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P. Winter

Urologische Universitätsklinik Bonn

A. Hesse

Experimentelle Urologie der Universitätsklinik Bonn

K. Klocke

Urologische Universitätsklinik Bonn

R. M. Schaefer

Urologische Universitätsklinik Bonn

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SCANNING ELECTRON MICROSCOPY OF 2,8-DIHYDROXYADENINE CRYSTALS AND STONES

P. Winter*, A. Hesse¹, K. Klocke, and R.M. Schaefer

Urologische Universitätsklinik Bonn, ¹Experimentelle Urologie der Universitätsklinik Bonn,
53105 Bonn, Federal Republic of Germany

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Abstract

The lack of purine salvage enzyme, adenine phosphoribosyltransferase (APRT), leads to 2,8-dihydroxyadenine stone formation and/or crystalluria because it is insoluble in urine. Urolithiasis composed of 2,8-dihydroxyadenine is not only formed in a complete defect of APRT, but also in a partial deficiency of this enzyme. The defect is inherited as an autosomal recessive trait, the homozygous state is associated with high urinary levels of 2,8-dihydroxyadenine and with crystalluria, calculus formation, and potential nephrotoxicity. Determination of the APRT activity will facilitate quantification of the enzyme deficiency and elucidation of the hereditary history. 2,8-dihydroxyadenine excretion in the 24-hour urine and its circadian rhythm were determined using a new method of high performance liquid chromatography determination. By means of a standard case presentation, we illustrate the analysis of urinary sediments and calculi as well as the scanning electron microscopic images of this kind of stone.

Key Words: 2,8-Dihydroxyadenine, stone analysis, urinary sediment, scanning electron microscopy.

*Address for correspondence:
Peter Winter
Urolog. Universitätsklinik Bonn
Sigmund-Freud Str. 25
53105 Bonn, FRG

Telephone Number: (0228) 280-2109
FAX Number: (0228) 280-3616

Introduction

In 1968, Kelly *et al.* described a formerly unknown disorder in purine metabolism caused by a deficiency of the enzyme adenine phosphoribosyltransferase (APRT) [8]. This defect is inherited as an autosomal recessive trait [1, 7].

The complete or partial lack of the salvage enzyme obstructs the conversion of adenine to adenosine monophosphate. The alternative metabolic route available for urinary excretion is the oxydation of the increased concentration of adenine to 8-hydroxyadenine (Fig. 1). The inadequate solubility of 2,8-dihydroxyadenine (2,8-DHA) in the normal pH-range of human urine (namely 4.8-8.0) leads to crystalluria and stone formation [12]. Homozygotes tend to develop urinary calculi in early childhood. As stone formation in children does not occur frequently, the possibility of 2,8-DHA stones should come to mind when X-ray negative stones are found in children [1, 6, 7, 13]. 2,8-DHA urolithiasis in homozygotes was first described in 1974 [2]. Since that time, 20 cases have been described in medical literature [9]. By means of a standard case presentation, we illustrate here the analysis of 2,8-DHA urinary sediments and calculi as well as the scanning electron microscopic (SEM) appearance of this kind of stone [11].

Materials and Methods

A 30-year-old male patient, who had produced X-ray negative calculi in his left kidney since infancy, lost the kidney at 8 years of age (no stone analysis). At the age of 22, first spontaneous excretion of stones from the remaining right kidney was observed. The first stone analysis by infrared (IR) spectroscopy was made after a further 3 years and showed 2,8-DHA. In 1987, 8 concrements in the right kidney were sonographically detected. The majority of the stones were removed percutaneously. The remaining concrements were passed spontaneously. The stone analysis by IR spectroscopy revealed also 2,8-DHA. There was no known family history of lithiasis.

Laboratory Investigations

Urinary sediment: Microscopic examination of

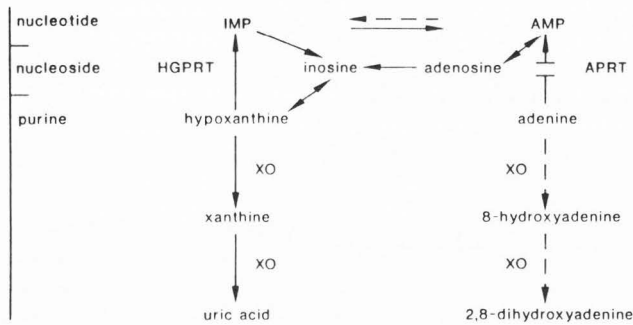


Figure 1. Diagram of purine metabolism. XO = xanthine oxidase, APRT = adenine phosphoribosyltransferase, HGPRT = Hypoxanthineguaninephosphoribosyltransferase, IMP = Inosinemonophosphat, AMP = Adeninemonophosphat.

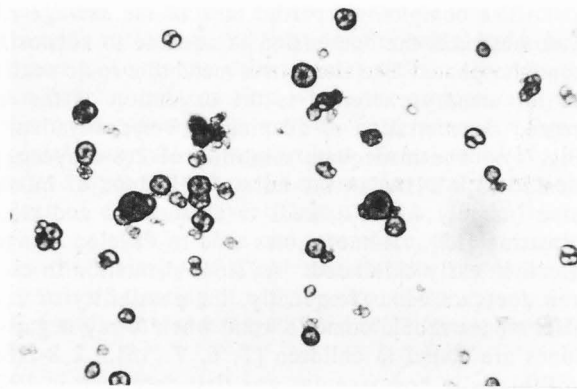


Figure 2. Microscopic examination of the crystalline urinary sediment containing 2,8-dihydroxyadenine spherical crystals in a radial corona. Bar = ??? μm .



Figure 3. Scanning electron micrograph of 2,8-dihydroxyadenine stone crystals in urinary sediment. Bar = 10 μm .

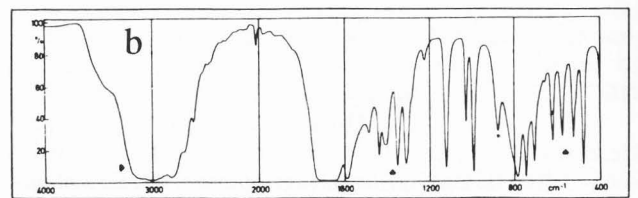
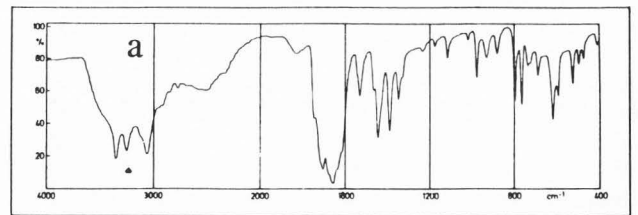


Figure 4. Infrared spectra of a 2,8-DHA (a) and uric acid (b). At wavelengths 3045, 3238 and 3338 cm^{-1} , there are characteristic absorptions which do not occur in uric acid.

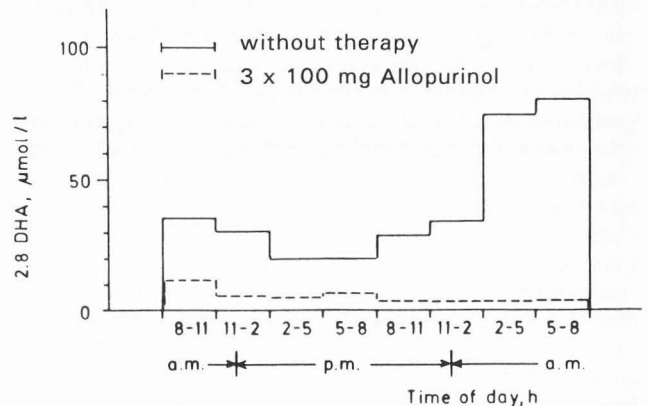


Figure 5. 2,8-DHA concentration in urine with (solid line) and without 3 x 100 mg allopurinol treatment (dotted line).

the crystalline urinary sediment was made after centrifugation. A large quantity of crystalline material was obtained for quantitative IR analysis. IR spectroscopic concrement analysis of the urinary sediment and the stone was performed using the KBr press process [14].

APRT activity: The activity of the enzyme was assayed in the erythrocyte hemolysat by a radiochemical technique [3]. Quantitative 2,8-DHA determination in the urine was performed using high performance liquid chromatography (HPLC) in conjunction with a separating system specially developed for purine [4].

SEM investigations: In order to study the morphology of the concrements, their surfaces, stone interspaces, fracture surfaces, and a commercially available synthetic test substance of 2,8-DHA were examined by scanning electron microscopy (SEM).

Results

In the presence of APRT deficiency, and dependent upon the scale of the enzyme deficiency, crystals were detected in the urine. Masses of crystals were always present; they were spherical in shape and brown-red in color. Under a light microscope, the crystalline arrangement was spherulitic (Figure 2). Under the SEM, nests of individual crystals were detected, some 10 μm in diameter, which also aggregated to form microliths (Figure 3).

2,8-DHA can only be detected using modern chemophysical methods of analysis [5, 10]. The chemical method will always produce a false result indicating uric acid. Infra-red spectroscopic analysis indicated that both urinary sediments and stones consisted of pure 2,8-dihydroxyadenine. The characteristic absorptions observed at wavelengths 3045, 3238 and 3338 cm^{-1} do not occur in any other type of urolith, e.g., uric acid (Figures 4a, 4b).

In order to obtain a clear picture of the extent of the enzyme deficiency, the lack of APRT activity in haemolysed erythrocytes must be determined. Caution is advisable if the patient has just received blood transfusions, which can lead to a temporary increase in APRT activity.

The determination of 2,8-DHA in urine was carried out by the HPLC method. In the 24-hour urines of healthy test persons, no 2,8-DHA could be traced. If 2,8-DHA is found, it can always be regarded as an indicator of APRT deficiency.

2,8-DHA excretion rates in our patient ranged between 125 and 160 $\mu\text{mol/l}$ (i.e., 245-414 μmol per 24 hours). Treatment with 300 mg allopurinol per day reduced the daily excretion to 40 $\mu\text{mol/day}$. The excretion of 2,8-DHA shows a characteristic day/night rhythm. Increased rates were registered at night and in the morning hours. The administration of the xanthinase inhibitor allopurinol reduced excretion rates to a constantly low level, while adenine concentration in urine increased (Figure 5).

SEM examination of the synthetic test material after fast recrystallization revealed plaque-shaped structures (Figure 6), and after slow recrystallization spherical, matted structures (Figure 7).

With increasing magnification, the 2,8-DHA stone shows a spherical surface, a felt-like surface and spherulite surface with matted needle-shaped crystals (Figures 8, 9, 10 and 11). The SEM examination of structures in stone interspaces revealed mostly needle-shaped crystals (Figure 12), although, sometimes lance-shaped crystals were also observed (Figure 13). After fragmentation of a 2,8-DHA stone and SEM examination of individual fragments, microliths with an irregular surface, and sometimes a fracture surface with peripheral regular crystals, were observed (Figures 14, 15, 16 and 17).

Discussion

Only the introduction of new methods of urinary stone analysis such as X-ray diffraction and IR spectroscopy have made it possible to identify 2,8-DHA calculi. There is no structural difference between 2,8-DHA and uric acid. Since there is still no way to heal this "inborn error of metabolism", recurrence of stone formation can only be prevented by strict life-long treatment.

The SEM has been instrumental in yielding valuable new data in the field of stone research. The variety of microscopic forms and the different structures of urine stones can be observed in details with the SEM. Whereas examinations have been carried out with the SEM on most types of urinary stones, an analysis of the 2,8-DHA stone, due to its extreme rarity, was lacking. This paper presents SEM analysis of the test substance after recrystallization, urinary sediment, stone surface, stone interfaces, and fracture surface of the 2,8-DHA stone. Once a 2,8-DHA stone has been diagnosed, the enzyme deficiency can be determined by measuring APRT activity in erythrocyte haemolysate. Making use of this relatively simple test, a possible hereditary factor may be determined in other family members.

The HPLC method permits a quantitative determination of 2,8-DHA excretion in urine. With this method, the monitoring of treatment is easy and reliable. In addition to allopurinol therapy, the dilution of urine throughout the day, sufficient fluid intake, and a low-purine diet are advised to eliminate 2,8-DHA excretion in urine.

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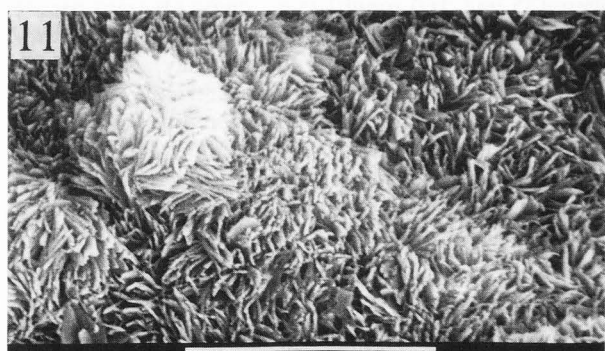
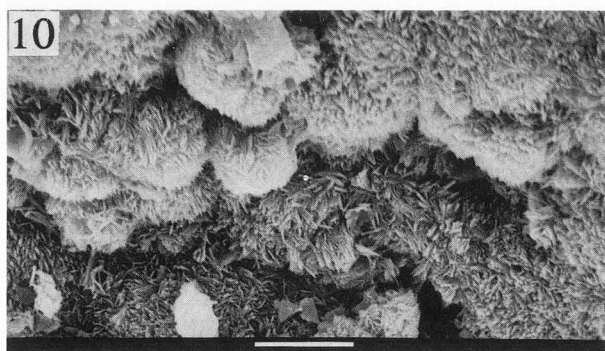
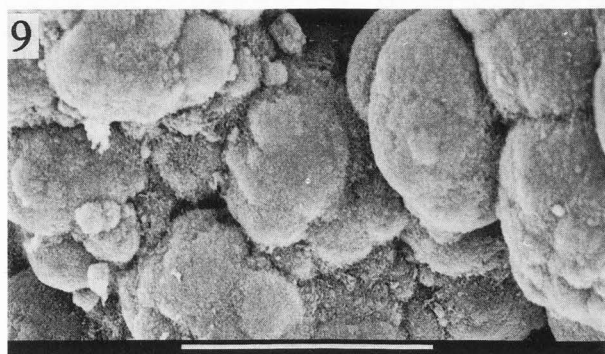
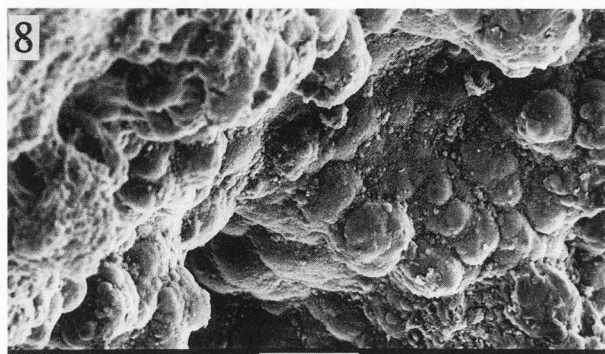
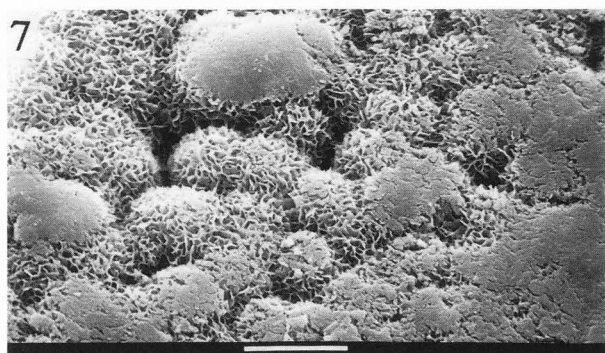
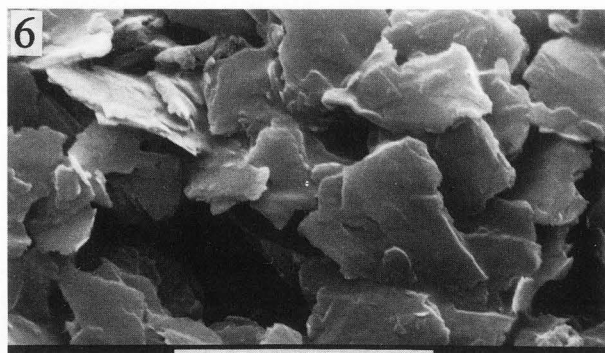


Figure 6. Synthetic test material. Plaque-shaped structure. Fast crystallisation. Bar = 10 μm .

Figure 7. Synthetic test material. Spherical, matted structure. Slow crystallisation. Bar = 10 μm .

Figure 8. Spherical surface. Bar = 100 μm .

Figure 9. Spherical, now felt-like surface. Bar = 100 μm .

Figure 10. Spherulite surface with matted needle-shaped crystals. Bar = 10 μm .

Figure 11. Similar structures with regular crystals. Bar = 10 μm .

2,8-Dihydroxyadenine crystals and stones

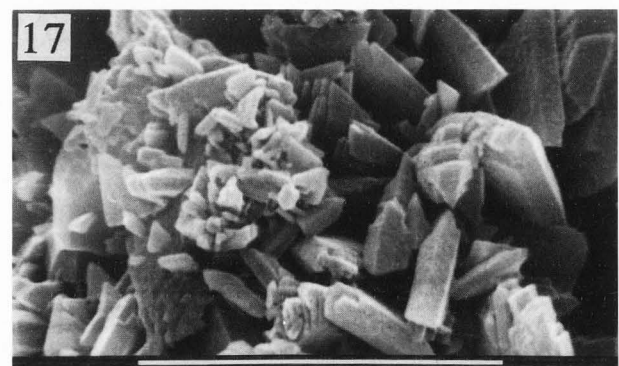
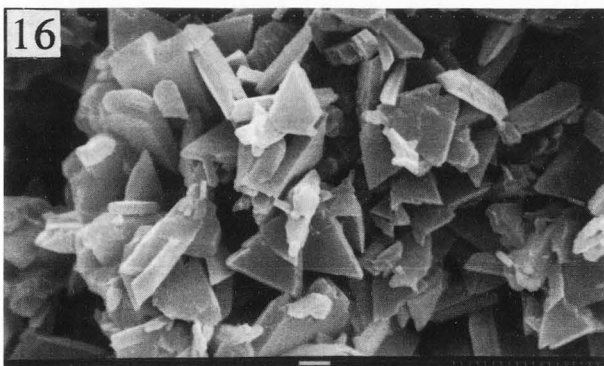
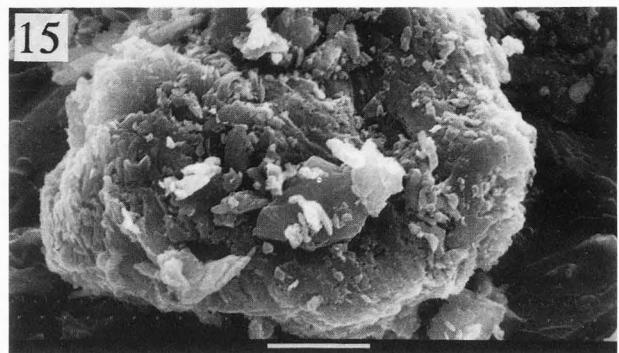
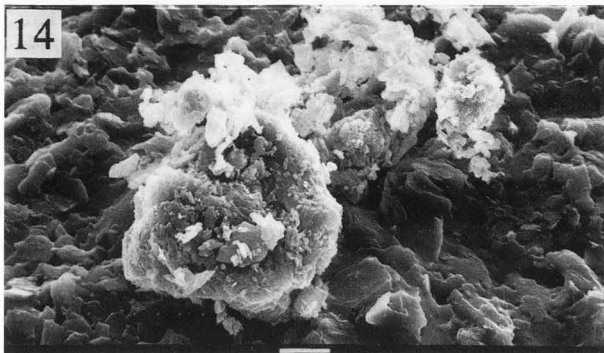
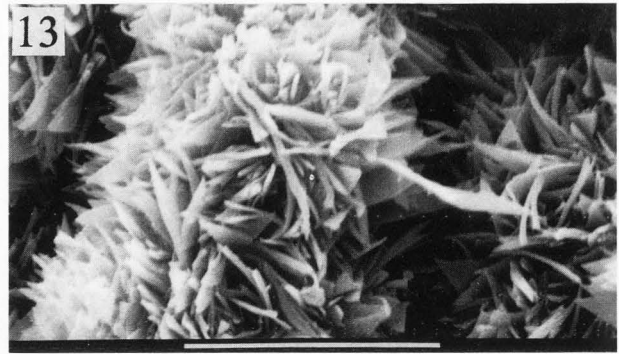
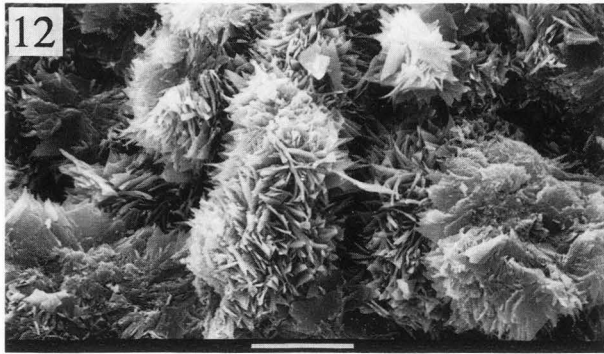


Figure 12. Needle-shaped crystals. Bar = 10 μm .

Figure 13. Lance-shaped crystals. Bar = 10 μm .

Figure 14. Microlith with irregular surface. Bar = 10 μm .

Figure 15. Doubled magnification shows a similar surface structure. Bar = 10 μm .

Figure 16. Fracture surface of a 2,8-DHA stone. Bar = 10 μm .

Figure 17. Fracture surface with peripheral regular crystals. Bar = 10 μm .

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Discussion with Reviewers

H. Iwata: Was dissolution carried out by heating or changing pH? Was the substance dissolved in pure water, buffer solution, and urine?

Authors: 2,8-DHA was obtained from Sigma (Article no. D7382). The substance was heated in pure water at neutral pH at 80°C. After filtration, one part was cooled down to room temperature (slow crystallization), and the other part in ice water (fast crystallization).

D.B. Leusmann: Is there a possibility to resolve 2,8-DHA stones either by oral medication (similar to uric acid stones) or by direct dissolution after pyelostomy and/or indwelling ureteral stents?

Authors: The dissolution of 2,8-DHA stones by oral medication is not possible. Solubility of 2,8-DHA stones in the pH-range of 4.8-8.0 varies between 3 and 5 mg/l. A considerable increase in solubility can be achieved at a pH-range less than 2 or more than 9.5; however, such extensive changes in human pH-values

cannot be achieved through oral medication. In any case, attempts to change the pH-value by means of percutaneous chemolitholysis will fail because of incompatible tissue tolerance; even if this problem was irrelevant, the many potential side effects of this kind of therapy would forbid its application.

S.R. Khan: What are the some of the other features associated with this disease?

Authors: Other features associated with APRT-deficiency on its own are not known. APRT-deficiency, when not treated, may lead to nephrocalcinosis with resultant renal failure.

A. Rogers: In the **Materials and Methods** section, you mention that the first stone analysis showed 2,8-DHA. What technique was used for this analysis?

Authors: We used infrared spectroscopy.

A. Rogers: Do you think that the brown red colour of the 2,8-DHA crystals is due to blood stains? Uric acid microliths are often red orange. Were the 2,8-DHA crystals similar in colour to uric acid crystals?

Authors: 2,8-DHA crystals of brown-red colour appeared in several patients and cannot be attributed to blood stains in our opinion. It is most likely that urine colouring matter may be adsorbed by 2,8-DHA, which causes this kind of stone to appear very similar to a uric acid stone.

A. Rogers: Figures 11, 12 and 13 show crystals which are very similar to the morphological features of sodium urate. Were no other stone components besides 2,8-DHA detected (e.g., uric acid and salts thereof)?

Authors: Analyses by IR proved 2,8-DHA stones to be extremely pure. Components of uric acid or salts thereof are rather unlikely.

A. Rogers: How sensitive are your IR measurements?

Authors: The sensitivity of IR spectroscopic measurements is dependent on the composition of substances investigated. All components exceeding 5% can be unmistakably determined.

A. Rogers: Did you consider verifying your results by subjecting the crystals to another analytical technique such as X-ray powder diffraction or mass spectrometry?

Authors: Results achieved through IR spectroscopy were confirmed by X-ray diffraction. Mass spectroscopy was not applied.

Editor: Am I correct in understanding that Figures 8 to 17 are from different stones collected from your 30 year old patient?

Authors: Yes; Figures 6 and 7 are scanning electron micrographs from synthetic crystals; while Figures 8 to 17 are from stones collected from the patient.