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Influence of Calcium-Sensing Receptor Gene on Urinary Calcium Excretion in Stone-Forming Patients

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Abstract. Calcium-sensing receptor (CaSR) is a plasma membrane protein that regulates tubular reabsorption of Ca. To establish its role in idiopathic hypercalciuria, the association of urinary Ca excretion with the polymorphisms of *CASR* gene has been studied in healthy subjects and in hypercalciuric and normocalciuric Ca stone formers. *CASR* exon 7 single nucleotide polymorphisms (SNP), G/T at codon 986, G/A at codon 990, and C/G at codon 1011, were evaluated by PCR amplification and direct sequencing in 97 normocalciuric stone formers, 134 hypercalciuric stone formers, and 101 normocalciuric healthy controls. Four haplotypes were defined on the basis of *CASR* gene SNP: haplotype 1 was characterized by the most frequent sequence; haplotypes 2, 3, or 4 by the presence of a single polymorphic variant at codon 986, 990, or 1011, respectively. The relative risk of hypercalciuria was calculated with multinomial logistic regression and was significantly increased only in individuals carrying haplotype 3 (Odds ratio, 13.0 [95% confidence interval, 1.7 to 99.4]). Accordingly, Ca excretion was higher in subjects bearing haplotype 3, whereas those bearing haplotype 2 showed a slight increase of plasma Ca concentration. Multiple regression analysis showed that haplotype 3 explained 4.1% of the total variance of Ca excretion and 12.6% of the variance explained by the variables considered in the study. In conclusion, *CASR* gene could be a component of the complex genetic background regulating Ca excretion. Arg990Gly polymorphism could facilitate activation of CaSR and increase Ca excretion and susceptibility to idiopathic hypercalciuria.

Calcium-sensing receptor (CaSR) is a plasma membrane protein that regulates parathyroid hormone (PTH) secretion by parathyroid cells and Ca reabsorption by kidney tubular cells (1–3). It is activated by the increase of extracellular concentration of Ca ions, which bind to the large extracellular N-terminal domain of the CaSR molecule (1,2). CaSR exerts its cellular activity through the stimulation of a G protein by its intracellular tail (2), which leads to the inhibition of PTH production (3) and tubular Ca reabsorption (2,4). In addition, CaSR influences intestinal Ca absorption (5), bone remodeling (6,7), and even nervous transmission (8).

Inactivating mutations of *CASR* gene account for disorders characterized by hypocalciuria and hypercalcemia due to the altered control of PTH release and tubular Ca reabsorption: heterozygotes develop benign familial hypocalciuric hypercalcemia, whereas homozygotes are affected by severe neonatal hyperparathyroidism (9,10). Through opposite mechanisms, mutations enhancing CaSR activity lead to disorders characterized by hypocalcemia and hypercalciuria (11).

Three clustered single nucleotide polymorphisms (SNP), causing nonconservative amino acid changes, have been described on exon 7, encoding the intracellular domain of CaSR (12,13). The most common SNP consists of the substitution of a guanine with a thymine at codon 986, leading to the Ala986Ser variant (12,13). The other two SNP are less frequent: an adenine/guanine substitution at codon 990 determines the Arg990Gly variant; a cytosine/guanine substitution at codon 1011 determines the Gln1011Glu variant. The functional effects of these amino acid changes are yet unknown, but a slight increase of plasma Ca concentrations, not associated with symptoms, was found in healthy subjects carrying the 986Ser allele (12).

Studies in humans (9) and in canine tubular cells (4) showed an inhibitory effect of CaSR on cellular Ca transport, suggesting that CaSR plays a key-role in the regulation of Ca excretion. In keeping with these observations, *CASR* gene has been

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considered a candidate for idiopathic hypercalciuria (14), a familial disorder predisposing to Ca kidney stones and characterized by increased Ca excretion in the presence of normal Ca concentrations in plasma (15,16). This view has been contrasted by a study in Ca stone-forming French-Canadian sibling pairs, finding no linkage between idiopathic hypercalciuria and *CASR* gene locus (17). However, even though *CASR* gene may not be a major determinant in idiopathic hypercalciuria, its polymorphisms may influence tubular Ca reabsorption capacity. Therefore, in the present study, the association of urinary Ca excretion with *CASR* gene SNP was evaluated in an Italian population of healthy subjects and of hypercalciuric and normocalciuric Ca stone formers.

Materials and Methods

Patients

Ninety-seven normocalciuric stone formers (65 male patients and 32 female patients; weight, 69 ± 1.2 kg; age, 45 ± 1.5 yr), 134 hypercalciuric stone formers (78 male patients and 56 female patients; weight, 69 ± 1.1 kg; age, 47 ± 1.1 yr), and 101 normocalciuric healthy controls (58 male patients and 43 female patients; weight, 72 ± 1.2 kg; age, 46 ± 1.2 yr) were included in the study. These subjects were white and were characterized by no polymorphic variants or by the presence of a polymorphic variant at one single *CASR* exon 7 SNP. According to these criteria, six individuals (two healthy subjects, one normocalciuric, and three hypercalciuric stone formers), who were double heterozygotes for two *CASR* exon 7 SNP, were not included among the subjects enrolled for the study.

Stone formers were recruited at their first visit at the Outpatient Stone Clinic of San Raffaele Hospital in Milan, Italy. To be included in the study, stone formers had to have produced at least one Ca kidney stone and their plasma creatinine, sodium, potassium, and Ca concentrations had to be normal. All of them had to be able to lower urinary pH under 5.5. Stones present in patient urinary tract had to have diameter smaller than 5 mm. None of them had to have obstructive nephropathy or urinary tract dilatation, evaluated by echographic examination. Most of the patients ($n = 150$; 65%) were addressed to the clinic after lithotripsy and were studied at least 3 mo after lithotriptic treatment. The hospital's protocol does not include any pharmacologic treatment for kidney stones immediately after lithotripsy, but only dietary counseling (increase of water intake). The other patients ($n = 81$; 35%) were sent to the Outpatient Clinic by attending physicians before starting any kind of treatment. In no case was a patient studied if he or she took any treatment for kidney stones (thiazide, citrate, or others) for at least 3 mo before the study. No patient had diseases other than kidney stone, and none took any long-term medications.

Healthy subjects were selected from the population recruited for the InCHIANTI study, an epidemiologic survey conduced in Greve and Bagno a Ripoli, two little towns near Florence. They were selected according to the following criteria: absence of hypercalciuria, age between 20 and 60 yr, no clinical diagnosis of hypertension, diabetes, dyslipidemia, stroke, coronary heart disease, kidney stones, and no treatment with drugs affecting Ca metabolism, like Ca salts or vitamin D. The rationale and methods of the InCHIANTI study have been previously reported (18).

Stone forming and healthy subjects were considered hypercalciuric when their 24-h Ca excretion was greater than 7.5 mmol in male patients or 6.25 mmol in female patients or greater than 0.1 mmol/kg of body weight independently of gender (19).

The study agrees with the indications of the Helsinki Declaration and was approved by San Raffaele Hospital Ethical Committee. All subjects gave informed consent to the study and genomic analysis.

Phenotyping

Total plasma concentration of Ca and 24-h Ca excretion were determined in healthy subjects and in stone-forming patients. In stone formers, 24-h excretion of phosphates and sodium, serum phosphate, plasma creatinine, and PTH and intestinal absorption of strontium were also measured. Intact PTH was determined by immunoradiometric assay (Nichols Institute). Strontium was used as a surrogate marker to assess intestinal Ca absorption. Intestinal strontium absorption was determined after an oral load, as described previously (20). Strontium (30.2 μmol of Sr per kg of body weight) was administered to patients in distilled water solution as chloride salt after overnight fast. Blood samples were drawn 60 min after strontium load ingestion, and strontium concentrations were measured in serum by atomic absorption spectrophotometer (Perkin-Elmer 4000; Perkin-Elmer, Norwalk, CT). Ion absorption was calculated as the incremental area (above baseline) under the serum strontium concentration-time curve, using the trapezoid method (mmol $\cdot$ L^{-1} $\cdot$ min).

Genotyping

Genomic DNA was isolated from peripheral blood cells by standard procedures. Mutation screening was carried out by denaturing gradient gel electrophoresis (DGGE) in hypercalciuric patients. The entire *CASR* coding sequence (exons 2 to 7) was amplified by the PCR. DNA amplification primers for each exon were designed on the basis of Pearce *et al.* and Vigouroux *et al.* (11,21); moreover, one of the two primers used in each PCR had the proper 5' end GC-tail to create a high melting temperature domain.

Samples were run on the Bio-Rad DCode denaturing gel electrophoresis system for mutation detection (Bio-Rad, Hercules, CA). The denaturing conditions were from 20% to 80%, whereas acrylamide concentration ranged from 6% to 10% depending on fragment length. Electrophoresis was performed at a constant temperature of 60°C at 120 V for 12 h. Gels were stained with ethidium bromide and were photographed with Kodak Digital Camera (Eastman Kodak, Rochester, NY).

Part of exon 7 of *CASR* gene, which comprises 3 SNP (G/T at codon 986, A/G at codon 990, and C/G at codon 1011), were evaluated by PCR amplification and direct sequencing. PCR was carried out using primers 5'CAGAAGGTCATCTTTGGCAGCGGCA3' and 5'TGCAGACCTGTTTCCTGGACGGTC3', sequences that flank the polymorphic site giving an amplimer of 206 bp. Purified PCR products served as template for the sequence reaction, which was performed using a ready reaction mix (ABI PRISM Big-Dye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, CA) and the same forward primer as for PCR. Sequence products were generated during 25 cycles of the following steps: 15 s at 96°C, 10 s at 50°C, and 2 min at 60°C. Unincorporated dye terminators were removed by ethanol/sodium acetate precipitation. Afterward, dyed samples were denatured in 20 μl of Template Suppression Reagent (TSR, Applied Biosystems) at 95°C for 10 min and cooled on ice for 15 min. The ABI PRISM 310 Genetic Analyzer (Applied Biosystems) was used for capillary electrophoresis. All sequences were analyzed for comparison using resident software.

Four haplotypes were defined on the basis of exon 7 SNP of *CASR* gene (Table 1): they were respectively characterized by the most common sequence (haplotype 1) or by the presence of one single polymorphic variant, respectively at 986 (haplotype 2), 990 (haplo-

Table 1. Patients and haplotypes[a]

	n		Base Change			Amino Acid Change		
			986	990	1011	986	990	1011
Group 1	186	Haplotype 1	GCC	AGG	CAG	Ala	Arg	Gln
		Haplotype 1	GCC	AGG	CAG	Ala	Arg	Gln
Group 2	101	Haplotype 2	**T**CC	AGG	CAG	**Ser**	Arg	Gln
		Haplotype 1	GCC	AGG	CAG	Ala	Arg	Gln
	17	Haplotype 2	**T**CC	AGG	CAG	**Ser**	Arg	Gln
		Haplotype 2	**T**CC	AGG	CAG	**Ser**	Arg	Gln
Group 3	15	Haplotype 3	GCC	**G**GG	CAG	Ala	**Gly**	Gln
		Haplotype 1	GCC	AGG	CAG	Ala	Arg	Gln
	1	Haplotype 3	GCC	**G**GG	CAG	Ala	**Gly**	Gln
		Haplotype 3	GCC	**G**GG	CAG	Ala	**Gly**	Gln
Group 4	12	Haplotype 4	GCC	AGG	**G**AG	Ala	Arg	**Glu**
		Haplotype 1	GCC	AGG	CAG	Ala	Arg	Gln

[a] Healthy controls and stone formers were divided according to their haplotypes for *CASR* exon 7. Four haplotypes were considered and defined by the most frequent wild-type sequence (haplotype 1) or by the polymorphic variant for one single SNP (haplotype 2, 3, and 4). Each polymorphism was assumed dominant, and group membership was assigned to patients carrying one or two alleles for each SNP. None was homozygous for Gln1011Glu polymorphism. Base and amino acid changes are indicated for each SNP.

type 3) and 1011 (haplotype 4). According to these criteria, six double heterozygotes at *CASR* exon 7 SNP were not considered, as already mentioned. Participants in the study were divided into four groups according to their haplotypes: group 1 was composed by Ala986, Azg990, and Gen1011 homozygotes; group 2 was composed by 986Ser homozygotes and Ala986Ser heterozygotes; group 3 was composed by 990Gly homozygotes and Arg990Gly heterozygotes; group 4 was composed by Gln1011Glu heterozygotes (Table 1).

Statistical Analyses

Distribution of *CASR* genotypes was compared in normocalciuric and hypercalciuric stone formers and healthy subjects by multinomial logistic regression. Relative risk to be hypercalciuric was estimated by the calculation of the odds ratio (OR) and its 95% confidence interval (95% CI).

Quantitative variables were reported in the text as mean ± SE. Differences of the means were tested by *t* test or one-way ANOVA with Scheffe's *post-hoc* multiple comparisons test.

Dependence of Ca excretion or plasma Ca concentration on *CASR* haplotypes was evaluated in stone formers by multiple regression analysis. To take into account the effect of possible confounders, we used a two-step multiple regression model in which the confounders were entered at the first step and *CASR* haplotypes at the second step. Statistical analyses were conducted at the $\alpha = 0.05$ level and were two-tailed. All analyses were performed using the SPSS 10 statistical package (SPSS Inc., Chicago, IL).

Results

Frequency of CaSR Polymorphisms

Preliminary analysis by DDGE showed no abnormal mobility pattern of *CASR* gene in either hypercalciuric and normocalciuric stone formers, but the already described shifts of exon 7 (data not shown). Genotype frequencies at polymorphic sites did not deviate from those expected according to Hardy-Weinberg equilibrium in both healthy subjects and stone formers. Furthermore, no linkage disequilibrium was observed among the three SNP (data not shown). In the whole population, haplotype 1 represented the most frequent sequence of *CASR* gene exon 7.

Participants were divided into four groups, each comprising homozygotes and heterozygotes for each *CASR* gene exon 7 haplotype (Table 1). Their distribution is shown in Table 2. To test the relationship between CASR haplotypes and clinical classification (healthy subjects, normocalciuric stone formers, and hypercalciuric stone formers), a multinomial logistic regression was performed with clinical classification as the de-

Table 2. Distribution of individuals with different *CASR* haplotypes was compared in healthy subjects and normocalciuric or hypercalciuric stone formers[a]

	Healthy Subjects	Hypercalciurics	Stone Formers Normocalciurics	Total
Group 1	53 (52.5%)	74 (55.2%)	59 (60.8%)	133 (57.6%)
Group 2	44 (43.6%)	43 (32.1%)	31 (32%)	74 (32.0%)
Group 3	1 (1%)	13 (9.7%)	2 (2.1%)	15 (6.5%)
Group 4	3 (3%)	4 (3%)	5 (5.2%)	9 (3.9%)

[a] Group composition is displayed in Table 1.

pendent variable and haplotype group as the factor chosen for the design. Healthy controls were the reference population in the analysis. Relative risk to be hypercalciuric (Table 3) was significantly increased in haplotype 3 subjects (OR, 13; 95% CI, 1.7 to 99.4).

Association between Haplotypes and Phenotypes in the Whole Population

The quantitative variables were compared in individuals with different haplotypes. At first, healthy subjects and stone formers were considered together (Table 4). Ca excretion was higher in individuals carrying haplotype 3 ($n = 16$; 9.18 ± 0.95 mmol/24 h) than in those not carrying haplotype 3 ($n = 316$; 6.16 ± 0.19 mmol/24 h; $P = 0.0005$, *t* test). Although one-way ANOVA showed no significant difference in plasma Ca concentration between individual groups (Table 4), it was slightly higher in individuals carrying the haplotype 2 ($n = 118$; 2.37 ± 0.01 mmol/L) than in those not carrying haplotype 2 ($n = 214$; 2.35 ± 0.01 mmol/L; $P = 0.047$, *t* test). This difference was particularly marked in the 17 homozygotes for haplotype 2 (2.41 ± 0.02 mmol/L; $P = 0.02$ *versus* individuals not carrying haplotype 2, *t* test), whereas their Ca excretion was not increased (5.46 ± 0.89 mmol/24 h).

Association between Haplotypes and Phenotypes in Stone Formers

Also when stone formers were considered alone (Table 5), Ca excretion remained significantly higher in haplotype 3 than in haplotype 1 patients or when compared with all patients not carrying haplotype 3 ($n = 216$; 7.23 ± 0.23 mmol/24 h; $P = 0.007$, *t* test). Stone formers carrying haplotype 2 had marginally significant higher plasma Ca concentration than those not carrying haplotype 2 ($n = 157$; 2.35 ± 0.01 mmol/L; $P = 0.05$, *t* test). A further increase of plasma Ca was observed in stone formers homozygous for haplotype 2 ($n = 8$; 2.41 ± 0.01), whereas urinary Ca excretion (7.06 ± 1.71 mmol/24 h) was not different from that of other groups.

Table 3. Results of multinomial logistic regression in hypercalciuric and normocalciuric stone formers, with healthy controls as the reference population in the analysis

	Odds Ratio	95% CI	Sig
Hypercalciuric stone formers			
haplotype 1	1.40	0.98 to 1.99	0.06
haplotype 2	0.98	0.64 to 1.49	0.92
haplotype 3	13.00	1.7 to 99.4	0.01
haplotype 4	1.33	0.3 to 5.96	0.71
Normocalciuric stone formers			
haplotype 1	1.11	0.77 to 1.61	0.57
haplotype 2	0.71	0.45 to 1.12	0.14
haplotype 3	2.00	0.18 to 22.06	0.57
haplotype 4	1.67	0.40 to 6.97	0.48

Forty-three hypercalciuric stone formers carried haplotype 2; their plasma Ca concentration (2.39 ± 0.01 mmol/L) and Ca excretion (10.38 ± 0.51 mmol/24 h) were higher than in the other 91 hypercalciuric stone formers (2.35 ± 0.01 mmol/L [$P = 0.026$] and 9.13 ± 0.26 mmol/24 h [$P = 0.016$, *t* test], respectively). Only three homozygotes for haplotype 2 were detected among hypercalciuric stone formers (plasma Ca: 2.43 ± 0.02 mmol/L; Ca excretion 11.97 ± 2.54 mmol/24 h).

Multiple Regression

The capacity of CaSR haplotypes to explain variance of Ca excretion was analyzed by multiple regression in stone formers. Independent variables were entered in the regression model in two subsequent steps. In the first step, potential confounders of the association between haplotype and Ca excretion were entered in the model: namely, they were body weight, age, plasma Ca, plasma PTH, serum phosphate, plasma creatinine, intestinal strontium absorption. and sodium excretion. In the second step, haplotype 2 or haplotype 3 was entered and added to the other independent variables.

Sodium excretion, body weight, plasma creatinine, and enteral absorption of strontium associated with Ca excretion ($r^2 = 0.284$; $F = 6.1$; $P = 0.0001$). Haplotype 3 significantly improved the percentage of explained variance by 12.6% (cumulative $r^2 = 0.326$; $F = 6.54$; $P = 0.0001$; r^2 change = 0.041; F change = 7.5, $P = 0.007$). Haplotype 2 did not contribute to the variance of Ca excretion (r^2 change = 0.007; F change = 1.21; $P = 0.27$). Regression analysis for plasma Ca was performed with the same model and the same independent variables used for calciuria analysis, but including calciuria at the place of plasma Ca concentrations. No variables other than haplotype 2 showed significant association with the plasma Ca concentration (cumulative $r^2 = 0.101$; $F = 1.52$; $P = 0.15$; r^2 change = 0.04; F change=5.58, $P = 0.02$).

Discussion

Several pieces of evidence indicate that CaSR plays an important role in the regulation of tubular Ca reabsorption (1,2,4). In keeping with these studies, *CASR* gene has been proposed as a candidate gene for idiopathic hypercalciuria (14). However, testing this hypothesis, Petrucci *et al.* (17) found no linkage of idiopathic hypercalciuria and Ca nephrolithiasis with *CASR* gene locus when using an affected sib-pair approach in a French-Canadian population. Indeed, DGGE analysis of the entire coding sequence of *CASR* gene failed to detect abnormal mobility patterns suggestive for mutations in hypercalciuric patients, with the exception of the already known SNP on exon 7 (10). Accordingly, a previous DNA sequencing study found no point mutations of *CASR* in members of French hypercalciuric families (22).

The functional importance of exon 7 polymorphisms has been suggested by the lower plasma Ca concentration in healthy subjects carrying the allele 986Ser (12) and by the lower plasma PTH level in Japanese uremic patients carrying the allele 990Gly (23). Genetic polymorphisms of *CASR* exon 7 could account for the phenotype variability of a quantitative multifactorial trait such as Ca excretion. Therefore, in the

Table 4. Urinary Ca excretion and plasma Ca concentration in stone formers and healthy controls considered together and divided according to the haplotypes of *CASR* gene

	Group 1[b]	Group 2	Group 3	Group 4	*F*	*P*
n	186	118	16	12		
Plasma Ca (mmol/L)	2.35 ± 0.01	2.37 ± 0.01	2.35 ± 0.02	2.38 ± 0.02	1.67	0.17
Urinary Ca excretion (mmol/24 h)[a]	6.05 ± 0.22	6.44 ± 0.35	9.18 ± 0.95	5.05 ± 0.86	4.86	0.003

[a] $P < 0.05$, group 3 *versus* each other haplotype group by Scheffè *post hoc* test. No significant difference among groups 1, 2, and 4.
[b] Group composition is displayed in Table 1.

Table 5. Values of phenotypes were compared in stone formers with different haplotypes of *CASR* gene

	Group 1[b]	Group 2	Group 3	Group 4	*F*	*P*
n	133	74	15	9		
Plasma Ca (mmol/L)	2.35 ± 0.01	2.38 ± 0.01	2.35 ± 0.02	2.38 ± 0.03	1.43	0.24
Urinary Ca excretion (mmol/24 h)[a]	6.91 ± 0.26	7.96 ± 0.46	9.68 ± 0.87	6.00 ± 0.91	4.46	0.005
PTH (pg/ml)	35 ± 1.6	37 ± 2.3	40 ± 4.3	36 ± 6.0	0.44	0.72
Plasma creatinine (mg/dl)	0.9 ± 0.02	0.90 ± 0.02	0.94 ± 0.04	1.0 ± 0.07	0.82	0.49
Serum phosphate (mmol/L)	1.04 ± 0.02	1.02 ± 0.03	1.07 ± 0.07	1.02 ± 0.05	0.31	0.82
Urinary phosphate excretion (mmol/24 h)	27.01 ± 0.84	28.41 ± 1.2	27.73 ± 2.01	27.28 ± 3.48	0.32	0.81
Urinary sodium (mmol/24 h)	171 ± 6.8	171 ± 8.9	169 ± 16.7	208 ± 24.8	0.63	0.59
Enteral Sr absorption ($mmol \cdot L^{-1} \cdot min$)	10.1 ± 0.34	11.5 ± 0.63	11.9 ± 0.93	1.07 ± 0.32	1.53	0.21

[a] $P < 0.05$ group 3 *versus* group 1 by Scheffè *post hoc* test. No other significant differences among groups.
[b] Group composition is displayed in Table 1.

present study, we evaluated the distribution of these SNP in healthy subjects and in a large cohort of stone formers, comprising a high number of hypercalciuric patients. We found that a polymorphic variant of the *CASR* gene was associated with the increase of Ca excretion and could contribute to the Ca excretion variability in our patients.

Genotypes of exon 7 variants were distributed according to the Hardy-Weinberg equilibrium both in stone formers and healthy subjects. Ala986Ser was the most frequent SNP, and its frequency in our populations was close to that previously reported in other white populations (12,13,25,26). Haplotype 3 was significantly more frequent in hypercalciuric stone formers than in normocalciuric patients or healthy subjects, so that it increased the probability to be hypercalciuric for the carriers. This suggests that Arg990Gly polymorphism affects Ca excretion, the 990Gly allele predisposing to idiopathic hypercalciuria. Indeed, haplotype 3 explains a small but significant proportion of Ca excretion variance in stone formers. It equals 12.6% of the explained variance and 4.1% of the total variance of urinary Ca excretion, in agreement with its multifactorial nature (27,28). The low explained variance excludes *CASR* as a major gene for idiopathic hypercalciuria (24,29).

Therefore, *CASR* gene is one of the genetic components modulating Ca excretion. The minor contribution of *CASR* gene to Ca excretion could explain why no linkage was shown between urinary Ca excretion and *CASR* locus in a study on French-Canadian stone-forming sib-pairs (17). However, the different genetic background in Canadian and Italian populations could modify the weight of *CASR* in Ca excretion determination (24,30). Furthermore, that study could be underpowered for the small genotype relative risk we observed (29). To reduce the influence of unknown genetic components or unevenness with controls, we compared hypercalciuric stone formers with two normocalciuric groups: healthy subjects and stone formers (30). Patients and controls were recruited in different areas: Lombardy and Tuscany. Previous studies with several polymorphic markers failed to disclose genetic heterogeneity among Italian regions, except for Sardinia (31). Therefore, our samples of healthy subjects and patients appear to be representative of Italian population, excluding the risk of genetic stratification.

The activation of CaSR by extracellular Ca inhibits tubular Ca reabsorption acting directly on ion transport (2,4). We propose that the 990Gly allele increases CaSR sensitivity or response to Ca ions. Accordingly, PTH secretion could be more inhibited by the 990Gly allele, in agreement with previous findings in uremic patients (23).

The contribution of Ala986Ser polymorphism to plasma Ca variance is confirmed in the present study (12). Although not corrected for serum albumin, plasma Ca was slightly but sig-

nificantly higher in individuals carrying 986Ser allele. This association suggests that the inhibitory activity of CaSR on tubular Ca reabsorption and PTH secretion be depressed in subjects carrying haplotype 2 (1). In spite of these effects, the increase of plasma Ca was coupled with elevated Ca excretion in hypercalciuric patients carrying haplotype 2. A possible interpretation of these findings is that the decreased tubular Ca reabsorption, characterizing hypercalciuric patients, could overcome the enhancing effect of haplotype 2 on tubular Ca transport. In addition, the larger glomerular filtration of Ca, associated with increased Ca concentration in blood, could facilitate Ca loss in these patients (1,4). Conversely, in normocalciuric subjects carrying haplotype 2, the increase of filtered Ca could be balanced by the increased reabsorptive capacity.

Previous studies revealed that patients with idiopathic hypercalciuria exhibited a decreased axial and peripheral bone density (32,33), increased bone resorption (34), and histologic features of high bone turnover (35). These findings suggest that an abnormality in bone remodeling could be involved in the mechanisms leading to excessive Ca excretion. Polymorphisms of *CASR* gene could exert a direct influence on Ca metabolism in bone cells (6,7), and two studies demonstrated the association of *CASR* gene polymorphisms with mineral density at different bone sites (26,36). This suggests a potential role of *CASR* gene variants on bone reabsorption in idiopathic hypercalciuria.

Enteral absorption of Ca is typically increased in hypercalciuric patients (26) and could be influenced by CaSR as well (5). Its alteration was proposed as the primary event in hypercalciuria (16), but our results do not support any relation among *CASR* gene polymorphisms and intestinal Ca absorption, tested using strontium as a surrogate marker.

In conclusion, *CASR* gene could be considered as a component of the genetic background regulating Ca excretion. Arg990Gly polymorphism could produce a gain-of-function for CaSR that could increase Ca excretion and the susceptibility to idiopathic hypercalciuria. Ala986Ser polymorphism appears to affect Ca concentration in blood, possibly through a decrease in CaSR activity, which could cause a further increase of Ca excretion in hypercalciuric patients. Future studies will attempt to evaluate the function of these polymorphisms through *in vitro* analyses.

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