Calcium Oxalate Crystallization Index (COCI): an Alternative Method for Distinguishing Nephrolithiasis Patients from Healthy Individuals

Bowei Yang1, Thasinas Dissayabutra1, Wattanachai Ungjaroenwathan2, Piyaratana Tosukhowong1, Monpichar Sri-Art3, Thavorn Supaprom4, Numpon Insin3, and Chanchai Boonla1

1Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, 2Division of Urology, Supasit Prasong Hospital, Ubon Ratchathani Province, 3Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, and 4Department of Biological Science, Faculty of Science, Ubon Ratchathani University, Ubon Ratchathani Province, Thailand

Abstract. Urinary supersaturation triggers lithogenic crystal formation. We developed an alternative test, designated calcium oxalate crystallization index (COCI), to distinguish nephrolithiasis patients from healthy individuals based on their urinary crystallization capability. The effect of urine volume, oxalate, phosphate, citrate, potassium, and sodium on COCI values was investigated. COCI values were determined in 24-hr urine obtained from nephrolithiasis patients (n=72) and matched healthy controls (n=71). Increases in urine oxalate and phosphate and decreases in urine volume and citrate resulted in significantly increased COCI values. The urinary COCI in nephrolithiasis patients was significantly higher than that in healthy individuals. Two healthy subjects who had elevated COCI values were found to have asymptomatic kidney calculi. The receiver operating characteristic analysis showed an area under the curve of the urinary COCI test of 0.9499 (95%CI: 0.9131-0.9868) for distinguishing between nephrolithiasis and healthy subjects. At the cutoff of 165 mg oxalate equivalence/day, the urinary COCI test provided sensitivity, specificity, and accuracy amounts of 83.33%, 97.18%, and 90.21%, respectively. Urinary COCI values were primarily dependent on urine volume, oxalate, and phosphate. The test provided high sensitivity and specificity for clinically discriminating nephrolithiasis patients from healthy controls. It might be used to detect individuals with asymptomatic kidney calculi.

Keywords: kidney stone, nephrolithiasis, calcium oxalate crystallization index, COCI, diagnosis

Introduction

Kidney stone disease or nephrolithiasis is a highly recurrent condition with a progressively increased prevalence worldwide, at least in part, due to the global warming [1]. In Thailand, nephrolithiasis is highly prevalent in the rural communities of the Northeast [2,3]. Large stone formation and multiple recurrences subsequently cause intrarenal inflammation, oxidative damage, and renal dysfunction [4-7]. Therefore, development of a simple and cost-effective method to screen individuals at risk of kidney stone formation would be helpful in order to begin the early counseling concerning the measures to prevent the onset of nephrolithiasis.

Address correspondence to Dr. Chanchai Boonla, Ph.D., Assistant Professor; Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok 10330 Thailand; phone: 66-2-2564482, fax: 66-2-2564482; e-mail: chanchai.b@chula.ac.th

It is well-known that urinary crystals are the building blocks of stones, and urinary supersaturation is an initial driving force for the formation of lithogenic crystals [8]. Globally, the major type of urinary calculi is calcium oxalate (CaOx) [9, 10]. Quantification of the potential for CaOx to crystallize in the urine, therefore, would provide adequate clinical utility for estimating the risk of kidney stone formation. In Thai patients, high urinary supersaturation for CaOx salt was demonstrated [11], and the CaOx crystals formed even in low specific gravity urine [12]. In addition, the level of urinary citrate in Thai nephrolithiasis patients was significantly lower than the level in healthy controls [13-16]. We hypothesized that the potential for CaOx crystallization in nephrolithiasis urine was higher than in healthy urine.
At least four methods have been established for assessing the CaOx crystal-forming potential in the urine. An Oxalate tolerance test is used to determine the upper limit of metastability [17-19]. The test measures the maximum amount of oxalate that can be added into the urine to not induce crystal nucleation; this amount of oxalate is called the permissible increment in oxalate. Later, Grases et al proposed a simpler test to evaluate the capacity of urine to crystallize calcium salts [20]. A non-protected, non-renewed surface was submerged in urine to initiate crystallization of supersaturated, lithogenic substances on the surface. They found that normal urine did not induce crystallization whereas lithogenic urine induced crystallization of calcium salts on the surface. The test provided a good sensitivity and specificity of 92% and 73%, respectively, but it required a large volume urine sample (at least 40 mL) [20]. In 1998, Sriboonlue et al developed an indirect method for estimating urinary oxalate based on the precipitation of CaOx crystals in urine under excessive calcium and ethanol conditions [21]. They measured the calcium level in the precipitated CaOx crystals (by atomic absorption spectrophotometry) and the urinary oxalate level in nephrolithiasis patients (by the oxalate oxidase method) and found that levels of calcium in the precipitated CaOx crystals were positively correlated with the urinary oxalate levels of the patients. The Bonn-risk Index (BRI), developed by Laube et al in 2000, is the most widely used method for estimating lithogenic potential in the unprepared native urine [22]. The BRI is calculated as a ratio of urinary free calcium ions concentration to concentration of ammonium oxalate that is added to initiate CaOx crystallization [22, 23]. The BRI is good to discriminate CaOx stone formers from healthy controls (sensitivity 69.7%, specificity 100%). However, the current BRI test is limited to research use, due to its complexity and the expense involved in performing the test [23, 24].

![Schematic diagram of the procedure for COCI method. Details of the COCI procedure were described in the Method Section. OD_w_{urine}: The actual absorbance of urine sample, OD_{Ox-spiked urine}: absorbance of urine sample that had been spiked with 2 mM Ox, OD_{STD 2mM Ox}: absorbance of 2 mM standard Ox.](image-url)
In this study, we aimed to establish an alternative, simple, and reliable method for quantifying the ability of CaOx precipitation in urine sample to be clinically useful in discriminating kidney stone patients from healthy individuals. It is called the CaOx crystallization index (COCI). How well the urinary COCI test was able to distinguish these two populations was evaluated by receiver operating characteristic (ROC) analysis. The effect of urine volume, oxalate, phosphate, citrate, potassium, and sodium on COCI values was investigated. Our COCI method shared the same principle as the method developed by Sriboonlue et al [21] since calcium was continuously added into the urine to induce CaOx crystallization. The difference in the two methods was that Sriboonlue et al measured the calcium content in the precipitated CaOx for an indirect estimation of the urinary oxalate concentration, but we used an absorption at 215 nm to determine the oxalate content in the precipitated CaOx in order to estimate the capability of urine to crystallize CaOx.

**Materials and Methods**

**COCI procedure.** Human urine is generally metastable with respect to CaOx, and it requires a substrate or a nucleation platform in order for crystallization to take place [25,26]. In the COCI procedure, we shifted the urinary saturation state from metastable supersaturation to unstable supersaturation by adding lithogenic substances (calcium and oxalate ions) to trigger the spontaneous formation of CaOx crystals. The procedure of the COCI method is shown in Figure 1.

**Figure 2.** Effects of urinary volume, oxalate, phosphate, citrate, potassium and sodium on the COCI values. Increased urine dilution significantly caused decreased COCI values (*P<0.001 for all vs. control). (a) Increases in oxalate concentrations caused significantly increases in COCI values (*P<0.001 for all vs. control). (b) Increases in phosphate concentrations caused significantly increases in COCI values (*P<0.001 for all vs. control). (c) The COCI values tended to decrease after gradual addition of citric acid, and at concentration of 20 mM citric acid, COCI value was significantly lower than the native urine control (*P<0.05). (d) Additions of varied concentrations of potassium (e) and sodium (f) had no significant effect on the COCI values. C.: Control, Ox: Oxalic acid, Cit.: Citric acid. Error bars indicate mean ± SD.

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Varied concentrations of oxalic acid (Ox) solution (0,1,2,3 and 4 mM) were used for generating a standard curve. Urine samples were filtered through 0.22 μm membrane filters for deproteination and decontamination. Every urine sample (950 μL) was spiked with 50 μL of 80 mM Ox solution. Subsequently, 1 mL of 100 mM CaCl2 solution was added. Final concentrations of added Ox and CaCl2 were 2 mM and 50 mM, respectively. The mixture was incubated at 37°C for one hr to complete crystallization. In order to harvest crystals, the mixture was centrifuged at 4,000 rpm for 15 minutes. The crystal pellet was promptly washed once with 0.5 mL PBS (pH 6.8) and then air dried at room temperature for 10 minutes. The dried crystals were re-dissolved in 8 N HCl (0.5 mL), and the absorbance at 215 nm (for oxalic acid detection) was measured [27,28]. The
standard curve of Ox concentrations vs. OD\textsubscript{215} nm was generated. The actual absorbance of urine sample (OD\textsubscript{urine}) was calculated from the absorbance of the urine sample that had been spiked with 2 mM Ox (OD\textsubscript{x-spiked urine}) subtracted by absorbance of 2 mM standard Ox (OD\textsubscript{STD2mMOx}). The COCI value for the urine sample was determined by comparison to the standard curve. Since Ox was used to generate the standard curve of COCI test, COCI values were expressed as mM Ox equivalence (equiv.). We multiplied by 90 for mg Ox equiv./L. COCI amount (per day) and normalized COCI (divided by urinary creatinine) were also calculated (Figure 1). Fresh 24-hr urine samples were the most appropriate specimens for the COCI analysis. However, a urine sample could be kept at 4°C up to 5 days without a significant change in the COCI value. Multiple freeze-thawed urine samples were not recommended.

Effects of urinary volume, oxalate, phosphate, citrate, potassium and sodium on the COCI values were explored. Twenty-four-hour urine specimens from healthy volunteers were used for the experiment. To investigate the effect of urine volume, the urine sample was diluted with deionized water to achieve 10%, 20%, and 40% diluted urine. Undiluted urine (100%) was used as the control. To investigate the effect of oxalate on COCI value, different concentrations of Ox (0.5, 1, 2.5 and 5 mM) were added into urine samples. These conditioned urine samples were then analyzed using the COCI method, and their COCI values were compared with the native urine control (without added of Ox). Likewise, for comparison of the different effects of phosphate, citrate, potassium, and sodium on COCI values, different concentrations of NaH\textsubscript{2}PO\textsubscript{4} (2.25, 4.5, 9.0 and 18 mM), citric acid (2.5, 5, 10 and 20 mM), KCl (37.5, 75 and 150 mM), and NaCl (65, 130 and 260 mM) were added into urine samples prior to performing the COCI analysis. Their COCI values were compared with their respective controls. Each experiment was repeated six times.

Subjects and 24-hr urine specimens. A total of 185 subjects were initially recruited for the study and divided into two groups: nephrolithiasis patients (n=96) and healthy controls (n=89). After being matched by age and gender, the numbers of subjects in nephrolithiasis and healthy groups were reduced to 72 and 71, respectively. The nephrolithiasis patients were recruited from the Sunpasit Prasong Hospital, Ubon Ratchathani province, Thailand. The presence of Kidney calculi were confirmed by a positive radiograph of the kidney-ureter-bladder (KUB x-ray) and/or intravenous urography (IVU). Patients with an anomalous kidney and other urinary tract diseases, i.e., horseshoe kidney, polycystic kidney, congenital vesicoureteral reflux, neurogenic bladder and any malignancies were excluded. Residents of rural communities in Ubon Ratchathani province, Thailand served as controls and were confirmed as healthy via direct interview and/or previous medical evaluation reports. They had no history or symptoms of nephrolithiasis.

The research protocol was reviewed and approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University and the Ethics Committee of the Sunpasit Prasong Hospital. Written and informed consents were obtained from all participants prior to specimen collection.

Single 24-hour urine specimens were collected from all subjects and thymol was used as a preservative. No restriction on oral intake was applied to the patients or control group prior to the collection of urine. The COCI values were determined in all urine specimens in duplicate. Urinary creatinine concentration was determined in all samples using the Jaffe method. To verify the mineral constituent of crystals generated by COCI method, the harvested crystals from six selected cases (three nephrolithiasis and three healthy) were subjected to the Fourier transform infrared spectroscopy (FTIR) analysis. All COCI crystals were primarily constituted of CaOx; however, in three cases (one nephrolithiasis and two healthy) calcium phosphate (CaP) was found as a minor component (Supplementary Figure 1).
Statistical analysis. Mean and standard deviations (SD) were used as representatives of the data. Comparisons of the mean between two and three groups of subjects were performed using independent t-tests and ANOVA tests with Bonferroni post-hoc tests, respectively. Mann-Whitney and Kruskal-Wallis tests, the equivalent non-parametric tests of independent t-test and ANOVA test, respectively, were performed, and similar conclusive results were obtained. A ROC curve analysis was used to evaluate how well the urinary COCI test could discriminate the nephrolithiasis patients from healthy subjects. An area under curve (AUC) of 1.0 indicates that the test can perfectly separate the two groups, while an AUC of 0.5 indicates that the test lacks discriminatory power. An appropriate cutoff was chosen to calculate sensitivity, specificity, and accuracy of the urinary COCI determination. All statistical analyses were performed by STATA version 12 (StataCorp, TX) and graphs were created using GraphPad Prism 5 (GraphPad Software, CA). A two-sided P<0.05 was considered statistically significant.

Results

Urine oxalate and phosphate increased, but volume and citrate decreased COCI values. Increased urine volume (by increase in dilution) caused decreased COCI values (Figure 2a). After dilutions to 40%, 20%, and 10% urine content (v/v with water), the measured COCI values were significantly lower than those measured in undiluted urine (P<0.001 for all). In contrast, increases in oxalate concentrations (0.5, 1.25 and 5 mM) caused significant increases in COCI values (P<0.001 for all) (Figure 2b). Also, increases in phosphate concentrations (2.25, 4.5, 9.0 and 18.0 mM) caused significant increases in COCI values (P<0.001 for all) (Figure 2c). The COCI values decreased after the gradual addition of citric acid (2.5, 5, 10 and 20 mM) (Figure 2d). At a concentration of 20 mM citric acid, the COCI value was significantly lower than that of the native urine control (P<0.05 by Turkey’s multiple comparison test). Gradual additions of potassium (Figure 2e) and sodium (Figure 2f) had no significant influence on COCI values.


**Patients with nephrolithiasis had higher urinary COCI levels than healthy individuals.** In all cases, urinary COCI in the nephrolithiasis group \( n=96, 456.50\pm401.30 \text{ mg Ox equiv./day} \) was significantly higher than that in the healthy \( n=89, 74.86\pm48.72 \text{ mg Ox equiv./day} \) group (Supplementary Figure 2). Two healthy individuals had a urinary COCI > 200 for both concentration and amount units, subject #1: 227.22 mg Ox equiv./L and 231.77 mg Ox equiv./day, and subject #2: 208.84 mg Ox equiv./L and 213.02 mg Ox equiv./day. Kidney stones in these asymptomatic individuals were suspected and were later confirmed by imaging results; both had kidney calculi sized between 7.9 and 12.5 mm (Figure 3).

Similar to the unmatched subjects, nephrolithiasis patients \( n=72, \text{ aged } 47.22\pm10.02 \text{ years, } 59.72\% \text{ males} \) had significantly greater urinary COCI than the aged- and sex-matched healthy individuals \( n=71, \text{ aged } 43.42\pm12.86 \text{ years, } 43.66\% \text{ males} \) (urinary COCI concentration: 270.32\pm205.82 vs. 67.83\pm29.83 mg Ox equiv./L, urinary COCI amount: 480.65\pm391.62 vs. 73.00\pm42.60 mg Ox equiv./day, normalized urinary COCI: 333.00\pm238.60 vs. 72.48\pm58.12 mg Ox equiv./mg creatinine, \( P<0.001 \) for all) (Figure 4). Mean age and sex distribution between these two groups were not statistically different.

Of 72 patients, 46 had stone specimens available for stone analysis by FTIR. Stone type was classified according to the major mineral component. Thirty-five (76.09\%), eight (17.39\%), two (4.35\%), and one (2.17\%) patients had CaOx, CaP, uric acid (UA) and magnesium ammonium phosphate (MAP) calculi, respectively. The urinary COCI values were not significantly different among different stone groups neither expressed as mg Ox equiv./L, mg Ox equiv./day nor mg Ox equiv./mg creatinine (Supplementary Figure 3).

Within- and between-day coefficient of variation (%CV) of the urinary COCI test were 2.16\% and 5.44\%, respectively, indicating that the COCI result was consistently reproduced.

**Urinary COCI test had high diagnostic performance to discriminate nephrolithiasis from healthy populations.** Based on ROC analysis, the AUC of 0.9229 (95\%CI: 0.8739-0.9720), 0.9499 (95\%CI: 0.9131-0.9868) and 0.9259 (95\%CI: 0.8838-0.9680) were obtained for the urinary COCI expressed in mg Ox equiv./L, mg Ox equiv./day and mg Ox equiv./mg creatinine, respectively (\( P<0.001 \) for all) (Figure 5).

The appropriate cutoff values of urinary COCI test were selected based upon the highest accuracy (Table 1). After normalizing by creatinine, the urinary COCI at cutoff of 126 mg Ox equiv./mg creatinine provided sensitivity, specificity, and accuracy of 72.22\%, 95.77\% and 83.92\%, respectively.

**Discussion**

The gold standard for the determination of urinary saturation and crystal-forming potential is EQUIL2 calculation using 12 urinary parameters including Na, K, Ca, Mg, NH\(_4\), Cl, PO\(_4\), SO\(_4\), UA, Ox, citric acid and pH [29]. More-convenient and cost-effective alternative methods have been developed, and the most cited method in the literatures is BRI [22]. However, the BRI method requires sophisticated equipment to kinetically detect the very first crystal nucleation. We established herein a novel simple endpoint crystallization approach with a clinical potency to use for screening individuals at risk of kidney stone formation.

The principle of the COCI test was for all crystallizable oxalate ions in urine to form CaOx crystals through the addition of excess calcium ions. Indeed from our FTIR data, CaP could be co-precipitated. As the amount of harvested COCI crystals was measured based on absorbance at 215 nm, other organic acids and ions in the COCI crystals could increase the optical density. Therefore, the obtained COCI values were indicative of total formation of calcium-containing crystals, primarily CaOx and CaP. In the lithogenic process, it is well recognized that CaP or hydroxyapatite crystals is a main component of interstitial Randall’s plaques that have been proved to be the origin of calcium oxalate monohydrate papillary stones [30]. Therefore, measuring the capability of urine to crystallize both CaOx and CaP would be a better approach to estimate the sole lithogenic potential of urine in order to accurately identify people at risk of kidney stone formation.
Our data showed that increases in urine oxalate and phosphate concentrations were significant determinants for increased COCI values. In contrast, decreased COCI values were caused by increases in urine dilution and citrate. We reasoned that more citrate in urine reduced the supersaturation of calcium salts. A weak effect of citrate at lower concentrations (Figure 2d) may be due to surplus amount of added calcium ions, and citrate could not significantly reduce the urinary supersaturation to cause a significantly decreased COCI value. At a very high concentration of citrate, there were sufficient free citrate ions to form soluble complexes with excessive calcium ions, thus lowering the urinary supersaturation and COCI value. This might be the explanation for the effect of citrate on COCI values; however, the reduction of urinary supersaturation by citrate in the COCI procedure needs further experimental proof. It was not surprising that urine volume caused reduced COCI values because increased water volume reduced urinary supersaturation. These suggested that our COCI method was mainly measuring the supersaturation of the calcium salts.

It should be noted that urinary COCI does not measure only urinary oxalate, but also other urinary factors that contribute to crystal formation, such as low urine volume, an increase in urinary phosphate, and a decrease in urinary citrate. Therefore, urinary COCI value is usually higher than the actual urinary oxalate.
The procedure of the COCI test was simple, and the total time needed to analyze the data was only an hour and a half. In terms of equipment, only an ultraviolet spectrophotometer was required for OD215 nm measurement. In addition, only 950 μL of urine was required for the test, and the only pretreatment necessary was filtration through the 0.22 μm membrane. For the original BRI assay, 200 mL of unprepared native urine, determination of calcium ions, and a sophisticated laser-probe crystal system analyzer for kinetically detecting the onset of crystallization are required [22]. However, a micromethod BRI has been recently developed that uses at least 1.5 mL urine [23]. In addition, a convenient a BRI-measuring device, called Urolizer®, was developed, and clinical usefulness in metabolic monitoring patients with CaOx calculi was demonstrated [29]. In this study, we successfully developed a cheaper and simpler tube-based endpoint method to estimate the CaOx crystal forming capability in 24-hr urine. Furthermore, this urinary method had a clinical utility for distinguishing patients with kidney stone formation from those without.

A few differences in procedure for CaOx precipitation between our COCI method and the method developed by Sriboonlue et al [21] should be mentioned. We used a final CaCl₂ concentration of 50 mM, but they used 3.53 mM CaCl₂.2H₂O. They used approx. 78% (v/v) ethanol to aid CaOx precipitation, but we added 2 mM Ox into urine sample in order to trigger the crystallization. For urine pre-treatment, they adjusted to pH 5, but we only did a filtration through 0.22 μM membrane.

Our data showed that patients with nephrolithiasis had significantly higher urinary COCI than those in healthy conditions. This underlined the clinical usefulness of urinary COCI for discriminating stone-formers from non-stone-formers. In addition, our preliminary data showed that the urinary COCI in patients with other kidney/urologic diseases (end-stage renal disease, chronic kidney disease, nephrotic syndrome, diabetic nephropathy, IgA nephropathy, hypertension, post kidney transplantation, membranous glomerulonephritis and muscle invasive bladder carcinoma) (n=30) was significantly lower than in nephrolithiasis patients, but nonetheless comparable to that of healthy subjects (data not shown). This data indicated that the elevated urinary COCI was specific for kidney stone disease.

The more important finding was that two subjects in the healthy group, who said they were healthy and had no history of kidney stone formation during a direct interview, had very high COCI values. The ultrasound imaging showed that these two subjects had asymptomatic stones sized between 7.9 and 12.5 mm in their kidneys (Figure 3). The urinary COCI test might be useful to detect asymptomatic calculi, but further studies in a larger population need to be conducted to verify this screening power.

For an overall performance of the diagnostic test, AUC between 0.9–1.0 are considered to be ‘excellent or highly accurate’ in separating disease from non-disease conditions [31]. Our urinary COCI test, expressed in mg Ox equiv./day had the highest AUC of 0.9499 (95%CI: 0.9131-0.9868), indicated that it had an excellent discriminatory power in separating nephrolithiasis from healthy individuals. A slightly higher AUC of the BRI test (0.981, 95%CI: 0.9561-1.000) has recently been reported as useful for separating children with CaOx stones (aged 5-18 years) from healthy children (aged 5-17 years). It has a sensitivity and specificity of 69.7% and 100%, respectively [24]. In this study, sensitivity, specificity, and accuracy of the urinary COCI test (cutoff: 165 mg Ox equiv./day) in discriminating adult nephrolithiasis patients from healthy subjects were 83.33%, 97.18% and 90.21%, respectively (Table 1). Although the diagnostic values are relatively high enough to be used in clinics, validating studies in a larger population as well as in prediagnostic urine specimens need to be conducted.

There are four commonly used radiological techniques for the diagnosis kidney calculi viz. KUB x-ray, IVU, ultrasound of the KUB, and CT scan. The sensitivity of KUB x-ray is relatively low (45%-58%). Sensitivity of ultrasound imaging is approximately 60% with a specificity of 90%. CT KUB has the highest sensitivity and specificity of 96% and 99%, respectively, and it is a gold standard imaging method for kidney calculi diagnoses [32].
Although the test principles are different, our urinary COCI test provides a much better diagnostic efficacy than the x-ray and ultrasound KUB. Despite the fact that the CT scan is more accurate than the urinary COCI test, there are more risks associated with CT scanning.

Limitations of the present study should be mentioned. Single 24-hr urine samples were used in our study. We did not have the urinary biochemical profile of the studied cohorts, and the stone analysis was not completed for all patients. Correlations between urinary COCI and urinary oxalate, phosphate and calcium were not assessable in this study. Urine with pH values >6.4 usually generates calcium phosphate deposits after 24-hr collection, and this might interfere with the COCI result. We could not directly compare the diagnostic efficacy of our urinary COCI test with the mentioned methods since we did not carry them out in parallel. We classified the healthy controls based on history and medical checkup report, but the non-stone forming state was not confirmed by imaging.

In conclusion, an alternative method for estimating the capacity of CaOx crystal formation in the urine, called COCI, was successfully developed. The primary determinants of urinary COCI values were urine volume, oxalate, phosphate, and citrate. The urinary COCI in nephrolithiasis patients was significantly greater than matched healthy controls. Self-reported healthy subjects with high urinary COCI values were confirmed to have asymptomatic kidney stones by ultrasound imaging. Based on ROC analysis, the urinary COCI test provided high diagnostic power for clinically discriminating nephrolithiasis patients from healthy controls. Therefore, the COCI test is a cheap, simple, non-invasive, and reliable method that has a clinical potential to identify individuals at risk of kidney stone formation and/or to detect those with asymptomatic urinary calculi.

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