# A novel polymorphism at the *GNAS1* gene associated with low circulating calcium levels

**Laura Masi**
**Francesca Del Monte**
**Alessia Gozzini**
**Maria Laura De Feo**[a]
**Riccardo Gionata Gheri**[a]
**Annasilvia Neri**[b]
**Alberto Falchetti**
**Antonietta Amedei**
**Rosario Imbriaco**
**Carmelo Mavilia**
**Annalisa Tanini**
**Maria Luisa Brandi**

Departments of Internal Medicine, [a] Clinical Physiopathology, and [b] Pediatrics, and Regional Center for Hereditary Endocrine Tumors, University of Florence, Florence, Italy

Address for correspondence:
Maria Luisa Brandi, M.D., Ph.D.
Regional Center for Hereditary Endocrine Tumors
Department of Internal Medicine
University of Florence
Viale Pieraccini 6, 50139 Florence, Italy
Ph. +39 055 4296586
Fax +39 055 4296585
E-mail: m.brandi@dmi.unifi.it

**Summary**

**The concentration of calcium in the extracellular fluid is crucial for several physiological functions in humans and in normal conditions its circulating levels are maintained between 8.5-10.5 mg/dl. Among the regulators of calcium homeostasis parathyroid hormone (PTH) acts though the G-protein coupled PTH receptor and a hormone-sensitive adenylate cyclase, with Gsα subunit (stimulatory guanine nucleotide-binding protein alpha-subunit) being responsible for the stimulation of the catalytic complex. Mutations of the Gsα encoding gene, *GNAS1*, are causal for some forms of congenital hypocalcemia. In the present study genetic variability in the *GNAS1* gene was analyzed in a group of hypocalcemic patients collected through the Italian Register of Primary Hypoparathyroidism (RIIP). We identified a new intronic variant of the *GNAS1* gene, consisting of a T>C polymorphism. This polymorphism was studied in a group of unrelated healthy subjects for a possible association with bone turnover biomarkers and bone mineral density. The T>C polymorphism was found in 18% of the studied populations, with 15% heterozygous TC and 3% homozygous CC (Pearson $\chi^2$ analysis: p=0.04). A significant association with low serum calcium levels was found in healthy subjects carrying the T > C polymorphism (ANCOVA analysis: p=0.04). These results support segregation of a novel *GNAS1* gene intronic variant with low calcium levels in primary hypoparathyroidism, pseudo-hypoparathyroidism and in the general population.**

*KEY WORDS: hypocalcemia; GSα Unit; GNAS1 gene; primary hypoparathyroidism; pseudo-hypoparathyroidism.*

## Introduction

Calcium (Ca) is an important bioinorganic ion performing a variety of intra- and extra-cellular functions with an important role in maintaining normal homeostasis. The concentration of Ca in the extracellular fluid is crucial for many physiological processes, ranging between 8.5 and 10.5 mg/dl (2.25 to 2.6 mmol/L). The total plasmatic pool of Ca consists of about 35% protein-bound (mostly albumin-bound), 15% complexed to phosphate and citrate, and 50% ionized. It is only the ionized form of Ca that is relevant in several physiological processes, as neuromuscular contractility, enzymatic activities, and coagulation (1).
Hypocalcemia is a disorder that can be asymptomatic or present as an emergency case (2). Generally it is not congenital, but more often the result of vitamin D deficiency or neck surgery. Genes responsible for the hereditary forms include the Gsα subunit gene (*GNAS1*) (3, 4), the parathyroid hormone gene (*PTH*) (5), the calcium-sensing-receptor gene (*CaSR*) (6), and the human orthologue gene of the *Drosophila glial cells missing gene dGCM* (*GCMB*) (7, 8).
The activity of the hormone-sensitive adenyl cyclase, the enzyme that catalyzes the formation of the intracellular second messenger cyclic AMP (cAMP), is under dual regulation by two guanine nucleotide-binding proteins (G-proteins) and, of these, Gsα mediates the action of PTH and cAMP accumulation and protein phosphorylation (9). The *GNAS1* gene is located in the chromosomal region 20q13.3 and encompasses 13 exons, with a cDNA sequence spanning a region of about 1.2 Kb and giving rise to maternally, paternally, or bi-allelically expressed transcripts (10, 11). Several of the naturally occurring mutations of the *GNAS1* gene have been identified through the entire coding region, being responsible of different congenital disorders (3, 12-14). Specific aminoacid substitutions resulting in constitutive activation of Gsα subunit have been firstly characterized in growth hormone secreting pituitary tumors (15, 16). In addition, mutations of *GNAS1* gene can also lead to a condition known as McCune-Albright syndrome (MAS; MIM # 174800) (17), characterized by polyostotic fibrous dysplasia, cafè-au-lait patches on the skin, and endocrine abnormalities such as hyperthyroidism and precocious puberty. Conversely, inactivating mutations of *GNAS1* gene result in a condition known as Albright's Hereditary Osteodystrophy (AHO;MIM # 103580) characterized by short stature, round face with a low flat nasal bridge, shortening of the fourth and fifth metacarpals and metatarsals, obesity, and subcutaneous and intracranial calcification (4). Small insertions/deletions and aminoacid substitutions represent the predominant type of mutations, but nonsense and point mutations, leading to altered translation initiation or aberrant mRNA, have also been documented (18). Marguet et al. (12) observed that in 51% of PHP-Ia (pseudo-hypoparathyroidism type Ia) patients *GNAS1* gene mutations were found in hypocalcemic siblings. Conversely, Linglart et al. (3) did not find *GNAS1* mutations in a small number of sporadic cases with isolated AHO, while *GNAS1* gene mutations are present in subjects within PHP-Ia kindreds where isolated AHO is present in family members (3).
Recent studies on the *GNAS1* locus indicated that this gene is extremely complex, with multiple alternatively spliced tran-

scripts encoding multiple protein products. Polymorphic markers of the *GNAS1* gene, currently employed in the genetic evaluation of congenital disorders, frequently result to be uninformative due to the low associated heterozygosity value (19). Nevertheless, *GNAS1* is actually the only gene encoding a G-protein that has been identified as target for mutations that unequivocally cause endocrine disorders, leading to the clinical phenotype of hormone defect or excess (15, 18-20). Moreover, the PTH receptor (PTHr) is coupled to G-proteins (21), with a specific α-subunit being responsible of both receptor and effectors interactions (22). It is, therefore, not surprising that inactivating mutations of the *GNAS1* gene have been identified as responsible of congenital primary hypocalcemia in patients with PHP and pseudo-PHP (PPHP) (23-26).
The aim of the present study was to evaluate the presence of *GNAS1* gene mutations that have been described in the literature and of potential new mutations responsible for various forms of hypocalcemia. In this study, the genetic screening of primary hypocalcemia made possible the identification of an intronic variant of the *GNAS1* gene with statistically significant polymorphism information content (PIC). This genetic marker was used to screen a population of 100 healthy volunteers and described as a new polymorphic site.

## Materials and methods

### *Clinical characteristics of the patients*

The Italian Register of Primary Hypoparathyroidism was created in 1996 in Florence and named RIIP (Registro Italiano Ipoparatiroidismo Primitivo: www.dmi.unifi.it/ipopara/default.htm). RIIP collects clinical records both on sporadic and familial cases of primary hypoparathyroidism. Information was obtained from Italian endocrine, neurological and pediatric Centers through the compilation of a form, that includes the identification code of the patient, the date of birth, the diagnosis of the type of hypoparathyroidism, the presence of other associated diseases (typical or not), data on organ and non-organ antibodies, and results of PTH infusion test. Each subject is requested to give informed consent and at any time the patient can ask to obtain his/her clinical data deleted from the official file. The primary goal is represented by collection of clinical information to obtain epidemiological data on primary hypoparathyroidism in Italy. The Register supports also the genetic test for Centers not equipped for molecular diagnostic procedures.
At present, 107 Caucasian hypocalcemic patients (61 males and 46 females, age range 6-71 yrs, mean age 42 ± 15 SEM yrs) have been registered in the RIIP.
Registered subjects encompassed 14 cases of PHP Ia, 8 cases of PHP Ib, 8 cases of PPHP, 57 cases of idiopathic primary hypocalcemia, 2 cases of DiGeorge syndrome, 8 cases of isolated parathyroid agenesis, and 10 case of Autoimmune Polyglandular Syndrome (APS) type 1. Among the 107 subjects, 39 patients, affected by clinically idiopathic hypocalcemia or by PHP (10 women and 29 men; age range 7-66 yrs, mean age 40 ± 22.4 SEM yrs) and 7 relatives, underwent genetic test to evaluate the presence of *GNAS1* gene mutations. Developmental status of the patients was appropriate. In all the subjects routine exams, serum Ca (corrected for levels of serum albumin), phosphorus, magnesium, bone alkaline phosphatase, PTH, vitamin D ($25OH_2D_3$ and $1\text{-}25OH_2D_3$), organ- and non-organ specific antibodies, TSH, fT3, fT4, FSH, and LH were evaluated. A 24 hrs urinary sample was collected in order to measure the excretion of calcium, phosphorus, magnesium, deoxypiridinoline, and cAMP. Lumbar spine- and femoral-BMD (LS-BMD and F-BMD) measured by DXA (Hologic QDR 4500), electrocardiogram, electromyography, ocular inspection, and skull X-ray and/or Computed Tomography were performed in all patients.
A population of Caucasian healthy volunteers from the Florence metropolitan area was recruited by mailing. The group was composed by 75 women and 25 men with an age of 47-76 yrs and a mean age (± SEM) of 57 ± 8 yrs. A large database was completed for all subjects with inclusion of a detailed medical history and of dietary Ca intake assessed by a sequential self-questionnaire that included foods accounting for the majority of Ca in the diet.
The Ethical Committee of the University of Florence approved the study and both patients and normal volunteers were requested to give informed consent for genetic evaluation.

### *Genetic analysis of the GNAS1 gene*

Genomic DNA was isolated from peripheral EDTA blood samples of the hypocalcemic and healthy population with the phenol/chloroform procedure. Exons 1-13 of the *GNAS1* gene were amplified in 50 ml by PCR (polymerase chain reaction) containing 67 mM Tris-HCl, 16.6 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 0.01% Tween-20, 200 µM each of the four deoxyribonucleotides, 0.4 µM of oligonucloetide primers and 1 U of Polytaq (Polymed, Florence, Italy) (23). The length of the PCR products was analyzed on 3% agarose gels, stained with ethidium bromide and visualized with UV light. In Table I are reported the

Table I - DNA sequences of the oligonucleotide primers used to amplify exons of the *GNAS1* gene.

| Exon | Upper primer | Lower primer | Annealing temperature (°C) |
|---|---|---|---|
| 1 | 5'-ATGGGCTGCCTCGGGAACAGTA-3' | 5'-CCCTTACCCAGCAGCAGCAGGC-3' | 58 |
| 2 | 5'-AAAATGCCTCCTTCATAACCTGA-3' | 5'-GCCCACCTATACTTCCTAAAG-3' | 55 |
| 3 | 5'-ATGGTTGAGGAATGTAGAGAGACTG -3' | 5'-CAGTATGATCTTCATGTTTGTTTG-3' | 55 |
| 4-5 | 5'-ATGAAAGCAGTACTCCTAACTGA-3' | 5'-TGGATGCTCCTGCCCATGTG-3' | 66 |
| 6 | 5'-ATTAGTTCAAGCTCTTGCCTTTC-3' | 5'-TTGTCTGTTTTATGTGCTGATGG-3' | 60 |
| 7-8 | 5'-TGCTGCATAACTGTGGGACG-3' | 5'-AGAAACCATGATCTCTGTTATA-3' | 60 |
| 9-11 | 5'-ACAGAGATCATGGTTTCTTG-3' | 5'-AGAACCACCGCAATGAACAGCC-3' | 60 |
| 12 | 5'-AGACTTCACGAGCTACAGAGA-3' | 5'-AGAGGAGGAACAAGAGAGGAA-3' | 60 |
| 13 | 5'-CATCAGAGGCTGGCTGACAGCG-3' | 5'-AAGGCTTTAATTAAATTTGGG-3' | 60 |

From Ref. 19 with minor modifications.

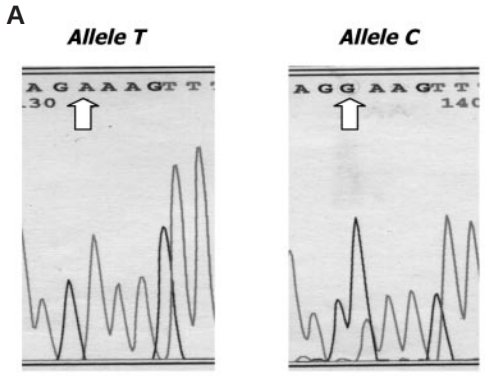
sequences of the oligonucleotide primers used to amplify exons of the *GNAS1* gene. In each subject both strands of each exon were sequenced. Sequencing of the PCR products using both sense and antisense primers was performed using AmpliTaq BigDye Terminator kit and 3,100 Genetic Analyzer (Applied Biosystems).

### *Statistical analysis*

Pearson chi-square ($\chi^2$) analysis was performed to evaluate the distribution of the genotypes in the population. ANOVA and ANCOVA analysis were performed and $p < 0.05$ was accepted as the value of significance. In order to know which means contributed to the effect, that is, which groups were particularly different from others, we performed post hoc LSD (least significant difference) test. The results were presented as mean ± SEM. $P < 0.05$ was accepted as the value of significance. The following covariates were considered for the ANCOVA analysis: age, weight, and Ca intake. All statistical tests were performed using Statistica 5.1 Program (Statsoft Inc., Tulsa, OK, USA).

## Results

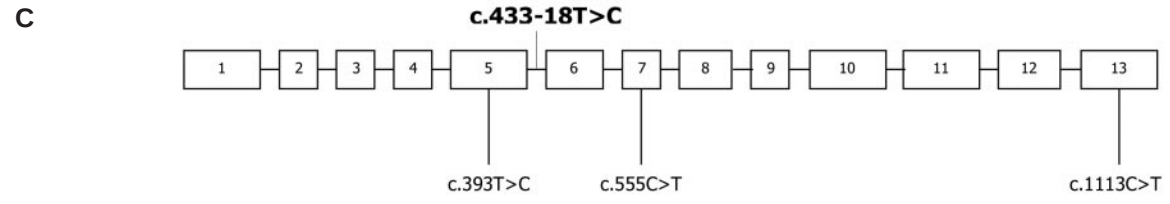
Direct sequencing of the amplified genomic DNA fragment showed a novel heterozygous T > C variant at the nucleotide position $433^{-18}$ (intron 5) in 7 of the 39 hypocalcemic patients and 4 relatives [reference sequence is the Gs-alpha 1 cDNA (Gene Bank association number SEG-HUMGNAS)] (Figs. 1 A and C). The T > C transition was present in 18% of the subjects indicated by A, B, C, D1, D2, D3, E1, F1, F2, G1, G2 and it was not associated with modifications of restriction endonuclease recognition sequences. The clinical characteristics of patients and their first-degree relatives are summarized in Table II. No subject with the exon 7 polymorphism already described in the literature (15) has been detected in our study. Of the 39 subjects analyzed, 13 had the polymorphism at the exon 5 previously described by Miric et al. (9), two being homozygous. In family D, the patient affected (D1) and his sons (D2 and D3) showed the heterozygous T > C mutation and no mutations were found in the mother (D4). Patient D2 had a polymorphism at the exon 13 together with the T > C polymorphism at the intron 5 (27). In family F, patient (F1) and her mother




B

| Genotype | F | M | Total Number (%) |
|---|---|---|---|
| **TT** | 64 | 18 | 82 (82) |
| Column % | 86.49 | 69.23 | |
| Row % | 78.05 | 21.95 | |
| Total % | 64 | 18 | |
| **TC** | 7 | 8 | 15 (15) |
| Column % | 9.46 | 30.77 | |
| Row % | 46.67 | 53.33 | |
| Total % | 7 | 8 | |
| **CC** | 3 | 0 | 3 (3) |
| Column % | 4.05 | 0 | |
| Row % | 100 | 0 | |
| Total % | 3 | 0 | |

**F**: Female; **M**: Male




| Exon/Intron | Mutation | Amino acid | Mutation type | Reference (in the text) |
|---|---|---|---|---|
| Exon 05 | c.393T>C | 131 | Polymorphism | Miric et al., 1993 (9) |
| **Intron 05** | **c.433-18T>C** | | **Polymorphism** | **This study** |
| Exon 07 | c.555C>T | 185 | Polymorphism | Aldred & Trembath, 2000 (15) |
| Exon 13 | c.1113C>T | N371N | Polymorphism | Waltman et al., 1994 (27) |

Figure 1 - **A:** Direct DNA sequencing analysis of *GNAS1* gene. The figure shows the A → G substitution at the intron 5 nucleotide position $433^{-18}$ [reference sequence is the Gs-alpha 1 cDNA (Gene Bank association number SEG.HUMGNAS)]. Right: sequencing analysis in subjects with the T > C polymorphic site. Left: sequencing analysis in subject without the T>C polymorphic site. **B:** The Table indicates the distribution of the T > C polymorphism in the 100 Caucasian unrelated subjects. The T > C polymorphism was found in 18% of the population [3% homozygous (CC) and 15% heterozygous (TC)]. Pearson $\chi^2$ analysis: df = 2; p = 0.04. The TT genotype was present in 82 subjects (82%) of the population with the distribution between females and males respectively of 64 females (78.05%) vs. 18 males (21.95%). The CC was present in 3 female subjects (3%) of the total population while the TC genotype was present in 15 subjects (15%) of the total population with a distribution between females and males respectively of 7 females (46.67%) and 8 males (53.33%). **C:** Schematic representation of *GNAS1* with the location of the polymorphic sites. The T > C polymorphic site has been localized at the intron 5 nucleotide position $433^{-18}$ [reference sequence is the Gs.alpha.1 cDNA (Gene Bank association number SEG.HUMGNAS)]. The Table indicates a schematic representation of the polymorphic sites described in the literature and of the new one.

(F2) carried out the T > C mutation with no mutations found in the father (F3). In family G patient (G1) and his brother (G2) carried out the T > C mutation with no mutations found in the mother. DNA from the G1 patient father was not available.
In addition, constitutional DNA from a population of 100 (200 chromosomes) unrelated Caucasian individuals (75 females and 25 males) who had no known history of hypocalcemia was amplified by PCR and sequenced to verify the frequency of the T > C variant. The clinical characteristics of the study population are reported in Table III and the allele distribution of the *GNAS1* gene polymorphism in this population is shown in Table IV. The T allele had a frequency of 0.895 and the C allele had a frequency of 0.105. The $\chi^2$ test was 1.24 with 1 degree of freedom. From Hartl table (28) we observed that

Table II - Clnical and biochemical data of the hypocalcemic patients.

| Code # | Sex | Age (Yr) | S-Ca (8.5-10.5 mg/dl)* | UrCa (100-300 mg/24h) | s-P (2.8-4.5 mg/dl) | PTH (10-60 ng/ml) | TSH (0.25-3.5 mU/ml) | Diagnosis and clinical signs | Imaging | *GNAS1* gene polymorphism |
|---|---|---|---|---|---|---|---|---|---|---|
| A1 | F | 65 | 6.8 | 362 | 4.8 | 26 | < 0.05 | Late idiopathic hypocalcemic crisis Hyperthyroidism | IC | Heterozygous |
| B1 | F | 59 | NA | NA | 3.8 | 99 | NA | Late idiopathic hypocalcemic crisis | IC | Heterozygous |
| C1 | M | 66 | 7.7 | 244 | 3.8 | 45 | NA | Late idiopathic hypocalcemic crisis | IC | Heterozygous |
| D1 | M | 47 | 5.9 | 235 | 4.68 | 137 | 3.6 | PHP Ib, Subclinical hypothyroidism Cataract, Br | IC | Heterozygous |
| D2 (S) | M | 15 | 9.8 | 200 | 4.7 | 42.3 | NA | N | NA | Heterozygous |
| D3 (S) | M | 17 | 9.7 | 230 | 4.5 | 58 | NA | N | NA | Heterozygous |
| D4 (W) | F | 45 | 10.1 | 245 | 3.5 | 62 | NA | N | NA | NP |
| E1 | M | 48 | NA | NA | NA | NA | NA | Late idiopathic hypocalcemic crisis | NA | Heterozygous |
| F1 | F | 7 | 7.9 | 288 | 4.5 | 143 | 6.64 | PHP Ia , Br, Ob, RF, SC | SM | Heterozygous |
| F2 (M) | F | 36 | 10 | 160 | 3.9 | 40 | NA | Br | NA | Heterozygous |
| F3 (F) | M | 50 | 9.8 | 185 | 3.5 | 65 | NA | N | NA | NP |
| G1 | M | 17 | 8.2 | 180 | 4.2 | 56 | 2.9 | Br, Osteopenia, Hyperprolactinemia, SM | NA | Heterozygous |
| G2 (B) | M | 21 | 9.6 | 213 | 3.9 | 57 | NA | N | NA | Heterozygous |
| G3 (M) | F | 62 | 9.5 | 228 | 2.9 | 60 | NA | N | NA | NP |

# Proband: bold characters
* Serum Ca levels were corrected for levels of albumin.
Abbreviations: F: Female; M: Male; Br: Brachidactily; IC: Intracranic Calcification; N: Normal; NA: Not Available; Ob: Obesity; NP: Not Polymorphism; PHP: Pseudohypoparathyroidism; RF: Round Face; SC: Subcutaneous Calcification; SM: Shortening of Metacarpals; (S): Son; (W): Wife; (M): Mother; (F): Father; (B): Brother.

Table III - Clinical characteristics of the healthy population.

| Genotype | TT | TC | CC |
|---|---|---|---|
| Number | 82 | 15 | 3 |
| Sex | M: 18<br>F: 64 | M: 7<br>F: 8 | M: 0<br>F: 3 |
| Age (yrs) | 56.1 ± 7 | 57.2 ± 7.9 | 51 ± 9.1 |
| Dietary Ca intake (mg/day) | 797 ± 320 | 801 ± 263 | 811 ± 296 |
| LS-BMD (g/cm$^2$) | M: 0.896 ± 0.13<br>F: 0.826 ± 0.15 | M: 0.932 ± 0.13<br>F: 0.829 ± 0.4 | M: 0.916 ± 0.14<br>F: 0.880 ± 0.3 |
| FN-BMD (g/cm$^2$) | M: 0.786 ± 0.13<br>F: 0.650 ± 0.5 | M: 0.770 ± 0.13<br>F: 0.690 ± 0.4 | M: 0.779 ± 0.12<br>F: 0.680 ± 0.5 |
| Height (cm) | M: 170 ± 8.4<br>F: 160 ± 2.3 | M: 169.8 ± 6.4<br>F: 162.9 ± 2.6 | M: 0.779 ± 0.12<br>F: 161.2 ± 1.8 |
| Weight (kg) | M: 76 ± 10.4<br>F: 63 ± 2.3 | M: 75.8 ± 6.3<br>F: 62.9 ± 2.7 | M: 75 ± 8.9<br>F: 63.5 ± 1.7 |
| Serum Ca (mg/dl) | 9.1 ± 0.9 | 8.3 ± 0.87* | 8.1 ± 0.25** |
| PTH (ng/ml) | 54 ± 8.1 | 65 ± 7 | 67 ± 5 |

Abbreviations: M: male; F: female; LS-BMD: Lumbar Spine BMD; FN-BMD: Femoral Neck BMD; P values: *TT genotype vs. TC genotype: p=0.03. **TT genotype vs. CC genotype p=0.04.

Table IV - Allelic distribution of the T > C polymorphism at the nucleotide 433$^{-18}$ of the *GNAS1* gene in an Italian population of 100 unrelated individuals with no history of hypocalcemia.

| Allele | Total (n=100) |
|---|---|
| T | 0.895 |
| C | 0.105 |

$\chi^2$: 1.24; p=0.35.
From Ref. 28 and 29.

the p value was 0.35 which is greater than 0.05. For this reason genotype frequencies could be considered in Hardy-Weinberg equilibrium (28, 29). Among a total of 200 independent chromosomes examined for the T > C polymorphism, 179 (89.5%) contained the T allele (164 TT and 15 TC genotypes) and 21 (10.5%) the C allele (15 TC and 6 CC genotypes). If used for linkage analysis, this polymorphism may be informative in 18% of cases (2 x 0.895 x 0.105). The T > C PIC (polymorphism information content) value at the *GNAS1* gene was 0.17 (30) and can, therefore, be considered useful as a genetic marker (www.biosci. ohio.state. edu).
The T > C polymorphism was found in 18% of the healthy Caucasian population with 3% homozygous and 15% heterozygous (Fig. 1B). There were three genotype groups and by Pearson $\chi^2$ analysis we observed: 82 (82%) homozygous TT [64 (78.05%) females vs. 18 (21.95%) males], 15 (15%) heterozygous TC [7 (46.6%) females vs. 8 (53.3%) males], and 3 (3%) homozygous CC [3 (100%) females] (Pearson $\chi^2$ analysis: df = 2; p = 0.04) (Fig. 1 B).
Applying ANCOVA analysis to evaluate potential relationships between *GNAS1* genotype and bone biomarkers, we observed that subjects with T > C polymorphism showed a significant lower serum Ca (ANCOVA analysis: p = 0.04) than subjects without this polymorphism. In particular, patients with TC and CC genotypes had respectively 8.79% and 10.98% lower serum Ca level in comparison with those with TT genotype [8.3 ± 0.87 and 8.1 ± 0.25 vs. 9.1 ± 0.9 mg/dl, p = 0.03 and p = 0.04, respectively] (Fig. 2). No statistically significant differences were found neither between genotypes and other biochemical markers nor between genotypes and LS and F-BMD (data not shown).

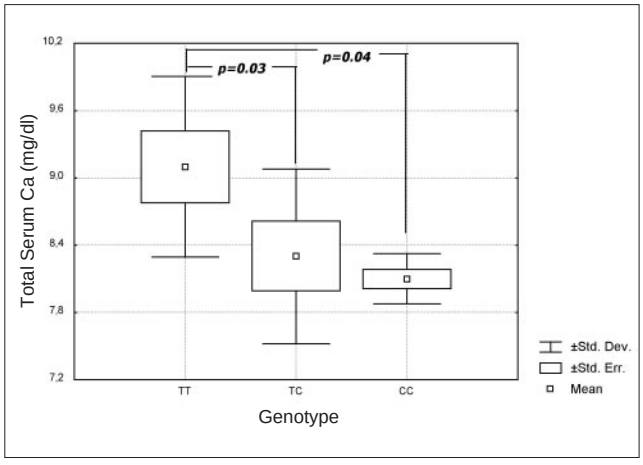



Figure 2 - Serum Ca levels in healthy subjects with various *GNAS1* genotypes. Ancova analysis and LSD test showed that subjects with CC genotype had significantly lower levels of serum Ca (p=0.04 and p=0.03, respectively) in comparison with TT genotype.

## Discussion

It has been well established that proteins involved in signal transduction are targets for mutations resulting in human diseases. The identification of mutations of G-proteins had major implications for understanding the structure and function of these signaling proteins (2, 14-15, 20-22). By direct DNA sequencing we analyzed the entire *GNAS1* gene coding sequence and identified a novel T > C transition polymorphism within intron 5. The novel polymorphism was evaluated in a population of 39 primary hypocalcemic patients and in 100 unrelated healthy subjects. Eighteen percent either of the hypocalcemic patients or the 100 unrelated subjects carried out the *GNAS1* gene T > C polymorphism. Interestingly, in the latter group of subjects the *GNAS1* gene T > C polymorphism was significantly associated with lower serum Ca levels when compared to the negative cases. A number of observations derive from these findings.
First, several cases of hypocalcemia remain still unsolved and approximately 20% of inactivating *GNAS1* gene mutations are undetected after mutation scanning with DGGE or direct sequencing (15). It is possible that this novel identified *GNAS1* gene polymorphism plays a role in some of these cases. In a recent report 8 new mutations of the *GNAS1* gene were described in Italian patients affected by PHP Ia (31). These subjects were younger than the subjects described in the present report (age range: 6-24 yrs vs. 7-66 yrs). It is possible to speculate that the intronic T > C polymorphism could confer the genetic susceptibility to develop lower circulating Ca levels in adulthood. An association between VDR (vitamin D receptor) polymorphism and intestinal calcium absorption is well documented in the literature (32). However, the genetic impact of the VDR polymorphism on calcium absorption is evidenced only in subjects with a relatively low calcium intake (32). It is interesting that in the healthy population analyzed in this study the daily calcium intake is lower than the recommended allowance in adults (33). This condition can evidence the genetic effect of *GNAS1* gene T > C polymorphism in adulthood.
Linglart et al. (3) observed in nine informative families that patients with hormonal resistance always had a mutation on the maternal allele and they assumed that mutations inherited from the father, or occurring in the paternal allele, are associated with a mild phenotype. In this study, male patient D1 developed a mild PHP Ib in adulthood and his sons (age 15 and 17 yrs) carried the T > C polymorphism without any clinical and biochemical abnormality. On the other hand, in the patient F1 a typical PHP Ia was detected at the age of 7 years with a T > C polymorphism maternally transmitted. It is possible that also for the present polymorphism, such as for other *GNAS1* gene mutations, the paternal imprinting of the Gsα could be an important mechanism in the variation of phenotypic expression of the genotype.
In addition, Jia et al. (34) found a polymorphism at exon 5 of the *GNAS1* gene as a predictor of blood pressure, suggesting that the *GNAS1* locus may carry a functional variant that influences blood pressure variation and response to β-blockade in essential hypertension. Similarly, the polymorphism described in the present paper may become useful as a genetic marker (PIC: 0.17) in predicting hypocalcemia.
The molecular mechanisms through which the T > C polymorphism acts in predisposing to hypocalcemia is not yet fully elucidated (11, 19). A possibility is that intronic *GNAS1* gene polymorphism itself produces an intrinsic effect on Gsα expression as described for other genes (35, 36). Rusin et al. (37) demonstrated that an intronic polymorphism of the heat shock protein 70 gene was associated with a significant reduction of the receptor gene activity. Recently, a functional in vitro study on fibroblasts demonstrated an association between an intronic

polymorphism of the aromatase gene and estrogen synthesis (38). The T > C polymorphism could alter gene transcription and protein synthesis influencing the responsiveness to PTH. It is also possible that the T > C polymorphism could be in linkage disequilibrium with *GNAS1* gene functional polymorphisms or alternatively with functional polymorphisms in genes neighboring the *GNAS1* locus (39). The functionality of the T > C variant needs to be evaluated considering all these various hypotheses.

Finally, could the identification of this *GNAS1* gene polymorphism be used in the diagnosis and treatment of hypocalcemic subjects without a PHP or Albright's Hereditary Ostedystrophy phenotype? So far three polymorphic sites have been described in the literature for the *GNAS1* gene (9, 15, 19, 27). However, none of these has been associated with hypocalcemia. The data presented here support the hypothesis that *GNAS1* gene T > C polymorphism confers susceptibility for hypocalcemia expression in adulthood. The evaluation of the intron 5 T > C polymorphism could become useful in excluding other causes of hypocalcemia and also in driving appropriate interventions.

The interaction between functional polymorphisms and environmental factors may determine both the risk of developing diseases and the response to treatment (40). The recognition of the pathogenetic basis of hypocalcemia and the discovery of the polymorphisms associated with hypocalcemia could be important for patient care, making possible: a) precocious diagnosis of patients affected by hypocalcemia; b) prevention of complications due to chronic hypocalcemia; and c) early treatment of associated disorders.

In conclusion, in this report we describe the segregation of a novel *GNAS1* gene polymorphism with lower levels of serum Ca. This sequence could represent a marker allele linked to other still unknown DNA variants of the *GNAS1* gene or to other genetic loci. Further studies will attempt both to evaluate functionality of the *GNAS1* gene polymorphism and to extend the analysis to a larger population of normal individuals.

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