The Prevalence of *GNAS* Deficiency-Related Diseases in a Large Cohort of Patients Characterized by the EuroPHP Network

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Context: The term pseudohypoparathyroidism (PHP) was coined to describe the clinical condition resulting from end-organ resistance to parathormone (rPTH), caused by genetic and/or epigenetic alterations within or upstream of *GNAS*. Although knowledge about PHP is growing, there are few data on the prevalence of underlying molecular defects.

Objective: The purpose of our study was to ascertain the relative prevalence of PHP-associated molecular defects.

Design: With a specially designed questionnaire, we collected data from all patients (n = 407) clinically and molecularly characterized to date by expert referral centers in France, Italy, and Spain.

Results: Isolated rPTH (126/407, 31%) was caused only by epigenetic defects, 70% of patients showing loss of imprinting affecting all four *GNAS* differentially methylated regions and 30% loss of methylation restricted to the *GNAS A/B:*TSS-DMR. Multihormone resistance with no Albright's hereditary osteodystrophy (AHO) signs (61/407, 15%) was essentially due to epigenetic defects, although 10% of patients had point mutations. In patients with rPTH and AHO (40/407, 10%), the rate of point mutations was higher (28%) and methylation defects lower (about 70%). In patients with multihormone resistance and AHO (155/407, 38%), all types of molecular defects appeared with different frequencies. Finally, isolated AHO (18/407, 4%) and progressive osseous heteroplasia (7/407, 2%) were exclusively caused by point mutations.

Conclusion: With European data, we have established the prevalence of various genetic and epigenetic lesions in PHP-affected patients. Using these findings, we will develop objective criteria to guide cost-effective strategies for genetic testing and explore the implications for management and prognosis. (***J Clin Endocrinol Metab*** **101: 3657–3668, 2016)**

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Abbreviations: AHO, Albright's hereditary osteodystrophy; DMR, differentially methylated region; Gsα, α subunit of the stimulatory G protein; iUPD, uniparental isodisomy; LOI, loss of imprinting; LOM, loss of methylation; NESP55, neuroendocrine secretory protein 55; OMIM, Online Mendelian Inheritance in Man; PHP, pseudohypoparathyroidism; pLOM, partial LOM; POH, progressive osseous heteroplasia; PPHP, pseudo-pseudohypoparathyroidism; UPD, uniparental disomy.

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The clinical condition resulting from end-organ resistance to PTH, first described in the early 1940s in patients with hypocalcemia and hyperphosphatemia in the absence of vitamin D deficiency or impaired renal function, is termed pseudohypoparathyroidism (PHP) (1). PHP was divided into types 1 and 2 according to measured serum and urinary levels of cAMP after the injection of bovine parathyroid extract. cAMP excretion is closely related to the parathyroid activity and is considered a useful test in the diagnosis of parathyroid diseases. In individuals with PHP type 1 (PHP1), urinary cAMP and phosphate excretion fail to increase in response to exogenous PTH, demonstrating the presence of a molecular defect causing a defective cAMP generation. In contrast, patients with PHP2 present a normal nephrogenous cAMP generation associated with a decreased phosphate excretion, indicating a defect downstream of the adenylate cyclase (2). To date, relatively few cases of PHP2 have been reported and the underlying molecular defect remains unknown (2). On the other hand, the clear phenotypic heterogeneity of patients with PHP1, in particular the presence of additional clinical features such as resistance to other hormones (TSH/GHRH/gonadotropins), Albright's hereditary osteodystrophy (AHO) (short stature, rounded face, and brachydactyly), and sometimes ectopic ossifications, obesity and/or mental retardation, led to the distinction of specific PHP1 subtypes; hence, the term PHP now refers to a group of rare, related, and deeply impairing metabolic diseases (3).

Deficient expression or activity of Gsα, the α subunit of the stimulatory G protein is, by far, the most frequent cause of PHP1 (4). Gsα is the main transcript encoded by the *GNAS* locus (OMIM#139320), a complex imprinted transcriptional unit generating several different monoallelic transcripts, including the extra-large Gsα variant, the neuroendocrine secretory protein 55 (NESP55), the noncoding antisense transcript, and the untranslated A/B transcript (5) through the use of differently methylated promoters located in differentially methylated regions (DMRs). From here on in this manuscript, PHP is used to refer to conditions associated with deficient Gsα expression or activity.

The main subtypes of PHP are caused by de novo or autosomal dominantly inherited inactivating genetic mutations, and/or epigenetic, sporadic, or genetic-based alterations within or upstream of *GNAS* (5). Most reported patients with PHP type 1a (PHP1A) (Online Mendelian Inheritance in Man [OMIM] #105380), who show AHO with resistance to multiple hormones, carry either point or structural mutations on the maternal allele (6–8). When affecting the paternal allele, the same mutations lead to pseudo-PHP (PPHP, OMIM #612463), in which AHO usually occurs in the absence of endocrine abnormalities (6, 7). In addition, paternally inherited *GNAS* mutations may lead to progressive osseous heteroplasia (POH, OMIM #166350), in which heterotopic ossifications progressively extend into skeletal muscle and deep connective tissues (9, 10).

On the other hand, most patients with PHP type 1b (PHP1B) (OMIM #603233), who classically display hormone resistance limited to PTH and TSH with no AHO signs, have methylation defects at GNAS DMRs (11). Broad or partial loss of imprinting (LOI) affecting all GNAS DMRs is frequently observed in sporadic cases (sporPHP1B), whereas in a small subset of patients, it has been associated with complete or segmental uniparental disomy (UPD) of chromosome 20 (12–15). Familial cases have an autosomal dominant mode of inheritance (AD-PHP1B) through the maternal line and most show LOI limited to the A/B DMR (*GNAS A/B:*TSS-DMR) secondary to deletions on the maternal allele of cis-acting control elements (imprinting control regions) within *STX16* (16) or *NESP55* (17), although other maternally inherited deletions have been identified in some rare familial cases affecting either an isolated *GNAS A/B:*TSS-DMR (18–20) or all four DMRs (*GNAS-NESP*:TSS-DMR, *GNAS-AS1*: TSS-DMR, *GNAS-XL*:Ex1-DMR, and *GNAS A/B:*TSS-DMR) (21, 22).

Although surveys of genetic contributions to PHP have been performed by single laboratories, up to now none comprehensively evaluated a large cohort of unselected patients (6–10, 21, 23–25). The European Network for the study of PHP (EuroPHP Network), supported by the European Society of Pediatric Endocrinology, designed the present work to quantify the contributions of identifiable genetic lesions to PHP subtypes and to develop objective criteria to guide a cost-efficient strategy for genetic testing for this heterogeneous entity.

Materials and Methods

A European network for the study of GNAS-related disorders was created in 2011 and partially supported by the European Society for Pediatric Endocrinology (EuroPHP Consortium). The aims of the network are to collect and share clinical information as well as biological samples for a better characterization of this group of rare metabolic disorders. Some of the members of this Consortium (clinicians and scientists) collected clinical and molecular data on 407 PHP index patients (Italy, n = 177; Spain, n = 99; and France, n = 131) studied over the past decade in their laboratories (2005–2015) or followed in their clinical center, to evaluate the prevalence of different PHP phenotypes and *GNAS* molecular defects (Supplemental Table 1A–D). Demographic data of the patients are resumed in the Table 1. Several patients reported herein (296 of 407) have already been described in previous publications (6, 8, 14, 19, 24, 26–28).

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Table 1. Demographic Data of the Studied Series of 407 Patients With PHP and Related Disorders

	Italy			Spain			France			Total			Molecular Diagnostic Age (Mean ±SD)		
	F	M	F + M	F	M	F + M	F	M	F + M	F	M	F + M	F	M	F + M
GNAS point mutation	53	32	85	21	22	43	21	21	42	95	75	170	10.9 ± 10.3	9.9 ± 12	10.5 ± 11
Structural rearrangement	1	3	4		2	2	2		2	3	5	8	19 ± 8.5	16.8 ± 14,5	17.6 ± 11.9
Broad methylation defects	39	32	71	23	21	44	25	26	51	87	79	166	23.8 ± 13.2	21.1 ± 14.2	22.5 ± 13.7
Isolated LOM	6	11	17	4	6	10	17	19	36	27	36	63	29.2 ± 15.9	21.7 ± 10.7	23.1 ± 13.7
A/B	99	78	177	48	51	99	65	66	131	212	195	407			

F, female; F + M, sum of females and males; M, male; molecular diagnostic age, age at which the molecular diagnosis was performed.

The inclusion criteria were the detection of GNAS genetic or epigenetic defects in patients referred for PHP, together with clinical data being available describing the presence of clinical features of PHP, namely, PTH resistance, resistance to other hormones (TSH, gonadotropins, and/or GHRH) and/or signs of AHO. To establish presence of AHO, patients were required to show at least two signs, brachydactyly and/or heterotopic ossifications being necessary for the diagnosis. Only probands were included in the study to prevent bias in the prevalence estimate due to describing the same mutation spread within families. Informed consent was obtained from all patients (or guardians) included in the study. This work was approved by the Basque Clinical Research Ethics Committee

Molecular analysis methods

The presence of point mutations was investigated by Sanger sequencing of *GNAS* exons and flanking intronic sequences from genomic DNA extracted from peripheral blood leukocytes, and pathogenicity defined as previously described: all novel nonsense mutations, frameshift mutations, and mutations of the first two bases of canonical intron splice sites as deleterious; for other mutations that could affect RNA splicing in silico softwares were used (GeneSplicer, http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml; Gene Finding and Intron Splice Site Prediction, http://www.cbs.dtu.dk/biolinks/pserve2.php); CRYPSKIP http://cryp-skip.img.cas.cz/); and, for novel missense mutations, we performed in silico analyses by Polyphen2 (http://genetics.bwh. harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html) (6, 26, 29). Structural rearrangements and previously reported *STX16* deletions were detected by multiplex ligand-dependent probe amplification (8).

The methylation status of GNAS DMRs was determined by methylation-specific multiplex ligand-dependent probe amplification and/or pyrosequencing (13, 24, 26, 28).

UPD was explored by microsatellite typing and/or SNP array.

The extensive and systematic molecular characterization of PHP patients was a gradual exploration, starting from the clinical presentation of patients. In particular, the molecular analysis of patients showing hormone resistance and an AHO phenotype started from direct sequencing, and, only if negative, epigenetic alterations and structural rearrangements were tested by Methylation Specific-Multiple Ligation Probe Amplification. On the contrary, in case of hormone resistance without AHO, the flowchart of molecular investigations was reversed, thus starting from the epigenetic testing followed but the search for genetic defects.

Results

Prevalence of different clinical subphenotypes

Most patients showed multihormone resistance, mainly to PTH and TSH, and AHO (38%), or only resistance to PTH with no additional features (31%), conditions classically referred to as PHP1A and PHP1B, respectively. Two subgroups of patients were diagnosed with PPHP (4%), showing only AHO features and no hormone resistance, and POH (2%), given the presence of ectopic ossifications progressing into deep connective and muscle tissues. The other patients had multihormone resistance without AHO (15%) or resistance limited to PTH together with AHO (10%).

As summarized in Figure 1A, isolated PTH resistance was associated only with epigenetic defects, whereas multihormone resistance with no AHO signs was mainly due to epigenetic defects, although 10% of these patients had a *GNAS* point mutation. In patients with PTH resistance and AHO, the rate of point mutations was higher, at 28%, and methylation defects lower, at about 70%. Multihormone resistance plus AHO was associated with a wider variety of molecular genetic and epigenetic defects: most patients had point mutations located in the Gsα coding region (81%), whereas the other cases were due to large deletions involving from one *GNAS* exon up to the entire GNAS locus (6%), broad GNAS methylation defects (10%), or isolated loss of methylation (LOM) at the GNAS *A/B:*TSS-DMR (3%). Finally, isolated AHO and POH were exclusively caused by paternal genetic *GNAS* alterations.

GNAS mutations

Direct sequencing of our cohort revealed that 170 patients (42% of those studied) had *GNAS* inactivating mu-

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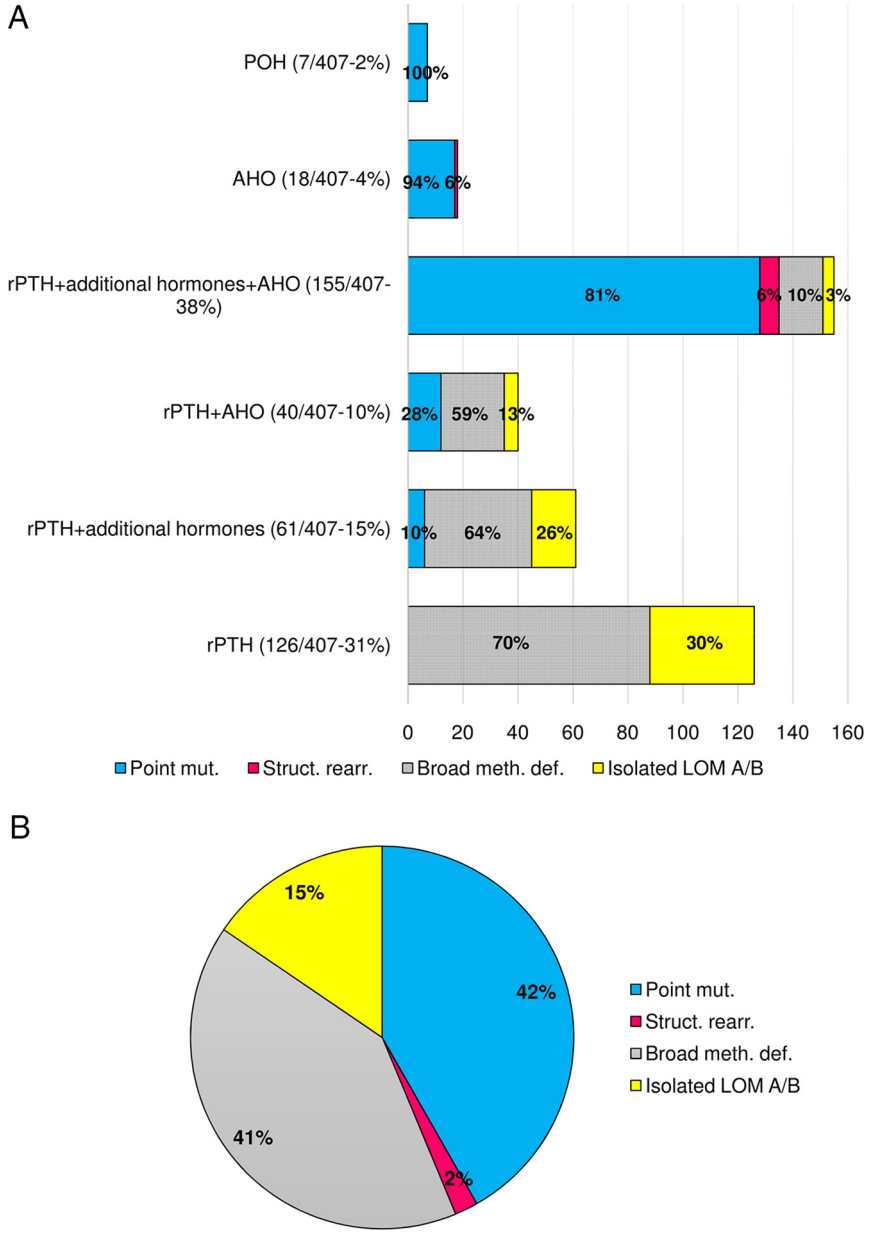

Figure 1. (A) This graph schematically summarizes *GNAS* molecular defects discovered in our cohort as a function of the relative clinical presentation of patients. The number and percentage of cases belonging to each clinical subtype are reported in parentheses. Percentages were determined for each individual subtype. Point mut.: point mutations, which are small genetic lesions affecting the Gsα coding region. Struct. rearr.: structural rearrangements affecting 20q involving the *GNAS* locus; in our series, only gross deletions were found. Broad meth. def.: broad methylation defects, which means LOI marks at all four *GNAS* DMRs. Isolated LOM A/B: loss of methylation affecting exclusively the *GNAS A/B:*TSS-DMR. (B) This chart shows the prevalence of *GNAS* molecular defects in our cohort of 407 patients. Point mutations (42%) and imprinting defects affecting all four *GNAS* DMRs (41%) were the most frequent molecular defects causative of PHP.

tations (Supplemental Table 1A; Figure 1B). Evaluating their frequency by inheritance pattern and mutation type, we observed that mutations resulting in an untranslated protein were present in 66% of mutation carriers (112/170, Supplemental Table 1A; Figure 2A), and hence, most patients are likely to show complete Gsα haploinsufficiency. On the other hand, inheritance pattern did not affect the prevalence or distribution of the mutation types.

Considering the clinical presentation of patients with mutations, as expected, most cases (76%) showed multihormone resistance together with AHO (ie, the classical PHP1A phenotype); similar rates of these mutations were maternally inherited, de novo, or of unknown origin (Figure 2B), and all mutation types were represented, except in-frame deletions (Figure 2C).

The second most frequently observed phenotype was AHO without hormone resistance (10%), which was associated with missense, nonsense, and frameshift mutations (Figure 2C). Except for one maternally inherited missense mutation, we were not able to determine the affected allele, which could explain why we did not discover any paternal mutations, these being expected to be PPHP-associated (Figure 2B). Moreover, we found one boy who had inherited the mutation from his father, but he displayed PTH resistance and AHO signs.

Notably, among patients with *GNAS* mutations, none had only PTH resistance, whereas 4% showed resistance to PTH and other hormones.

POH-related mutations (4% of cases) were all paternal or de novo, and in about half of the de novo samples it was possible to confirm that the mutation arose on the paternal allele (Figure 2B). Surprisingly, mutations associated with POH seem to have a highly damaging effect on transcript stability and protein function, whereas almost all patients showing only hormone resistance had a missense mutation (Figure 2C).

Regarding mutation localization, all exons were affected at different frequencies. In particular, exons 1 and 7 hosted about 20% of the mutations each; exon 5, 14%; and exon 6, 9%; whereas smaller percentages were found on other exons (Figure 3A). Exon 1, the only *GNAS* Gsα-specific exon, was the most affected, both considering the number of patients with mutations and the number of different mutations.

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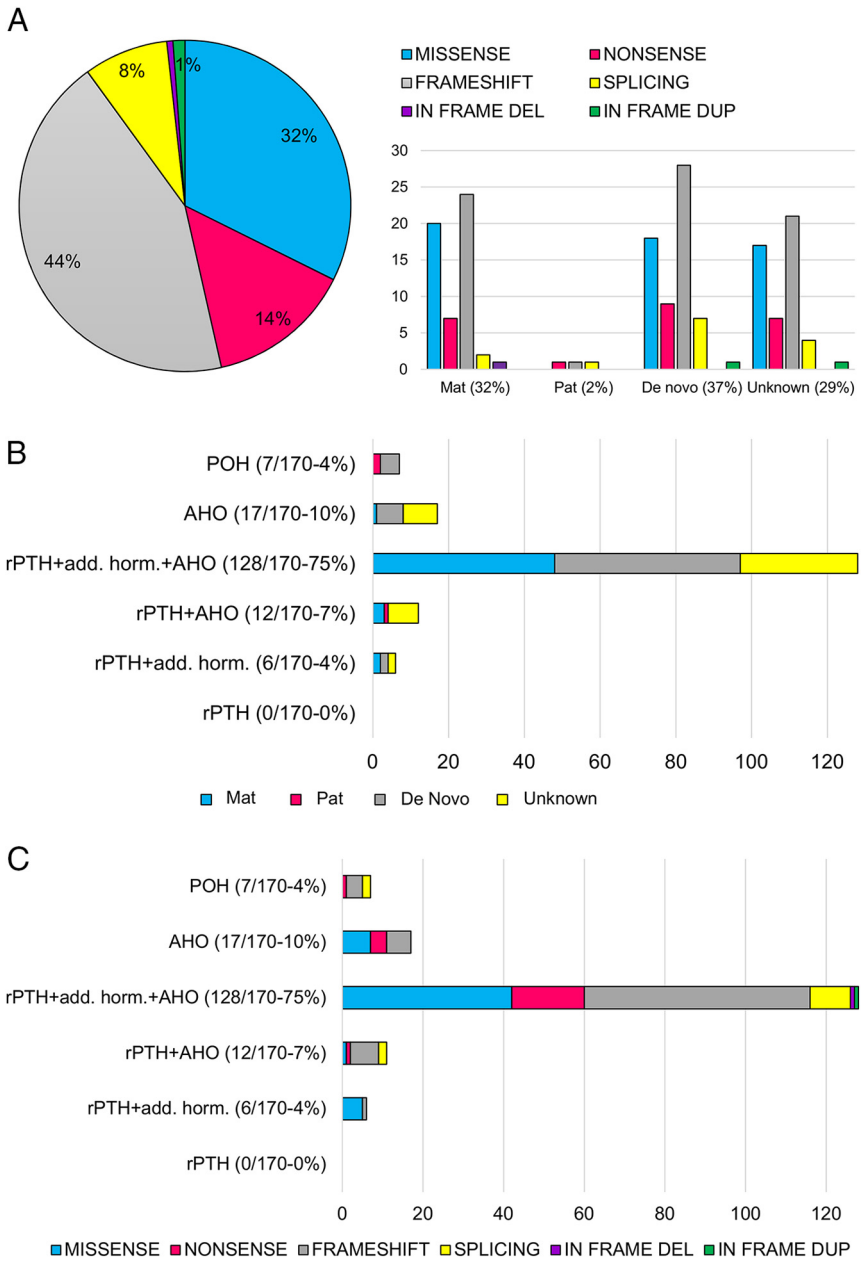

Figure 2. (A) The chart on the left illustrates the frequency of different point mutations affecting the Gsα-coding region. Most defects are frameshift and missense mutations, accounting for 77% of cases, followed by nonsense and splice site mutations and in-frame duplications. On the right, the graph shows that the inheritance pattern does not alter either the prevalence or the distribution of the different types of mutation. (B) This chart describes the relation between the inheritance pattern of *GNAS* point mutations and the clinical presentation in our 170 patients with mutations. De novo, mutation carried by the index case; Mat, maternal inheritance; Pat, paternal inheritance; unknown, the inheritance of the genetic lesion was not tested or determined. (C) This chart describes the relation between the specific mutation type and the clinical presentation in our 170 patients with mutations.

The most common mutation type in exon 7 was frameshift (19% of all studied mutations).

Missense mutations arose in all exons except exon 3, and half of the nonsense mutations were in exon 1, whereas frameshift mutations were evenly distributed throughout the gene, defects around splice sites more frequently affecting exons 1 to 5, whereas the two in-frame duplications were in exon 1 (Figure 3B).

Structural rearrangements involving the GNAS gene

Eight of the 407 PHP patients (2%) were affected by structural rearrangements involving the *GNAS* gene, namely broad deletions removing part of or the entire gene. Of these, five were maternally inherited, two de novo, and one of unknown origin (Supplemental Table 1B). Clinically, seven of these patients displayed resistance to PTH and other hormones together with features of AHO, the other patient showing an AHO phenotype without hormone resistance (Figure 1A).

Methylation defects affecting all GNAS DMRs

Broad GNAS methylation defects, that is LOI at all four GNAS DMRs, were found in 166 patients (41% of the cohort). As summarized in Figure 4A and Supplemental Table 1C and according to the guidance proposed by the EuroPHP for the molecular diagnosis of PHP not caused by genetic variants at *GNAS* (13), 61% of samples showed broad severe LOI. Most of these severe cases carried primary epimutations (84%), whereas 10% had segmental or complete uniparental isodisomy (iUPD) of 20q, although this could be a slight underestimate because we lacked parental samples to test for uniparental disomy in 6% of patients.

On the other hand, 25% of patients with GNAS epimutations displayed broad partial LOI (ie, pLOI at all *GNAS*-DMRs), which was mainly primarily epigenetic (91%), whereas the rate of confirmed mosaic iUPD was only 2% (one patient).

The remaining 14% (n = 23) of patients with broad imprinting defects showed the combined presence of both severe and pLOI, further confirming the existence of mixed patterns of methylation defects. As many as 83% (n = 19) of these patients with combined patterns showed partial LOM (pLOM) at the *GNAS-XL*:Ex1-DMR with complete LOI at the other three DMRs, supporting the view that this is the most prevalent mixed *GNAS* meth-

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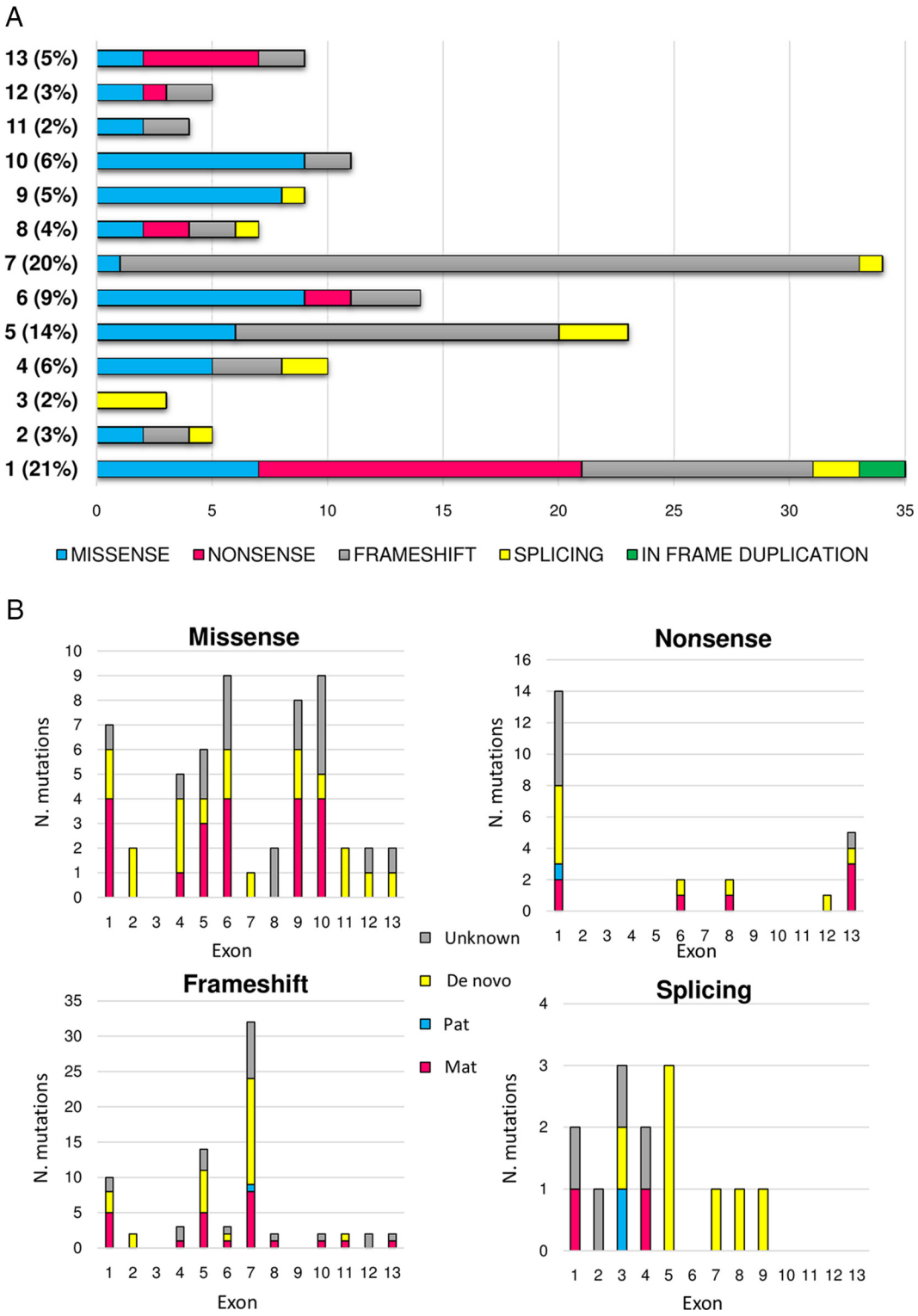

Figure 3. (A) This graph illustrates the *GNAS* mutational spectrum according to the number of patients with mutations. Exons 1–13 encoding Gsα are plotted on the y-axis (with the rate of mutations detected in our cohort of 170 patients in parentheses). Exon 1 is the most affected, considering the numbers of affected patients and of different genetic defects. (B) These four panels show the inheritance pattern along the *GNAS* gene of different mutation subtypes (exons 1–13 encoding Gsα are plotted on the x-axis).

ylation defect pattern, followed by pLOM at both the *GNAS-XL*:Ex1-DMR and *GNAS-AS1:*TSS-DMR with complete LOI at the *GNAS-NESP:*TSS-DMR and *GNAS A/B:*TSS-DMR in two patients (9%). Last, we identified one patient with pLOM at the *GNAS-AS1:*TSS-DMR and complete LOI at the other four DMRs, and another with partial gain of methylation at the *GNAS-NESP:*TSS-DMR and complete LOI at the remaining three DMRs (Figure 4B).

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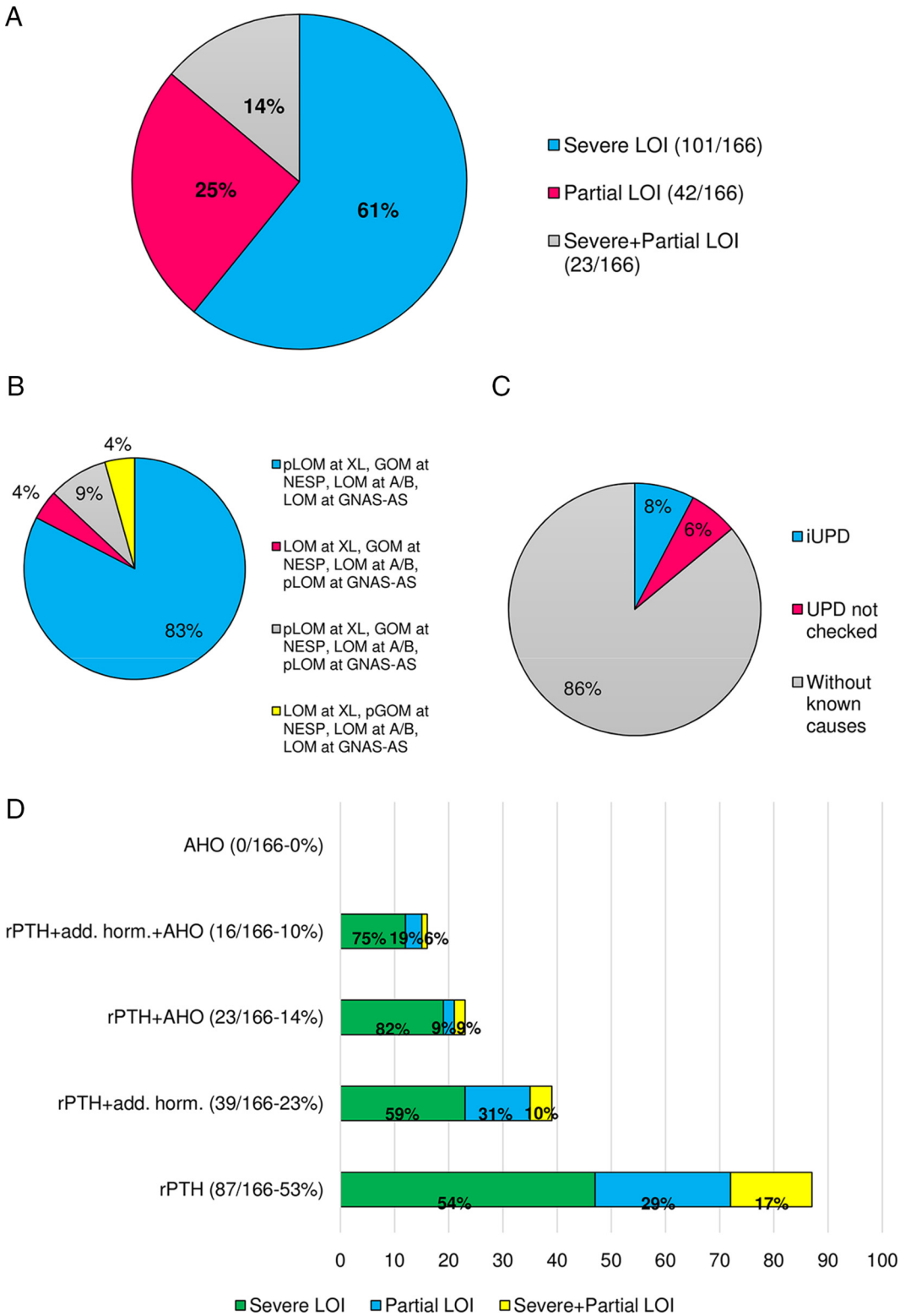

Figure 4. (A) This chart summarizes the prevalence of different subtypes of methylation defects affecting all four *GNAS* DMRs. Patients were categorized as complete, partial and complete + partial according to the methylation levels measured at each *GNAS* DMR. LOI, loss of imprinting. (B) This chart summarizes the prevalence of different subtypes of mixed methylation defects affecting all four *GNAS* DMRs. The most prevalent pattern is characterized by a complete loss of imprinting at the *GNAS-NESP:*TSS-DMR, *GNAS-AS1:*TSS-DMR, and *GNAS A/B:*TSS-DMR associated with partial loss of methylation at the *GNAS-XL* Ex1-DMR. GOM, gain of methylation; pGOM, partial GOM. (C). In this chart, we show that isodisomy at chromosome 20 (iUPD20) is the primary cause of the *GNAS* loss of imprinting in 8% of our patients. When parental DNA was available, we also excluded uniparental heterodisomy, hUPD20. The presence of UPD was not tested in 6% of the cohort. (D) The graph illustrates the relation between the methylation pattern defect and clinical presentation in our 166 patients. Note that the percentage of patients with a complete loss of imprinting at all four *GNAS* DMRs increases with the appearance of AHO signs.

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Furthermore, when considering both severe and partial LOI (n = 143), we attempted to define the overall frequency of 20q UPD associated with GNAS methylation defects, and observed that iUPD explained 8% of cases (Figure 4C).

Considering the clinical presentation of patients with methylation defects at GNAS DMRs, none had AHO features without resistance to PTH (Figure 4D). Overall, 76% of these patients were affected by hormone resistance only, either limited to PTH (53%) or also to other hormones acting through G protein-coupled receptors (23%), the others (24%) showing hormone resistance and AHO signs.

The prevalence of the different methylation defect subtypes (severe, partial, and severe plus partial) was similar in patients with hormone resistance without AHO. However, in patients with an AHO phenotype and hormone resistance, complete LOI affecting all four *GNAS* DMRs was the most common imprinting defect (about 80%).

Isolated LOM at the GNAS A/B:TSS-DMR

Our cohort also included 63 patients (15%) affected by isolated LOM at the GNAS *A/B:*TSS-DMR (Figure 1B, Supplemental Table 1D). Of these patients, 87% (55 patients) had autosomal dominant *STX16* deletions (the classical 3-kb deletion in 54 and a larger 24.6-kb deletion in 1 patient), whereas no underlying primary genetic defect was detected the others (13%, 8/63 patients) (Figure 5A). We identified both complete (90%) and partial (10%) methylation defects associated with the LOM at the GNAS *A/B:*TSS-DMR (Figure 5B). Notably, five of eight patients without known *STX16* deletions but only one of 54 patients with the 3-kb deletion showed pLOM.

The 86% of patients presented with hormone resistance only (60% with isolated resistance to PTH and 26% with multihormone resistance), whereas 14% showed hormone resistance together with the AHO phenotype (Figure 5C).

Discussion

Phenotype-genotype correlation

PHP is a heterogeneous group of rare endocrine disorders characterized by resistance to PTH, manifesting with hypocalcemia, hyperphosphatemia, and elevated PTH, and that includes the subtypes PHP1A, PHP1B, PHP2, and PPHP. In this work, we sought to assess the prevalence of PHP (both clinical and molecular aspects) in (epi)genetically confirmed cases.

Demographic data analysis showed no biased sex-ratio distribution in the whole cohort, which comprised 212 females and 195 males. The same result was observed considering patients according to their *GNAS* molecular defect, the age at diagnosis, or their nationality. Noteworthy was that point mutations are more frequent in females than in males (95 vs 75), especially in Italian females (53 vs 32) (Table 1). We calculated the mean age at molecular diagnosis (± standard deviation) on available data (10.5 years ± 11 for *GNAS* point mutations [n = 168], 17.6 years ± 11.9 for structural rearrangements [n = 8], 22.5 years ± 13.7 for broad methylation defects [n = 145] and 23.1 years ± 13.7 for isolated LOM A/B [n = 37]). We confirmed what different groups, including ours have reported, although our study was not designed for genotype/phenotype comparisons: that the diagnosis is done earlier in presence of AHO features.

We have observed a similar prevalence for PHP1A (individuals with AHO associated with isolated PTH resistance or with PTH and TSH resistance, 48%) and PHP1B (those with isolated resistance to PTH or with multihormone resistance in the absence of additional features, 46%). Because the researchers contributing to the cohort are all members of referral groups for this disease, we believe that these similar percentages are due to avoiding the bias associated with age-related genetic/epigenetic characteristics (patients diagnosed in the infancy, usually with AHO features, carry point mutations, whereas when they are diagnosed in adulthood, they usually present methylation defects) (26). Isolated phenotypic syndromes (ie, PPHP and POH) are less frequent (4% and 2%, respectively). Further, severity of the AHO phenotype is variable, some patients presenting the complete phenotype, whereas others present only subtle manifestations (3).

We observed that isolated PTH resistance in the absence of AHO phenotype was exclusively associated with epigenetic defects, as previously reported in a single series (11), and just 10% of patients with multihormone resistance but no AHO phenotype carried a *GNAS* point mutation. These data raise the eventuality that there could be PHP1A patients with an underdiagnosed AHO phenotype, or we should assume that PHP1B could be also associated with structural alterations. At the other end of the spectrum, PHP1A (ie, multihormone resistance in the presence of the AHO phenotype (1)) was mostly associated with mutations in the *GNAS* gene (87%), as expected (6); however, methylation defects were observed in 13% of patients, confirming an overlap in the molecular causes of the disease (23, 30). Surprisingly, 72% of patients with isolated PTH resistance and the AHO phenotype also had epigenetic defects, which again leads us to believe that the diagnosis of AHO is somewhat variable and subjective.

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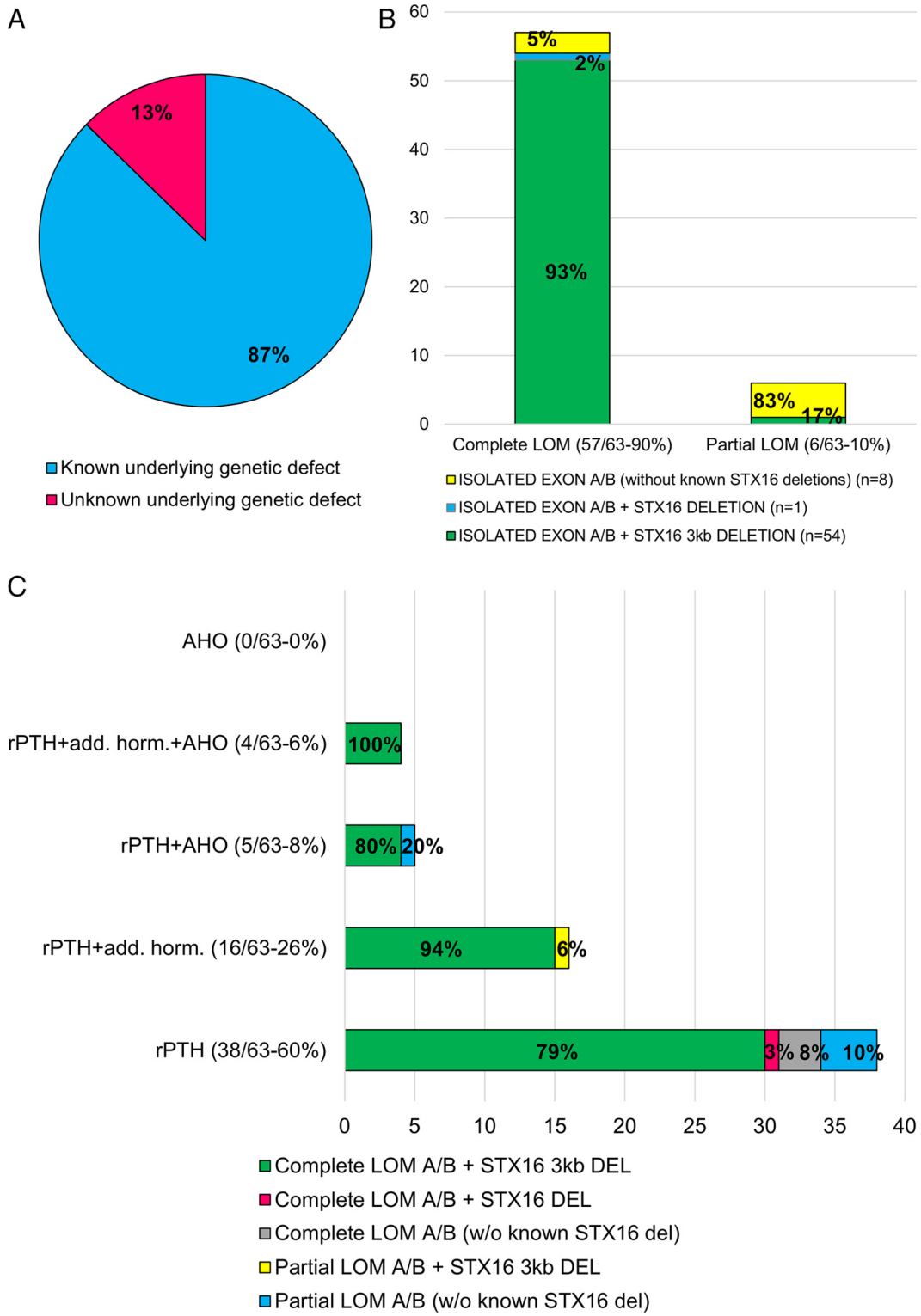

Figure 5. (A)This graph shows the prevalence of patients with an autosomal dominant imprinting defect who have an isolated LOM at the *GNAS A/B:*TSS-DMR due to the deletion of an imprinting control region. (B) The panel describes the observed frequency of different subtypes of methylation defects, according to the measured methylation level at the *GNAS A/B:*TSS-DMR. Most patients with partial LOM at the *GNAS A/B:*TSS-DMR are currently considered sporadic cases, as no primary genetic lesion has yet been discovered. (C) The chart describes the relation between different *GNAS A/B:*TSS-DMR methylation defects and clinical presentation in our 63 patients. *STX16* 3kb del, recurrent *STX16* deletion; *STX16* del, private *STX16* deletions; w/o, without.

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GNAS inactivating mutations

To date, 200 inactivating *GNAS* mutations have been described. Most of them are summarized in the Leiden Open Variant Database (http://databases.lovd.nl/shared/genes/GNAS). The mutations are distributed throughout the gene. In our series, all exons were affected at different frequencies similar to previous reports (6, 31).

Regarding mutation type, as in previous reviews (6, 31), most (66%) were frameshift, nonsense, or splicing mutations, which are expected to lead to a truncated protein or nonsense-mediated decay (32). The pattern of distribution along the gene according of different mutation types possibly reflected different underlying molecular mechanisms of formation, influenced by phenomena such as gene size, the nucleotide sequence itself, and chemical changes. As previously suggested (31), the lack of missense mutations in the first exon could be due to 1) amino acid changes with lower pathogenicity and hence remaining unidentified or 2) amino acid changes poorly tolerated.

Efforts have been made in recent years to identify relationships between mutations (type or location) and clinical manifestations (6, 7, 26, 31). Our data revealed that mutations were most often associated with multihormone resistance in the presence of AHO, followed by the isolated AHO phenotype. Within this group, two families deserve to be mentioned. The first opens the possibility of a new clinical overlap between PHP1A and PPHP because the mutation was maternally inherited, in contrast with the expected inheritance pattern (33), because the patient presented AHO without hormone resistance. The second family presented a paternally inherited mutation with PTH resistance and AHO, as recently observed by Turan and colleagues (25). On the other hand, six of 61 (nearly 10%) of patients with multihormone resistance but no AHO phenotype carried an inactivating mutation (most being missense mutations). These observations 1) confirm the need to look for genetic mutations in the absence of epigenetic defects in individuals with a classical PHP1B phenotype and 2) suggest that some missense mutations may not reach a threshold effect for the development of AHO features. Further studies are needed to confirm this putative correlation.

GNAS inactivating mutations were also related to POH (9, 10). In our series, all POH patients carried truncating mutations, as observed in a previous review (31). This supports the hypothesis that these highly disruptive mutations are responsible for the greater severity of heterotopic ossifications in POH respect to those found in PPHP and PHP1A.

Epigenetic defects at the GNAS locus

As mentioned previously, patients with PHP1B display epigenetic defects of the GNAS locus involving some or all of the DMRs (11, 34), with complete or partial methylation changes; however, the DMR comprising the promoter and the first exon of the A/B transcript is always affected. In our series, 41% of patients had a methylation defect affecting all four DMRs: 61% of them had a complete overall LOI; in 25%, there was a partial overall methylation defect; and in 14%, a mixed pattern of complete and partial methylation defects coexisted, the most common pattern being partial at the GNAS-*XL:* Ex1-DMR and complete for other DMRs. The differences in methylation levels at the GNAS-*XL:* Ex1-DMR and the GNAS-*AS1:*TSS-DMR could support the hypothesis of Court and colleagues suggesting that they are located in independent DMRs (35), unlike previous data (reviewed in (36)).

Since the first description of UPD(20q)pat as a cause of sporPHP1B (37), 10 other patients have been reported (14, 15, 38–40). Within our series, 10% of patients with a broad complete methylation defect had segmental or complete iUPD, quite similar to the rate in smaller series, suggesting that all racial backgrounds are similarly affected. On the other hand, 2% of those with broad partial methylation defects presented iUPD in a mosaic state. However, these figures on disomy prevalence could be an underestimate as heterodisomy could not be ruled out in all other patients due to a lack of parental samples.

The 15% of patients (63 cases) showed isolated LOM at the GNAS *A/B:*TSS-DMR, associated with a *STX16* deletion in 87% of cases (13.5% of the total series). In eight patients, we were unable to identify the reported deletions (16, 18, 19); in most of these patients, the methylation defect was partial, and we hypothesized that point alterations within *STX16* should be investigated.

Clinically, among patients with epigenetic defects, 79% were affected by hormone resistance (limited to PTH or multihormone resistance), whereas the others (21%) presented hormone resistance with AHO signs, as described previously (23, 30).

In brief, this prevalence study of 407 patients with molecular defects at the GNAS locus revealed that, clinically, 48% presented PHP1A, 46% PHP1B, 4% PPHP, and 2% POH. Focusing on the genetic/epigenetic features, which are fundamental to correctly define the recurrence risk, and hence for the genetic counseling, 41.4% of patients carried point mutations, 2.4% structural rearrangements involving the *GNAS* gene, 13.5% isolated LOM at the GNAS *A/B:*TSS-DMR associated with a *STX16* deletion, and 2.7% 20qiUPD. Unfortunately, we are still unable to estimate the risk of recurrence in the 2% of patients showing isolated LOM at the GNAS *A/B:*TSS-DMR and in the

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38% of patients carrying broad GNAS methylation defects.

Moreover, our findings underline the pressing need for a novel classification due to the overlap between clinical and molecular forms and the risk of diagnostic errors. For instance, if we would have considered a purely clinical diagnosis, we wouldn't have characterized 19% of the genetic diagnoses in patients with multihormone resistance and AHO and, more importantly, 72% of those with isolated PTH resistance and AHO. On the other hand, if only a molecular diagnosis had been considered, patients with point mutations manifesting the hormonal resistance only (without AHO), and patients with LOI manifesting as hormonal resistance associated with AHO would have been underdiagnosed. So, the authors propose to create an inclusive and specific nomenclature that involves both the clinical and genetic aspects of the disease.

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References

1. **Albright F, Burnett CH, Smith PH, Parson W.** Pseudohypoparathyroidsm—an example of "Seabright syndrome." *Endocrinology*. 1942;30:922–932.
2. **Drezner M, Neelon FA, Lebovitz HE.** Pseudohypoparathyroidism type II: a possible defect in the reception of the cyclic AMP signal. *N Engl J Med*. 1973;289:1056–1060.
3. **Mantovani G, Elli FM, Spada A.** GNAS epigenetic defects and pseudohypoparathyroidism: time for a new classification? *Horm Metab Res*. 2012;44:716–723.
4. **Patten JL, Johns DR, Valle D, et al.** Mutation in the gene encoding the stimulatory G protein of adenylate cyclase in Albright's hereditary osteodystrophy. *N Engl J Med*. 1990;322:1412–1419.
5. **Juppner H, Bastepe M.** Different mutations within or upstream of the GNAS locus cause distinct forms of pseudohypoparathyroidism. *J Pediatr Endocrinol Metab*. 2006;19(Suppl 2):641–646.
6. **Elli FM, deSanctis L, Ceoloni B, Barbieri AM, et al.** Pseudohypoparathyroidism type Ia and pseudo-pseudohypoparathyroidism: the growing spectrum of GNAS inactivating mutations. *Hum Mutat*. 2013;34:411–416.
7. **Thiele S, Werner R, Grotzinger J, et al.** A positive genotype-phenotype correlation in a large cohort of patients with pseudohypoparathyroidism Type Ia and pseudo-pseudohypoparathyroidism and 33 newly identified mutations in the GNAS gene. *Mole Genet Genom Med*. 2015;3:111–120.
8. **Garin I, Elli FM, Linglart A, et al.** Novel microdeletions affecting the GNAS locus in pseudohypoparathyroidism: characterization of the underlying mechanisms. *J Clin Endocrinol Metab*. 2015;100:E681–E687.
9. **Shore EM, Ahn J, Jan dB, et al.** Paternally inherited inactivating mutations of the GNAS1 gene in progressive osseous heteroplasia. *N Engl J Med*. 2002;346:99–106.
10. **Adegbite NS, Xu M, Kaplan FS, Shore EM, Pignolo RJ.** Diagnostic and mutational spectrum of progressive osseous heteroplasia (POH) and other forms of GNAS-based heterotopic ossification. *Am J Med Genet A*. 2008;146A:1788–1796.
11. **Liu J, Litman D, Rosenberg MJ, Yu S, Biesecker LG, Weinstein LS.** A GNAS1 imprinting defect in pseudohypoparathyroidism type IB. *J Clin Invest*. 2000;106:1167–1174.
12. **Liu J, Nealon JG, Weinstein LS.** Distinct patterns of abnormal GNAS imprinting in familial and sporadic pseudohypoparathyroidism type IB. *Hum Mol Genet*. 2005;14:95–102.
13. **Garin I, Mantovani G, Aguirre U, et al.** European guidance for the molecular diagnosis of pseudohypoparathyroidism not caused by point genetic variants at GNAS: an EQA study. *Eur J Hum Genet*. 2015;23:438–444.
14. **Fernandez-Rebollo E, Lecumberri B, Garin I, et al.** New mechanisms involved in paternal 20q disomy associated with pseudohypoparathyroidism. *Eur J Endocrinol*. 2010;163:953–962.
15. **Takatani R, Minagawa M, Molinaro A, et al.** Similar frequency of paternal uniparental disomy involving chromosome 20q (patUPD20q) in Japanese and Caucasian patients affected by sporadic pseudohypoparathyroidism type Ib (sporPHP1B). *Bone*. 2015;79:15–20.
16. **Bastepe M, Frohlich LF, Hendy GN, et al.** Autosomal dominant pseudohypoparathyroidism type Ib is associated with a heterozygous microdeletion that likely disrupts a putative imprinting control element of GNAS. *J Clin Invest*. 2003;112:1255–1263.
17. **Bastepe M, Frohlich LF, Linglart A, et al.** Deletion of the NESP55

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differentially methylated region causes loss of maternal GNAS imprints and pseudohypoparathyroidism type Ib. *Nat Genet*. 2005; 37:25–27.
18. **Linglart A, Gensure RC, Olney RC, Juppner H, Bastepe M.** A novel STX16 deletion in autosomal dominant pseudohypoparathyroidism type Ib redefines the boundaries of a cis-acting imprinting control element of GNAS. *Am J Hum Genet*. 2005;76:804–814.
19. **Elli FM, de Sanctis L, Peverelli E, et al.** Autosomal dominant pseudohypoparathyroidism type Ib: a novel inherited deletion ablating STX16 causes loss of imprinting at the A/B DMR. *J Clin Endocrinol Metab*. 2014;99:E724–E728.
20. **Richard N, Abeguile G, Coudray N, et al.** A new deletion ablating NESP55 causes loss of maternal imprint of A/B GNAS and autosomal dominant pseudohypoparathyroidism type Ib. *J Clin Endocrinol Metab*. 2012;97:E863–E867.
21. **Chillambhi S, Turan S, Hwang DY, Chen HC, Juppner H, Bastepe M.** Deletion of the noncoding GNAS antisense transcript causes pseudohypoparathyroidism type Ib and biparental defects of GNAS methylation in cis. *J Clin Endocrinol Metab*. 2010;95:3993–4002.
22. **Rezwan FI, Poole RL, Prescott T, Walker JM, Karen Temple I, Mackay DJ.** Very small deletions within the NESP55 gene in pseudohypoparathyroidism type 1b. *Eur J Hum Genet*. 2015;23:494–499.
23. **Perez de Nanclares G, Fernandez-Rebollo E, Santin I, et al.** Epigenetic defects of GNAS in patients with pseudohypoparathyroidism and mild features of Albright's hereditary osteodystrophy. *J Clin Endocrinol Metab*. 2007;92:2370–2373.
24. **Elli FM, de Sanctis L, Bollati V, et al.** Quantitative analysis of methylation defects and correlation with clinical characteristics in patients with pseudohypoparathyroidism type I and GNAS epigenetic alterations. *J Clin Endocrinol Metab*. 2014;99:E508–E517.
25. **Turan S, Thiele S, Tafaj O, et al.** Evidence of hormone resistance in a pseudo-pseudohypoparathyroidism patient with a novel paternal mutation in GNAS. *Bone*. 2015;71:53–57.
26. **Fernandez-Rebollo E, Lecumberri B, Gaztambide S, Martinez-Indart L, Perez de NG, Castano L.** Endocrine profile and phenotype-(epi)genotype correlation in Spanish patients with pseudohypoparathyroidism. *J Clin Endocrinol Metab*. 2013;98:E996–E1006.
27. **Maupetit-Mehouas S, Azzi S, Steunou V, et al.** Simultaneous hyper- and hypomethylation at imprinted loci in a subset of patients with GNAS epimutations underlies a complex and different mechanism of multilocus methylation defect in pseudohypoparathyroidism type 1b. *Hum Mutat*. 2013;34:1172–1180.
28. **Maupetit-Mehouas S, Mariot V, Reynes C,et al.** Quantification of the methylation at the GNAS locus identifies subtypes of sporadic pseudohypoparathyroidism type Ib. *J Med Genet*. 2011;48:55–63.
29. **Linglart A, Carel JC, Garabedian M, Le T, Mallet E, Kottler ML.** GNAS1 lesions in pseudohypoparathyroidism Ia and Ic: genotype phenotype relationship and evidence of the maternal transmission of the hormonal resistance. *J Clin Endocrinol Metab*. 2002;87:189–197.
30. **Mantovani G, de Sanctis L, Barbieri AM, et al.** Pseudohypoparathyroidism and GNAS epigenetic defects: clinical evaluation of Albright hereditary osteodystrophy and molecular analysis in 40 patients. *J Clin Endocrinol Metab*. 2010;95:651–658.
31. **Lemos MC, Thakker RV.** GNAS mutations in Pseudohypoparathyroidism type 1a and related disorders. *Hum Mutat*. 2015;36:11–19.
32. **Rickard SJ, Wilson LC.** Analysis of GNAS1 and overlapping transcripts identifies the parental origin of mutations in patients with sporadic Albright hereditary osteodystrophy and reveals a model system in which to observe the effects of splicing mutations on translated and untranslated messenger RNA. *Am J Hum Genet*. 2003; 72:961–974.
33. **Wilson LC, Oude Luttikhuis ME, Clayton PT, Fraser WD, Trembath RC.** Parental origin of Gs alpha gene mutations in Albright's hereditary osteodystrophy. *J Med Genet*. 1994;31:835–839.
34. **Bastepe M, Pincus JE, Sugimoto T, et al.** Positional dissociation between the genetic mutation responsible for pseudohypoparathyroidism type Ib and the associated methylation defect at exon A/B: evidence for a long-range regulatory element within the imprinted GNAS1 locus. *Hum Mol Genet*. 2001;10:1231–1241.
35. **Court F, Tayama C, Romanelli V, et al.** Genome-wide parent-of-origin DNA methylation analysis reveals the intricacies of human imprinting and suggests a germline methylation-independent mechanism of establishment. *Genome Res*. 2014;24:554–569.
36. **Bastepe M.** The GNAS locus: quintessential complex gene encoding Gsalpha, XLalphas, and other imprinted transcripts. *Curr Genom*. 2007;8:398–414.
37. **Bastepe M, Lane AH, Juppner H.** Paternal uniparental isodisomy of chromosome 20q–and the resulting changes in GNAS1 methylation–as a plausible cause of pseudohypoparathyroidism. *Am J Hum Genet*. 2001;68:1283–1289.
38. **Bastepe M, Altug-Teber O, Agarwal C, Oberfield SE, Bonin M, Juppner H.** Paternal uniparental isodisomy of the entire chromosome 20 as a molecular cause of pseudohypoparathyroidism type Ib (PHP-Ib). *Bone*. 2011;48:659–662.
39. **Jin HY, Lee BH, Choi JH, et al.** Clinical characterization and identification of two novel mutations of the GNAS gene in patients with pseudohypoparathyroidism and pseudopseudohypoparathyroidism. *Clin Endocrinol (Oxf)*. 2011;75:207–213.
40. **Dixit A, Chandler KE, Lever M, et al.** Pseudohypoparathyroidism type 1b due to paternal uniparental disomy of chromosome 20q. *J Clin Endocrinol Metab*. 2013;98:E103–E108.

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