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ORIGINAL ARTICLE

Gender-related effects on urine L-cystine metastability

Andrea Masotti · Chiara Laurenzi · Sara Boenzi · Anna Pastore · Anna Taranta · Francesco Bellomo · Maurizio Muraca · Carlo Dionisi-Vici · Pierfrancesco Bertucci · Luca Dello Strologo · Francesco Emma

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Abstract Cystinuria is an autosomal recessive disease that causes L-cystine precipitation in urine and nephrolithiasis. Disease severity is highly variable; it is known, however, that cystinuria has a more severe course in males. The aim of this study was to compare L-cystine metastability in first-morning urine collected from 24 normal female and 24 normal male subjects. Samples were buffered at pH 5 and loaded with L-cystine (0.4 and 4 mM final concentration) to calculate the amount remaining in solution after overnight incubation at 4 °C; results were expressed as *Z* scores reflecting the L-cystine solubility in each sample. In addition, metabolomic analyses were performed to identify candidate compounds that influence L-cystine solubility. L-cystine solubility *Z* score was $+0.44 \pm 1.1$ and -0.44 ± 0.70 in female and male samples, respectively ($p < 0.001$). Further analyses showed that the L-cystine solubility was independent from urine concentration but was significantly associated with low urinary excretion of inosine ($p = 0.010$), vanillylmandelic acid (VMA) ($p = 0.015$), adenosine ($p = 0.029$), and guanosine ($p = 0.032$). In vitro L-cystine precipitation assays confirmed that these molecules induce higher rates of L-cystine precipitation in comparison with their corresponding dideoxy molecules, used as controls. In silico computational and modeling analyses confirmed higher binding energy of these compounds. These data indicate that urinary excretion of nucleosides and VMA may represent important factors that modulate L-cystine solubility and may represent new targets for therapy in cystinuria.

Keywords Cystinuria · Gender · Metastability

A. Masotti and C. Laurenzi have equally contributed.

A. Masotti (✉)
Gene Expression-Microarrays Laboratory, Bambino Gesù Children's Hospital - IRCCS, Piazza S. Onofrio 4, 00165 Rome, Italy
e-mail: andrea.masotti@opbg.net

C. Laurenzi · A. Taranta · F. Bellomo · L. D. Strologo · F. Emma
Division of Nephrology and Dialysis, Bambino Gesù Children's Hospital - IRCCS, Rome, Italy

S. Boenzi · A. Pastore · C. Dionisi-Vici
Division of Metabolism, Bambino Gesù Children's Hospital - IRCCS, Rome, Italy

M. Muraca
Division of Laboratory Medicine, Bambino Gesù Children's Hospital - IRCCS, Rome, Italy

P. Bertucci
Department of Clinical Biochemistry, Tor Vergata Hospital, Tor Vergata University, Rome, Italy

Introduction

Cystinuria [OMIM: 220100] is an autosomal recessive disorder characterized by defects in the epithelial reabsorption of cystine (L-cystine) and dibasic amino acids in the proximal tubule of the kidney and in the gastrointestinal tract (Palacin et al. 1995). To date, mutations in two genes have been identified allowing for classification of the disease in two distinct genetic defect that share very similar clinical features (Dello Strologo et al. 2002). Type A cystinuria is caused by recessive mutations in the *SLC3A1* gene, located on chromosome 2, that encodes for the amino acid transporter *SLC3A1* (solute carrier family 3, member 1), previously referred also as rBAT (Calonge et al. 1995; Zhang et al. 1994). Type B cystinuria is caused by recessive mutations in the SLC7A9 gene, located on

chromosome 19, that encodes for the amino acid transporter SLC7A9 (solute carrier family 7, member 9), previously also referred as $b^{0,+}$AT (Feliubadalo et al. 1999). The products of the *SLC3A1* and *SLC7A9* genes form a heterodimeric complex that mediates the sodium-coupled apical transport of L-cystine and dibasic amino acids in proximal tubular cells (Fernandez et al. 2003).

Patients with cystinuria generally develop multiple L-cystine nephrolithiases that in most cases relapse within 5 years after surgical removal (Chow and Streem 1998; Palacin et al. 1995). A majority of patients present with symptoms related to renal stones in the first years of life; nearly all patients have experienced at least one episode of symptomatic nephrolithiases by the age of 30 (Romero et al. 2010). Medical treatment can reduce the rate of recurrences. Strategies include reduction of urine L-cystine concentration with forced dieresis, attempts to decrease L-cystine excretion with low-protein and sodium diets, and to increase L-cystine solubility with alkali and with sulfhydroxyl compounds, such as D-penicillamine and mercaptopropionyl-glycine (tiopronin) (Dello Strologo et al. 2007).

Despite these treatments, a significant proportion of patients experiences severe relapsing renal stone disease.

To date, the mechanisms that influence L-cystine precipitation in urine remain incompletely understood. In particular, differences among patients in the severity of their clinical picture cannot be entirely explained by differences in urine L-cystine excretion (Coe et al. 2005). Moreover, and similarly to other renal stone diseases, a clear gender predisposition has been reported; 28 % of boys develop a first urinary stone before the age of 3 years, as opposed to 15 % of girls (Dello Strologo et al. 2002); likewise, in a large cohort of 224 patients, males produced on average 0.42 stones/year, as opposed to 0.21 stones/year in females (Dello Strologo et al. 2002).

Based on these observations, we have compared the solubility of L-cystine in urine samples collected from healthy Caucasian men and women to test if we could reproduce in vitro these gender-related clinical observations and identify factors that promote L-cystine solubility.

Methods

Urine samples

First morning urine samples were collected from 48 healthy subjects, including 24 females and 24 males, aged 25–49 years. All had been healthy, denied any drug intake for at least 1 month, and gave their written informed consent. This study was approved by the ethical committee of the Bambino Gesù Children's Hospital. First morning voiding samples were treated anonymously (approximately 50 ml per subject), after dividing specimens into two groups, according to gender. A first aliquot was used immediately for the L-cystine precipitation assay. The remaining urine was divided into 2 ml aliquots and stored at −80 °C for subsequent analyses.

L-Cystine precipitation assay

L-Cystine precipitation was assessed using a modified version of the protocol described by Parvex et al. (2003). Briefly, 48 µl of 0.5 M Hepes was added to 12 ml of urine to a final concentration of 2 mM. Urine pH was then adjusted to 13.3 with concentrated NaOH and samples were split into 4 ml aliquots. One aliquot served to measure the basal L-cystine concentration, while the remaining two samples were used for L-cystine precipitation assays. To this end, 40 µl of freshly prepared L-cystine solution (solution A or solution B) was added to each urine sample. These solutions were prepared as follows:

- Solution A: L-cystine 400 mM, Hepes 0.2 M, pH 13.3 (final L-cystine concentration: 4 mM).
- Solution B: L-cystine 40 mM, Hepes 0.2 M, pH 13.3 (final L-cystine concentration: 0.4 mM).

The pH of all specimens was then lowered to 5.0 using concentrated HCl solutions; samples were then stored overnight at 4 °C to allow for L-cystine precipitation. The next morning, specimens were centrifuged at 3,000*g* (RCF) for 10 min and the amount of L-cystine that remained in solution was measured in the supernatant of each sample. Results were expressed as the percentage of L-cystine in solution, after dividing the measured amount of L-cystine in the supernatants by the total amount of L-cystine of each urine sample. This was calculated by adding basal L-cystine content to the amount of L-cystine that had been added using either solution A or B. A dilution factor of 3 % was used to account for solutions used for sample alkalinization and acidification.

L-Cystine measurements

Quantitative L-cystine determination was performed by HPLC after sample derivatization and chromatography, following a previously described protocol (Palacin et al. 1995), with minor modifications. Analyses were performed with an Agilent Technologies 1,200 series apparatus, which includes an HPLC system, a sample processor and solvent delivery system, and it is equipped with a fluorescence detector operating at excitation wavelength 390 nm and emission wavelength 478 nm (Agilent Technologies, Santa Clara, California, CA). Data were analyzed with the HP ChemStation for Windows NT software (Agilent Technologies, Santa Clara, California, CA).

Briefly, 10 µl of 4 M $NaBH_4$ (in 0.33 % DMSO and 66 mM NaOH), 5 µl of 2 mM EDTA/DTT (1:1), 5 µL of 1-octanol, and 5 µL of 2 mM HCl were injected into the derivatization vial containing 10 µl of urine sample. After 1 min at room temperature, the autosampler added 25 µl of 2 M N-ethylmorpholine buffer and 10 µl of 25 mM bromobimane dissolved in acetonitrile:H_2O (1:1). After 1 min, 20 µl of the admixture were injected into a 150 × 4.6 mm HypersiL-ODS HPLC column (Thermo Fisher Scientific Inc. Waltham, MA) that had been equilibrated with 30 mM ammonium nitrate and 40 mM ammonium formate buffers, at pH 3.6. Calibration curves for L-cystine (0–500 µmol/L) were obtained using L-cystine solution dissolved in 100 mM HCl and 0.1 mM DTT.

Mass spectrometry

Organic acids and other organic compounds, such as purines and pyrimidines, were analyzed by ElectroSpray Ionization (negative-mode without prior derivatization) and chromatography. Multiple reaction monitoring (MRM) was based on the analysis of metabolite-specific transition. Quantification was performed with a modified procedure adapted from a previously published protocol by Pitt et al. (2002). Tandem mass spectrometry was performed on a Sciex API365 triple-quadrupole mass spectrometer (PE Sciex Instrument, Applied Biosystems, Concord, ON, Canada) equipped with a IonSpray source and a syringe pump. Sample injection was automated with a HPLC PE series 200 autosampler (PerkinElmer, Waltham, MA). Nitrogen was used as the curtain and collision gas.

Ion intensities of the specific metabolites were compared with labeled internal standards by computer analysis (Neonatal Script, PE Sciex, Concord, ON, Canada). Ratios of MRM signals relative to the internal standards were used to generate calibration curves. Labeled internal standards were obtained from Cambridge Isotopes Laboratories (Andover, MA).

Other urine measurements

Urine ions, creatinine, BUN, and osmolarity were measured in the Bambino Gesù Children's Hospital clinical laboratory, using standard, quality-certified routine methods.

Urine citrate, isocitrate, oxalate, nitrite, nitrate, and sulfate were measured on a liquid chromatographic system (Dionex, Sunnyvale, CA, USA), equipped with an anion-exchange column and a conductivity detector. Background conductivity was reduced with the anion suppressor unit ASRS-300 4 mm (Dionex). Urine samples were diluted in bi-distilled water (1:20 v/v) and injected into the system. Anion separation was performed on a Ion-Pack AS11 analytical column (4 × 250 mm, Dionex) connected to a Ion-Pack AG11 guard column (5 × 50 mm, Dionex), using a linear gradient of the following two solvents: solvent A = bi-distilled water and solvent B = 50 mmol/l NaOH (Sigma-Aldrich, St Louis, MO, USA) (constant flow of 1.0 ml/min, 98:2 ratio solutions A:B at t_0, with a total run of 30 min). Concentrations were quantified by peak area analysis using external calibration curves.

Fluorimetric L-cystine precipitation assay

A real-time fluorimetric L-cystine precipitation assay was developed using a saturated suspension of L-cystine that was transiently brought into solution with HCl and that spontaneously precipitated after few minutes. Under these conditions, fluorescence increases substantially when L-cystine precipitates. A turbidimetry assay was also tested using real-time spectrophotometric light measurements, but was found to be less sensitive and to yield more variable results on repeated pilot experiments. Calibration curves using broad wavelength excitation and emission spectra (250–750 nm) and variable gates were used to identify conditions that optimize signal-to-noise ratios. The time that L-cystine remained in solution was used as an indicator of L-cystine stability in the aqueous phase. Measurements were performed with a PerkinElmer LS50B Luminescence Spectrometer (Waltham, MA), equipped with a 37 °C warming circuit and a magnetic stirrer.

The assay was performed at 37 °C using a freshly prepared PBS-L-cystine solution that contains phosphate-buffered saline, 1.7 mM $CaCl_2{\cdot}2H_2O$, 3.32 mM $MgSO_4$ and 4 mM L-cystine, pH 9.0. Two additional solutions were freshly prepared in PBS:

- Solution 1: adenosine, inosine, guanosine, vanillylmandelic acid: 20 mmol/l.
- Solution 2: adenine, guanine, hypoxantine, homovanillic acid: 20 mmol/l.

Before each run, 20 µl of solutions 1 or solution 2 or 20 µl of PBS (blank) was added to 2 ml of modified PBS-L-cystine in a fluorimeter cuvette. The cuvette was placed in the fluorimeter and stirred at constant speed at 37 °C. Fluorescence was measured at 390 nm after excitation at 350 nm. After 20 s to allow stabilization of baseline fluorescence, 100 µl of HCl 37 % was added to bring L-cystine into solution. The average emitted fluorescence per second was recorded to detect L-cystine precipitation. Each experiment lasted approximately 5 min.

Preparation of input files for docking

The crystal structure of L-cystine hydrobromide (LCHB, ID No. CCDC 744552) (Anbuchezhiyan et al. 2010) was

downloaded from Cambridge Crystallographic Data Center (CCDC, http://www.ccdc.cam.ac.uk/). The molecular packing of LCHB was created and visualized with Mercury 2.3 software from CCDC. For molecular docking, the input files containing atomic coordinates and charges were prepared using Chimera, a visualization tool developed by the Resource for Biocomputing, Visualization, and Informatics (UCSF, https://cgl.ucsf.edu/chimera/). Molecular docking was performed using DOCK 6.3 from the Molecular Design Institute of University of California San Francisco, (UCSF, http://dock.compbio.ucsf.edu/DOCK_6/index.htm) (Ewing et al. 2001; Lang et al. 2009). Coordinate files (.mol files) of adenosine, adenine, guanosine, guanine, inosine, hypoxantine, vanilmandelic acid, and homovanillic acid (the ligands) were created with ACD/Chemsketch (http://www.acdlabs.com), imported in Chimera, and converted in PDB or MOL2 files. All the procedures were performed on a multiprocessors PC running a linux-compatible OS (Ubuntu v.12.04 LTS).

Docking procedure

To perform a docking calculation the coordinate files of LCHB was used to compute the molecule surface using DMS package (Richards 1977). Spheres were generated and selected using sphgen and sphere_selector commands in DOCK 6.3. The box and the grid containing the parameters for calculation were created with showbox and grid commands. Docking was performed using flexible ligand parameter files and ligand coordinates according to general docking procedures.

Energy scoring

The score is a summation of the heavy atom contacts (every atom except hydrogen) between the ligand and receptor. A contact is defined as an approach of two atoms within some cutoff distance (usually 4.5 angstroms). If the two atoms approach close enough to bump (as identified with the bump grid), then the interaction can be penalized by a pre-determined amount. The attractive score in grid is negative and a repulsive score is positive. The energy scoring component of DOCK is based on the implementation of force field scoring. Force field scores are approximate molecular mechanics interaction energies, consisting of van der Waals and electrostatic components according to the following equation:

$$E = \sum_{i=1}^{\text{lig}} \sum_{j=1}^{\text{rec}} \left(\frac{A_{ij}}{r_{ij}^{a}} - \frac{B_{ij}}{r_{ij}^{b}} + 332 \frac{q_i q_j}{D r_{ij}} \right),$$

where each term is a double sum over ligand atoms i and receptor atoms j. A_{ij} and B_{ij} are the repulsion and attraction parameters, a and b the repulsive and attractive exponents (with $a > b$) and D is the dielectric constant.

Statistical analysis

Statistical analysis was performed with SPSS for Windows 11.0 software (SPSS Inc, Chicago, IL). Continuous data are expressed as mean ± SD after passing normality tests (Kolmogorov–Smirnov and Shapiro–Wilk) and were compared by ANOVA and MANOVA. All p values are two-sided and considered significant if <0.05.

Results

Urinary L-cystine solubility

To measure the solubility of L-cystine in urine, L-cystine was added at two different concentrations (0.4 and 4.0 mmol/l) to each sample, as described in the "Methods" section. As expected, more L-cystine remained in solution when less L-cystine was added to the urine samples (Fig. 1a). Specifically, 66.7 ± 13.3 % of the calculated total L-cystine remained in solution when L-cystine was added at a final concentration of 0.4 mM, as opposed to 28.7 ± 7.1 % when L-cystine was added to a final concentration of 4 mM. There was a significant consistency within subjects in their capacity to maintain L-cystine in solution when urine samples were loaded with different amounts of L-cystine (black lines in Fig. 1a). Data dispersion was consistent with a normal distribution, which allows calculating a Z score for each individual by averaging the SD score obtained after adding 0.4 and 4 mmol/l of L-cystine. Higher Z scores indicate higher L-cystine solubility.

Z scores were then compared according to gender. As shown in Fig. 1b, L-cystine solubility was markedly higher in female samples (+0.44 ± 1.1) than in male samples (−0.44 ± 0.70) ($p < 0.001$). As a whole, no significant relationship between L-cystine solubility and urine osmolarity was observed by regression analysis ($R = 0.06$, $p = 0.11$), indicating that gender differences were not related to differences in urine concentration (Fig. 1c). Interestingly, L-cystine solubility and urine osmolarity were significantly correlated in males ($r = 0.48$, $p = 0.017$) but not in females ($r = -0.22$, $p = 0.305$). Urine osmolarity was preferred to urine creatinine as an indicator of urine concentration, because creatinine excretion is dependent on muscular mass and is lower in females. Nonetheless, even when Z scores were expressed as a function of urine creatinine, no significant relationship was observed (data not shown).

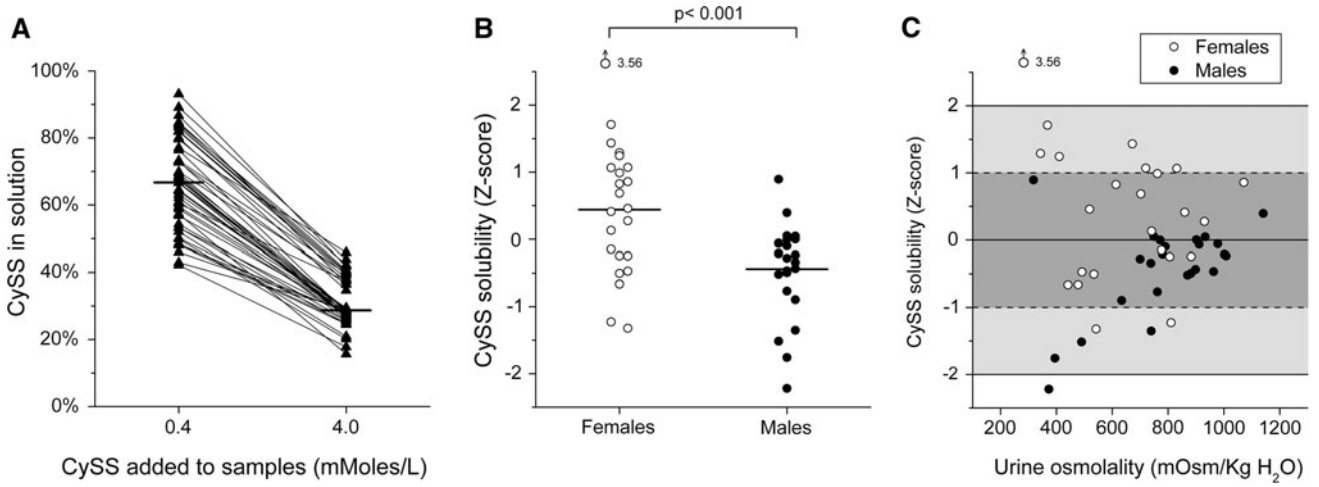

Fig. 1 Gender effect on L-cystine solubility. **a** Urines were loaded with L-cystine (CySS) to a final concentration of 0.4 or 4.0 mmol/l; CySS solubility was expressed as the percentage of CySS recovered in solution (see text for details). As expected, less CySS remained in solution at higher CySS concentrations. When comparing the percentage of CySS in solution, results were highly consistent within subjects, but showed marked differences between subjects. Data followed a normal distribution, allowing to calculate a CySS solubility score (Z score). **b** Z scores were significantly higher in female than in male subjects. **c** CySS solubility was not significantly influenced by urine concentration

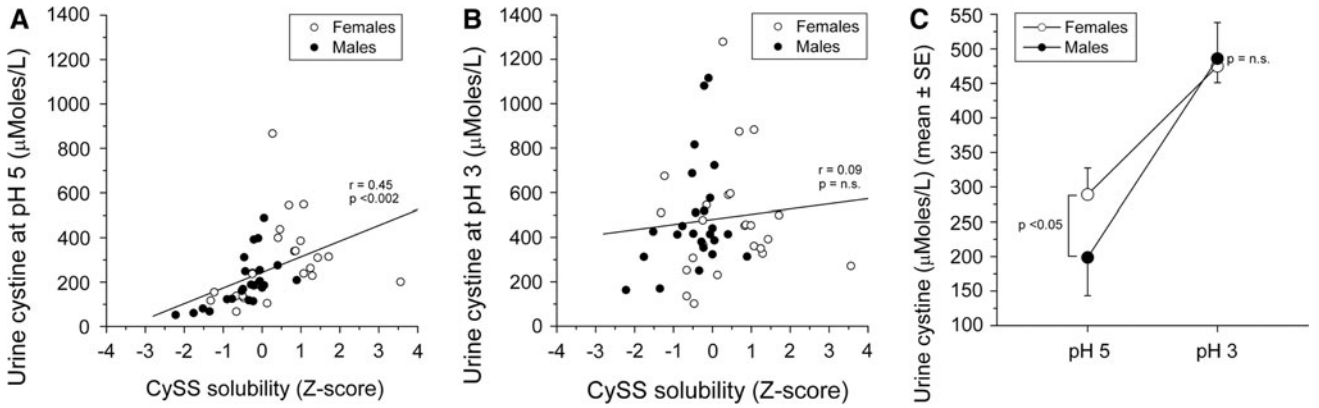

Fig. 2 Gender effect on L-cystine solubility. **a** Urine L-cystine (CySS) solubility correlated with baseline CySS concentrations at pH5. When samples were acidified to pH 3 (**b**), CySS concentration increased in all samples, indicating solubilization of part of the CySS that was not in solution at pH 5 and CySS solubility score no longer correlated with CySS solubility. Differences between genders at pH 5 disappeared when urine samples were acidified to pH 3 (**c**)

Analysis of urine metabolites

To search for parameters that influence L-cystine solubility, samples were divided according to gender and to their L-cystine solubility. To this end, subjects were divided into a low-solubility (Z score < 0, $n = 27$) and a high-solubility group (Z score > 0, $n = 21$) and then compared by ANOVA. A strong correlation was observed between urinary L-cystine concentration and L-cystine solubility (Fig. 2a), suggesting that more L-cystine remained in solution at baseline (prior to adding L-cystine as in Fig. 1) in samples with high solubility. Urine pH was, therefore, lowered to a value of 3 with HCl, in order to bring into solution all L-cystine molecules contained in each sample. As shown in Fig. 2b, the correlation observed at pH 5 was no longer present at lower pH values; differences in baseline L-cystine concentrations between males and females were abolished at pH 3 (Fig. 2c). This latter result was observed also when expressing L-cystine per mOsm/Kg H_2O or per mmol of creatinine (data not shown).

We then searched for organic and inorganic molecules that were associated with L-cystine solubility and with gender. As shown in Fig. 3, L-cystine solubility was significantly associated with urinary excretion of inosine ($p = 0.010$), vanillylmandelic acid (VMA) ($p = 0.015$), adenosine ($p = 0.029$), and guanosine ($p = 0.032$). Inosine, adenosine, and guanosine excretions were

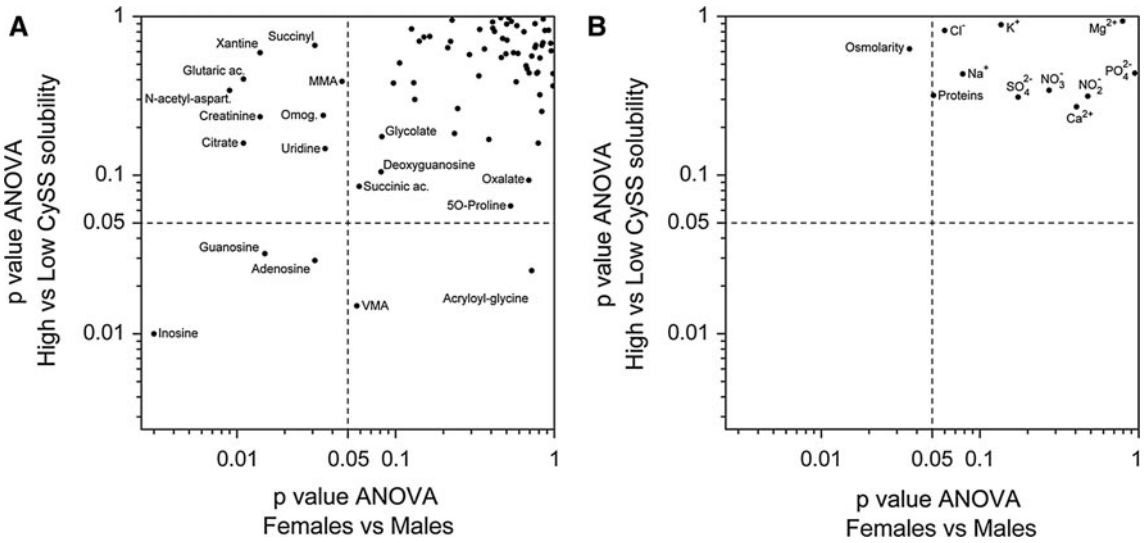

Fig. 3 Univariate analysis of variance (ANOVA) comparing gender and L-cystine-solubility groups. The *p* value for the ANOVA comparing excretion of urine metabolites in female versus male subjects and in high- versus low-L-cystine (CySS) solubility subjects are reported in the *X* and *Y* axis, respectively. **a** Results for organic compounds, **b** shows results for inorganic molecules, total urine proteins, and urine osmolarity. Solutes that are significantly associated ($p < 0.05$ *dashed lines*) with CySS solubility are represented in the *bottom* quadrants; solutes that are significantly associated with both CySS solubility and with gender are represented in the *left-lower* quadrant. Metabolites indicated in the *upper-right* quadrant of **a** include acetyl glycine, acryloyl glicine, adenine, adenosine, adipic ac., adipil glycine, azelaic ac., blood urea nitrogen, butyric + isobutytic ac., deoxyadenosine, deoxyguanosine, (ethylmalonic + methylsuccinic + glutaric ac.), (eptanoyl + 4-methylesanoyl glycine), eptenoyl glycine, (esanoyl + 4-methylesanoyl glycine), phenylacetyl glycine, phenylpropionyl glycine, fumaric ac., furoyl glycine, glyceric ac., glycolic ac., glycolate, urinary glucose, glutaryl glycine,, homovanillic ac., isocitrate, (isovaleryl glycine + 2-methylbutyryl glycine), lactic ac., malonic ac., 2-methylcrotonyl glycine, (3-methylglutaric ac. + 2-ketoglutaric ac.), methylcrotonyl glycine, 3-methyl glutaric ac., mevalonic acid lactone, *N*-acetylaspartic ac., octanoyl glycine, 3-hydroxy-methylglutaric ac., hydroxy-butyryl glycine, hydroxy-valeryl glycine, 2-hydroxy-glutaric ac., 2-hydroxy-isovaleric ac., 2-hydroxy-3-methylvaleric ac., 3-hydroxy-glutaric ac., 3-hydroxy-isovaleric ac., 3-hydroxy-propionic ac., 4-hydroxy-butyric ac., 4-hydroxy-phenyl lactic ac., 4-hydroxy-phenylacetyl glycine, 7,8-dihydroxy-octanoyl glycine, (4-hydroxy-butyric + 2-hydroxy-isobutyric ac.), orotic ac., oxalate, pyroglutamic ac., propionyl glycine, sebacic ac., sialic ac., suberic ac., suberic glycine, succinic ac., succinylacetone, sulfo-cysteine, thymine, uric ac., uracil, uridine, vanillylmandelic ac. *ac.* acid, *MMA* methylmalonic ac

Table 1 Urinary excretion of adenosine, inosine, guanosine, and VMA excretion in males and females

Metabolite	Mean	Median	SD	SE
Males ($N = 24$)				
Adenosine (μmol/l)	0.88	0.47	1.27	0.26
Inosine (μmol/l)	0.67	0.50	0.63	0.13
Guanosine (μmol/l)	0.13	0.10	0.11	0.02
VMA (μmol/l)	0.49	0.26	0.72	0.15
Females ($N = 24$)				
Adenosine (μmol/l)	0.44	0.25	0.57	0.12
Inosine (μmol/l)	0.40	0.34	0.24	0.05
Guanosine (μmol/l)	0.10	0.08	0.05	0.01
VMA (μmol/l)	0.33	0.12	0.53	0.11

Table 2 Logistic regression (dependent variable = solubility *Z* score <0)

Variable	OR	95 % CI	*p*
Univariate analysis			
Gender, female	0.20	0.06–0.69	0.011
Osmolality, mOsm/Kg H_2O	1.00	0.99–1.01	0.616
Adenosine (A), μmol/l	1.28	1.06–1.56	0.012
Inosine (I), μmol/l	1.32	1.04–1.67	0.020
Guanosine (G), μmol/l	2.99	1.05–8.49	0.040
Vanillylmandelic acid (VMA), μmol/l	1.65	1.12–2.43	0.012
A + I + G + VMA, μmol/l	1.14	1.03–1.25	0.010
Multivariate analysis			
Gender, female	0.26	0.06–1.15	0.075
Osmolality, mOsm/Kg H_2O	1.00	0.99–1.01	0.157
A + I + G + VMA, μmol/l	1.17	1.03–1.33	0.015

significantly associated with gender, while VMA excretion failed to reach statistical significance ($p = 0.057$). Table 1 summarizes the excretion levels of these metabolites within gender. VMA was included in further analyses because it had borderline statistical significance for gender, was highly associated with cystine solubility, and has a similar molecular structure to nucleosides.

Table 3 Pearson's correlations

	Adenosine (μmol/l)	Inosine (μmol/l)	Guanosine (μmol/l)	VMA (μmol/l)	Sum (μmol/l)
Inosine (μmol/l)					
Pearson's corr. coeff.	0.844				
p	**0.000**				
Guanosine (μmol/l)					
Pearson's corr. coeff.	0.772	0.665			
p	**0.000**	**0.000**			
VMA (μmol/l)					
Pearson's corr. coeff.	0.913	0.754	0.689		
p	**0.000**	**0.000**	**0.000**		
Sum (μmol/l)					
Pearson's corr. coeff.	0.990	0.893	0.779	0.943	
p	**0.000**	**0.000**	**0.000**	**0.000**	
Solubility (*Z* score)					
Pearson's corr. coeff.	−0.270	−0.319	−0.188	−0.278	−0.294
p	0.063	**0.027**	0.201	0.056	**0.043**

Significative *p*-values (<0.05) are indicated in bold

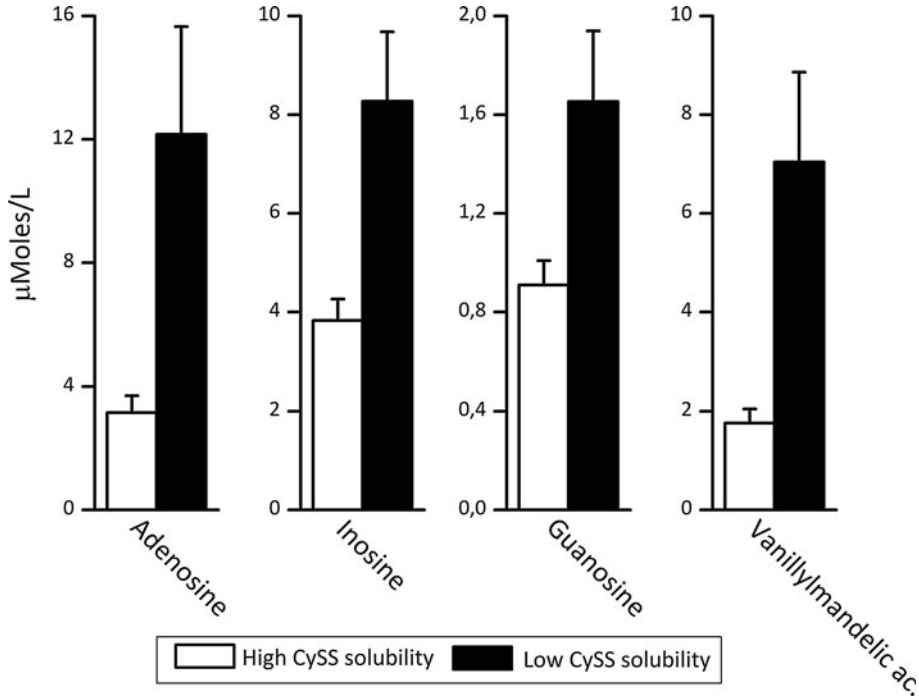

Fig. 4 Urinary concentrations of adenosine, inosine, guanosine, and vanillylmandelic acid in the high- and low-L-cystine (CySS) solubility groups (mean ± SE)

This analysis was refined by logistic regression (Table 2). By univariate analysis, the sum of the four above-indicated metabolites was highly associated with the L-cystine solubility group ($p = 0.010$); when normalized for gender and urine osmolarity, this association remained highly significant ($p = 0.015$).

Correlation analysis between these metabolites and L-cystine solubility expressed as *Z* score similarly showed a negative correlation between the sum of these four metabolites and L-cystine solubility ($p = 0.043$) (Table 3). Overall, inosine was the metabolite that was most significantly associated with L-cystine solubility, both in the logistic model and in the correlation analyses. Correlation between adenosine, guanosine or VMA and L-cystine solubility *Z* score showed a negative trend, but failed to reach statistical significance. Mean concentrations of adenosine, inosine, guanosine, and VMA in the two solubility groups are reported in Fig. 4.

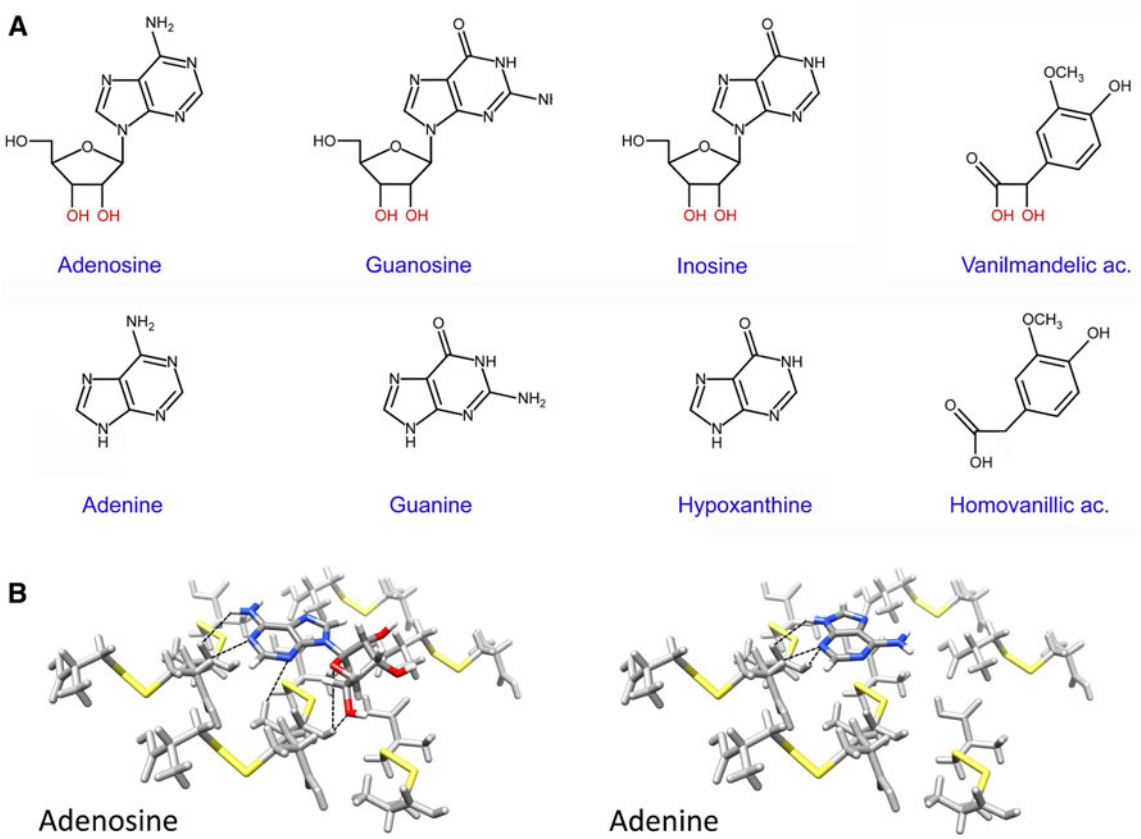

Fig. 5 Structure of adenosine, inosine, guanosine, and vanillylmandelic acid. **a** The structure of compounds identified by ANOVA are reported and compared with their corresponding nitrogenous bases (for nucleosides) or homovanillic acid (for VMA). As shown, adenosine, inosine, guanosine, and VMA are characterized by the presence of two neighboring highly hydrophilic OH groups (indicated in *red*). **b** Modeling of the interactions between adenosine (*left*) and adenine (*right*) with a L-cystine (CySS) crystal. Interactions are indicated with *dashed black lines*. Nitrogen and oxygen atoms of adenosine and adenine are represented in *blue* and *red*, respectively. Sulfur atoms of CySS are indicated in *yellow* (color figure online)

Impact of nucleosides and VMA on L-cystine metastability

We then tested the contribution of nucleosides and VMA on L-cystine solubility in silico and in vitro. These organic molecules are characterized by the presence of two hydroxyl groups; nucleosides have a 3,4-diol group on their ribose moiety, while VMA is an α-hydroxy acid that exposes 2 hydroxyl residues (Fig. 5a). On these bases, we have speculated that these groups are particularly important in initiating or stabilizing L-cystine crystals by electrostatic interactions. The correspondent nitrogenous bases and homovanillic acid (HVA), which all lack these hydroxyl groups (Fig. 5a), have been used for comparison in a subsequent set of experiments that are detailed below (indicated as "control molecules").

Interactions with L-cystine crystals were first analyzed by in silico modeling (molecular docking). In general terms, "docking" is the identification of the low-energy binding modes of small molecules with a macromolecular compound of known structure; the docking procedure allows to place each metabolite in optimal positions within the L-cystine crystal, as defined by Anbuchezhiyan et al. (2010). The total binding energy and the contributions of

Table 4 In silico analysis of molecular interactions

Molecule name	Total energy	Van der Waals contribution	Electrostatic contribution
Adenosine (A) (kcal/mol)	−31.8	−25.7	−6.1
Adenine (a) (kcal/mol)	−15.8	−14.9	−0.9
Guanosine (G) (kcal/mol)	−29.9	−25.3	−4.6
Guanine (g) (kcal/mol)	−17.4	−16.0	−1.4
Inosine (I) (kcal/mol)	−32.4	−23.8	−8.6
Hypoxantine (i) (kcal/mol)	−25.2	−20.4	−4.8
Vanillylmandelic acid (VMA) (kcal/mol)	−27.3	−22.2	−5.1
Homovanillic acid (HVA) (kcal/mol)	−15.8	−15.2	−0.6

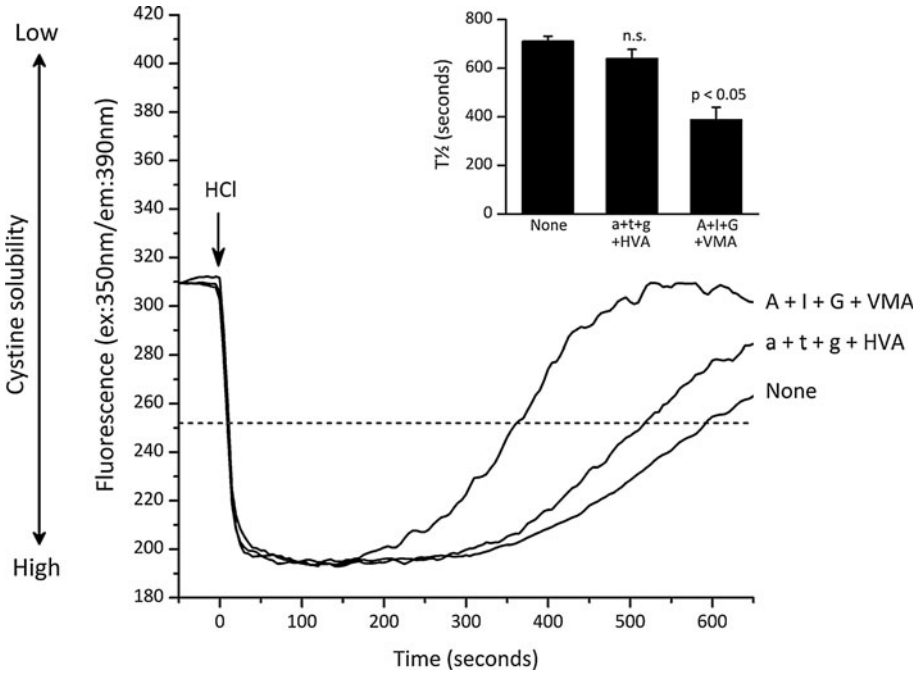

Fig. 6 L-Cystine (CySS) precipitation assay. A real-time fluorimetric L-cystine (CySS) precipitation assay was developed using a saturated suspension of L-cystine that was transiently brought into solution with HCl and spontaneously precipitated after few minutes. CySS precipitation out of solution causes increased fluorescence emission (390 nm) after excitation at 350 nm. Experiments were performed using PBS-modified solutions, as detailed in the methods section. Blank tests were performed after HCl addition to PBS-modified samples without CySS and showed no detectable fluorescence above background (water) (data not shown). Fluorescence was sampled at 1-s intervals. Each *line* represents the average of five experiments. The ½ time ($T½$) to recover half of the baseline fluorescence was used to compare CySS re-precipitation rates after HCl (*insert*). CySS precipitated significantly faster when a mixture of adenosine, inosine, guanosine, and VMA (A + I + G + VMA) was added to samples, in comparison with samples added with adenine, hypoxantine, guanine, and HVA (a + h + g + HVA) or to PBS ("none")

Van der Waals and of electrostatic forces were then calculated. As shown in Table 4, the binding energy of adenosine, guanosine, inosine, and VMA is more negative, indicating stronger binding, than the energy of control molecules. The patterns of molecular interactions between an L-cystine crystal and adenosine or adenine are reported in Fig. 5b; higher number of interactions reflects the more favorable energy score of adenosine. Similar 3D models were obtained with the other studied compounds (data not shown).

We then tried to decrease the precipitation of L-cystine of urines by adding directly nucleosides or VMA to samples and exposing them to the same precipitation test as detailed above, but failed to see significant changes (data not shown). We, therefore, raised the hypothesis that nucleosides and VMA are primarily implicated in promoting the nucleation of L-cystine crystals in the lumen of renal tubules, leading to the excretion of higher amounts of microcrystals; these microaggregates of L-cystine would then promote cystine precipitation in our in vitro assay.

To begin testing this hypothesis, real-time precipitation experiments were developed as detailed in the "Methods" section, allowing to bring in contact high concentrations of solubilized L-cystine molecules with small amounts of nucleoside or VMA (relative concentration 20:1, respectively). As shown in Fig. 6, fluorescence decreases after L-cystine solubilization with HCl and increases again after 300–400 s, indicating L-cystine precipitation. The same experiment was performed after adding a mix of adenosine, guanosine, inosine, and VMA, demonstrating a significant increase in the precipitation rate of L-cystine that was not observed using control molecules.

Discussion

Cystinuria is an autosomal recessive disorder secondary to impaired transport of cystine, lysine, ornithine, and arginine in the proximal renal tubule and in epithelial cells of the gastrointestinal tract, that has a prevalence ranging 1:2,500 in the Libyan Jewish population to 1:100,000 in Northern Europe (Palacin et al. 1995).

The disease is primarily diagnosed in non-familial cases after detecting renal stones in patients presenting with renal colic or macroscopic hematuria. In recent years, widespread use of abdominal ultrasonography has significantly

increased the number of asymptomatic patients diagnosed with cystine stones, including young children, prior to any clinical symptoms.

Disease evolution is variable and is characterized by relapsing episodes of nephrolithiases, which generally appear before the age of 15 years, and frequently as early as infancy, but rarely lead to chronic renal insufficiency (Dello Strologo et al. 2002). The quality of life of many patients with cystinuria, however, is severely compromised by recurrent episodes of renal colic that often result in hospital admissions and repeat surgical or endoscopic procedures for stone removal.

To date, no satisfactory therapy has been identified. Patients are primarily treated with lifelong hyperhydration and alkali therapy; diet modifications are poorly effective (Rodman et al. 1984). Sulfhydroxyl compounds, such as D-penicillamine or α-mercaptopropionylglycine, can reduce the formation of cystine crystals by reacting with L-cystine and generating more soluble mixed disulfide compounds; however, they do not prevent completely renal stone recurrences and may have significant side effects, including foul odor, nausea, fever, fatigue, skin allergies, premature skin aging, and hypersensitivity (Dello Strologo et al. 2007; Dolin et al. 2005; Goodyer 2004; Pietrow et al. 2003). In an attempt to overcome some of these side effects, alternative approaches based on in vitro studies of cystine crystal growth inhibition using L-cystine methyl- and dimethyl-ester (L-CME, L-CDME), for example, are currently being evaluated (Rimer et al. 2010); however, studies in other disease models have shown that L-CDME, for example, can be toxic to cells (Wilmer et al. 2007).

Overall, renal stone disease is a frequent condition in the general population, which has been shown in several cohort studies to be consistently more frequent in male than in female subjects (Alexander et al. 2012; Pearle et al. 2005; Stamatelou et al. 2003; Yasui et al. 2008). In these studies, hypercalciuria represents the most common risk factor for nephrolithiases (Cirillo et al. 2003) and is closely related to dietary habits, in particular to sodium intake; low-salt diet and hyperhydration represent, therefore, the mainstay approach for the prevention of non-cystinuric kidney stones (Borghi et al. 2002).

Similarly, male patients with cystinuria are also more likely to develop symptoms earlier in life and to exhibit a higher stone emission rate (Dello Strologo et al. 2002), although no relationship with a specific urinary excretion pattern underlying male predisposition has been demonstrated to date. In particular, in our review of more than 200 cystinuric patients collected in the European cystinuria database, we failed to observe a relationship between urinary calcium and disease severity (unpublished data). Of notice, male predominance was evident since early childhood, suggesting that hormonal factors are unlikely to play a significant role (Dello Strologo et al. 2002). Previous work has documented a significant association between urine cystine and sodium excretion rates in cystinuric urines (Goldfarb et al. 2006).

In an attempt to develop new strategies for preventing cystine stone formation, we have hypothesized that an unsupervised analysis of urine samples obtained from normal male and female subjects would help identifying gender-related urinary factors that influence L-cystine metastability in urines.

Among natural amino acids, L-cystine has the highest propensity to form crystals (Moe 2006; Rimer et al. 2010). L-cystine solubility is considerably dependant on pH. In vivo, the limit of solubility at pH 6 is approximately 1.3 mmol/l, while at pH values >7 solubility increases steeply (14); in vitro, L-cystine can also be brought back into solution by lowering pH < 4 (Apruzzese et al. 2002). In cystinuric patients, impaired proximal tubular reabsorption increases urine L-cystine concentration above its solubility threshold (often >3 mmol/l), leading to urine supersaturation and ultimately to nucleation of L-cystine crystals and to the development of stones (Nakagawa et al. 2000). Unlike calcium oxalate and uric acid, few in vitro systems have been developed to assess L-cystine metastability in solutions (Coe et al. 2001; Parvex et al. 2003). Moreover, it is important to recognize that in vitro systems, which often rely on regression analyses and association studies, may not be appropriate to detect threshold phenomena and that systems aimed at assessing cystine solubility in vitro require steps to bring cystine into solution that may not represent the physiological conditions in vivo.

L-Cysteine is a polar hydrophilic amino acid that interacts with H_2O molecules in part through its sulfhydryl residues (-SH). Conversely, water solubility of L-cystine decreases substantially, as a result of the oxidation of thiol side-chains that form a disulfide bridge linking together two molecules of L-cysteine. This generates a symmetrical compound, which further facilitates the formation of ordered crystals. In vitro, L-cystine can be crystallized at physiological pH by slow evaporation, acidification of basic L-cystine solutions, or gradual cooling of solutions supersaturated with L-cystine (Dahaoui et al. 1999; Fujiki et al. 2006; Girija et al. 1995). Under these conditions, zwitterionic L-cystine molecules form hexagonal plates with large basal surfaces that can achieve a width of 400 μm and a thickness of 30 μm (Dahaoui et al. 1999; Moggach et al. 2005; Oughton and Harrison 1959; Rimer et al. 2010); more rarely they also form tetragonal crystals (Chaney and Steinrauf 1974).

No in vivo data are available on factors that facilitate the formation of cystine crystals. Importantly, L-cystine molecules that remain in the lumen of renal tubules as a consequence of impaired transport are exposed to significant

physicochemical changes (other organic and inorganic compound concentration, changes in osmolarity and pH) as they are progressively concentrated along the nephron, before being excreted in urines. These intra-renal changes may in fact be essential to trigger the nucleation of cystine crystals and may not be entirely reproducible in vitro.

Hexagonal crystals contain helices spanning six molecules of L-cystine that interact with each other with strong $NH_3^+\cdots{}^-O(C=O)$ hydrogen and S···S disulfide bonds (Oughton and Harrison 1959; Rimer et al. 2010). Other molecules facilitating the initial interaction between molecules of L-cystine may have a prominent role in promoting nucleation. Our results show a significant association between L-cystine solubility and urine concentration of adenosine, inosine, guanosine, and VMA, suggesting that these compounds influence the nucleation of crystals and/or the interaction of L-cystine with the microenvironment at the surface of renal tubular cells, which is thought to play a critical role in the formation of other renal stones (Sheng et al. 2005).

Our in vitro fluorimetric precipitation assay allowed us to test the precipitation of a super-saturated L-cystine suspension that was transiently brought into solution in buffered medium by adding HCl. This probably mimicked the initial conditions for the nucleation of L-cystine crystal; similar strategies are in fact used to generate L-cystine crystals in vitro for crystallography studies (see above). Our results allow speculating that adjacent hydroxyl groups of nucleosides or VMA engage in molecular interactions that bring together L-cystine molecules and initiate the formation of crystals or stabilize L-cystine multimers. These molecules were more concentrated in male than in female urines, which may explain the observed differences in L-cystine metastability between normal male and female subjects. Conversely, control molecules sharing the same organic structure without hydroxyl groups had remarkably lower effect on L-cystine precipitation.

Also, nucleosides or VMA had little impact when added directly to urine samples, suggesting that they promote the initial formation of L-cystine microaggregates in vivo, which are then excreted into urines causing lower L-cystine solubility in vitro when more L-cystine is added to samples. This view is further supported by our acidification data, showing that lowering urine pH to 3 increases L-cystine concentration by more than twofold and abolishes differences between males and females (Fig. 2c). Further tests are clearly needed to assess if similar factors play a role in urines of cystinuric patients. Remarkably, however, cystine solubility was not dependent in our experiments on urine concentration in females, indicating that the balance between factors promoting or preventing L-cystine precipitation could be more important than their absolute concentration. This is also in agreement with recent data on cystinuric urine samples showing that the solubility of L-cystine was weakly correlated with 24 h L-cystine excretion rates but not with urinary volumes (Goldfarb et al. 2006).

The concentration of urinary nucleosides is dependent on the degree of RNA degradation, which reflects the human body metabolic state (Struck et al. 2013). A number of diseases, including hepatitis, obstructive lung disease, cirrhosis, acute cholecystitis, acute pancreatitis, regional enteritis, rheumatoid arthritis, ulcerative colitis, psoriasis, urinary tract infections, and other bacterial infections have been associated with changes in urine nucleoside levels, although for the most part these changes are modest (Heldman et al. 1983; Schram 1998). Very few data exist on the excretion of nucleosides in normal subjects; in general, these are thought to be relatively constant in time and to be slightly higher in females when normalized to urine creatinine, as a result of higher urinary creatinine excretion in men (Szymanska et al. 2010). In the present work, urine concentration of nucleosides was directly used in the analyses, based on the assumption that cystine metastability is primarily related to the absolute concentration of surrounding molecules. Importantly, however, nucleoside concentrations remained significantly associated with lower cystine solubility after correction for gender and for urine concentration, as indicated in the multivariate analysis reported in Table 2.

From a pharmacological stand point, it is noteworthy that purines are structural components of several drugs such as 6-mercaptopurine, azathioprine, allopurinol, and several other synthetic nucleoside analogues that are used in anti-tumoral and anti-viral therapies; in addition, theophylline and caffeine, which are frequent component of most adult diets, also have a purine moiety in their structure (Rashidi and Pashaei-Asl 2012). Some of these molecules may modify solubility, which may be relevant in prescribing drugs and diets to cystinuric patients.

Conflict of interest The authors declare that they have no conflict of interest.

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