study is that R53H normal volunteers had significantly lower serum ionized Ca concentrations after a 2-h furosemide infusion than did R53R participants (genotype x furosemide interaction: \( P = 0.0002 \)). This effect was observed twice (i.e., on both diets) with a similar magnitude. Seeming discrepancy between total and ionized Ca concentration variations during furosemide infusion can be explained by increase in protein concentration (and thus in Ca bound) as a result of Na depletion that counteracts the decrease in free Ca concentration. These balanced effects can explain (1) the smaller decrease in total Ca concentration than in ionized Ca concentration under the LowNa/HighK diet and (2) the constancy in total Ca concentration under the HighNa/LowK diet.

Because the daily Ca intake during our study was low, the serum concentration of ionized Ca is a direct consequence of both renal Ca reabsorption and net bone resorption. In this condition, variations in Ca excretion after an acute furosemide infusion should reflect variations in renal Ca reabsorption. If we assume that the net bone resorption was similar between the two genotypes, then we would expect a low serum ionized Ca concentration in R53H participants to be associated with a high urinary Ca excretion.

We found that postfurosemide urinary Ca excretion was slightly higher in R53H participants than in R53R participants \(( P = 0.14)\) and was more marked on the LowNa/HighK diet (+11%) than on the HighNa/LowK diet (+3%). This could suggest that adaptation to LowNa/HighK diet may depend partly on distal TK activity stimulation. The influence of the R53H polymorphism on urinary Ca excretion, however, did not reach statistical significance \(( P = 0.14)\). The lack of statistically significant detectable hypercalciuria in R53H participants may be due to (1) the low statistical power of the study for urinary Ca excretion and (2) the large between-subject variability in postfurosemide calciuria in each genotype group, which is much larger than that of serum ionized Ca concentration. We calculated that we would have needed 60 participants per genotype to achieve statistical significance with a between-genotype difference in postfurosemide urinary Ca excretion of 13 \( \mu \)mol/L\( _{\text{Ca}} \), a SD of 25 \( \mu \)mol/L\( _{\text{Ca}} \), and an \( \alpha \) risk of 5% and a \( \beta \) risk of 20%. Volume contraction as a result of a low-Na diet and furosemide infusion might have affected Ca excretion by stimulating Na and Ca proximal reabsorption and consequently Na and Ca delivery to Henle’s loop. This effect plays a major role in hypocalciuric effect of thiazides (21). However, there was no effect of genotype on Na excretion in our study, which suggests that an effect of genotype on Ca reabsorption, if present, occurs in a segment where Na and Ca reabsorption can be regulated independently. In addition, as noticed above, the tendency toward higher Ca excretion in R53H participants was observed only under furosemide infusion and not at baseline.

**Conclusion**

Overall, our results suggest an increased Ca reabsorption in the TAL under baseline conditions that counteracts a defect in distal tubule Ca reabsorption in individuals with constitutively low UKLKa levels. As with all genetic association studies, our data do not allow us to conclude that the abnormal Ca regulation in R53H individuals is linked causally to the kallikrein mutation. However, in our study, the causality is supported further by the inactivating effect of the polymorphism on the catalytic activity of kallikrein and the observation that mice with an inactivated TK gene have a defect in renal Ca handling (6). Investigation of homozygous H53H individuals would help to address further the role of TK in Ca metabolism, but such homozygous individuals are rare (approximately 3 to 4 per 1000 in the white population). Our study of partially TK-deficient individuals provides for the first time data suggesting that TK is an intrarenal modulator of renal Ca reabsorption in humans.

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Disclosures
None.

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