Calcium Oxalate Crystallization in Urine of Healthy Men and Women: A Comparative Study

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CALCIUM OXALATE CRYSTALLIZATION IN URINE OF HEALTHY MEN AND WOMEN: A COMPARATIVE STUDY

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Abstract

This study aimed to compare calcium oxalate (CaOx) crystallization in undiluted urine from healthy men and women with the object of clarifying the difference in stone incidence between the two sexes. Twenty-four hour urine specimens were collected from 37 men and 28 women. Urinary pH, and concentrations of Ca, oxalate and urate were measured, and indices of crystallization determined by Coulter Counter particle analysis following induction of CaOx crystallization by addition of oxalate. The amount of oxalate required to induce crystallization was significantly ($p < 0.01$) higher in females than in males, as was the overall particle volume deposited after 90 minutes incubation ($p < 0.006$). Scanning electron microscopy revealed larger individual crystals in female urine, and a greater degree of crystal aggregation in male urine, although the average overall size of the precipitated crystal particles did not differ between the two sexes. There were no significant differences between men and women with regard to median pH, or Ca and oxalate concentrations, but the median urate concentrations were slightly, but significantly, higher ($p < 0.05$) in the women's urines than in the men's. It was concluded that the greater risk of CaOx stones in men is related to an increased propensity to nucleate CaOx crystals *per se*, rather than to a tendency to form larger crystalline particles.

Key Words: Urolithiasis, gender differences, calcium oxalate crystallization, crystal growth, crystal aggregation.

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Introduction

Although an increase in the incidence of CaOx stone formation in women in developing countries has been reported to have occurred during the past three decades [2], for many years it has been generally accepted on the basis of extended epidemiological studies that the male:female ratio for the formation of non-infective urinary CaOx stones is approximately 2-3:1 [1, 21, 36]. The general predominance of these stones in males may perhaps be explained by sex-dependent differences in concentrations of substances that inhibit or promote lithogenesis in urine, which, in turn, may be regulated by sex hormones [30, 33]. A previous study in this laboratory [30] showed that female urine inhibits CaOx crystal aggregation more potently than male urine, whereas there was no difference in their respective effects on crystal growth. That study, however, tested the effects of the urine samples in an inorganic, aqueous crystallization system: the physiological relevance of results obtained from such systems have rightly come to be questioned, since it is widely acknowledged that they cannot reproduce the complex conditions operating in urine itself. Therefore, the aim of the current study was to study parameters of CaOx crystallization in undiluted urine of healthy men and women in order to determine whether the disparity in the incidence of stone formation between the two sexes can be explained by differences in the inhibitory effects of their urine on CaOx crystallization. The urinary pH and concentrations of calcium, oxalate and urate were also determined in order to relate any observed differences in crystallization parameters to factors known to influence CaOx crystallization.

Materials and Methods

Materials

All reagents were of analytical purity. All solutions were prepared with the highest quality water from a "Hi Pure" water purification system fitted with a 0.2 μm pore-size filter (Permutit Australia, Brookvale, NSW, Australia).
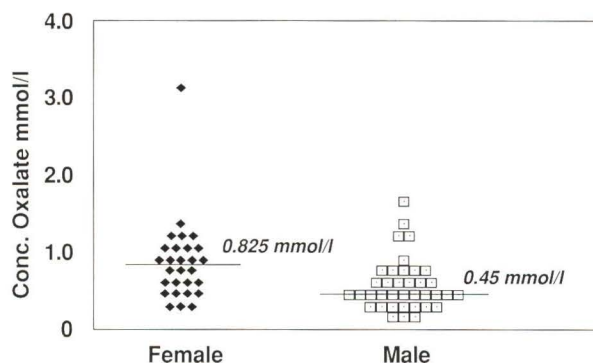
Figure 1. The minimum amount of oxalate required to induce detectable CaOx crystallization in 20 ml of urine of healthy women and men (metastable limit) in mmol/l. Horizontal lines indicate the median values.

Figure 2. The total particle volume 90 minutes after induction of crystallization in urines of healthy women and men. Horizontal lines indicate median values.

Subjects and urine collection

Twenty-four hour urine specimens were obtained from 28 healthy women (median age 26: range 17-62 years) and 37 healthy men (median age 34: range 18-62 years); the difference in age between the men and women was not significant. Urine samples were refrigerated during the collection period and during storage prior to use (always within 12 hours of completion of the collection), each specimen was tested to exclude the presence of blood using Multistix test strips (Miles Laboratories, Mulgrave, Victoria, Australia). Data reported in this study consisted of a combination of previous and recent work. Therefore, although complete crystallization data were available for all individuals, values for pH, and calcium, urate and oxalate concentrations were not available for all subjects.

Crystallization experiments

Urines were centrifuged at 20°C for 30 minutes at 10,000 x g in a J2-21M/E centrifuge (Beckman Instruments, Palo Alto, CA), and then filtered through 0.22 μm pore-size filters (GSWP04700; Millipore Corp.; Bedford, MA). The minimum amount of oxalate required to induce crystallization and the urinary inhibitory activity were then assessed by the oxalate load method of Ryall *et al.* [28], using a Coulter Counter model TAII fitted with a 70 μm orifice and Population Count accessory (Coulter Electronics Ltd, Herts., U.K.). The crystallization parameters measured were: (1) the metastable limit; defined as the minimum amount of oxalate necessary to induce detectable (> 2 μm) CaOx crystals in 20 ml of urine; (2) the total particle volume deposited at 90 minutes; and (3) the particle size, expressed as the peak of the volume distribution curve at 90 minutes.

Six estimates of each parameter were obtained for each urine and the mean of these values was used for comparative purposes. In order to compare the overall
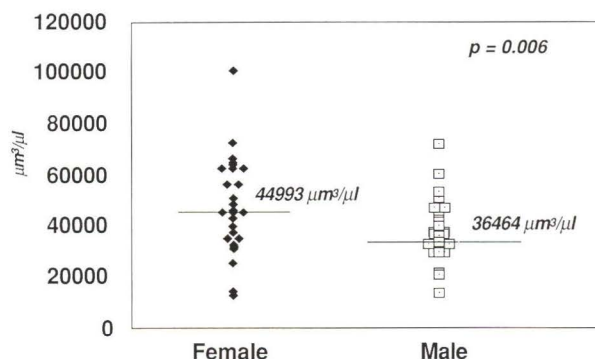
distribution of particle volume and size at 90 minutes, the volumes of particles falling within a certain channel of the Coulter Counter were combined for all the men and for all the women, and the median values plotted in relation to particle size. Increase in particle volume with time was expressed as the median value of all values obtained at each time interval, for men and for women. In order to test whether the crystal volume deposited was a function of the concentration of oxalate in solution at the commencement of the incubation period, the total oxalate concentration was calculated by adding the endogenous amount of oxalate to that used to induce CaOx crystallization.

Scanning electron microscopy (SEM)

At the end of seven of the experiments (3 females, 4 males), 2.0 ml aliquots of urine were filtered through 0.22 μm Millipore filters, and the filters were prepared for SEM according to the method used by Grover *et al.* [12]. The filtered crystals were then visualised by SEM, using an ETEC AutoScan electron microscope (Siemens AG, Stuttgart, Germany). To avoid bias, the operator was blinded with regard to the sex of the individual from whom the crystals had been obtained.

Analytical procedures

Analytical procedures were carried out on unprocessed, 24 hour whole urine samples with the exception of the urinary pH which was measured immediately before the beginning of the experiments in centrifuged and filtered (0.22 μm Millipore) urine, at room temperature, and using a glass electrode. Calcium was determined by atomic absorption spectroscopy, oxalate by the technique of Mazzachi *et al.* [20], and urate by a standard autoanalyser technique.

Statistics

Values were compared using the Mann-Whitney-U test. A p-value < 0.05 was regarded as statistically

Table 1. Urinary pH and concentrations of calcium, oxalate and urate in the urine samples from the men and women, and their daily calcium excretion. Values are expressed as the median and range. Numbers in parentheses denote the number of values available for calculation.

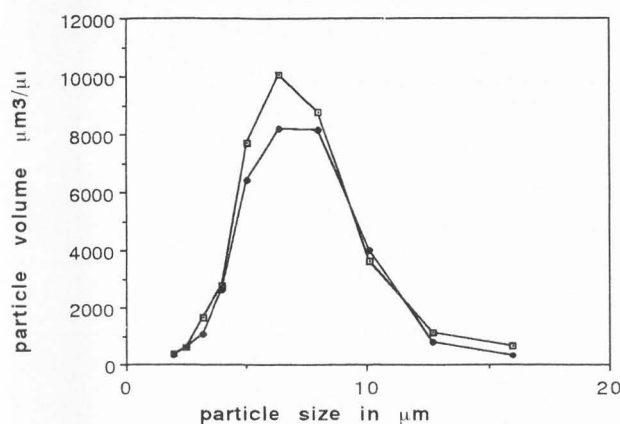|		men	women	significance
pH		6.24: 5.55-6.82 (n = 13)	6.21: 5.04-7.50 (n = 23)	none
Ca	mmol/l	3.7: 0.6-7.8 (n = 29)	2.8: 0.9-7.2 (n = 25)	none
Ca	mmol/day	3.95: 0.6-7.8 ((n = 25)	2.82: 0.9-7.2 (n = 24)	p < 0.0001
Ox	mmol/l	0.29: 0.09-0.61 (n = 26)	0.33: 0.10-0.68 (n = 23)	none
Urate	mmol/l	2.9: 1.4-5.5 (n = 34)	3.3: 1.8-7.4 (n = 24)	p = 0.046

Figure 3. Particle volume distribution in urines of healthy women and men. The median volumes of the female urines are represented in the upper curve, the median volumes of the male urines in the lower curve. The similar peak at 7.1 μm indicates a similar overall particle size in both sexes.

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significant. The Spearman Rank Order Correlation technique was used to test for the existence of a relationship between crystal volume and the urinary pH, and the total urinary concentrations of oxalate, calcium, and urate. These parameters were also tested for a possible relationship with the metastable limit.

Results

The metastable limit of the urines was significantly higher (p = 0.009) in the women (median 0.825: range 0.15-3.15 mmol/l oxalate), than in the men (median 0.45: range 0.15-1.65 mmol/l in men; Fig. 1). Figure 2 shows the total particle volume 90 minutes after the induction of crystallization: whereas women had a significantly (p = 0.006) greater total particle volume (44,993: range 12679-100,230 μm^3/μl) than the men (36,464: range 13532-71567 μm^3/μl), the average over-

all particle size was similar for both sexes, being 7.1 μm (Fig. 3). SEM confirmed this similarity in overall size by showing that although the **individual** crystals precipitated from the men's urines (Fig. 4) tended to be smaller than those from the women (Fig. 5), they were clustered into larger aggregates than those occurring in the women's urines. Thus, the overall sizes of the crystalline particles precipitated from the women's and men's urines, expressed as the positions of the peak of the particle volume distribution curves, were indistinguishable. There were no correlations between the volume of crystalline material deposited and the urinary pH, the total urinary concentration of oxalate, and the urinary concentrations of Ca and urate. Similarly, no correlation was found between the metastable limit and the urinary pH, the total urinary oxalate concentration, and the urinary urate concentration. However, as we have shown in a previous study already [29], an inverse correlation existed between the urinary Ca concentration and the metastable limit (women: r = -0.41, n = 25, p < 0.001; men: r = -0.58, n = 29, p < 0.001; all subjects: r = -0.54, n = 54, p < 0.001). Likewise, as we have previously shown in another study [30], the total daily Ca output was significantly (p < 0.001) less in the women (2.82 mMol/day) than in the men (3.959 mMol/day).

The median values and ranges of urinary pH, and concentrations of calcium, oxalate, and urate are presented in Table 1.

Discussion

The well documented disparity in the incidence of CaOx stones between men and women suggests that sex hormones may play a determinant role in stone formation, either by influencing the urinary conditions which dictate the likelihood that CaOx will precipitate, or once this has occurred, by affecting the probability that the precipitated crystals will be retained in the urinary collecting system. This notion is supported by the results of one study which indicated that a higher estrogenic activity during their reproductive years appears to protect

Figure 4. Scanning electron micrograph of typical crystals in the urine of a male subject. Bar = 5 μm.
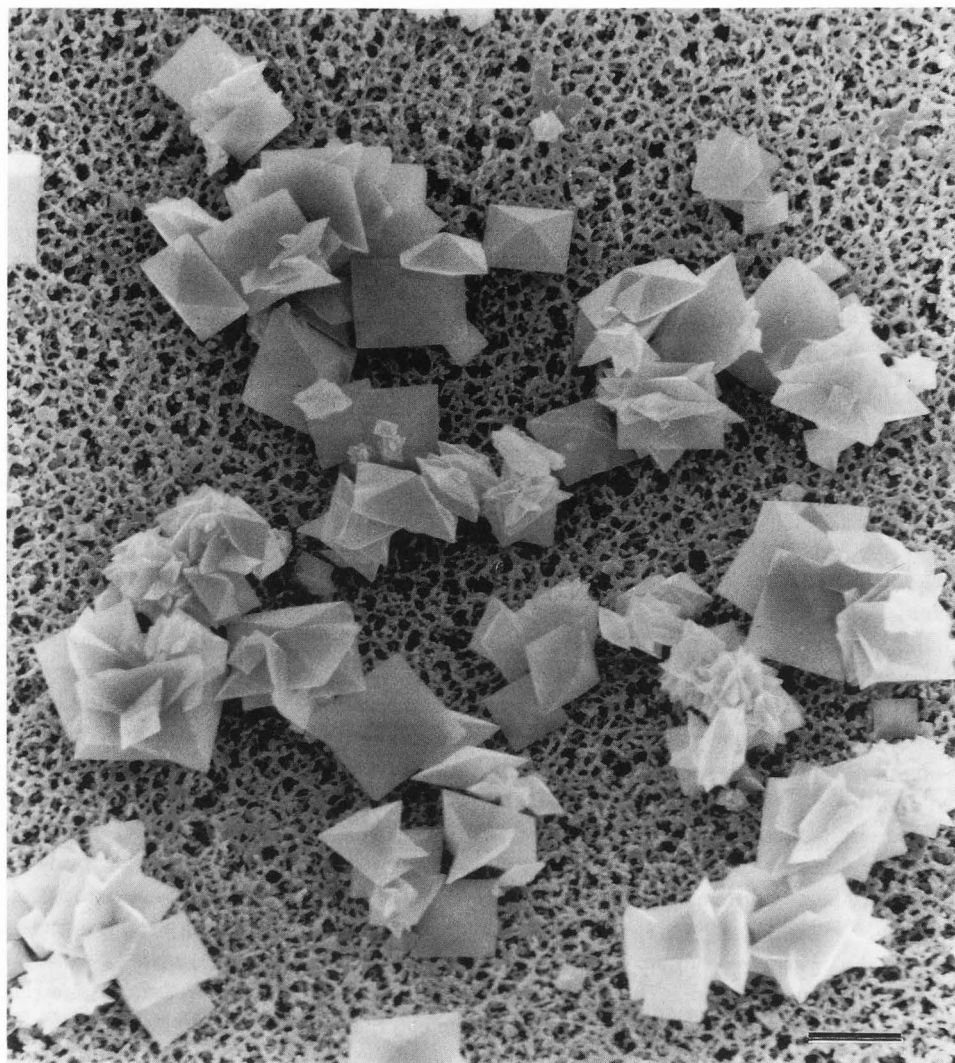
women from stone formation [33]; by corollary, administration of testosterone has been reported to promote CaOx stone formation in rats [19]. One possible mechanism by which sex hormones might influence the likelihood of stone formation could be by determining the concentration and/or activity of urinary inhibitors. Indeed, sex-dependent differences in the inhibitory effect of urine on CaOx crystallization in a seeded metastable crystallization system have previously been reported by our laboratory [30]. Moreover, it has also been demonstrated that consumption of oral contraceptives increases the inhibitory effect of female urine on CaOx crystallization in a similar medium [35]. However, the shortcomings of such experimental systems are now well recognised, and it is generally accepted that an organic, undiluted real urine system would more accurately reflect the complexity of physiological conditions, and thereby provide data more likely to indicate the true effects of urinary inhibitors *in vivo*. In this study, therefore, parameters of crystallization were assessed in undiluted urines

of healthy men and women. No attempt was made to standardise the subjects' diets because we wished to compare urines of women and men under their normal living conditions. However, all urines were standardised insofar as a standard load of oxalate was added to all urines at their measured metastable limits.

The metastable limit, expressed as the minimum amount of oxalate necessary to induce crystallization, is an empirical parameter that measures the propensity of a urine to nucleate CaOx crystals, and was found to be inversely related to the prevailing urinary Ca concentration, as we have already shown in a previous study [29]. The metastable limits of the female urines were significantly higher than those of the men, probably reflecting their lower urinary concentrations of Ca, and indicating that they were less likely to undergo precipitation of CaOx. *Prima facie*, this might be considered sufficient grounds to explain the difference in stone incidence between the two sexes, since the precipitation of insoluble crystals is the first, and absolute, requirement for stone
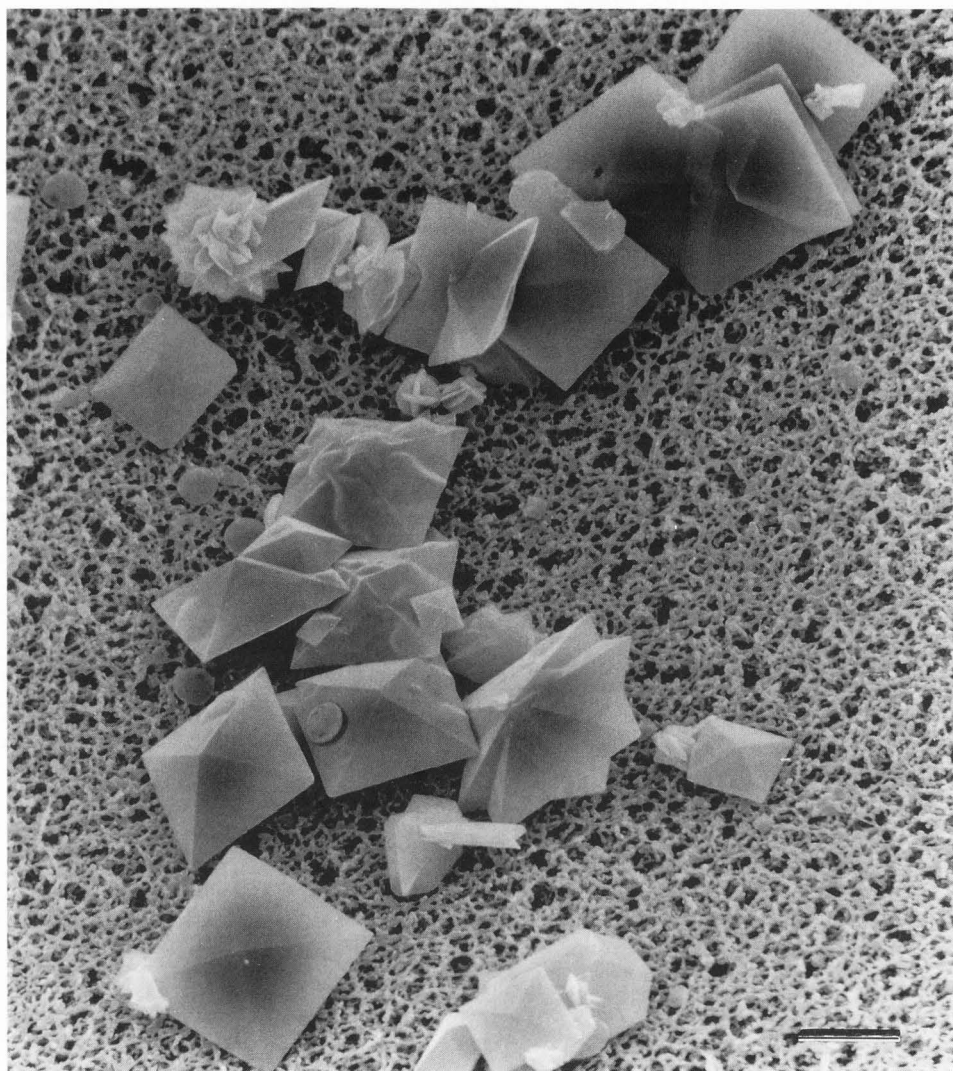
Figure 5. Scanning electron micrograph of typical crystals in the urine of a female subject. Bar = 5 μm.

pathogenesis. However, nucleation of crystals is, of itself, insufficient to induce CaOx stone formation, which also requires retention of those crystals within the urinary tract. Intuitively, it is apparent that the mass and size of precipitated crystalline particles will directly affect the probability that they will be retained in the renal collecting system, and this is borne out by the observation that individuals who have never formed a kidney stone pass small, single CaOx crystals, while recurrent stone formers excrete a greater mass of crystals clustered into large aggregates [22]. Thus, male and female urines were also compared with regard to both the total volume and the average size of CaOx crystals precipitated.

Coulter Counter analysis showed that urines from females precipitated significantly larger volumes of crystalline particles during the 90 minute incubation period following induction of crystallization, and this finding was complemented by the results of SEM which clearly revealed that the individual crystals precipitated from the female urines were larger than those from the males. There are two obvious mechanisms by which larger crystals could be formed. First, they could result from a greater amount of crystal growth, which might occur in the presence of higher concentrations of calcium and oxalate. The urinary concentrations of calcium were generally **lower** in the female urines; nonetheless, their endogenous oxalate concentrations were slightly higher, and their metastable limits significantly so, which means that the total concentration of oxalate at the commencement of the 2 hour incubation period was significantly higher in the women's urines than in the men's. It has been recognised for more than 20 years that the prevailing concentration of oxalate is approximately 15 times more influential than that of calcium in determining the likelihood that precipitation of CaOx will occur in urine, a consequence principally of the greater concentration of calcium in urine relative to oxalate [9]. It is possible therefore that the increased volume of CaOx crystals observed in the women's urines may have resulted from

the higher concentrations of oxalate required to induce crystal nucleation. However, this is unlikely because we were unable to detect any correlation between crystal volume and total oxalate concentration.

An alternative explanation for the larger crystal volume in the women's urines could be an enhanced inclusion of organic material into the crystalline structure, which would increase the volume, but not the mass of the precipitated crystals. In two other studies, in which CaOx deposition was also measured using ^{14}C-oxalate, we showed that a similar increase in volume was not the result of an enhanced deposition of solute, but was probably caused by the inclusion into the crystals of organic matrix material [7, 32]. We therefore assume that the increased crystal volume and larger individual crystal size that we observed in the material precipitated from the women's urines may have resulted from an increased inclusion of organic matrix macromolecules into the crystals. This is supported by the simultaneous observation that the crystals deposited from the men's urines, although smaller than those from the women's, were more highly aggregated: the organic matrix of urinary CaOx crystals is known to inhibit CaOx crystal aggregation occurring in undiluted, ultrafiltered human urine [7]. Moreover, this inhibitory effect can probably be attributed principally to the predominant protein in this matrix, urinary prothrombin fragment 1 (UPTF1), which has been shown to be the most potent macromolecular inhibitor of both CaOx crystal growth and aggregation in this experimental crystallization system [32].

However, although the reduced degree of aggregation of the crystals precipitated from the women's urines might suggest that they would be less likely to retain precipitated crystals of CaOx in their kidneys, such an advantage would be offset to some extent by their formation of larger individual crystals, which as discussed above, occupy a greater total volume. In fact, the results presented here indicate that the potential benefit offered by a reduced degree of crystal aggregation was completely negated by the corresponding increase in the size of the individual crystals and total particle volume. Coulter Counter analysis demonstrated that the position of the mode of the volume size distribution curve of the crystalline particles precipitated from the women's urines was indistinguishable from the corresponding male value, being 7.1 μm for both. This would suggest that, once formed, crystalline particles precipitated from both sexes are equally likely to be retained in the urinary tract, and that physicochemical events subsequent to crystal nucleation do not explain the higher incidence of CaOx stone disease in men.

Although it is well accepted that the overall degree of supersaturation of urine with CaOx depends upon a number of factors, including pH and the concentrations of chelating agents such as citrate and Mg, the probability that CaOx crystal nucleation will occur obviously depends to a great extent on the prevailing concentrations of Ca and oxalate: measurements of their urinary concentrations have long constituted the spearhead of stone investigation. Although we were unable to demonstrate a statistically significant difference in urinary Ca concentrations between the male and female groups in the current study population, the median Ca concentration was considerably lower in the women (2.8 mmol/l) than in the men (3.7 mmol/l). When these results were expressed as daily Ca output, the women (median 2.82 mMol/day) were found to excrete significantly less Ca than the men (median 3.959 mMol/day). These findings are in agreement with a previous report from our laboratory [30] and to other reports [4, 23, 33, 34]. We were also unable to demonstrate a significant difference between the urinary oxalate concentrations between the two sexes (0.29 in men and 0.33 mmol/l in women), which is in agreement with other reports in the literature [3, 5, 10, 15, 22, 30]. Only one study reported a lower urinary oxalate concentration in females [34].

Another urinary factor thought to influence the likelihood of CaOx stone disease is the urinary excretion of uric acid. Several mechanisms have been advanced to explain the apparent relationship between the two, including epitaxial deposition of CaOx upon crystals of sodium urate [6] and mitigation of urinary glycosaminoglycan inhibitory activity by colloidal particles of sodium urate [24]. However, direct supporting evidence for both these theories is lacking [26]. Nonetheless, other work has shown that increasing the concentration of urate in urine promotes CaOx crystallization [12, 13, 14, 16], and that the effect is probably wrought by the process of "salting out" [12, 13, 14, 16]. The urinary excretion of urate has been reported to be similar in the two sexes [18], and to be increased in males [8, 30]. Contrary to our earlier observations [30], results presented in the current study reveal an increased median urinary uric acid concentration in females, which, though significant, was nonetheless only minor. The median urinary concentration in women was 3.3 mmol/l and in men 2.9 mmol/l: salting out of CaOx by urate requires increases in urate concentration of the order of 2-3 mmol/l [12, 13, 14], which is many times greater than the difference in concentration between men and women found here. Thus, it is unlikely that our finding of a higher urate excretion in women is of any significance in determining the incidence of CaOx stone formation. This is supported by the fact that we were unable to document any correlation between urinary urate and either the deposited crystal volume or the metastable limit.

Although urinary pH is known to affect the solubility of CaOx, we could not show any difference in pH

between men and women, which is in agreement with the findings of Robertson *et al.* [25]. To exclude pH changes during storage, pH was measured immediately before the beginning of each experiment, thus, reflecting the true experimental pH conditions. Furthermore, there was no correlation between pH and any of the measured crystallization parameters.

Another important urinary constituent which is known to influence urinary saturation with CaOx is citrate, which possesses the ability both to chelate Ca ions in solution and to inhibit CaOx crystal deposition in undiluted human urine [28], probably by binding to the crystal surface. Unfortunately, owing to the partly retrospective nature of this study, data for urinary citrate concentrations were not available for our patients. Urinary citrate concentration is reportedly higher in women during their reproductive years than in men. Estrous phase-related alterations in urinary concentration and a marked decrease in citrate excretion in menopausal women suggest that urinary citrate concentration is influenced by female sex-hormones, i.e., estrogen, thus, offering another possible mechanism for the relative protection of females from urinary stone formation [33, 34]. However, we believe that such an explanation is not supported by the results of the present study, since there was no difference between men and women with respect to the total volume of CaOx precipitated in their urines. Moreover, although citrate has been shown to affect CaOx crystal nucleation and aggregation [11, 17, 27], those studies were performed in inorganic solutions whose ionic conditions bear little resemblance to those of human urine. In fact, the lack of difference between the size of the crystalline particles precipitated in the urines from the men and women is in accord with the fact that urinary citrate does not affect the aggregation of CaOx crystals in undiluted human urine using the same experimental system as was employed here [31].

In summary, the results of this study have confirmed the findings of our previous study [30] that urine from women inhibits CaOx crystal aggregation more strongly than that from men. However, although this would serve to protect females from stone disease, the advantage is offset by the tendency of their urines to precipitate larger individual crystals. On the other hand, there was a clear distinction between the sexes with regard to the propensity of their urines to undergo spontaneous CaOx crystal nucleation in response to a fixed oxalate challenge, which is probably attributable to the fact that men excrete urine containing higher concentrations of Ca. The reason why men have a higher incidence of CaOx stone formation remains far from clear, but it seems to be associated with an increased tendency of their urines to nucleate CaOx crystals *per se*, rather than to factors predisposing to the formation of larger crystal-line particles more likely to be retained in the renal collecting system.

Acknowledgement

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Discussion with Reviewers

R.P. Holmes: Would control of fluid intake prior to and during the urine collection be useful to amplify the inherent differences in excretions and crystallization properties between the sexes?

Authors: Standardising fluid intake might certainly magnify any differences between the urinary calcium concentrations in men and women. However, the object of this study was to compare CaOx crystallization parameters in the urine of men and women under normal, everyday conditions. Normalising fluid intake would simply serve to introduce an artificial, confounding factor.

R.P. Holmes: Results from your laboratory [37] suggest that UPTF1 will be the major protein incorporated into the crystals formed in the experiments reported here. Recent studies by Dussol *et al.* [38] of a limited

number of calcium oxalate stones indicate that this protein is not a major component. Does this disparity in composition suggest that inducing crystallization with an oxalate load creates artefactual results, or is stone matrix predominantly inter-crystalline and differs considerably from intra-crystalline proteins?

Authors: Dussol *et al.* [38] did not detect the presence of UPTF1 in stones for the simple reason that they did not look for it. In fact, we have detected UPTF1 in 9 of 10 CaOx stones, thereby confirming the validity of the experimental model we use and providing indirect evidence that proteins in stones are intra-crystalline [42].

B. Hess: How do the authors explain that there is no correlation between total urinary oxalate concentrations and crystal volumes?

Authors: If crystal mass (and volume) were determined solely by the mineral content then we would expect a direct correlation between total crystal volume and total oxalate concentration (and total calcium concentration). The fact that they are not correlated suggests that other factors contribute to crystal volume. Because proteins are known to be included into CaOx crystals and to occupy a significant space, we have speculated that this is the reason for the lack of correlation, as has been previously discussed [7, 32].

B. Hess: What are the small rounded crystals in the woman's urine (Fig. 5) which cannot be observed in the man's specimen? How do the authors explain the somewhat irregular surfaces of the calcium oxalate dihydrate (COD) crystals in the woman's urine (Fig. 5) which cannot be found in the man's sample (Fig. 4)?

Authors: The small rounded particles are very small calcium oxalate monohydrate (COM) crystals. We often observe both COD and COM crystals in the same urine specimen, although one type usually predominates. The presence of the COM crystals here should therefore be regarded as a normal occurrence.

We also often observe mixed aggregates of COD and COM. It is not uncommon to see COM crystals bound to the surface of COD crystals, and these tend to become enveloped by the advancing crystal growth front and become embedded inside the structure. These are visible as lumps on the surface of the larger crystals, and this is the source of the surface irregularities in Figure 5. In fact, careful scrutiny of Figure 4 shows that in the center of the micrograph there is a COD crystal to which has bound a smaller COD crystal which has then been overgrown with CaOx. The effect is more common when there is a mixture of small and large particles, and is not confined specifically to either sex.

H.-G. Tiselius: The authors have previously stated that centrifugation and filtration of urine remove important macromolecules from solution [37]. Could such a mechanism have affected the result?

Authors: Removal from urine of Tamm-Horsfall mucoprotein by both low speed centrifugation and filtration is now widely acknowledged, and this protein would have been absent from both male and female urines alike. Other macromolecules can be removed from urine by filtration through certain types of membranes (Type GS Millipore), but this does not occur with the membranes used in this study (Type GV). The urines from the male and female subjects were therefore equivalent in that they were both free of Tamm-Horsfall mucoprotein, but still contained their normal complements of other macromolecules.

H.-G. Tiselius: The addition of oxalate increases the supersaturation (the ion activity product) of calcium oxalate, and if the crystal nucleation was exactly the same in urine from men and from women, the size in crystal volume should be expected to be similar. Although the Coulter Counter technique is useful for assessing the early steps of the crystallization, it has definite limitations, attributable to the fact that crystals with a size below 2 μm escape detection. The concentration of oxalate is thus increased until the crystals attain a size exceeding this limit. If the initially formed nuclei are more numerous in urine A than in urine B, it is likely that the supersaturation in sample A has to be brought to a higher level than in B in order to produce crystals detectable in by the Coulter Counter. Such a mechanism might subsequently affect the further size and volume of the crystals. We have thus observed a higher mean crystal volume in urine from normal subjects than in urine from stone formers following a similar titration experiment. Although differences in the aggregation is a possible explanation for this observation, differences in nucleation cannot be excluded. It would be valuable to get some comments by the authors on the methodological problems with this technique.

Authors: If crystal nucleation were exactly the same in two different urine specimens then we would certainly expect similar crystal volumes and size, **all other things being equal**. However, differences between the urinary content of inhibitors or promoters could affect the subsequent growth of crystals and cause large differences between crystal size or volume. We do, nonetheless, agree that formation of a larger number of initial nuclei would require a greater deposition of calcium oxalate to enable detection using the Coulter Counter, and this would inevitably result in an increased total deposition of crystal volume. However, crystal aggregation, which we know occurs in this system, will also have a very

marked effect, as Prof. Tiselius acknowledges. Because of detection limits with the Coulter Counter, it is not possible to measure crystal nucleation. It is important to note that this same deficiency applies to **any method** for measuring particle size; estimates of nucleation rates are always arrived at by extrapolation. We recognised this problem ten years ago when we pointed out that the detection limit of the Coulter Counter introduced inaccuracies in the experimental estimation of inhibition, and proposed the use of a computer model [40]. Unfortunately, this model cannot be used in the self-nucleating crystallization system we use, and we abandoned the use of crystal numbers as an estimate of crystal aggregation in this technique many years ago. The technique does, however, still provide useful estimates of the bulk of calcium oxalate precipitated and the size of the aggregated particles.

J.M. Baumann: Differences in urine with respect to calcium oxalate metastability may be explained by a different activity of promoters and inhibitors. Glycosaminoglycans (GAGs) are such substances but they are also secreted in the female genital tract. Therefore, the question arises how 24 hour urine was collected in women, and if the difference in crystallization conditions between men and women could not be explained by a contamination of the female urine from the genital tract.
Authors: Urine was collected from the women with the use of a plastic collecting funnel: collection of midstream urine specimens would have been impractical and would have resulted in the loss of significant quantities of the 24 hour volume. It is doubtful that GAGs from the genital tract would explain the observed differences, in view of the fact that the most common GAG, chondroitin sulphate, has no appreciable effect on CaOx crystallization in our experimental system [41].

J.M. Baumann: What was the reason for the six-fold repetition of the crystallization test in each urine. How is the reproducibility of the test system used and what is the coefficient of variation, respectively?
Authors: The experiments are performed in hextuplicate in order to guarantee acceptable statistical reliability. The coefficient of variation for the estimation of the metastable limit is 5.6% and for crystal volume, is 6.7% [28].

J.M. Baumann: Crystal nucleation in tissue seems to be responsible for kidney calcification, which is thought to be important for stone formation. However, in urine of healthy controls compared to stone formers, Kavanagh [39] found an increased nucleation rate and a decreased metastability with respect to calcium oxalate. How do the authors interpret their findings of an increased urinary metastability in women suffering less from calcium oxalate stone disease than men in the light of the results of Kavanagh [39]?
Authors: It is important that nucleation rate is not confused with our measurement of metastable limit. One is the rate at which nucleation proceeds; the other is simply an empirical measure of the amount of oxalate necessary to begin and allow detection of the process. We have previously shown that the amount of oxalate required to induce observable crystal nucleation is inversely proportional to the endogenous calcium concentration: because the women's urines had lower concentrations than the men's, their metastable limits were higher, which is consistent with their having a decreased tendency to precipitate calcium oxalate spontaneously. We have also shown previously that stone formers have **lower** metastable limits than healthy controls, so using the same methodology, our data on women and men are in accord with those of stone formers and normals. We can only assume therefore that the apparent disparity in the findings relates to differences in methodology. We do not measure nucleation rates (see reply to Dr. Tiselius above) and so cannot make any direct comparison with Dr. Kavanagh's results [39].

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