CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Vitamin D and Calcium Supplementation Accelerates Randall's Plaque Formation in a Murine Model

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Most kidney stones are made of calcium oxalate crystals. Randall's plaque, an apatite deposit at the tip of the renal papilla, is considered to at the origin of these stones. Hypercalciuria may promote Randall's plaque formation and growth. We analyzed whether long-term exposure of *Abcc6*^{-/-} mice (a murine model of Randall's plaque) to vitamin D supplementation, with or without a calcium-rich diet, would accelerate the formation of Randall's plaque. Eight groups of mice (including *Abcc6*^{-/-} and wild type) received vitamin D alone (100,000 UI/kg every 2 weeks), a calcium-enriched diet alone (calcium gluconate 2 g/L in drinking water), both vitamin D supplementation and a calcium-rich diet, or a standard diet (controls) for 6 months. Kidney calcifications were assessed by 3-dimensional microcomputed tomography, μ -Fourier transform infrared spectroscopy, field emission-scanning electron microscopy, transmission electron microscopy, and Yasue staining. At 6 months, *Abcc6*^{-/-} mice exposed to vitamin D and calcium supplementation developed massive Randall's plaque when compared with control *Abcc6*^{-/-} mice ($P < 0.01$). Wild-type animals did not develop significant calcifications when exposed to vitamin D. Combined administration of vitamin D and calcium significantly accelerates Randall's plaque formation in a murine model. This original model raises concerns about the cumulative risk of vitamin D supplementation and calcium intakes in Randall's plaque formation. (*Am J Pathol* 2019, 189: 2171–2180; <https://doi.org/10.1016/j.ajpath.2019.07.013>)

Renal stone incidence is increasing, currently affecting almost 10% of the population.^{1,2} More than two thirds of all kidney stones are made of calcium oxalate.^{3,4} Alexander Randall⁵ hypothesized more than 80 years ago that apatite (calcium phosphate) deposits at the tip of the renal papilla would initiate stone formation. Evan et al⁶ reported that these “Randall's plaque” begin in basement membranes of thin loops of Henle. The same group reported that increased urine calcium excretion could promote Randall's plaque formation.⁷ Stoller et al⁸ and Verrier et al⁹ showed that Randall's plaque also could

originate from vasa recta, capillaries surrounding renal tubules, in the deepest part of the papilla. These observations highlight that calcium phosphate supersaturation, predicted to

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Table 1 Serum Biological Parameters

Serum	WT				<i>Abcc6</i> ^{-/-}			
	Control	Calcium	Vitamin D	Calcium + vitamin D	Control	Calcium	Vitamin D	Calcium + vitamin D
Urea, mmol/L	9.34 ± 0.55	10.78 ± 0.51	9.40 ± 0.21	10.12 ± 0.43	10.67 ± 1.80	9.59 ± 0.37	8.72 ± 0.59	8.32 ± 0.38
Calcium, mmol/L	2.51 ± 0.1	2.53 ± 0.07	2.23 ± 0.08	2.49 ± 0.11	2.49 ± 0.06	2.65 ± 0.15	2.5 ± 0.06	2.4 ± 0.1
Phosphate, mmol/L	1.83 ± 0.06	2.16 ± 0.39	1.83 ± 0.18	2.38 ± 0.25	2.73 ± 0.41	1.94 ± 0.08	2.29 ± 0.14	2.29 ± 0.08
Vitamin D, ng/mL	82.5 ± 6.6	87.6 ± 6.9	123.4 ± 6.9*	144.8 ± 14.5*	88.9 ± 6.9	74.9 ± 3.0	124.4 ± 5.8*	114 ± 5.9*

**P* < 0.05 versus control and calcium group.

WT, wild-type.

be increased around the vasa recta in the papilla, would promote Randall's plaque formation.¹⁰

Several recent studies and meta-analyses have highlighted that vitamin D and calcium supplementation may increase urine calcium excretion and stone formation, especially in individuals with hypercalciuria.^{11,12} A recent randomized controlled trial showed that the recommended upper serum level of vitamin D, when associated with calcium supplements, frequently results in hypercalciuria.¹³ Vitamin D, with or without calcium supplements, is prescribed to prevent bone

mass loss, although randomized and controlled studies have failed to show a protective role of vitamin D against fractures.^{14,15} A few reports have suggested that kidney stone formers with Randall's plaques may have an increased biological response to vitamin D, but there is little evidence that vitamin D promotes or aggravates Randall's plaque formation to date.¹⁶

The combined administration of high calcium intakes and/or vitamin D has been tested in rats, leading to kidney stone formation, defining a model of kidney stone disease.¹⁷

Table 2 Urine Biological Parameters

Urine	WT				<i>Abcc6</i> ^{-/-}			
	Control	Calcium	Vitamin D	Calcium + vitamin D	Control	Calcium	Vitamin D	Calcium + vitamin D
At baseline								
Calcium/creatinine mmol/mmol	0.97 ± 0.23	0.66 ± 0.09	0.59 ± 0.06	0.71 ± 0.06	1.06 ± 0.15	1.02 ± 0.11	0.99 ± 0.2	1.23 ± 0.31
Phosphate/creatinine mmol/mmol	15.5 ± 3.3	16.5 ± 1.5	19.6 ± 2.2	24 ± 2.5	23.2 ± 4.6	14.7 ± 1.3	17.4 ± 1.1	20.3 ± 3.2
Pyrophosphate/creatinine μmol/mmol	1.89 ± 0.32	3.84 ± 0.79	2.71 ± 0.46	2.96 ± 0.57	1.82 ± 0.3	1.85 ± 0.4	1.86 ± 0.15	1.42 ± 0.42
At 3 months								
Calcium/creatinine mmol/mmol	0.87 ± 0.08	0.70 ± 0.11	0.94 ± 0.11	0.85 ± 0.09	0.84 ± 0.09	0.89 ± 0.19	1.08 ± 0.13	1.06 ± 0.15
Phosphate/creatinine mmol/mmol	14.6 ± 1.6	15.2 ± 1.5	16.9 ± 1.8	12.6 ± 0.5	16.1 ± 0.8	9.3 ± 1.5	13.7 ± 2.1	17.1 ± 1.8
Pyrophosphate/creatinine μmol/mmol	2.8 ± 0.36	3.25 ± 0.19	2.62 ± 0.3	2.76 ± 0.26	1.83 ± 0.36	1.9 ± 0.41	1.8 ± 0.29	2.68 ± 0.66
At 6 months								
Calcium/creatinine mmol/mmol	0.77 ± 0.17	0.71 ± 0.13	1.03 ± 0.24	0.91 ± 0.12	0.76 ± 0.11	0.63 ± 0.03	1.06 ± 0.1	0.93 ± 0.06
Phosphate/creatinine mmol/mmol	14.3 ± 0.2	13.1 ± 1	17.8 ± 1.9	18.8 ± 2.1	16 ± 0.9	13.3 ± 1.1	13.2 ± 0.9	14.8 ± 0.7
Pyrophosphate/creatinine μmol/mmol	3.92 ± 0.43	3.26 ± 0.47	3.3 ± 0.28	3.17 ± 0.58	2.68 ± 0.43	3.01 ± 0.41	1.83 ± 0.18	2.32 ± 0.5

WT, wild-type.

However, rats did not develop tissue calcifications mimicking Randall's plaques.

Recently, patients affected by pseudoxanthoma elasticum (PXE) have been described as affected frequently by kidney stones. PXE is a genetic (autosomal-recessive) disease caused by mutations in the *ABCC6* gene. PXE patients are affected by vascular (arterial), retinal, and skin calcifications.^{18,19} ATP binding cassette subfamily C member 6 (ABCC6) is a transporter expressed in the liver that facilitates the release of extracellular ATP, rapidly converted to inorganic pyrophosphate by ectonucleotide pyrophosphatase phosphodiesterase-1 in vessels. ABCC6 and ectonucleotide pyrophosphatase phosphodiesterase-1 are actually the main source of systemic pyrophosphate, an inhibitor of calcification limiting ectopic hydroxyapatite crystalline deposits.²⁰ Patients with PXE, as well as *Abcc6*^{-/-} mice, have reduced plasma pyrophosphate level, which explains their mineralization disorder.^{21,22} PXE patients develop massive Randall's plaques and are prone to develop kidney stones.²³ Interestingly, kidneys from *Abcc6*^{-/-} mice develop interstitial calcifications with aging (especially after 1 year), surrounding the loop of Henle and the vasa recta, made of calcium phosphate, and developed in the deepest part of the renal papilla, all features of Randall's plaques.²³ Taken together, these observations suggest that pyrophosphate deficiency is a critical determinant of Randall's plaque formation, by increasing calcium phosphate supersaturation in renal papilla.²³

Because *Abcc6*^{-/-} mice represent a unique model of Randall's plaque, we hypothesized that calcium and/or vitamin D supplementation would accelerate Randall's plaque formation in young *Abcc6*^{-/-} animals before the spontaneous development of these papillary calcifications.

Material and Methods

Animal Studies

Mice knock-out for the *Abcc6* gene usually are named *Abcc6*^{tm1Aabb}. They were generated on a 129/Ola background and then backcrossed into a C57Bl/6J more than 10 times in the Netherlands Ophthalmic Research Institute (Amsterdam, the Netherlands). These mice are designated *Abcc6*^{-/-} in the current article.

Forty-eight 10-week-old female mice were housed and bred at the INSERM Unité Mixte de Recherche S 1155 Mouse Facility, with a 12-hour dark/light cycle. Mice received standard chow, containing 1000 IU vitamin D3/kg and 1.05% calcium *ad libitum*. Six *Abcc6*^{+/+} [wild type (WT)] and six *Abcc6*^{-/-} mice had free access to water containing 80 mg/L of calcium (control group). Six WT and six *Abcc6*^{-/-} mice had free access to water containing 2 g of calcium chloride (calcium group). Considering that a mouse's daily drinking intake is approximately 5 mL/day (25 g C57Bl/6J), the daily calcium intake was 10 mg/mouse per day or 0.4 mg/g per day in the calcium groups. Six WT and six *Abcc6*^{-/-} mice received vitamin D (2500 IU ergocalciferol/mouse,

Sterogyl 15H; DB Pharma, La Varenne Saint-Hilaire, France) every 2 weeks (eg, 2.5 IU/g every 2 weeks) for 6 months by s.c. injection, and had free access to water containing 80 mg/L of calcium (vitamin D group). Six WT and six *Abcc6*^{-/-} mice received similar vitamin D injections and had free access to water containing 2 g/L calcium (calcium + vitamin D group).

All studies were performed in accordance with the European Union and NIH guidelines (Comité d'Ethique en Experimentation Charles Darwin C2EA-05). The project was authorized by the Health Ministry and local Ethics Committee (authorization 11420 2017092015335292).

Biological Samples and Biochemistry

Urine was collected in metabolic cages. Mice had free access to water (calcium-enriched or not) before the first administration of vitamin D and at 3 and 6 months. Mice were sacrificed 14 days after the last injection of vitamin D and blood was collected at that time.

Several parameters have been measured in urine: diuresis volume and calcium, phosphate, pyrophosphate, and creatinine concentrations. The blood samples have been analyzed for calcium (total), phosphate, urea, and vitamin D (at 6 months).

Urinary creatinine, serum urea, and phosphate levels have been analyzed on IDS-iSYS automat (Immunodiagnostic Systems Holdings PLC, Pouilly-en-Auxois, France). Calcium serum and urinary levels were measured with the PerkinElmer 3300 atomic absorption spectrometer (Perkin-Elmer, Villebon-sur-Yvette, France). Vitamin D serum levels were measured by the IDS-iSYS 25-hydroxy vitamin D immunoassay (IS-2700S; Immunodiagnostic Systems Holdings PLC) to assess 25-hydroxy vitamin D (D2 and

Figure 1 Urinary pyrophosphate excretion at baseline and at 3 and 6 months. Urine pyrophosphate excretion, indexed to urine creatinine ($\mu\text{mol}/\text{mmol}$), is decreased significantly in *Abcc6*^{-/-} mice in comparison with wild-type (WT) animals. $n = 24$ animals per group. Data are expressed as means $\pm$ SEM. * $P < 0.05$ (U-test). PPI, inorganic pyrophosphate.

Figure 2 Renal calcification assessment by X-ray tomography. Micro-computed tomography analyses allow the identification of kidney calcifications. The global volume of the calcifications (in red) has been assessed after three-dimensional kidney reconstruction. Wild-type (WT) mice do not develop significant calcifications (**A–D**), whereas *Abcc6*^{−/−} mice develop calcifications (**E–H**), especially at the tip of the papilla.

D3) levels. Pyrophosphate urine levels were measured using an ATP sulfurylase (M0394; New England Biolabs, Évry, France) to convert pyrophosphate into ATP in the presence of excess adenosine 5′phosphosulfate (A5508; Sigma-Aldrich, Sain-Quentin Fallavier, France). Generated ATP then was quantified using the ATP Determination Kit (ATPlite 6016941; PerkinElmer).

X-Ray Microtomography and Three-Dimensional Modeling

Left kidneys were fixed in formaldehyde and embedded in paraffin. X-ray computed tomography imaging was performed using a Skyscan 1272 (Bruker, Anvers, Belgium) at the Lariboisière Hospital imaging platform (Paris, France). A 6-μm resolution scale was obtained. Shadow images were obtained using an X-ray energy of 65 kV and 150 mA without filter exposition. The angular step between image acquisitions was 0.5°, and each image was averaged after 2 frames. Data

were reconstructed using Nrecon software (Bruker), and then exported into a 16-bit Tag Image File Format stack of virtual slices. The Mimics Innovation suite 20.0 (Materialise, Leuven, Belgium) was used for the quantification analysis of the papilla’s calcification volume, and three-dimensional modeling of Randall’s plaques and kidneys.

Histology and Yasue Staining

Kidney tissues were fixed in formaldehyde and embedded in paraffin. Four-micron tissue sections were performed and stained by the Yasue procedure to show tissue calcifications.

Field Emission Scanning Electron Microscopy

A Zeiss SUPRA55-VP Field Emission Scanning Electron Microscope (Zeiss France, Marly-le-Roi, France) was used to study tissue sections (4 μm), at low voltage (1.4 KeV).

Figure 3 Renal calcification global volume. The global volume of the calcifications was quantified (μm^3) after three-dimensional kidney reconstruction. The global renal calcification volume was increased significantly in *Abcc6*^{-/-} mice in comparison with wild-type (WT) animals, but there was no significant difference among *Abcc6*^{-/-} mice subgroups. Data are expressed as means $\pm$ SEM. $n = 6$ per group.

Transmission Electron Microscopy

Right renal papilla were fixed in 2.5% glutaraldehyde in 0.1 mmol/L cacodylate buffer (pH 7.4) at 4°C. Fragments were fixed in 1% osmium tetroxide, dehydrated using alcohol series, and then embedded in epoxy resin. Semithin sections (0.5 μm) were stained using toluidine blue. Ultrastructure sections (80 nm) were contrast-enhanced using UranylLess

Figure 4 Papillary calcifications. The volume of the calcifications was quantified (μm^3) in each papilla after three-dimensional kidney reconstruction. Global papillary calcification volume was increased significantly in *Abcc6*^{-/-} mice exposed to both calcium and vitamin D versus *Abcc6*^{-/-} control mice. Data are expressed as means $\pm$ SEM. $n = 6$ animals per group. * $P < 0.05$ (*U*-test). WT, wild type.

staining (Delta Microscopies, Mauressac, France). A JEOL 1010 electron microscope (JEOL, Ltd., Tokyo, Japan) with a MegaView III camera (Olympus Soft Imaging Systems GmbH, Munster, Germany) was used to analyze tissues.

μ Fourier Transform InfraRed Spectroscopy

Microcalcifications were characterized using μ -Fourier transform infrared (FT-IR) spectrometry on 4- μm tissue sections deposited on low-emission microscope slides (MirrIR; Keveley Technologies, Tienta Sciences, Indianapolis, IN). FT-IR hyperspectral images were analyzed with a Spectrum spotlight 400 FT-IR imaging system (PerkinElmer), at a spatial resolution of 6.25 μm and a spectral resolution of 8 cm^{-1} . The spectra were recorded in the 4000 to 700 cm^{-1} mid-infrared range.

Statistical Analyses

Data are expressed as means $\pm$ SEM. Data were analyzed with nonparametric tests (*U*-test), using Statview (SAS Institute, Atlanta, GA) and GraphPad Prism software version 7 (GraphPad Software, Inc., San Diego, CA). The level of significance was set at $P < 0.05$.

Results

Serum Biochemistry

No significant difference in serum calcium, phosphate, or urea levels was observed between the different groups at 6 months (Table 1). An expected but limited increase in vitamin D serum levels (approximately $1.5 \times$ control serum levels) was observed in the groups receiving vitamin D (ergocalciferol) supplementation (Table 1).

Urine Biochemistry

Urine calcium, phosphate, and pyrophosphate excretion was assessed before treatment, and at 3 and 6 months. No significant difference in urine calcium excretion was observed between WT and *Abcc6*^{-/-} mice, although there was a systematic trend toward increased urine calcium excretion at 3 and 6 months in animals exposed to vitamin D $\pm$ calcium (Table 2). When taken together (considering all *Abcc6*^{-/-} and WT mice at each time), *Abcc6*^{-/-} mice had a significantly lower urinary excretion of pyrophosphate in comparison with WT animals at each time point (Figure 1) ($n = 24$ per group; $P < 0.01$). When considering subgroups of *Abcc6*^{-/-} and WT mice, the difference in urine pyrophosphate was inconsistently significant because of the low number of animals ($n = 6$ per group) (Table 2).

Figure 5 Characterization of papillary calcifications by Yasue staining. Yasue staining shows intratubular and interstitial calcifications predominating in the renal papilla. **A–D:** WT mice develop very sparse and rare tubular plugs, even after exposure to calcium and vitamin D. **E–H:** By contrast, *Abcc6*^{−/−} mice develop papillary calcifications, tubular plugs, and interstitial calcifications, mimicking the human Randall's plaque. **G** and **H:** The exposure to vitamin D, especially in addition to calcium, increases interstitial calcifications. Scale bars: 200 μ m. Original magnification: $\times 100$. WT, wild-type.

Figure 6 Characterization of papillary calcifications by transmission electron microscopy. Transmission electron microscopy shows that the interstitial deposits observed in *Abcc6*^{-/-} mice exposed to vitamin D are a round-shaped, concentric, and electron-dense structure, surrounding the vasa recta and loop of Henle, with features similar to the incipient Randall's plaque observed in humans. **Boxed areas** in the panels in the **left column** are shown at higher magnification in the panels in the **right column**. Scale bars: 1 μ m.

Quantification of Renal Calcification at Six Months

Micro-computed tomography analyses were performed to assess global kidney calcifications (Figures 2 and 3). *Abcc6*^{-/-} mice had an increased calcification volume ($1,443,548 \pm 174,767 \mu\text{m}^3$) in comparison with control mice ($198,145 \pm 42,226 \mu\text{m}^3$, $n = 24$ per group; $P < 0.01$). In subgroup analysis, there was a nonsignificant trend toward an increased renal calcification volume in *Abcc6*^{-/-} mice exposed to calcium and vitamin D ($1,939,003 \pm 484,442 \mu\text{m}^3$) when compared with *Abcc6*^{-/-} control mice ($1,051,010 \pm 166,189 \text{ sem } \mu\text{m}^3$, $n = 6$ per group) (Figure 3).

Micro-computed tomography analyses focused on the kidney papilla were performed next (Figures 2 and 4). WT animals did not develop significant papillary calcifications, even after 6 months' exposure to both calcium and vitamin D (Figure 4). *Abcc6*^{-/-} mice had an increased papillary calcification volume ($434,910 \pm 77,943 \mu\text{m}^3$) in comparison with control mice ($62,722 \pm 23,811 \mu\text{m}^3$, $n = 24$ per group; $P < 0.01$). In subgroup analysis, there was significantly increased calcification volume in the renal papilla of *Abcc6*^{-/-} mice exposed to calcium and vitamin D ($892,410 \pm 172,006 \mu\text{m}^3$) when compared with *Abcc6*^{-/-} control mice ($248,937 \pm 101,734 \mu\text{m}^3$, $n = 6$ per group; $P < 0.01$) (Figure 4).

Histopathologic Analysis and Transmission Electron Microscopy

Yasue staining showed the presence of rare tubular calcifications (tubular plugs) in WT animals and massive tubular and interstitial calcifications in the renal papilla in *Abcc6*^{-/-} mice, similar to human Randall's plaque (Figure 5). Of note, vitamin D and calcium supplementation dramatically increased the interstitial papillary calcified surface in *Abcc6*^{-/-} mice (Figure 5). Electron microscopy showed the massive presence of round-shaped, electron-dense structures in the interstitial tissue, around the vasa recta and the loop of Henle, especially in *Abcc6*^{-/-} mice exposed to calcium and vitamin D (Figure 6).

Field Emission Scanning Electron Microscopy and μ FT-IR Spectroscopy

Field emission scanning electron microscopy has shown that tubular plugs observed in *Abcc6*^{-/-} mice exposed to vitamin D and calcium were made of aggregated spherulites (Figure 7A) and showed to a lesser extent the presence of interstitial spherulites embedded in the renal tissue and corresponding to interstitial calcifications (Figure 7A). To go further in the characterization of the crystalline phases, μ FT-IR spectroscopy was performed

Figure 7 Characterization of papillary calcifications by field emission scanning electron microscopy images and Fourier transform infrared spectroscopy. **A:** Field emission scanning electron microscopy images show tubular plug structure (**black arrow**), and, to a lesser extent, interstitial deposits (**white arrows**). Calcifications are made of spherulite aggregates, a typical feature of calcium phosphate (apatite) deposits. **B:** These deposits are made of apatite and, to a lower extent, amorphous carbonated calcium phosphate as evidenced by Fourier transform infrared spectroscopic imaging analyses (typical spectrum of a mixture of apatite and amorphous calcium phosphate and a Fourier transform infrared microspectroscopy hyperspectral image of a papilla from an *Abcc6*^{-/-} exposed to calcium and vitamin D).

with an imaging system. The analysis of the absorption spectrum and its second derivative showed some features specific for the presence of different absorption bands of the apatite [Ca₅(PO₄)₃(OH)], including the ν_3 phosphate–oxygen stretching vibration mode measured at 1035 to 1045 cm⁻¹ (Figure 7B). Carbonate ions were detected together with apatite by their ν_3 carbon–oxygen stretching vibration mode at around 1420 cm⁻¹ and the ν_2 carbon–oxygen bending mode at 875 cm⁻¹. The presence of amorphous calcium phosphate, shown by the partial disappearance of the shoulder of the ν_3

phosphate–oxygen absorption band of apatite, has been observed in both interstitial deposits and intratubular calcifications (Figure 7B).

Discussion

The long-term administration of vitamin D, especially in addition to calcium supplementation, dramatically accelerates Randall's plaque formation in *Abcc6*^{-/-} mice. These 8-month-old animals were affected by massive papillary calcifications whereas unexposed control *Abcc6*^{-/-} mice

developed tiny interstitial calcifications and tubular plugs. In parallel, wild-type mice exposed to vitamin D, with or without calcium supplementation, developed very sparse tubular calcifications and no Randall's plaque. *Abcc6*^{-/-} had significantly decreased urinary pyrophosphate levels but calcium and phosphate urine (and serum) levels were similar in WT and *Abcc6*^{-/-} mice. In contrast to our seminal observations, no difference in phosphate metabolism was seen between WT and *Abcc6*^{-/-} mice.²³ Mice exposed to both calcium and vitamin D had a nonsignificant trend toward higher urine calcium excretion, despite high doses of calcium and vitamin D, in comparison with doses used in humans in clinical practice. It seems difficult to compare calcium homeostasis in mice and humans, but the modest increase in vitamin D serum levels, the nonsignificant increase in urine calcium excretion, and the absence of hypercalcemia in animals exposed to calcium and vitamin D rules out the hypothesis of an intoxication. Nevertheless, in mice affected by pyrophosphate deficiency, even a mild increase in urine calcium excretion was sufficient to accelerate the papillary calcification process.

Interestingly, Sprague-Dawley rats exposed to high calcium intake did not develop renal calcifications, and rats exposed to vitamin D alone developed hypercalciuria but only tiny calcium phosphate kidney stones.¹⁷ By contrast, the synergistic administration of calcium and vitamin D promoted the development of large stones but no Randall's plaque. The genetic hypercalciuric stone-forming rat has increased expression of the vitamin D receptor in intestine, bone, and kidney.²⁴ These animals develop calcium phosphate kidney stones, however, again, no Randall's plaque. It appears therefore that vitamin D, especially in combination with calcium, is not sufficient to induce papillary calcifications, whose initiation may be owing to a calcification inhibitor defect, but dramatically accelerates the development of Randall's plaque in *Abcc6*^{-/-} mice.

Predisposed individuals may be at risk to form kidney stones when exposed to vitamin D, especially when vitamin D and calcium intakes are combined. In the Women's Health Initiative study, a controlled randomized study, 36,282 postmenopausal women received 1 g calcium and 400 IU vitamin D daily or a placebo during 7 years.¹¹ The treatment did not significantly reduce bone fractures, but an increased kidney stone risk has been described in women who received calcium and vitamin D. Recent studies confirmed that vitamin D, especially when prescribed in addition to calcium supplementation, increases urine calcium excretion significantly, and in some studies the risk to develop kidney stones.^{12,13} By contrast, some studies did not identify an increased risk of kidney stones after exposure to vitamin D alone (without calcium supplementation) during a median follow-up period of 3.3 years.²⁵ Interventional studies remain sparse, but recent observations have shown that some kidney stone formers may develop hypercalciuria after vitamin D "repletion."^{26,p.539,27} An important point to take into consideration is the time

required to develop Randall's plaque, which precedes stone formation. Actually, vitamin D increases urine calcium excretion, which may accelerate plaque formation and promote calcium oxalate stone formation several years later.⁷ The situation may have worsened during the past decades because of the increase in vitamin D prescriptions, although there is no evidence that vitamin D supplementation may protect against fractures or other conditions, including cancers, cardiac and vascular diseases, obesity, diabetes, depression, falls, infectious diseases, and maternal/perinatal conditions.²⁸ Interestingly, Ferraro et al²⁹ analyzed the association of vitamin D intake and the risk of developing kidney stones and found no association in the Health Professionals Follow-up Study and the Nurses' Health Study I, but found an increased risk in the more recent Nurses' Health Study II, which included women who received an increased amount of vitamin D supplements.

A synergistic effect of combined vitamin D/calcium supplementation was observed in experimental models, consistent with clinical studies. Of note, calcium intake in the absence of vitamin D did not increase the risk of Randall's plaque formation in the murine model or in humans: on the contrary, higher dietary calcium was associated with a lower risk of developing kidney stones.³⁰ This results from the calcium-oxalate complex formation in the intestine, which limits oxalate absorption and eventually calcium oxalate stone formation.

These results raise concerns about the prescription of vitamin D and calcium to patients affected by PXE. Massive Randall's plaque and frequent kidney stones have been observed in these patients and it may be hypothesized that a vitamin D prescription could accelerate plaque and stone formation in patients affected by pyrophosphate deficiency.²³ A deficiency in urine pyrophosphate has been reported in kidney stone formers, but the pyrophosphate level in urine is difficult to assess in routine practice. This probably explains why few studies have focused on the importance of pyrophosphate in kidney stone formation, and no study has highlighted its role in Randall's plaque formation until recently.³¹⁻³³

Most kidney stones are made of calcium oxalate and many of them originate from Randall's plaque. Some observations have suggested that patients affected by Randall's plaque could be more "sensitive" to vitamin D.¹⁶ The dramatic acceleration of papillary calcifications by the combined administration of vitamin D and calcium in *Abcc6*^{-/-} mice suggests that in some genetically predisposed individuals, combined calcium and vitamin D intake may accelerate Randall's plaque formation, and deserve specific clinical studies to address this concern.

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