Arrate Pereda Agirre

European Doctoral Thesis

Vitoria-Gasteiz, 2015

Universidad del País Vasco
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Characterisation of patients with brachydactyly type E associated or not with hormonal resistance: clinical, genetic and radiological approach

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Universidad del País Vasco
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Facultad de Ciencia y Tecnología

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Caracterización de pacientes con braquidactilia tipo E asociada o no a alteraciones hormonales: abordaje clínico, genético y radiológico

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EUROPEAN DOCTORAL THESIS

2015

Osakidetza

BIO ARABA
Instituto de Investigación Sanitaria

El trabajo de esta tesis doctoral ha sido subvencionado por el Fondo de Investigación Sanitaria - Instituto Carlos III (FI11/0309; PI10/0148, PI13/00467).

"Discovery consists of seeing what everybody has seen, and thinking what nobody has thought"

Albert Szent-Györgyi (1893-1986)

ACKNOWLEDGEMENTS - AGRADECIMIENTOS - ESKERRAK

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Agradezco a todas las personas e instituciones que habéis contribuido durante estos años al desarrollo de esta tesis doctoral.

Debo un especial agradecimiento al Fondo de Investigación Sanitaria - Instituto de Salud Carlos III por confiar en mí al concederme la beca predoctoral para la formación de personal investigador en salud, sin la cual no hubiese sido posible realizar este trabajo.

De la misma forma quiero agradecer a mi directora, la Dra. Guiomar Pérez de Nanclares Leal, por haber confiado tanto en mí para este trabajo. Desde muy joven, mi deseo había sido trabajar y participar en la investigación en genética de enfermedades raras y Guiomar ha cumplido mi deseo. Además de ello, le agradezco toda su dedicación y constancia, tanto en mi trabajo como en lo personal. También se merecen un especial agradecimiento mi codirectora y compañera, la Dra. Intza Garin Elcoro, y también compañera, la Dra.Elena Beristain Mendizabal, no solo por el tiempo invertido en mi formación sino también por preocuparse por mí. Eskerrik asko Guiomar, Intza eta Elena!

Por otra parte, quisiera agradecer a todos los pacientes y familiares que han participado en este estudio, así como a los clínicos que nos han derivado estos casos y que muchas veces han participado como coautores en la elaboración de los artículos que componen esta tesis. Gracias por haber confiado en nuestro grupo, porque sin vuestra colaboración no hubiese sido posible este trabajo.

I would also like to thank, the group of Dr. Caroline Silve, especially Dr. Claire Briet and Dr. Catherine Le-Stunff, where I worked for eight months in Paris. Thank you for giving me the opportunity to learn new techniques and directing the experiments, which have been necessary to conclude this work.

ESKERRAK

Nire familia osoari baita ere eskertu gura deutsat, beti nire alboan egoteagaitik, batez ere nire guraso eta nebari. Nire amatxu eta aitatxuri, Sorkunde eta Jesuseri, beraiek ez izandako aukera guzti honeek oparitzeagatik baita baloratzen erakusteagatik be, eurangaitik naizelako gaur naizena. Gaur hemen nirekin ezin egon arren, bere indar, maitasun eta hurbiltasuna sentitzen dodalako inoiz baino gehiago eta badakit nitaz arro sentitzen dirala dagozan leku horretatik begiratzen nabenean. Nire neba Ikertxuri, beti nire alboan egoteagaitik eta bere besarkada eta muxuekin gure etxea betetzeagaitik; erreferentetzat hartzen nauela sentiarazteagaitik eta baita nire erreferentea izateagaitik ere.

Nire koadrilari be eskertu nahi deutsat, lan osteko momentu beharrezko horreek betetzeagaitik, batez ere nire lau neskei, Irune, Maider, Naiara eta Patriciari, baita Mertxe eta Monicari be. Eskerrik asko zuen maitasunagatik, beti ni laguntzeko prest egoteagatik, baita irribarre bat ateratzeko prest be. Honeek bezain garrantzitsuak izan dira nire ikasketetan zehar ezagutu dodazan eta nire alboan jarraitzen daben lagun on guzti horreek: Itsasne, Maite, Izaskun, Amaia, Belén eta Ane, hainbeste elkar ez ikusi arren, beti hor dagozala sentiarazten nabelako.

Azkenik, eta ez horregatik garrantzi gutxiagokoak, eskerrak eman nahi deutseet azken urte honeetan nire bigarren familia izandako Izaskun, Javi, Nagore, Iñaki eta batez ere Ikertxuri. Eskerrik asko behar izan nebanean zuen etxeko ateak horrela zabaltzeagaitik eta bereziki Ikertxuri, zure laguntasun eta maitasunagaitik; zure muxu eta besarkadengaitik; baita gure ezagutza elkarrekin konpartitzean, gozatu doguzan hainbeste momentu polit horiengaitik eta geratzen direnengaitik be.

Eskerrik asko guztioi bihotz-bihotzez,

Arrate

GLOSSARY

AC	Adenylate Cyclase	Adenilato ciclasa
ACRDYS1	Acrodysostosis type 1, with hormonal resistance	Acrodisostosis tipo 1 con resistencia hormonal
ACRDYS2	Acrodysostosis type 2, without hormonal resistance	Acrodisostosis tipo 2 sin resistencia hormonal
ACTH	Adrenocorticotropic hormone	Hormona adrenocorticotropa
AD	Autosomal dominant	Autosómico dominante
ADH	Antidiuretic hormone	Hormona antidiurética
ADOHR	Acrodysostosis with hormonal resistance	Acrodisostosis con resistencia hormonal
ADOP4	Acrodysostosis type 2, without hormonal resistance	Acrodisostosis tipo 2 sin resistencia hormonal
AHO	Albright's hereditary osteodystrophy	Osteodistrofia hereditaria de Albright
ATP	Adenosine triphosphate	Adenosin trifosfato
BD	Brachydactyly	Braquidactilia
BDA	Brachydactyly type A	Braquidactilia tipo A
BDE	Brachydactyly type E	Braquidactilia tipo E
BDMR	Brachydactyly-mental retardation syndrome	Síndrome de braquidactilia con retraso mental
BLC	Blomstrand' disease	Enfermedad de Blomstrand
cAMP	3',5'-cyclic adenosine monophosphate	Adenosin monofosfato cíclico
cDNA	Complementary DNA	ADN complementario
CHO	Chinese hamster ovary cells	Células derivadas de ovario de hámster chino
CpG island	Cytosine-phosphate-guanine island	Islas citosina fosfato guanina
CREB	cAMP response element-binding protein	Elementos de respuesta a AMPc
dATP	Deoxiadenosine	Deoxiadenosina
ddNTPs	2',3'-dideoxynucleotide triphosphates	2'3'-dideoxinucleótidos trifosfato
DMR	Differentially methylated region	Región diferencialmente metilada
DNA	Deoxyribonucleic acid	Ácido desoxirribonucleico
DNAse	Deoxyribonuclease	Desoxirribonucleasa
dNTPs	Deoxynucleotide triphosphates	Deoxinucleótidos trifosfato
EDTA	Ethylenediaminetetraacetic acid,	Ácido etildiaminotetraacético
FSH	Follicle-stimulating hormone	Hormona folículoestimulante
GH	Growth hormone	Hormona de crecimiento
GHRH	Growth hormone releasing hormone	Hormona estimuladora de la hormona de crecimiento
GNAS	Guanine Nucleotide-binding-protein, alpha Stimulating	Proteina de unión al nucleotido Guanina, alfa estimuladora
GNAS-AS	GNAS antisense transcript	Transcrito antisentido de GNAS
GPCR	G protein-coupled receptor	Receptor acoplado a proteína G
$G_s\alpha$	Alpha-subunit of the stimulatory guanine nucleotide-binding protein	Subunidad alfa de la proteína G estimuladora
GTP	Guanosine triphosphate	Guanosina trifosfato
HA	Human influenza hemagglutinin epitope	Epítopo Hemaglutinina
HDAC4	Histone deacetylase 4	Histona deacetilasa 4
HEK293	Cell line derived from human embryonic kidney	Línea celular transformada derivadas de riñón embrionario humano
HOXD13	Homeobox D13	Proteína homeótica D13

HRP	Horseradish peroxidase	Peroxidasa del rábano
HTNB	Hypertension and brachydactyly syndrome	Síndrome de hipertensión y braquidactilia
IHH	Indian hedgehog protein	Proteína Indian hedgehog
IP	Immunoprecipitation	Inmunoprecipitación
IUGR	Intrauterine growth restriction	Restricción del crecimiento intrauterino
JMC	Jansen's metaphyseal chondrodysplasia	Condrodisplasia metafisaria tipo Jansen
LH	Luteinizing hormone	Hormona luteinizante
LOM	Loss of methylation	Pérdida de metilación
MLPA	Multiplex ligation-dependent probe amplification	Amplificación múltiple dependiente de ligación
MR	Mental retardation	Retraso mental
mRNA	Messenger RNA	ARN mensajero
MS-MLPA	Methylation-specific multiplex ligation-dependent probe amplification	MLPA específico de metilación
MW	Molecular weight	Peso molecular
NESP55	Neuroendocrine secretory protein 55	Proteína neuroendocrina secretora-55
NMD	Nonsense-mediated mRNA decay	Degradación del ARNm mediada por mutaciones terminadoras
patUPD20q	Paternal 20q disomy	Disomía paterna 20q
PCR	Polymerase chain reaction	Reacción en cadena de la polimerasa
PDE3A	Phosphodiesterase 3A	Fosfodiesterasa 3A
PDE4D	Phosphodiesterase 4D, cAMP-specific	Fosfodiesterasa 4D, específica de AMPc
PFE	Primary failure of tooth eruption	Fallo Primario de Erupción en la dentición
PHP	Pseudohypoparathyroidism	Pseudohipoparatiroidismo
PKA	Protein kinase A	Proteína quinasa A
POH	Progressive osseous heteroplasia	Heteroplasia ósea progresiva
PPHP	Pseudopseudohypoparathyroidism	Pseupseudohipoparatiroidismo
PRKAR1A	cAMP-dependent protein kinase type 1 regulatory subunit protein	Subunidad reguladora de la proteína quinasa tipo 1, dependiente de AMPc
PTH	Parathyroid hormone	Hormona paratiroidea o parathormona
PTHLH	Gene encoding parathyroid hormone-related protein	Gen codificante de la proteína relacionada con la parathormona
PTHR1	Receptor for PTH/PTHrP	Receptor 1 de la PTH/PTHrP
PTHrP	Parathyroid hormone-related protein	Proteína relacionada con la parathormona
RNA	Ribonucleic acid	Ácido ribonucleico
RT-PCR	Reverse transcription polymerase chain reaction	Reacción en cadena de la polimerasa con transcriptasa inversa
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis	Electroforesis en gel de poliacrilamida con dodecilsulfato sódico
SNP	Single nucleotide polymorphism	Polimorfismo de un solo nucleótido
STX16	Syntaxin 16	Sintaxina 16
TRPS	Tricho-rhino-phalangeal syndrome	Síndrome trico-rino-falángico

TRPS1	Zinc finger transcription factor TRPS1	Factor de transcripción TRPS1, tipo dedos de zinc
TS	Turner syndrome	Síndrome de Turner
TSH	Thyroid stimulating hormone	Hormona estimuladora de tiroides
UPD	Uniparental disomy	Disomia uniparental
WT	Wild type	Silvestre
XLαs	Extra-large Gsα	Transcrito de la forma larga de la proteína Gsα

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1. INTRODUCTION

Albright's hereditary osteodystrophy (AHO) is a heterogeneous clinical entity classically associated with pseudohypoparathyroidism (PHP)[1,2]. This peculiar phenotype was initially described including small stature, obesity with rounded face, subcutaneous ossifications, mental retardation and brachydactyly. PHP involves a heterogenic group of rare disorders associated with genetic and/or epigenetics alterations at Guanine nucleotide binding protein, alpha stimulating (GNAS) locus, and AHO phenotype is manifested in a wide spectrum in the different subtypes[3]. PHP prevalence is estimated to be about 1-9/1.000.000 (Orphanet).

PHP was described as the first hormone resistance syndrome by Albright in 1942. He appreciated a reduced calcemic and phosphaturic response to injected bovine parathyroid extract in patients with normal renal function. So initially the parathyroid hormone (PTH) resistance was included as a feature of AHO phenotype[1]. However, ten years later more patients with this phenotype but without hormone resistance were described, those known as pseudopseudohypoparathyroidism (PPHP). Consequently, this hormonal resistance was included as a non-obligatory manifestation of AHO[4].

The discovery of PTH receptor and adenylate cyclase (AC) signal transduction pathway made an important breakthrough in the understanding of the pathophysiology of PHP[2,5,6]. PTH receptor is a G protein-coupled receptor (GPCR), which stimulates the production of 3',5'-cyclic adenosine monophosphate (cAMP), thus the measurement of the serum and urinary levels of cAMP after injection of bovine parathyroid extract allowed for the differentiation between pseudohypoparathyroidism type 1 (PHP1) and pseudohypoparathyroidism type 2 (PHP2, OMIM%203330). The PHP1 shows a failure to increase urinary cAMP and urinary phosphate excretion in response to exogenous PTH administration. In contrast, PHP2 presents a normal nephrogenous cAMP generation with a decreased phosphate excretion, which indicates a defect downstream to AC[7].

To date, only a few cases of PHP2 have been reported and its subjacent molecular defect remains unknown[7,8]. The fact that treatment with calcium and vitamin D normalizes the phosphaturic response in some patients[9–12] suggests that PHP2 could be an acquired defect secondary to vitamin D deficiency. However, this hypothesis has not been confirmed yet and alternative diagnosis of the PTH-Parathyroid hormone-related protein (PTHrP)-protein kinase A (PKA) pathway has been included and subdivided within this category[13,14].

Similarly, PHP1, more deeply investigated than PHP2, has also been subdivided into different subtypes relying on clinical characteristics and molecular determinants. Thus, these disorders associated with GNAS locus can be a consequence of a genetic alteration as well as of an epigenetic alteration affecting the correct methylation pattern of the locus subjected to imprinting (better defined below). Consistent with the parent-of-origin specific expression of Gsα in some tissues, depending on the parental transmission of the alteration, they cause different manifestations named as PHP1A, PHP1B, PHP1C or PPHP[15]. *GNAS* loss-of-function mutations in the maternal allele are responsible for PHP1A, which is manifested as AHO phenotype with multihormonal resistance[1]. When the mutation is localized in the paternal allele, patients only manifest with the AHO phenotype and it is denominated PPHP[9,10]. If the alteration is produced by a defect in methylation pattern at GNAS locus, it causes PHP1B and, usually, patients only manifest PTH resistance without AHO phenotype[16,17]. Mentioned molecular defects, explain the 70% of PHP1 patients[18,19] (and personal data). Hence, this work will mainly focus on those patients with clinical diagnosis of PHP1, who did not present any of previously described molecular alterations.

First of all, to place PHP1 in context, GNAS locus will be briefly described as well as its implication in the pathophysiology of the disorders.

1. GNAS locus

GNAS locus maps to the telomeric end of the long arm of chromosome 20 (20q13.2-20q13.3) and it exemplifies a locus of such complexity. First because it

gives rise to multiple gene products; including transcripts that encode the alpha-subunit of the stimulatory guanine nucleotide-binding protein (Gsα), extra-large Gsα (XLαs), and neuroendocrine secretory protein 55 (NESP55)[20–23]. Two additional non-coding transcripts are also derived from GNAS: the A/B (also referred to as 1A or 1') (some evidence suggests that A/B could be translated[24]) and the GNAS antisense transcript (GNAS-AS)[25,26] (Figure 1.1.).

Gsα is encoded by exons 1–13 (*GNAS* gene) which give rise to four functional domains: guanosine triphosphate (GTP) hydrolase (GTPase) activity domain (exons 1 and 2), the adenylate cyclase activity domain (exons 4 and 5), the GTP-dependent conformational change domain (exon 9), and the G-protein coupled receptor interaction domain (exons 12 and 13)[27,28]. Furthermore, due to

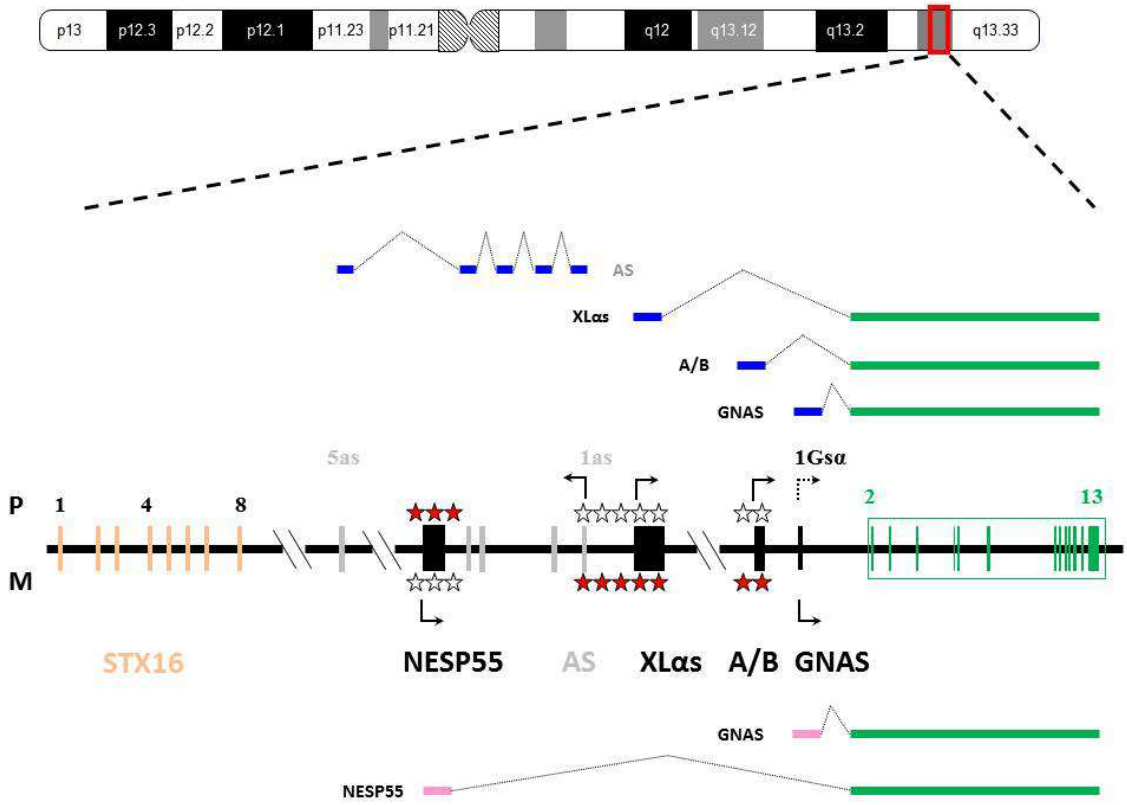

Figure 1.1. Organization and imprinting of the GNAS complex locus. The general organization and imprinting patterns of the paternal (above) and maternal (below) GNAS alleles are shown, with the exons of sense transcripts (NESP55, XL, A/B, and Gαs) depicted as black boxes, the common exons 2 to 13 represented as green boxes, the five exons of the antisense transcript (AS) represented as grey boxes and the eight exons of the *STX16* gene represented as orange boxes. The active sense and antisense promoters (arrows), as well as the splicing patterns of their respective paternal (blue) and maternal (pink) transcripts, are shown above and below the paternal and maternal exons, respectively. The dotted arrow for the paternal Gαs transcription indicates that the promoter is fully active in most tissues but is presumed to be silenced in some tissues, such as renal proximal tubules. Regions that are differentially methylated are represented as stars (red, methylated and white, unmethylated).

alternative pre-mRNA splicing, the Gsα transcript has several isoforms, such as, two long Gsα-L (Gsα-1 y Gsα-2) and two short Gsα-S (Gsα-3 y Gsα-4) forms (Figure 1.2.); they differ from the inclusion or exclusion of the exon 3, which gives rise to further 15 aminoacids[29,30]. The expression of both variants has been detected in nearly all tissues, as 52- and 45-kDa protein bands on Western blots, respectively[31]. In addition, Gsα-2 y Gsα-4 contain a serine residue encoded by a CAG tri-nucleotide at the start of exon 4[29,30]. Small, but potentially important, differences between the activity of each variant has been reported in several studies[31–39]. Finally, another variant termed Gsα-N1 was found out in the brain[40]. It is truncated in the C-terminus due to splicing of exon 3 (or exon 2 in the case of Gsα-S) onto a distinct exon between exons 3 and 4 containing an in-frame termination codon. Thus, it lacks most functional domains of Gsα, consequently it is probably unable to function in a similar way to the latter. Its cellular role and its biological significance remains unknown[39,40].

Regarding to the other transcripts encoded by GNAS locus, NESP55, XLαs, and A/B individually contain their unique first exon, each of them regulated by their own promoter. This first exons splice onto exon 2–13 of *GNAS* to give rise to different transcripts[20–23]. On the other hand, GNAS-AS transcript is derived from the antisense strand and traverses the promoter and the first exon of *NESP55*[25,41]. Each mentioned product presents complex transcriptional and splicing profiles carefully reviewed in some manuscripts[39,42,43] (Figure 1.1.).

Second feature which gives more complexity is the fact that GNAS locus has a parent-specific expression, since GNAS transcripts promoters are regulated by three differentially methylated regions (DMRs)[25,26,44–47] (Figure 1.1.). The DMR in which *NESP55* is immersed is established during the postfertilization and it is methylated in the paternal allele, thus it is expressed from maternal allele. While the DMR in which A/B is localized and the other one in which XLαs and GNAS-AS are immersed, are established during gametogenesis and are methylated in maternal allele, hence these transcripts are derived exclusively from the paternal non-methylated allele[25,26,39,42,43,45,48,49]. In contrast, Gsα promoter is not immersed in a cytosine-phosphate-guanine island (CpG island), accordingly it

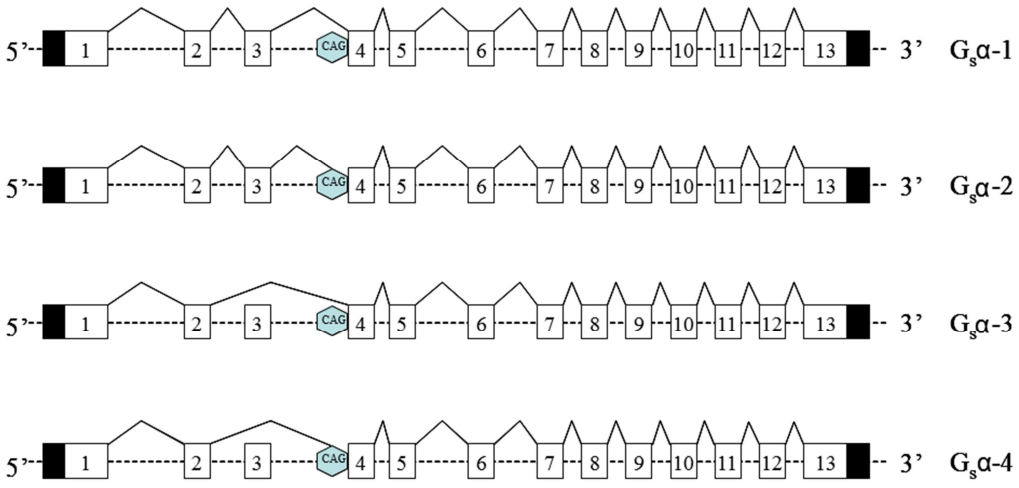

Figure 1.2. Gsα isoforms. Two long (Gsα-1 and Gsα-2) and two short (Gsα-3 and Gsα-4) forms of Gsα result from alternative splicing of exon 3. Use of an alternative splice acceptor site for exon 4 leads to insertion of an extra serine residue (CAG) in Gsα-2 and Gsα-4. Introns are represented as dash lines, exons as white boxes; UTRs as black boxes, triplet for serine residue as grey hexagons and splicing pattern as a solid line.

lacks methylation and it is biallelically active in most of tissues. Nevertheless, in some specific tissues including proximal renal tubule, neonatal brown adipose tissue (BAT), thyroid, gonads, paraventricular nucleus of the hypothalamus and pituitary, the Gsα expression is predominantly maternal[50–56]. Indeed, some studies have suggested that also in the tissues with biallelic expression, the maternal allele expression is moderately increased[50,57,58]. This characteristic plays an important role in the development of phenotypes associated with *GNAS* mutations, but underlying mechanisms of the tissue-specific paternal silenced expression of Gsα are poorly defined[48,50–52,54,57,59,60].

2. PHP pathophysiology and multihormonal resistance

In the preceding section, in addition to Gsα, other transcripts originated from GNAS locus have been mentioned. Some implications of these GNAS products in PHP/PPHP have been elucidated, but they remain poorly defined[61]. Therefore, in this section, Gsα role will be more deeply explained.

Gsα is a heterotrimeric G protein subunit and couples to diverse receptors known as GPCR. Generally, when the ligand binds to the heptahelical transmembrane receptor, such as the receptor for PTH/PTHrP (PTHR1), it induces

a GDP-GTP exchange, leading to the dissociation of Gsα from Gβγ subunits. The GTP-bound Gsα is able to stimulate different effectors and thereby initiates various responses depending on the cellular context. The best-known effector stimulated by Gsα is adenylyl cyclase, which catalyses the conversion of adenosine triphosphate (ATP) into the second messenger cAMP. Gsα is a GTP hydrolase and this activity ensures that the activation of Gsα is short-lived by converting the latter back into its GDP-bound state, which then reassembles with Gβγ and becomes inactive until the cycle is reinitiated[62].

Gsα is widely expressed and, apart from PTH, there are some more hormones acting through GPCR receptors, such as, thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), growth hormone releasing hormone (GHRH), antidiuretic hormone (ADH), glucagon, adrenocorticotropic hormone (ACTH) or calcitonin[63,64]. Accordingly, even if the mostly affected signalling is the one that implicates the PTH/PTHrP receptor[65], the resistance is not restricted to PTH hormone, but extends to several hormones when an alteration occurred in the maternal GNAS locus. Moreover, some neurosensory functions and mental disturbances might be attributable to the same mechanism[48,64]. This diversity of multihormonal resistance contributes to the various phenotypes of the disease. PTH and TSH resistance are implicated in almost all types of PHP1, but the severity of the resistances are heterogenic depending on the disorder[65]. In fact, the deficient endochondral bone formation that results from the PTHrP (which manifested as AHO)[19,65], the epinephrine, calcitonin, the gonadotropin and the GHRH resistance occur less frequently[65–68]. Very frequently, hypothyroidism is one of the first clinical manifestations of PHP1, it can be appreciated even before the hypocalcaemia and very often, it is already presented at birth (it is easily appreciated by neonatal screening programs)[65]. However, the resistance to PTH is the most typical and evident alteration of PHP1.

2.1. PTH resistance

As mentioned before, the classical feature of PHP1, is a failure to increase urinary cAMP and urinary phosphate excretion in response to exogenous PTH

administration[69]. Briefly, in normal conditions, when low serum calcium is detected by the parathyroid glands, these promote the production of PTH. As explained before, PTH acts through the PTH1R receptor, abundantly expressed in kidney and bone, and it activates the PTH1R–Gsα–cAMP–PKA signalling pathway[70]. Through this pathway, PTH promotes the homeostasis of serum calcium in different regulation points: (a) it stimulates osteoclast activity in the bone; consequently, osteoclasts dissolve bone, then calcium and phosphate move into the serum[71]. (b) PTH also increases calcium reabsorption and phosphate excretion (to prevent the increase of phosphate in serum) in the kidneys[72]. (c) In addition, it promotes the conversion of 25-hydroxyvitamin D (inactive form) to 1,25-dihydroxyvitamin D3 (calcitriol, active form) by the activation of 1-alpha-hydroxylase enzyme in proximal tubules, hence this increment in the levels of active vitamin D produces the increase of reabsorption of calcium and phosphate in the small intestine[72]. Bone resorption added to renal and intestinal reabsorption results in the upturn of serum calcium levels that inhibit further production of PTH via negative feedback.

Therefore, the PTH resistance is manifested as hypocalcaemia, hyperphosphatemia and elevated serum levels of PTH, although the hyperphosphatemia and elevated serum levels of PTH frequently appear before hypocalcemia[73] and even some PHP1A patients maintain normocalcaemic for life[11]. PTH resistance normally appears latter than TSH resistance, in early childhood[65,73]

As note, the resistances to hormones that bind to a Gsα-coupled heptahelical transmembrane receptor are not always consequence of Gsα alterations; alterations in other protein that affect the transduction of the signal through Gsα–cAMP–PKA signalling pathway can be the responsible of this defect[65]. That is the case of acrodysostosis type 1 (ACRDYS1) with PTH resistance associated with mutations in the cAMP-dependent protein kinase type 1 regulatory subunit (PRKAR1A)[13]. This will be explained later at the end of the introduction section.

3. Molecular alterations affecting GNAS locus

As mentioned, disorders associated with GNAS locus can be consequence of a genetic alteration as well as of an epigenetic defect affecting the correct methylation pattern. In addition, consistent with the parent-of-origin specific expression of Gsα in some tissues, several inherited disorders have been associated to this locus, depending on the parental transmission of the mutation.

3.1. Mutations in Gsα-coding GNAS exons

Germline as well as somatic alterations, and activating either inactivating alterations affecting the Gsα-coding sequence have been reported in the literature, and each of them produces different disorders.

3.1.1. Disorders associated to somatic activating mutations in Gsα-coding exons

Two main diseases associated with somatic activating mutations in Gsα have been described: McCune-Albright syndrome and endocrine and non-endocrine tumours. In both cases a constitutive activation of Gsα is detected[74]. In McCune-Albright syndrome, patients are mosaics for missense mutations in Arg201 or Gln227 residues[75–77], possibly acquired during early embryonic development[78]. These residues play an important role because they affect to the hydrolase activity of the protein, causing a prolonged existence of Gsα in its active GTP-bound state[74]. On the other hand, apparently, Gsα protein can also act as a "gsp oncogene" giving rise to endocrine and non-endocrine tumours[74]. It remains to be determined whether the gsp is a driver mutation in these tumours.

3.1.2. Germline inactivating mutations in Gsα-coding exons

To date, over 200 inactivating mutations have been reported through all *GNAS* gene, but proportionally more mutations have been described within exon 1. Generally, the mutations are specific of the family, but a hot-spot has been identified in exon 7, which consisted of a deletion of four nucleotides (c.565_568delGACT)[79,80] (www.lovd.nl/GNAS).

All type of mutations have been identified: missense, nonsense, insertions, inversions, intragenic deletions and deletions comprising a part of or all the long arm of chromosome 20[79,80] (www.lovd.nl/GNAS).

About the origin of the mutations, *de novo* as well as inherited mutations has been reported but due to the parent-of-origin specific expression of Gsα in some tissues, these mutations lead to different clinical manifestations. As previously mentioned, mutations in the maternal allele are associated with PHP1A/PHP1C (PTH resistance and AHO phenotype), while mutations in the paternal allele result in AHO phenotype without hormone resistance (PPHP).

Most of mutations in Gsα are not expressed because they lead to nonsense-mediated messenger ribonucleic acid (mRNA) decay (NMD) or because the subcellular localization is disrupted. Consequently, approximately a 50% reduction of Gsα activity is detected in the erythrocytes. However, this reduction can be only appreciated when the mutation affect the ability of the protein to stimulate the adenylate cyclase (PHP1A). If the mutation impairs the receptor coupling, a 100% of Gsα activity will be detected (PHP1C)[19,81].

3.1.2.1. Manifestations associated to inactivating mutations in Gsα-coding exons

PHP1A vs. PHP1C

Consistent with the parent-of-origin specific expression of Gsα in some tissues, patients with heterozygous inactivating mutations within Gsα-encoding *GNAS* exons located in the maternal allele, apart from AHO phenotype, also show multihormonal resistance; this disorder is known as pseudohypoparathyroidism type 1A (PHP1A)[1]. The failure to increase urinary cAMP and urinary phosphate excretion after exogenous PTH administration in PHP1A reflects PTH resistance at renal proximal tubules (Table 1.1.). As aforementioned, the paternal allele is silenced in some tissues, such as renal proximal tubules, thus the inactivation of the maternal allele produces a dramatic reduction in Gsα expression/activity, resulting in an impaired PTH action in this organ. As the expression is biallelic in most tissues, the

haploinsufficiency of Gsα in lymphocytes, adrenal, white adipose tissue, bone, and growth plate chondrocytes is thought to be the responsible of AHO phenotype[50,82,83].

The 50% reduction of Gsα activity in patients with inactivating *GNAS* mutations can be utilized as a diagnostic test. This test is not extensively used in practice, but it is necessary if the subtype of PHP1 want to be established[84,85]. In PHP1C subtype, Gsα normal activity is detected by the test in patient-derived erythrocytes, since these mutants are able to stimulate adenylyl cyclase but are defective in receptor coupling (Table 1.1.). In these cases, mutations are located near the C-terminal end of the Gsα molecule, hence this finding confirmed the importance of this region in receptor interactions[19,81]. Generally, a genotype-phenotype correlation does not exist in diseases caused by inactivating *GNAS* mutations, but some special cases have been described. For example, Ala366Ser mutation causes testotoxicosis due to constitutively Gsα activation at lower temperature, although it shows instability at body temperature causing the haploinsufficiency[86,87]. In addition, transient neonatal diarrhoea has been reported in two siblings who harboured a mutation leading to an AVDT amino acid repeat. This mutation produces the instability of mutant Gsα, contrary, in the gut it shows enhanced constitutive activity producing enhanced cAMP signalling[88].

PPHP vs. PHP1A

As shown, maternally inherited inactivating mutations in *GNAS* exons 1–13 lead to PHP1A, whereas paternal inheritance of the same mutations leads to PPHP[9,10]. Theoretically, the Gsα expression/activity keeps normal in tissues with maternal expression, but the haploinsufficiency is still present in biallelic expression tissues, resulting in AHO phenotype (Table 1.1.). However, evidence for subclinical PTH resistance, TSH resistance, and subnormal growth hormone (GH) stimulation response have been detected in few cases with paternally inherited Gsα-coding *GNAS* mutations[84,89–93]. The reason for these exceptions remains unclear. PHP1A and PPHP are frequently show in the same kindred, but never in the same sibship[9,10].

On the other hand, some differences have been appreciated in the manifestation of AHO phenotype concerning to parental origin of the Gsα mutations; obesity and cognitive impairment occur predominantly in patients with PHP1A, rather than PPHP[94,95]. The difference in these manifestations are consistent with the paternal silencing of the Gsα in some parts of the brain[55]. In addition, PPHP patients usually present lower birth weights (or intrauterine growth restriction, IUGR) than PHP1A ones and patients carrying mutation in exon 2 to 13 are even smaller than those with exon 1 defects[90,96,97]. Looking at the fact that exons 2–13 of *GNAS* are shared by XLαs, NESP55 and A/B transcript, it could be considered that mutations in those exons lead to deficiency of not only Gsα but also of these other products. In addition, studies in mice have demonstrated that the paternal ablation of XLαs results in low birth weight[98,99]. In contrast, bone dysplasia and the development of subcutaneous ossifications observed in AHO phenotype seems to be similar even if the mutation has a maternal or paternal origin. Heterozygous ablation of Gsα in mouse growth plate chondrocytes results in accelerated differentiation into hypertrophy regardless of the parental origin of this genetic manipulation[83]. In the same way, mice with ablation of *Gnas* exon 1 develop subcutaneous ossifications in spite of whether the disrupted allele is inherited from the mother or the father[100].

POH vs. PPHP

Progressive osseous heteroplasia (POH) is a rare manifestation of AHO characterized by severe heterotopic ossifications, which, unlike those typically observed in AHO patients, are progressive and affect deep connective tissue and skeletal muscle[101]. POH has a mild manifestation during infancy, it presents with dermal and subcutaneous ossifications and latterly it becomes more invasive during childhood. AHO phenotype and also hormone resistance are absent in most cases[96,101–103]. In agreement with this, generally, paternal germline heterozygous inactivating mutations in Gsα, have been detected in patients with POH[104]. Curiously, the same *GNAS* mutations have been identified in patients with POH, PHP1A, or PPHP, but the reason why some patients develop POH is unclear[101,103–105].

Table 1.1. Classification of PHP disorders according to the presence/absence of hormonal resistance and the AHO phenotype. *The mutation effect is not detected by the test, because the mutations in this subtype cause a defect in receptor coupling, but they are still able to stimulate.

TYPE	SERUM PTH	AHO	CALCEMIA	PHOSPHATEMIA	PTH EXOGENOUS ADMISTRATION		$G_s\alpha$ ACTIVITY	HORMONAL RESISTANCE	GNAS ALTERATION
					URINARY cAMP RESPONSE	PHOSPHATURIA			
PHP1A	⇧	YES	⇩	⇧	⇩	⇩	⇩	PTH/TSH/Gn	MATERNAL MUTATION
PHP1B	⇧	NO/mild	⇩	⇧	⇩	⇩	⇩	PTH/TSH	IMPRINTING
PHP1C	⇧	YES	⇩	⇧	⇩	⇩	NORMAL*	PTH/TSH/Gn	MATERNAL MUTATION
PHP2	⇧	NO	⇩	⇧	NORMAL	⇩	NORMAL	PTH	?
PPHP	NORMAL	YES	NORMAL	NORMAL	NORMAL	NORMAL	⇩	NO	PATERNAL MUTATION

3.2. Disorders associated with alterations in the imprinting of GNAS locus, PHP1B

Classically, when the hormone resistance is confined to the renal proximal tubular action of PTH, without AHO phenotype (Table 1.1.), the disorder is denominated pseudohypoparathyroidism type 1B (PHP1B)[16,17]. However, some patients show mild resistance to TSH[54,67], also some mild features of AHO[106–110] and a slightly diminished Gsα activity[111].

Patients with PHP1B lack coding Gsα mutations but display epigenetic defects of the GNAS locus; including loss of methylation (LOM) at the A/B, GNAS-AS, and XLαs DMRs and a gain of methylation at the NESP55 DMR[17,112]. These methylation changes can be complete or partial, and involve some or all of the DMRs. However, the DMR comprising the promoter and the first exon of the A/B transcript is always affected. A/B DMR probably regulates negatively the Gsα tissue-specific expression imprinting. Consequently, when the A/B is hypomethylated, the increased expression of this transcript presumably leads to a decreased Gsα expression in tissues where this gene is predominantly transcribed from the maternal allele[11].

Autosomal Dominant PHP1B

Frequently an isolated LOM at the A/B DMR is presented in the familial form of PHP1B, which is maternally transmitted in an autosomal dominant

manner (AD-PHP1B)[17,112], and it is typically associated with maternal deletions at the syntaxin 16 gene (*STX16*). The 3-kb deletion[113–116] is the most frequent but a 4,4-kb[117] and a 24,6-kb[118] deletions have also been reported. Interestingly, in a single kindred with an isolated LOM of A/B DMR, a 19-kb deletion was reported outside this region as it removed NESP55 and the upstream genomic region[119]. This suggests that apart from STX16 imprinting control element there is another one within the region deleted in this last family[48].

In addition, other maternally inherited microdeletions that comprise NESP55 and GNAS-AS have been identified, causing AD-PHP1B with methylation defects in multiple GNAS DMRs[64,120–122]. Neither *STX16* deletions nor NESP55 deletions yield any methylation changes following paternal transmission, but curiously, when the described deletion encompassing GNAS-AS exon 3-4 but not NESP55 is paternally inherited, results in gain of methylation in A/B DMR and partial LOM in NESP55[121]. Consequently, Gsα expression showed an increase in this patient, instead of a decrease, as in those patients who inherited the deletion from the mother. Therefore, it is likely the existence of a *cis*-acting element regulating imprinting of both parental alleles in this region[48].

Sporadic PHP1B

Most PHP1B cases are sporadic (spor-PHP1B) and they show imprinting abnormalities of GNAS that involve multiple DMRs, however in these cases no genetic lesion responsible for these imprinting abnormalities has been discovered[115]. These methylation defects, probably happen stochastically or are induced by environmental factors during pre- or post-implantation, as occur in other imprinting disorders[123,124].

In some spor-PHP1B patients paternal 20q disomy [UPD(20q)pat] has been described[125–129]. In addition, a possible autosomal recessive inheritance form has been proposed in five families[130].

4. PHP1 and AHO phenotype

As introduced previously, AHO phenotype is a group of physical features classically associated to PHP1 with a variable expression. These features (accompanied or not by multihormonal resistance) are mainly: brachydactyly, short stature (height below the third percentile for chronological age), obesity (body mass index > 30 kg/m2 in adults and >97th centile in children), round face, subcutaneous ossifications, and mental retardation[109].

Brachydactyly type E (BDE) is the most specific feature of AHO together with subcutaneous ossifications. IV and V metacarpals and I distal phalanx are the most commonly shortened bones but a wide variability exits within this feature[131–133].

Subcutaneous and intracranial calcifications (or Fahr disease) are rare clinical symptoms, so these could be good indicators of impaired calcium homeostasis[134]. Subcutaneous calcifications or ossifications are frequently appreciated in physical examination of AHO patients and the number and spread are variable[73,135,136]. As annotated before, in patients with POH; these ossifications progress to deep tissues such as the muscle[101]. When calcifications appear on the posterior longitudinal ligament of the spinal, can rarely cause spinal cord compression symptoms[137]. On the other hand, intracranial calcifications (preferentially localized on basal ganglia and cerebellum), are shown in the 50% of the patients with PHP1[138–140] and PPHP[141]. Intracranial calcifications can cause several complications such as neurocognitive and psychiatric alterations, extrapyramidal symptoms as parkinsonism, choreoathetosis and dyskinesia, walking problems (cerebellar ataxia) and also in the speech, with variable severity[140,142].

Obesity and short stature are also typical features of AHO. As mentioned before, obesity seems to be more specifically associated with PHP1A than with AHO[95,143]. Some studies in mice suggest that the obesity is a consequence, in part, of the Gsα impairment in the hypothalamus hunger-satiety centre. Gsα is also imprinted in the paraventricular nucleus of the hypothalamus, so maternal

mutations in Gsα affect to the pathway mediated by melanocortin (acts through GPCR receptors) to stimulate energy expenditure, but not affect to the food intake[55]. Moreover, short stature is an effect of the premature closure of the growth plate. This premature closure is a consequence of Gsα haploinsufficiency in PTH/PTHrP signalling pathway in the chondrocytes[83,144,145] in addition to GHRH resistance[63]. Generally the short stature is more appreciated in adulthood; affected children show normal stature until the age of 10-15 years, moment when the growth velocity stops prematurely, resulting in small stature[95,146].

Finally, mental retardation has been classically included as an AHO feature[147], but some studies suggest that the cognitive impairment is more restricted to PHP1A, whilst patients with PPHP typically have normal intelligence[94]. It is proposed that as happens with obesity, the cognitive impairment is more marked in these patients because of the fact of the imprinting of Gsα in certain brain regions. As remark, no PHP1B patients present cognitive impairment[94]. Further studies implementing standardized tests are required in wider populations of patients to better define the spectrum of cognitive function in PHP1/PPHP.

4.1. PHP misdiagnosis

PHP is a rare genetic disease with a high rate of misdiagnosis in initial diagnosis, especially in cases in which there is no family history[14,148] (and personal data). As mentioned before, the patients may show AHO phenotype, but its manifestations are variable. Some of them present complete phenotype, while others present only subtle manifestations[149]. In addition, AHO phenotype features are not specific, so frequently a similar phenotype is also present in other syndromes. In fact, AHO-like syndrome includes a group of patients who show several features of AHO but with normal Gsα levels and do not manifest any endocrine abnormality compatible with PHP[150]. As note, the BDE and mental retardation are the most remarkable features of this syndrome, giving the name of brachydactyly and mental retardation syndrome (BDMR, OMIM#600430). These patients frequently present deletions of 2q37 chromosome or mutation in the gene coding for histone deacetylase 4 (*HDAC4*), localized in this locus[150,151].

In addition, biochemical alterations (hypocalcaemia and hyperphosphatemia) shown in PHP are similarly presented in other syndromes associated to calcium homeostasis, like hypoparathyroidism[152]. PTH resistance as well as brachydactyly (but in a more severe form) are also present in acrodysostosis with multihormonal resistance ACRDYS1 (OMIM#101800 or ADOHR), associated with mutation in *PRKAR1A* gene[13]. Another type of acrodysostosis exists (ACRDYS2, OMIM#6146139), caused by mutation in the gene coding for phosphodiesterase 4D (*PDE4D*), in contrast this type appears not to show hormone resistance[153,154].

As stated before, brachydactyly is the most widely recognized feature of AHO, so in order to make a correct differential diagnosis, other conditions with BDE or similar types should be consider; such as, (i) acrodysostosis associated with mutation in the gene *PRKAR1A*[13] or *PDE4D*[153,154] (ii) isolated BDE in which the gene for homeobox D13 (*HOXD13,* OMIM#113300) has been often implicated[155,156]; (iii) brachydactyly type E with short stature, PTHLH type (OMIM#613382), caused by mutation in the gene coding for parathyroid hormone-related protein (*PTHLH*)[157–159]; (iv) hypertension with brachydactyly syndrome (OMIM #112410), in which very recently the responsible gene phosphodiesterase 3A, (*PDE3A*) has been identified[160]; (v) tricho-rhino-phalangeal syndrome type I and III (TRPS-I, OMIM#190350; TRPS-III, OMIM#190351), caused by mutations in the gene for zinc finger transcription factor TRPS1 (*TRPS1*)[161] or the more severe form, type II (OMIM#150230), which is a contiguous gene syndrome on 8q24.1, involving loss-of-functional copies of the *TRPS1* and *EXT1* genes[162]; (vi) brachydactyly mental retardation syndrome (OMIM#600430) associated with mutations in the gene encoding histone deacetylase 4 gene (*HDAC4*) or deletions in 2q37[151]. Finally, even if Turner syndrome chromosomal disorder (frequently, 45,X) is a quite well known entity, patients also show BDE[131] and short stature, thus these could give rise to some misdiagnosis.

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2. HYPOTHESIS AND OBJECTIVES

1. HYPOTHESIS

PHP is a rare disease whose phenotypic features are rather challenging to appreciate in some cases. In fact, the complex clinical characterization of PHP syndrome often leads to a misdiagnosis of the pathology. The confusion of this syndrome with other pathologies could have important implications in the treatment, the follow up and proper genetic counselling.

Therefore, the hypothesis of this thesis is that some patients with a clinical diagnosis of PHP but no genetic or epigenetic abnormalities in the GNAS locus, actually suffer another syndrome that can be clinically confused with PHP.

2. OBJECTIVES

The main objectives of this thesis are:

1. To perform a literature review comparing clinical and radiological characteristics of PHP and related disorders to facilitate the genetic diagnosis, correct clinical approach and proper genetic counselling.

2. To elaborate an algorithm to guide the correct clinical diagnosis of patients with syndromes associated with BDE and others features present in AHO phenotype, which facilitates the correct classification of the patients and the genetic diagnosis of the most probable candidate gene in each case.

3. To search for mutations in other candidate genes, based on the pattern of brachydactyly and the other features shown in patients in whom GNAS abnormalities had been previously ruled out.

4. To identify the specific and distinctive features of each disorder.

5. To characterise the functional effect of the novel genetic alterations identified.

3. PATIENTS

In this project patients referred to the Molecular (Epi)Genetics Laboratory at Hospital Universitario Araba for molecular diagnosis, with a clinical suspicious of PHP1A or AHO phenotype, were included. First, GNAS locus abnormalities were studied. After that, patients with negative result for GNAS locus abnormalities underwent a candidate gene approach. Therefore, their clinical features were reviewed to classify them according to the brachydactyly pattern and other clinical features.

The established inclusion criteria were:

- Clinical suspicious of AHO phenotype with or without PTH resistance.
- Presence of brachydactyly type E or similar pattern.

The exclusion criteria were:

- Epigenetic defects at GNAS locus (these patients were classified as PHP1B and were included in a separated research project).
- PTH resistance with decreased levels of vitamin D, without other characteristics of AHO phenotype. In this case, PTH resistance was supposed to be an acquired defect secondary to vitamin D deficiency.

In total 53 families were included in the study.

The project was approved by CEIC-E (2010-021; PI2013124). Patients were informed about this study and informed consent was obtained from all patients and relatives included in the study. Clinical history of the patients, including hand and feet radiographies and clinical photos (in case of availability), was requested to the physicians who referred the samples for the genetic study.

4. METHODOLOGY

The methodologies performed throughout this thesis are briefly detailed in each publication. This section aims to describe the commonly used techniques in more detail.

1. Nucleic acid extraction

Deoxyribonucleic acid (DNA) or Ribonucleic acid (RNA) was extracted from peripheral blood with QIAamp DNA Mini Kit and PAXgene Blood RNA Kit (Qiagen, Düren, Germany) by the robotic workstation QIAcube (Qiagen). Nucleic acids extraction involves the rupture of plasmatic membrane and nuclear envelope for explosion of the nucleic acids followed by isolation and purification. Moreover, RNA extraction requires the elimination of DNA with deoxyribonucleases (DNAses) to avoid its interference in the experiments.

2. Analysis of point mutations

2.1. Polymerase Chain Reaction (PCR)

PCR consists on an *in vitro* enzymatic amplification of a specific DNA fragment defined by oligonucleotides or primers. It allows amplifying a single or few copies of a sequence of DNA across several orders of magnitude to generate thousands to millions of copies of this fragment. For this reaction: template (DNA), buffer, Mg^{2+} (cofactor of DNA polymerase), nucleotides triphosphates (dNTPs), primers and DNA polymerase are needed. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting, primers hybridization and enzymatic replication of the DNA by DNA polymerase. Primers are oligonucleotides complementary to a target region along genomic DNA, and the ones that provide DNA polymerase with the free 3'-hydroxyl group already base-paired to the template, necessary to initiate the elongation reaction. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified[1].

PCR was performed to amplify the intron/exon(s) of the gene(s) implicated in each pathology. Normally only coding exons were amplified but in some cases 5'- and 3'-untranslated regions were also analysed. The specific primers sequences and conditions for each PCR were dependent of the study, so this information is described or referenced individually in each publication. All of primers had a universal M13 oligonucleotide tag on the 5' of the forward and reverse PCR primers (Forward M13 tail 5'-TGTAAAACGACGGCCAGT-3' and 5'-3' Reverse M13 tail 5'-CAGGAACACGCTATGACC-3'). These universal primers were used in the sequencing reactions, in order to remove the need for individual primers at the sequencing stage.

2.2. Allele-specific PCR

It is a variant of conventional PCR. In this case, at least one of the primers is chosen from a polymorphic area to specifically amplify only one of the both alleles. Under stringent conditions, a mismatched primer will not initiate replication, whereas a matched primer will[2]. The presence of an amplification product therefore indicates the genotype linked only to this allele. In publication 1 this technique was carried out in order to elucidate the parental allelic origin (maternal or paternal) when *de novo* mutation.

2.3. PCR products purification

Before sequencing reaction, PCR products should be clean-up to remove remaining single-stranded primers and dNTPs. For this, ExoSAP-IT® (Affimetrix USB, Ohio, EEUU) ready reagent was used, which contains Exonuclease I, for degradation of single stranded primers and Shrimp Alkaline Phosphatase, to remove unwanted dNTPs[3].

2.4. Dye-terminator Sequencing

Direct sequencing is based on Sanger dideoxy sequencing. This process takes advantage of the ability of DNA polymerase to incorporate 2', 3'-dideoxynucleotides (nucleotide base analogous that lack the 3'-hydroxyl group essential in phosphodiester bond formation) apart from nucleotides[4]. Direct sequencing requires a DNA template, a sequencing primer (forward or reverse),

DNA polymerase, nucleotides (dNTPs), dideoxynucleotides (ddNTPs), and reaction buffer. For this purpose BigDye® Terminator v3.1 Cycle Sequencing ready reaction kit (Applied Biosystems, California, EE.UU) was used, which contain DNA polymerase, dNTPs, ddNTPs labelled with different fluorescent dyes, and buffer. Moreover, before mentioned M13 universal primers were utilized (forward and reverse in separated reaction for each template) which hybrid with the complementary M13 tag added in PCR primers. The reaction of sequencing consist on a thermal cycling protocol with these steps: an initial 60 s (denaturation) at 96°C; followed by 25 cycles of 96°C (denaturation) for 10 s; 50°C (hybridization of primers) for 5 s; and 60°C (elongation) for 75 s.

After that, extension products were purified by ethanol/EDTA/sodium acetate precipitation following manufacturer recommended protocol (BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol, Part Number 4337035 Rev. B, Chapter 4, page 4.7-4.8; California, EE.UU). Purified products were then separated by capillary electrophoresis in an ABI3500 Genetic Analyzer (Applied Biosystems). Extension products enter in capillary as a result of electrokinetic injection. A high voltage charge applied to the buffered sequencing reaction forces the negatively charged fragments into the capillaries. The speed at which a DNA fragment moves through the medium is inversely proportional to its molecular weight. This process of electrophoresis can separate the extension products by size at a resolution of one base. Before reaching the positive electrode, the laser beam causes the dyes on the fragments to fluoresce, thus, an optical detection device on genetic analyser detects the fluorescence, and the data collection software converts the fluorescence signal into digital data. This software gives an electropherogram, a sequence of peaks in four colours and each colour represents the base called for that peak. Electropherograms were analysed by Sequencing Analysis Software (Applied Biosystems) and mutations/single nucleotide polymorphisms (SNPs) were detected by Variant Reporter™ software (Applied Biosystems).

2.5. *In silico* analysis tools

The potential pathogenic effect of all novel missense mutations was analysed *in silico*, using SIFT[5] (http://sift.jcvi.org/www/SIFT_enst_submit.html) and PolyPhen-2[6] (http://genetics.bwh.harvard.edu/pph2/) software packages, which are able to predict whether an amino acid substitution may affect protein function, based on sequence homology and physical properties of substituted amino acids. Novel mutations that could affect mRNA splicing were analysed *in silico* using Human Splice Finder v2.4.1 online tool[7] (http://www.umd.be/HSF/#). This tool calculates the consensus values of potential splice sites and search for branch points, based on algorithms.

3. Study of structural alterations: deletions and duplications

3.1. Multiplex Ligation-dependent Probe Amplification (MLPA) and Methylation-specific MLPA (MS-MLPA)

In those patients without point mutations or small insertions/deletions identified by direct sequencing, MLPA or MS-MLPA (in the case of the studied gene was subjected to genomic imprinting) was carried out for gene dosage analysis or detection of epigenetic alterations, respectively (MRC-Holland, Amsterdam, Netherlands). This technique is based on the semi-quantitative polymerase chain reaction principle and can be applied for detecting copy number changes and methylation quantification of the genomic DNA, of up to 40 sequences in a single reaction[8]. This methodology utilizes a series of synthetic oligonucleotide probes designed in pairs. Pairs of probes are composed by different fragments: the hybridization sequences, which hybridise one immediately adjacent to its pair; universal primers sequences, for the amplification of probes; and stuffer sequence, only present in one probe of the pair to achieve a specific size to each pair. Briefly, genomic DNA is employed as a template. Like in the PCR, the reaction mix is exposed to a thermal cycling. First, it is heated for denaturation (98ºC), when the temperature is lowered (25ºC),

probes are added to hybridise adjacent to each other on their target DNA sequence (16–20 hours at 60°C). After, ligation reaction is carried out to ligate immediately adjacent probes in order to have a single probe. During the subsequent PCR reaction, all ligated probes are amplified simultaneously using the same PCR primer pair. One PCR primer is fluorescently labelled, enabling the amplification products to be visualized during fragment separation. This was performed by capillary electrophoresis in an ABI3500 Genetic Analyzer. The genetic analyser gives an electropherogram composed by peaks of different sizes corresponding to each pair of probe. The relative height of each individual probe peak, is compared to the relative probe peak height in three reference DNA samples, this reflects the relative copy number of the corresponding target sequence in the sample. Thus, a deletion of one or more target sequence becomes apparent as a relative decrease in peak height while an increase in relative peak height reflects a copy number gain. Electropherograms were visualised by GeneMapper software (Applied Biosystems) and analysed in homemade Excel worksheet templates.

The MS-MLPA protocol is very similar, except that after the hybridisation step the reaction mix is split into two samples: one undigested sample for copy number detection and one digested sample for methylation detection. The first sample is processed as a standard MLPA. In the second sample, apart from the ligase, a methylation-sensitive endonuclease (*HhaI*) is added which digests the template when it is not methylated[9], so the pair of probes will neither ligated nor amplified, hence will not produce a signal during capillary electrophoresis. The electropherograms are analysed similarly, but in this case, relative decrease in methylation specific peak height reveals a loss of methylation in the target sequence; on the contrary, if an increase is detected, reflects a gain of methylation.

3.2. Microsatellite analysis

Microsatellites are highly polymorphic tandem repeating units of 1-6 base pairs in length. They are typically co-dominant and are used as molecular markers[10]. Throughout this thesis, microsatellite markers were utilized to

confirm family relationships or uniparental disomy (specific details are provided in each publication). Chromosome specific microsatellites information (location, size, primers) were taken from previously published papers or by genome browsers like Ensembl (http://www.ensembl.org) or USCS (http://genome.ucsc.edu/). Microsatellites were amplified under standard PCR condition with an extra elongation step of one hour at 60ºC at the end. Some thermostable DNA polymerases, including Taq, add a single nucleotide base extension to the 3' end of amplified DNA fragments. These polymerases usually add a deoxiadenosine (dATP), leaving an "A" overhang. So this extra step permits DNA polymerase to add an extra dATP to all amplified DNA fragments to avoid errors in the interpretation of microsatellite size[11]. The primers were tagged with a 5' fluorescent dye that was detected by capillary electrophoresis in ABI3500 Genetic Analyzer (Applied Biosystems) and analysed by GeneMapper software (Applied Biosystems).

4. Characterization of the pathogenic effect of novel mutations

4.1. Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction is based on the ability of reverse transcriptase enzyme to create a complementary DNA (cDNA) transcripts using mRNA as template. It was used to qualitatively detect gene expression of a target mRNA. In this case, particularly, One-Step RT-PCR kit (Qiagen, Düren, Germany) was applied using specific primers to amplify only the target mRNA and then sequence to confirm or discard the expression of the mutant allele (publication 7).

4.2. Functional studies

In publication 5, transient transfection of CHO and HEK293 cells was used to compare the activity of PDE4D3 mutants against WT. CHO (Chinese Hamster Ovary) cells belong to a cell line derived from the ovary of the Chinese

hamster[12,13] and HEK293 are a specific cell line originally derived from human embryonic kidney[14].

4.2.1. E. coli transformation for plasmid production and purification

Plasmids are replicons, a unit of DNA capable of replicating autonomously within a suitable host[15]. In publication 5 pcDNA™3.1 plasmid was used to express the cDNA encoding the cAMP-specific human phosphodiesterase PDE4D3 in CHO and HEK293 cells, as it is designed for high-level stable and transient expression in mammalian hosts. High-level stable and non-replicative transient expression can be carried out in most mammalian cells. The vectors contain the following elements: human cytomegalovirus immediate-early (CMV) promoter, for high-level expression of recombinant protein in a wide range of mammalian cells; multiple cloning sites in the forward (+) and reverse (–) orientations, to facilitate cloning of the gene which want to be expressed; pUC origin, to high-copy number replication and growth in *E. coli;* ampicillin resistance gene, to selection of vector in *E. coli,* etc.

Transformation is the process by which foreign DNA is introduced into a prokaryotic cell[16]. This permits the storing and replicating of plasmids. There are different ways to make competent the bacteria; in this case, One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, California, EE.UU) were used in which a calcium chloride solution and heat shock method is applied to make them competent. The addition of $CaCl_2$ serves to neutralize the unfavourable interactions between the negative charge of DNA and the polyanions of the membrane. The DNA and competent cells are further incubated on ice to stabilize the lipid membrane and allow for increased interactions between calcium ions and the negative components of the cell. The reaction mixture is then exposed to a brief period of heat-shock at 42ºC. The change in temperature alters the fluidity of the semi-crystalline membrane state achieved at 0ºC thus allowing the DNA molecule to enter the cell through the zone of adhesion[16]. Subsequently, the mixture of treated cells is cultured on media that contain the antibiotic so that only transformed cells are able to grow. After 12 h of

incubation in agar plate, an isolated colony is picked and inoculated in Lysogeny Broth (LB) broth medium to induce the replication of bacteria and thus increase the amount of plasmid. Finally, 12 h after the incubation in LB, the purification of the plasmid is performed. After the freezing of the bacteria precipitates, in this case, QIAGEN Plasmid Maxi Kit was used (Qiagen) to perform the purification. This kit provides gravity-flow, anion-exchange tips for isolation and washing of plasmid and then it is purified by precipitation with isopropanol and ethanol.

4.2.2. Cell culture and transfection

Mammalian cell transfection is a technique commonly utilized to express exogenous DNA or RNA in a host cell line[17]. There are many different ways to transfect mammalian cells, depending on the cell line characteristics, desired effect, and downstream applications. In publication 5, transient (temporary gene expression) transfection was performed, transfecting the designed pcDNA™3.1 plasmid, mentioned before, by X-tremeGENE DNA transfection reagent (Roche, Basel, Switzerland). These reagent complexes with nucleic acids, promoting efficient DNA transfer across cellular membranes, with minimal physiological and morphological cell changes. Details of these cells cultures and transfections are detailed in publication 5.

4.2.3. Immunoprecipitation

Immunoprecipitation (IP) is the technique of precipitating a protein antigen out of solution such as cell lysate, using an antibody that specifically binds to that particular protein. Then immunocomplex is immobilized onto an insoluble support, like agarose or magnetic beads[18]. In this case Protein G Sepharose™ 4 Fast Flow (GE Healthcare, Life Sciences, Little Chalfont, UK) were employed. This process permits to isolate and concentrate one particular protein from a sample containing many thousands of different proteins[18]. The details of the protocol are explained in publication 5.

4.2.4. Protein dephosphorylation

Dephosphorylation means removing of phosphate groups by a phosphatase enzyme, such as alkaline phosphatase (FastAP Thermosensitive

Alkaline Phosphatase, Life technologies, California, EE.UU)[19]. Dephosphorylation was carried out in publication 5 to analyse if phosphorylation affected the mobility of the PDE4D3 protein, in SDS-PAGE

4.2.5. SDS-PAGE and Western Blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a method for determining the molecular weight (MW) of an unknown protein, since the migration rate of a protein coated with SDS is inversely proportional to the logarithm of its MW[20,21], as the SDS is a denaturing agent which makes protein become fully denatured and dissociated from each other. Commonly used technique is SDS-PAGE, in which an electric field is applied across the gel, submerged in a buffer, causing the negatively charged proteins or nucleic acids to migrate across the gel from the negative electrode (the cathode) towards the positive electrode (the anode)[22]. Depending on their size, each biomolecule moves differently through the gel matrix: small molecules more easily fit through the pores in the gel, while larger ones have more difficulty. Protein samples are run in parallel to a protein molecular weight standard marker to make possible to determine the weight of the unknown proteins. Following electrophoresis, the proteins in the gel need to be transferred to a nitrocellulose membrane where they are hybridised with specific antibodies to the target protein. The transfer of the proteins is carried out by electroblotting, which uses the electric current to pull proteins from the gel into the nitrocellulose membrane. The proteins move from within the gel onto the membrane while maintaining the organization they had within the gel[23]. The method involves a sandwich system: the membrane is placed on top of the gel and a stack of filter papers on top of that. The entire stack is placed in a buffer solution that moves up the paper by capillary action, bringing the proteins with it. Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Then the membrane is blocked with a solution of protein to avoid non-specific binding of the antibody. This reduces the background in the final product of the Western blot, leading to clearer results, and eliminates false positives. Later, the membrane is incubated with the specific antibody, denominated

primary antibody, which will bind to the target protein. After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to the secondary antibody, consequently this secondary antibody will attach to the primary one. The secondary antibody is linked to a reporter enzyme such as horseradish peroxidase (HRP), that when exposed to an appropriate substrate, it cleaves a chemiluminescent agent and the reaction product produces luminescence in proportion to the amount of protein. A camera will detect the light from the reaction and give an image of the antibodies bound to the blot (the camera used in publication 5 was LAS-4000mini, Fujifilm, Minato, Tokyo, Japan).

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5. RESULTS

Fifty-three non-related families with clinical suspicion of PHP1A/PPHP were recruited between 2011 and 2015.

After *GNAS* gene structural study in this series of patients, 19 point mutations were identified in 27 families, 10 mutations had not been previously described in the literature nor in the *GNAS* mutation database (www.lovd.nl/GNAS) (Figure 5.1.). i) Three of these novel mutations were maternally inherited. ii) Five of them occurred *de novo,* and pathogenicity of the missense and splicing mutations was established based on prediction softwares (Table 5.1. and Table 5.2.). The mutation identified in patient PHP1015, as it is a frameshift mutation, is supposed to follow NMD. iii) In one case, parental samples were not available. iv) And in the last case we could only discard the mother as a carrier; however as this case presented with PHP1A, we would not expect a paternal inheritance of the mutation (Table 5.1.). None of these novel mutations was present neither in 200 normal control chromosomes nor in any of the unaffected family members analysed.

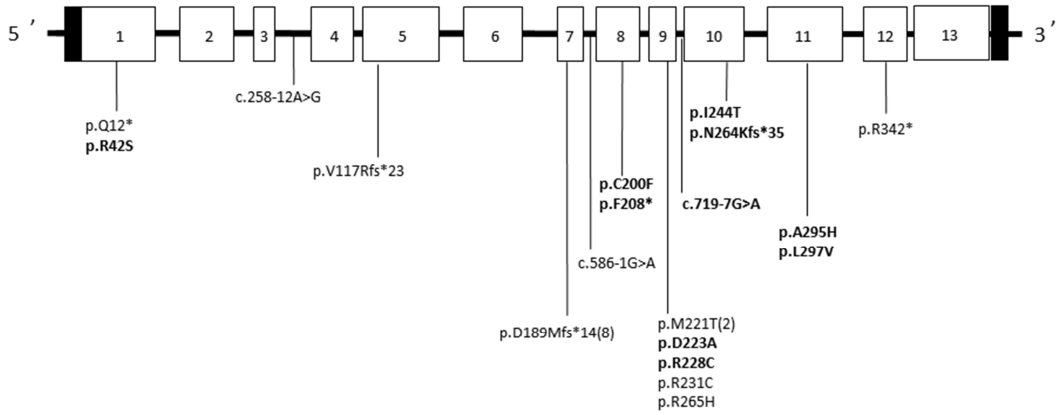

Figure 5.1. Distribution of identified point mutations in *GNAS* gene in the present study (in bold, novel mutations. In brackets, the number of families carrying the mutation). GNAS-015 ENST00000371085 was used as reference transcript.

Regarding clinical characteristics of these 27 patients, 24 of them showed multihormonal resistance (PTH included) and AHO phenotype with variable manifestations; confirming the clinical suspicious of PHP1A. In 5 of these 24 patients, maternal origin of mutations was confirmed. In all of the carrier mothers, a phenotype compatible with PPHP was appreciated after the genetic diagnosis. In the remainder cases, the parental allele carrying the mutation could

not be confirmed either because those mutations happened *de novo* (11 cases) or because progenitors' samples were not available (lack of paternal sample in 4 cases and lack of both parental samples in 2 cases) (Table 5.1.).

Two of the 27 index cases were classified as PPHP and a single one as POH. The three ones showed AHO phenotype without PTH resistance. In one of the PPHP patients and in the POH one, the mutation happened *de novo*. In the reminder PPHP patient, we could only discard that the mother was non-carrier of the mutation, (father's sample was not available) (Table 5.1.). A study was designed to confirm the parental origin of this mutation and as only one of them was informative for the intragenic SNPs, we could confirm that the mutation of one of the PPHP patients happened *de novo* in the paternal allele. Based on the fact that PPHP and POH are clinical entities that rarely are described without clinical history of PHP, we considered interesting to develop a manuscript comparing both clinical entities (publication 1).

Allele dosage was then studied in those families in which point mutations in *GNAS* gene were discarded. Three gross microdeletions involving complete GNAS locus and one intragenic microdeletion at *GNAS* gene were detected (Table 5.1.). Further details of this study and clinical description of these patients were published together with other cases of microdeletions involving GNAS locus, associated with PHP1A/PPHP, in a collaborative work within EuroPHP consortium (publication 2).

On the other hand, our hypothesis was that some patients of our series of patients with clinical suspicious of PHP/PPHP for whom genetic and epigenetic alteration in GNAS locus were discarded, were probably misdiagnosed (22 cases without genetic diagnosis, Table 5.3.). In most cases, brachydactyly E (or similar pattern), together with short stature and obesity, were the main features which suggested PHP/PPHP. Consequently, we decided to extend our research to try to find the underlying genetic defect in each case. Firstly, a review summarising the most common syndromes associated with BDE (or similar pattern) was developed (publication 3). An algorithm was proposed within this review, to

guide the clinical diagnosis of these patients and to facilitate their classification according to their brachydactyly pattern and other features present in these syndromes, to finally suggest the most probable candidate gene in each case.

Following this algorithm, 11 additional patients were genetically diagnosed. First of all, in four patients with PTH resistance a severe and generalized brachydactyly was appreciated, which is one of the main characteristics of acrodysostosis. Accordingly, the gene encoding cAMP-dependent protein kinase type I-α regulatory subunit (*PRKAR1A*), associated with acrodysostosis with hormonal resistance (ACRDYS1) was studied. In three of these patients the recurrent c.1101C>T/p.Arg368* mutation was found, and a novel mutation in the fourth one (c.854A>G/p.Gln285Arg). Three of these cases were published in collaboration with the EuroPHP consortium in a manuscript about a series of patients with acrodysostosis, with the aim of finding phenotype-genotype correlations at clinical, hormonal and radiological level (publication 4). In addition, in another patient with an initial PTH resistance (normal after vitamin D treatment) and acrodysostosis phenotype who also presented mental retardation (more clinical data in, Table 5.3.), a new mutation (c.934C>G/p.L312V) was found in the gene encoding phosphodiesterase 4D cAMP-specific (*PDE4D*), associated with acrodysostosis without hormone resistance (ACRDYS2). Information about this patient will be published in another article in collaboration with the EuroPHP on a large series of patients with acrodysostosis associated with *PDE4D* mutations (manuscript in preparation).

Functional studies were performed in eukaryotic cells transfected with WT-PDE4D or four different PDE4D mutants reflecting the mutations described in ACRDYS2 acrodysostosis patients, to try to understand the effect of mutations in this protein and to elucidate the underlying mechanisms these mutations use to cause a similar phenotype as mutations in *PRKAR1A*. Results of the study are summarized in the draft of the manuscript, which will be proposed to be published (publication 5).

Within our series of patients without genetic diagnosis, four patients with features suggestive of tricho-rhino-phalangeal syndrome I (TRPS-I) were identified. Consequently, the gene encoding zinc finger transcription factor TRPS1 (*TRPS1*) was analysed. Two patients presented the recurrent c.2762G>C/p.Arg921Gln mutation. In the other two patients different mutations were identified (c.2830delA/p.Arg944fs*3 and c.3159_3160delAAinsT/p.K1035Nfs*11, respectively); none of them was previously described in the literature. The cosegregation with the pathology in other family members suggested that they were pathogenic. Two of the cases were reported in an article trying to establish the distinguishing characteristics between TRPS-I/TRPS-III and PHP/PPHP (publication 6). The reminder cases are shown in Table 5.3.

Other cases that were resolved thanks to the study of candidate genes implicated in BDE were two patients who presented BDE with advanced bone maturation for their chronological age. Two novel *de novo* mutations (c.101+3delAAGT and c.166C>T/p.Arg56*, respectively) in *PTHLH* gene, encoding parathyroid hormone-related protein (PTHrP) were observed in these patients. The characteristics of the mutations (frameshift and nonsense, respectively) suggested that they were causative of the pathology. Expression of mutant allele will probably follow a NMD process leading to haploinsufficiency of PTHrP protein in the patients. One of these cases together with another familiar case identified by other members of EuroPHP has been reported in a manuscript, which is currently under review (publication 7). The clinical data of the reminder patient with *PTHLH* mutation is included in Table 5.3.

The summary of the genetic results found in our series of 53 patients is shown in Figure 5.2.

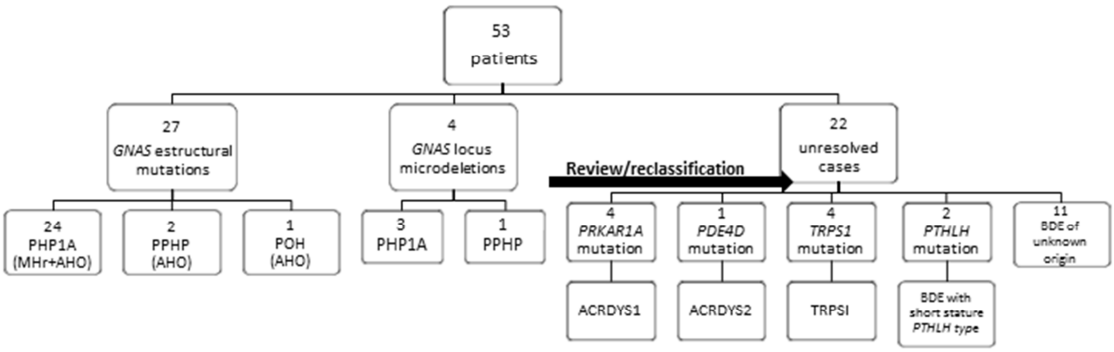

Figure 5.2. Outline of the results of genetic studies in the series of patients with clinical suspicious of PHP/PPHP (MHr: multihormonal resistance; AHO: with AHO phenotype).

Finally, as a result of this reclassification and acquired knowledge on new genetic alterations in patients with initial clinical diagnosis of PHP, we developed a new review, in this case in Spanish (publication 8), with the aim of reach out to clinicians who send us the most of the samples for the genetic study.

Table 5.1. Details of the mutations identified in PHP1A, PPHP and POH patients. Novel mutations are indicated in bold. Additional information about novel mutations is included: cosegregation and/or prediction software results (*: lack of paternal sample; **: lack of parental sample; NMD; nonsense mediated decay; HSF: Human Splice Finder software; delet: deleterious; PSD: possibly damaging; PD: probably damaging).

										Additional information about novel mutations			
PATIENTS	**PROTEIN MUTATION (ENSP00000360126)**	**cDNA MUTATION (ENST00000371085)**	**PROTEIN MUTATION (ENSP00000346328)**	**cDNA MUTATION (ENST00000354359)**	**EXON/INTRON**	**MUTATION TYPE**	**INHERITANCE**	**CLINICAL DIAGNOSIS**	**DESCRIBED**	**COSEGREGATION**	**SIFT**	**POLYPHEN**	**HSF**
PHP0092	p.Asp189Metfs*14	c.565_568delGACT	p.Asp190Metfs*14	c.568_571delGACT	7	Frameshift	*De novo*	PHP1A	HOT SPOT		-	-	
PHP0093	**p.Arg42Ser**	**c.124C>A**	**p.Arg42Ser**	**c.124C>A**	1	Missense	Unknown**	PHP1A	**NOVEL**		delet (0,01)	PSD (0,779)	
PHP0094	p.Asp189Metfs*14	c.565_568delGACT	p.Asp190Metfs*14	c.568_571delGACT	7	Frameshift	Unknown**	PHP1A	HOT SPOT		-	-	
PHP0108	p.Val117Argfs*22	c.348_349insC	p.Val118Argfs*22	c.351_352insC	5	Frameshift	Unknown**	PHP1A	YES		-	-	
PHP0109	**p.Asn264Lysfs*35**	**c.791_792insA**	**p.Asn265Lysfs*35**	**c.794_795insA**	10	Frameshift	Maternal	PHP1A	**NOVEL**	Yes	-	-	
PHP0111	**p.Arg228Cys**	**c.682C>T**	**p.Arg229Cys**	**c.685C>T**	9	Missense	Maternal	PHP1A	**NOVEL**	Yes	delet (0,02)	PD (0,989)	
PHP0115	p.Arg231Cys	c.694C>T	p.Arg232Cys	c.697C>T	9	Missense	Unknown*	PHP1A	YES		-	-	
PHP1003	p.Asp189Metfs*14	c.565_568delGACT	p.Asp190Metfs*14	c.568_571delGACT	7	Frameshift	Unknown*	PHP1A	HOT SPOT		-	-	
PHP1006	p.Arg342*	c.1024C>T	p.Arg343*	c.1027C>T	12	Nonsense	Unknown**	PHP1A	YES		-	-	
PHP1015	**p.Phe208***	**c.623_624delTT**	**p.Phe209***	**c.626_627delTT**	8	Nonsense	*De novo*	PHP1A	**NOVEL**		-	-	
PHP1016	-	**g.?-56,987,684_59,350,455del**	-	**g.?-56,987,684_59,350,455del**	Complete GNAS	Gross deletion	Maternal	PHP1A	NOVEL		-	-	
PHP1021	p.Asp189Metfs*14	c.565_568delGACT	p.Asp190Metfs*14	c.568_571delGACT	7	Frameshift	*De novo*	PHP1A	HOT SPOT		-	-	
PHP1022	p.Asp189Metfs*14	c.565_568delGACT	p.Asp190Metfs*14	c.568_571delGACT	7	Frameshift	*De novo*	PHP1A	HOT SPOT		-	-	
PHP1025	**p.Asp223Ala**	**c.668A>C**	**p.Asp224Ala**	**c.671A>C**	9	Missense	*De novo*	PHP1A	**NOVEL**		delet (0,02)	PD (0,977)	
PHP1026	-	**g.57,224,346_59,795,557del**	-	**g.57,224,346_59,795,557del**	Complete GNAS	Gross deletion	Maternal	PHP1A	NOVEL		-	-	
PHP1046	p.?	c.258-12A>G	p.?	c.258-9A>G	intron 3	Splicing	*De novo*	PHP1A	YES		-	-	
PHP1047	**p.Asp295His**	**c.883G>C**	**p.Asp296His**	**c.886G>C**	11	Missense	*De novo*	PHP1A	**NOVEL**		delet (0)	PD (0,994)	
PHP1049	**p.Ile244Thr**	**c.731T>C**	**p.Ile245Thr**	**c.734T>C**	10	Missense	*De novo*	PPHP	**NOVEL**		delet (0)	PD (0,999)	
PHP1051	p.?	**c.719-7G>A**	p.?	**c.722-7G>A**	intron 9	Splicing	*De novo*	PHP1A	**NOVEL**		-	-	New splice acceptor (Table 5.2.)
PHP1061	**p.Cys200Phe**	**c.599G>T**	**p.Cys201Phe**	**c.602G>T**	8	Missense	Unknown*	PHP1A	**NOVEL**		delet (0)	PD (1)	
PHP1063	-	**g.55,926,305_57,675,815del**	-	**g.55,926,305_57,675,815del**	Complete GNAS	Gross deletion	Paternal	PPHP	NOVEL		-	-	
PHP1064	-	**g.57475,484_57,481,914del**	-	**g.57475,484_57,481,914del**	intron 2_exon 6 of *GNAS*	Intragenic deletion	Maternal	PHP1A	NOVEL		-	-	
PHP1065	p.Arg265His	c.794G>A	p.Arg266His	c.797G>A	9	Missense	Maternal	PHP1A	YES		-	-	
PHP1078	p.-?	c.586-1G>A	p-.?	c.589-1G>A	intron 7	Splicing	*De novo*	PHP1A	YES		-	-	
PHP1088	**p.Leu297Val**	**c.889C>G**	**p.Leu298Val**	**c.892C>G**	11	Missense	Maternal	PHP1A	**NOVEL**	Yes	delet (0)	PD (0,999)	
PHP1089	p.Asp189Metfs*14	c.565_568delGACT	p.Asp190Metfs*14	c.568_571delGACT	7	Frameshift	*De novo*	POH	HOT SPOT		-	-	
PHP1095	p.Gln12*	c.34C>T	p.Gln12*	c.34C>T	1	Nonsense	Unknown*	PPHP	YES		-	-	
PHP1097	p.Met221Thr	c.662T>C	p.Met222Thr	c.665T>C	9	Missense	Unknown**	PHP1A	YES		-	-	
PHP1106	p.Met221Thr	c.662T>C	p.Met222Thr	c.665T>C	9	Missense	Maternal	PHP1A	YES		-	-	
PHP1108	p.Asp189Metfs*14	c.565_568delGACT	p.Asp190Metfs*14	c.568_571delGACT	7	Frameshift	*De novo*	PHP1A	HOT SPOT		-	-	
PHP1114	p.Asp189Metfs*14	c.565_568delGACT	p.Asp190Metfs*14	c.568_571delGACT	7	Frameshift	*De novo*	PHP1A	HOT SPOT		-	-	

Table 5.2. Human Splice Finder software prediction result about the novel mutation c.719-7G>A described within *GNAS* in patient PHP1051. The results suggested that as a consequence of the mutation in the splice site, a new potential acceptor appears with a higher consensus value in comparison to the WT potential acceptor. This mutation would add 5 nucleotide more to the start of the exon, changing the reading frame of the sequence, and probably causing NMD.

Splice site	Motif	New splice site	If cryptic site use, exon length variation	Consensus value (%)		
				WT	Mutant	Variation (%)
New potential acceptor	tgctctctttggtt	tgctctctttagTT	5	53.63	**82.58**	+53.97
WT potencial acceptor	tctttggttaagAT	tctttagttaagAT	0	**78.26**	78.3	+0.06

Table 5.3. Patients without genetic alterations at *GNAS* gene. Biochemical studies, description of the brachydactyly, other clinical characteristics and studied candidate genes in each patient are indicated. The final genetic diagnosis is specified, if a mutation was identified (P: patient; PTHr: PTH resistance; Vit D: vitamin D; Ca: calcemia; P: phosphoremia; BD: brachydactyly; MR: mental retardation; N: normal; ND: no data; MT: metacarpals; TP: telophalange; MP; medialphalange; BP: basophalange; Rx: radiography; SS: short stature; $^{\xi}$: additional studies of this variation pending.

PATIENTS	PTHr	Ca/P	Vit. D	BD	MR	Others features	MOLECULAR STUDIES							Genetic Diagnosis
							PRKAR1A	*PDE4D*	*PTHLH*	*HOXD13*	*TRPS1*	*HDAC4*	2q37 microsatellites	
PHP0034 (Publication 6: P1)	Yes and low GH	N	N	MT: III-V outcarving cones of MP & TP	learning difficulties (no test)	SS, obesity, round face, sparse hair, thin upper lip and prominent lower lip, pear-shaped nose, tooth hypoplasia, stubby fingers and toes, polyarthrosis, arthralgias of both hips and knees	N				c.2830delA/ p.R944Gfs*3			**TRPS -I**
PHP0068	No	N	ND	Severe (especially, IV & V MT) (no Rx)	Yes	SS, fine and sparse hair, sparse eyebrows, small saddle nose, prominent forehead, epicanthal folds, upward slanting palpebral fissures, low and dysplastic ears, café au lait spots	N	N			N	N	N	
PHP0070 (Publication 4: P9)	Yes and TSH	N	ND	Severe and generalized brachydactyly	Behavioural disorder	Heights in the range of lower normal, broad face with widely, spaced eyes, maxillonasal hypoplasia, clinical peripheral dysostosis, severe hypoplasia of the skull, thickened calvarium (PUBLICATION 4:P9)	c.1101C>T/ p.R368X							**ACRDYS1**
PHP1004 (Publication 4: P8)	Yes	P↑	ND	Severe and generalized brachydactyly	No	Heights in the range of lower normal, broad face with widely, spaced eyes, maxillonasal hypoplasia, clinical peripheral, dysostosis, severe hypoplasia of the skull, thickened calvarium, pigmented skin spots (PUBLICATION 4:P8)	c.1101C>T/ p.R368X							**ACRDYS1**
PHP1008	No	N	ND	MT: V (no Rx)	ND	SS , clinodactyly, cone-shaped phalangeal epiphyses			N	N	N			
PHP1009	Yes	N		Severe and generalized (no Rx)		Flat round face, osteoporosis, genu valgum, Madelung deformity	c.1101C>T/ p.R368X							**ACRDYS1**
PHP1014	No	N	ND	MT: IV (no Rx)	No	Normal stature, round face, facial asymmetry, hypothyroidism			N	N				
PHP1019	No (after Vit D treatment)	N	Low levels at first	Severe and generalized	Yes	Obesity, round face, short neck, hyperinsulinism	N	c.934C>G/ p.L312V						**ACRDYS2**
PHP1020	No (after Vit D treatment)	N (after Vit D treatment)	Low levels at first	MT: IV & V (no Rx)	ND	Obesity, acanthosis nigricans, dental malformations, short neck, hyperinsulinism			N	N				
PHP1023 (Publication 4: P14)	Yes and TSH	N	N	Severe and generalized	No	Heights in the range of lower normal, broad face with widely, spaced eyes, maxillonasal hypoplasia, clinical peripheral, dysostosis, severe hypoplasia of the skull, thickened calvarium (PUBLICATION 4: P14)	c.845A>G / p.Q285R							**ACRDYS1**
PHP1067	N	N	N	MT: II-V outcarving cones of MP & BP	N	Overweight, sparse hair, laterally sparse eyebrows, long flat philtrum, and thin upper vermillion border, pear-shaped nose, protruding ears					c.2762G>A/ p.R921Q			**TRPS-I**
PHP1068 (Publication 7: P3)	N	N	N	MT: IV	N	Advanced bone age			c.101+3delAAGT					**BDE, with short stature PTHLH type**
PHP1074	No	N	ND	MP: II-V, at least. (BDA?)	ND	SS, obesity, rhizomelia, prominent forehead, depressed nasal root		N	N			N	N	
PHP1075	No	N	N	Stubby digits MT:III-IV at least	No	SS, stocky build, hip hypoplasia, horizontalised acetabulum, varum deformity, shortened tibia and femur, decreased interpedicular distance, scoliosis Bone dysplasias		N	N					
PHP1076	No	N	N	MT: IV	No	SS			N	N	N			
PHP1083	No	N	N	MT: IV & V	No	SS, obesity, delayed puberty			N	Variant of unknown significance$^{\xi}$				
PHP1085	(TSH mildly increased)	N	ND	MT: IV; TP: I	No	N stature, prominent forehead			N	N				
PHP1086	No	N	ND	Generalized shortening, severe outcarving of the epiphysis		Sparse hair, thin upper lip, long philtrum, pear-shaped nose, sparse, eyebrows, prominent forehead					c.2762G>A/ p.R921Q			**TRPS-I**
PHP1096				MP:II & V (BDA?)	No	Bilateral cubitus valgus, short forearms, bicornuate uterus, overweight			N	N				
PHP1099	No	N	ND	MT: II-V TP:I & III	No	SS, obesity, anteverted nostrils, long philtrum, depressed nasal root, depressed temples, sort neck, low set ears		N	c.166C>T/p.R56*					**BDE, with short stature PTHLH type**
PHP1101	No	N	ND	Generalized shortening, stubby fingers (no Rx)	ND	Sparse hair, thin upper lip, long philtrum, pear-shaped nose, strabismus					c.3159_3160delAAinsT/ p.K1035Nfs*11			**TRPS-I**
PHP1104	No	N	N	MT: IV & V (mild)	Mild	Obesity, round face, sparse hair, thin upper lip, pear-shaped nose, sparse, arched eyebrows					N			

PUBLICATION 1

Seudoseudohipoparatiroidismo *vs* heteroplasia ósea progresiva en ausencia de historia familiar

Pseudopseudohypoparathyroidism *vs* progressive osseous heteroplasia in absence of family history

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*Ambas autoras colaboraron de forma similar en el desarrollo del presente trabajo.

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Medicina Clínica (Barcelona) **2015;S0025-7753(15):00111-00116**

Sr. Editor,

El fenotipo de osteodistrofia hereditaria de Albright (OHA), fue descrito como la asociación de braquidactilia, talla baja, cuello corto, obesidad, facies redondeada y retraso mental[1]. Este fenotipo puede presentarse en síndromes como el seudohipoparatiroidismo (PHP)[1], el seudoseudohipoparatiroidismo (PPHP)[2], la heteroplasia ósea progresiva (HOP)[3] y el OHA-like (o braquidactilia asociada a retraso mental, BDMR)[4]. Dado que tanto el PPHP como el HOP son enfermedades raras y sólo excepcionalmente se describen casos aislados (sin historia familiar), consideramos de interés clínico presentar estos dos casos con mutaciones en gen *GNAS* pero fenotipos diferentes.

Caso 1: niña de $2^{8/12}$ años que consulta por talla baja. Padres sanos de 176 y 157 cm; talla diana 160cm (-0.66 DE). Embarazo doble mediante ICSI *(intracitoplasmatic sperm injection),* que cursó con retraso del crecimiento intrauterino (RCIU). Cesárea en semana $32^{+2.}$ con Apgar 7/9, peso 1.170g (p3), longitud 41,5cm (p50) cm, perímetro craneal 32,5cm (p75). Ingreso neonatal por ileo meconial resuelto con enema. Hermano mellizo sano, peso: 1.760g (p50), Apgar 8/9. La paciente recibió estimulación precoz por prematuridad asociada a bajo peso; alta a los 42 meses con un cociente de desarrollo de 100. A los 3 años: Test Weschler con CI de 103. Escolarizada sin problemas curriculares. Evolución de talla: ($2^{8/12}$ años) 81,6cm (-3,7DE), peso 11kg (-2,1DE), perímetro craneal 47.5cm. A los 8 años: 117cm (-2,1DE), peso de 27,7kg (-0,2 DE), (IMC 20,24kg/m^2, +0,94DE). Fenotipo: ojos almendrados, apiñamiento dental, tórax discretamente ensanchado. RX de mano a los 4 años normal (Figura 1A); actualmente se aprecia metacarpianos y metatarsianos acortados (Figura 1B, 1C). Cariotipo 46 XX. Sin antecedentes de calcificaciones ectópicas ni problemas renales. Ecografía renal normal. Calcio 10,4 mg/dl, fósforo 4,76 mg/dl, Mg 2.11 mg/dl, TSH 3.21 mUI/L, fT4 1.33 ng/dl, IGF-I 284 ng/dl, PTH 30.1 ng/dl, Vitamina D3: 37 ng/ml. A los 9 años, talla 121,5cm (-2,12DE), peso 30kg (-0,35 DE), IMC de 20,4Kg/m^2 (+0,67DE), edad ósea de 10,6 años. Se sospechó seudoseudohipoparatiroidismo.

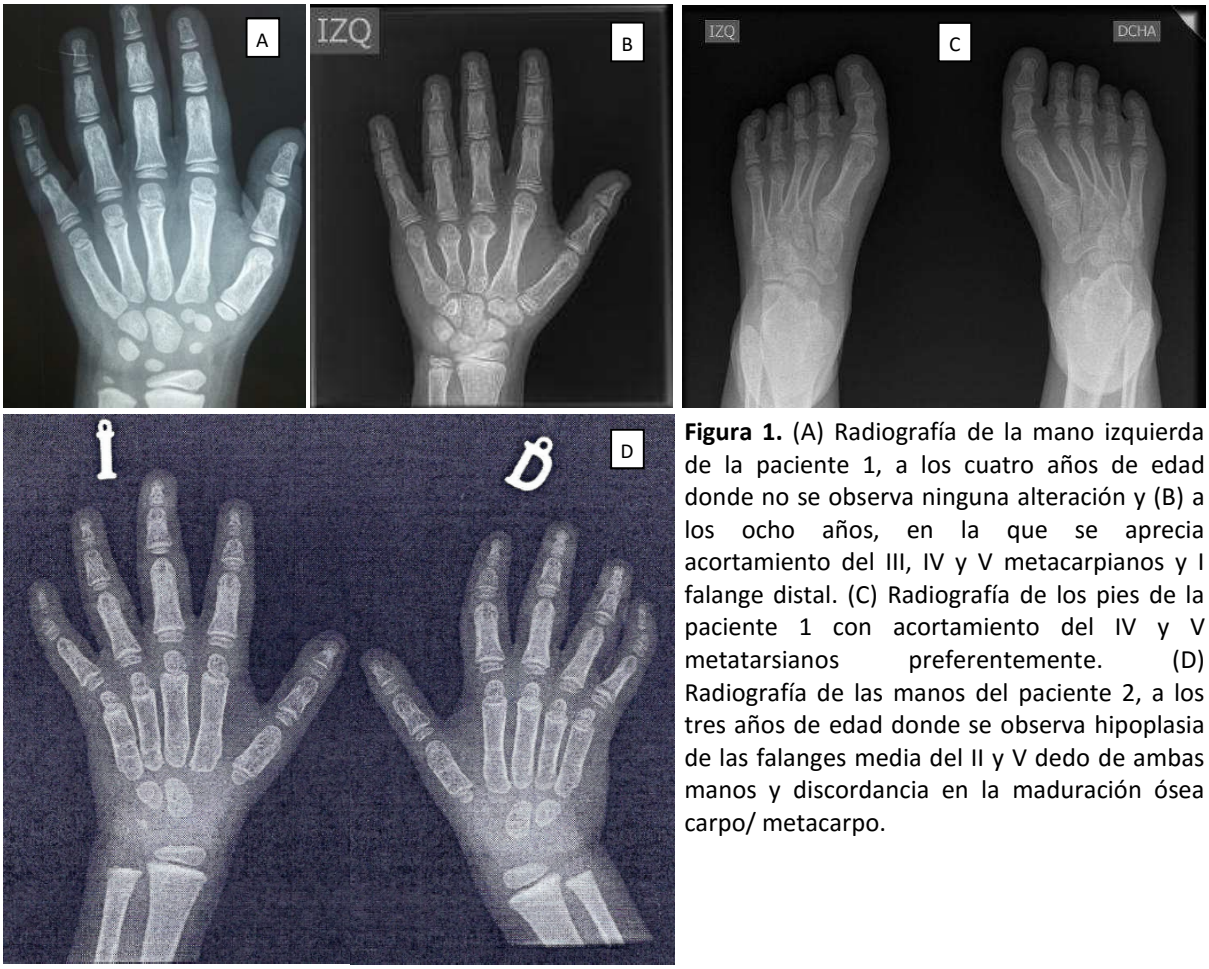

Figura 1. (A) Radiografía de la mano izquierda de la paciente 1, a los cuatro años de edad donde no se observa ninguna alteración y (B) a los ocho años, en la que se aprecia acortamiento del III, IV y V metacarpianos y I falange distal. (C) Radiografía de los pies de la paciente 1 con acortamiento del IV y V metatarsianos preferentemente. (D) Radiografía de las manos del paciente 2, a los tres años de edad donde se observa hipoplasia de las falanges media del II y V dedo de ambas manos y discordancia en la maduración ósea carpo/ metacarpo.

Caso 2: niño de $3^{3/12}$ años que consulta por retraso ponderoestatural. Madre y padre sanos con tallas de 161cm y 162cm, respectivamente. Embarazo con oligoamnios en tercer trimestre. Parto a las 38 semanas. Apgar 7/8, al nacer peso 2.170g (-2,4DE), longitud 43cm (-3,8DE), perímetro craneal 33cm (-1,4DE). Buen desarrollo psicomotor, aunque realiza cinesiterapia por disminución de la movilidad de extremidad superior derecha. Se observaron, calcificaciones difusas subcutáneas (abdomen y pie de aparición hacia los 3 meses de vida,) e intrarticular (hombro derecho), también hipocrecimiento, osteopenia y anomalías óseas inespecíficas en manos (hipoplasia falange media del II y V dedo de ambas manos) (Figura 1D), con disminución de la función muscular del dedo pulgar más acusadamente en el lado derecho. En su primera visita ($3^{3/12}$ años) tenía una talla de 81,5cm (-4,7DE), peso de 9,100kg (-2,9DE), con PC de 47,5cm (-2,4DE), activo. A los $5^{4/12}$ años, presentaba una talla de 94.8cm (-4,1DE), peso de 12,15kg (-2,4DE), IMC: 13.52Kg/m^2 (-1,2DE). No se observó avance de las calcificaciones abdominales y se descubrieron focos de calcificación en talones.

En la última consulta, a los $6^{3/12}$ años su talla fue de 99,1cm (-3,9DE), peso de 13,4kg (-2,4DE), IMC: 13,64 Kg/m^2 (-1,2DE). Según las pruebas bioquímicas realizadas, no presentó disfunción del metabolismo fosfocálcico ni tampoco otras resistencias endocrinas. El cariotipo fue normal, 46 XY. En vista de la falta de resistencia hormonal y la presencia de osificaciones heterotópicas se estableció como sospecha diagnóstica heteroplasia ósea progresiva.

El estudio genético del gen *GNAS* permitió identificar en el caso 1 la mutación novel c.733T>C (p.Ile244Thr) (exón 10) predicha tanto por Polyphen como SIFT como patológica; y en el caso 2 la deleción frecuente c.568_571del (p.Asp190Metfs*14) (exón 7), asociada a PPHP[5], PHP1A[5] y HOP[6] (http://www.LOVD.nl/GNAS). En los dos casos, el estudio familiar confirmó el origen *de novo* de las mutaciones. La PCR de alelo específica permitió confirmar en el caso 1 que la mutación se había originado en el alelo paterno. En el segundo caso no pudo confirmarse, porque ninguno de los polimorfismos estudiados fue informativo.

El fenotipo OHA puede ocurrir tanto asociado a resistencia a la PTH (PHP)[1], como en su ausencia (PPHP)[2]. El PPHP puede encontrarse en familias con PHP-Ia y, ocasionalmente, de forma aislada[5,7,8], estando ambos causados por mutaciones inactivantes en el gen *GNAS*. En el caso del PHP-Ia las mutaciones se heredan por vía materna y en el PPHP por paterna. En ocasiones, las mutaciones en el alelo paterno producen el fenotipo HOP que es relativamente diferente al PPHP ya que se manifiesta con osificaciones heterotópicas intramembranosas, que progresan a tejidos más profundos (musculo esquelético)[3], como las que presenta el segundo caso, que ya habían sido descritas en otro paciente de HOP con la misma alteración genética[6]. Se recomienda el seguimiento de las osificaciones e intervención sólo en el caso de impedir la correcta movilidad de las articulaciones, por su alto índice de recurrencia[3]. De las características típicas de OHA, aquellas que son más relevantes para la correcta identificación de las formas aisladas son: (i) La talla baja, motivo de consulta de ambos pacientes y principal causa de derivación a la consulta de endocrinología[7]; (ii) La braquidactilia, implica los III, IV y V metacarpianos y I falange distal, pero este

acortamiento puede no ser detectado hasta los seis años de edad[9], como en el caso 1, manifestado a los 8 años. La talla baja y la braquidactilia son el resultado del cierre prematuro de las epífisis óseas a causa de la haploinsuficiencia de Gsα que afecta a la señalización de la PTH/PTHrP en los condrocitos[10] y (iii) El RCIU observado ambos casos, se debe a que los transcritos derivados del alelo paterno del *GNAS* son necesarios para el crecimiento y desarrollo fetal y para el desarrollo de la placenta[5].

CONFLICTO DE INTERESES

Los autores declaran no tener ningún conflicto de intereses.

AGRADECIMIENTOS

Los autores agradecen a los pacientes y sus familiares su implicación en este estudio, al Profesor Joaquín Fernández Toral por el estudio clínico-genético de uno de los pacientes y al Profesor Fernando Aleixandre por su orientación en la escritura del manuscrito.

Este estudio ha sido parcialmente financiado por el Instituto Carlos III (becas PI10/0148, PI13/00467 a GPdN). Arrate Pereda posee una beca financiada por el Ministerio de Sanidad Español (FI11/00309); y la Dra. Guiomar Pérez de Nanclares está parcialmente financiada por el programa I3SNS del Ministerio de Sanidad Español (CP03/0064; SIVI 1395/09).

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PUBLICATION 2

Novel microdeletions affecting the GNAS locus in pseudohypoparathyroidism: characterization of the underlying mechanisms

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Journal of Clinical Endocrinolology & metabolism **2015 Apr;100(4):E681-7**

ABSTRACT

Context: Pseudohypoparathyroidism type Ia (PHP1A) is a rare endocrine disorder characterized by hypocalcemia, hyperphosphatemia, multiple hormonal resistance and features of Albright hereditary osteodystrophy. When the phenotype is present but not associated to hormonal resistance, it is called psedopseudohypoparathyroidism (PPHP). Both entities have been associated to *GNAS* haploinsufficiency, and are mostly caused by inherited inactivating mutations at *GNAS* gene that codes for the stimulatory alpha subunit of G protein (Gsα), though the cause remains unidentified in about 30% of patients.

Objectives: The aims of our work were (i) to identify GNAS locus defects in 112 patients with clinical diagnosis of PHP1A/PPHP and no point mutations at *GNAS,* to improve molecular diagnostic and genetic counseling; (ii) to outline the underlying molecular mechanism(s).

Methods: MS-MLPA, quantitative PCR, aCGH and long-PCR were used to search for genomic rearrangements at chromosome 20q and to identify their boundaries. We used different bioinformatic approaches to assess the involvement of the genomic architecture in the origin of the deletions.

Results: We discovered seven novel genomic deletions, ranging from 106-bp to 2.6-Mb. The characterization of 5 of 7 deletion breakpoints and the definition of the putative molecular mechanisms responsible for these rearrangements revealed that Alu sequences play a major role in determining the genetic instability of the region.

Conclusion: We observed that deletions at GNAS locus represent a significant cause of PPHP/PHP1A and that such defects are mostly associated with Alu-mediated recombination events. Their investigation revealed to be fundamental as, in some cases they could be misdiagnosed as imprinting defects.

INTRODUCTION

Pseudohypoparathyroidism type Ia (PHP1A, MIM#103580) is a rare genetic disorder, whose hallmark is end-organ resistance to parathyroid hormone (PTH) due to partial deficiency of the alpha subunit of the stimulatory G protein (Gsα), encoded by the complex imprinted GNAS locus (MIM#139320). PHP1A patients are characterized by PTH resistance, defined as raised serum PTH levels in the presence of hypocalcemia and hyperphosphatemia, and show features of obesity, mental retardation and Albright hereditary osteodystrophy (AHO)[1]. PHP1A patients may also develop resistance to thyroid-stimulating hormone (TSH), gonadotropins and growth-hormone-releasing hormone (GHRH)[2,3].

The disease is inherited in an autosomal dominant manner and, generally, is caused by heterozygous maternally-derived inactivating mutations of Gsα coding exons[1,4]. This pattern of inheritance is consistent with Gsα tissue-specific imprinting. Indeed Gsα predominant maternal expression has been demonstrated in specific human tissues, comprising proximal renal tubules, pituitary, gonads, and thyroid[5].

In 1990, Patten *et al.* described the first *GNAS* loss-of-function mutation responsible for PHP1A[4] and, to date, numerous different Gsα-coding mutations have been identified, with a detection rate of about 70%[1,6] (and personal data). Most defects are private mutations and few recurrent mutations in unrelated patients probably derived from the presence of a common molecular mechanism rather than a founder effect[7]. The detection of a mutation is associated with the development of the disease and 50% of occurrence in the offspring, thus the genetic test provides a diagnosis together with the possibility of genetic counseling in relatives.

Nevertheless, the molecular determinants of 30% patients still remain unclear and, in the last years, studies papers described GNAS epigenetic defects, similar to those classically found in PHP−Ib patients, in a subset of patients with

PHP and variable degrees of AHO, suggesting a molecular overlap between PHP1A and PHP1B[8].

Recently, some cases of PHP1A patients with deletions of 20q, including part or the whole *GNAS* gene, and an inversion at *GNAS* were reported, demonstrating that chromosomal rearrangements may also cause PHP1A and that investigation for these less common genetic defects is needed[9]. Moreover, deletions ablating GNAS DMRs mimic an imprinting defect during the methylation analysis and may lead to a misdiagnosis of PHP1B[10]. These defects, undetectable by Sanger sequencing and often also by karyotype investigation, are considered clearly distinct from the small-scale gene mutations, both for the size of the rearranged DNA and the causing mechanisms[11,12].

In order to ameliorate molecular diagnostic and genetic counseling, the aims of our work were to identify and characterize novel GNAS locus defects, such as micro-deletions or –duplications, associated with PHP1A/PPHP using methylation specific-multiplex ligation-dependent probe amplification (MS-MLPA) and custom made CGH array and unravel putative molecular mechanisms responsible for these rearrangements.

PATIENTS AND METHODS

A total of 112 patients with PHP1A/PPHP phenotype and no *GNAS* mutations, all born from non-consanguineous parents, were studied. The clinical diagnosis was based upon the presence of at least two of the six AHO manifestations: brachydactyly (shortening of fourth and/or fifth metacarpals), short stature (height below the 3th percentile for chronological age), obesity (BMI >30 kg/m^2 in adults and >97th centile in children), round face, subcutaneous ossifications (either clinically evident or at X-ray), and mental retardation; when these signs were observed in the presence of PTH resistance (i.e. hypocalcemia, hyperphosphatemia and raised serum PTH levels), PHP1A diagnosis was made. All PHP1A patients showed also resistance to TSH, documented by raised serum

TSH levels, absence of anti-thyroid antibodies and presence of a normal thyroid scan.

Patients were studied in different labs, using different techniques. A detailed description of methods is provided as supplementary data (section 1 Supplementary data, Supplementary Table 1). Informed consent was obtained from all patients and relatives included in the study.

RESULTS

Allele dosage and methylation analysis by MS-MLPA or custom aCGH revealed the presence of 7 heterozygous deletions of part or whole GNAS locus, in a cohort of 112 GNAS point mutation-negative PHP1A/PPHP probands (detection rate 6.25%) (Table 1, Figure 1A/B). Six of the seven patients (2 females and 4 males) were affected by PHP1A, and one female patient by PPHP (patient 6). When possible, the parental origin of the deletion was identified and the family members studied. Main clinical features of patients with deletion and affected relatives are described in Table 1.

The deletions ranged from 106-bp to 2.6-Mb. Specifically, defects detected in patients 2, 3, 4 and 7 were short *GNAS* deletions, affecting only one to seven Gsα-coding exons. The methylation analysis confirmed the absence of imprinting defects for patients 2, 4 and 7. In patient 3 we observed a deletion encompassing exon A/B to exon 1 revealing a methylation defect limited to the A/B DMR, an imprinting error classically associated with the autosomal dominant inherited form of PHP1B. Deletions found in patients 1, 5 and 6 comprised the entire GNAS locus. Methylation analysis of patients 1 and 5 displayed a pattern of loss of imprinting (LoI) similar to that detected in sporadic PHP1B cases (loss of methylation at AS, XL and A/B DMRs and gain of methylation at NESP DMR), indicating that the maternal allele was ablated. On the contrary, patient 6 presented the opposite methylation pattern, as the deleted allele was the

paternal one. A comprehensive description of novel rearrangements is available in the supplementary data, section 2.

Deletions' breakpoints were subjected to bioinformatic analyses to explore underlying mechanisms and to assess the contribution of the genomic architecture. We analyzed the extent of microhomology at breakpoints and investigated for the presence of known sequence motifs, purine-pyrimidine repeats, AT-content and repetitive elements. An overview of all these results can be found in supplementary data, section 3 (Supplementary Table 2)

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Table 1: Clinnical charcateristics and molecular analysis of patients included in the present study-

Pt	Sex	Age 1[c]	Age 2[c]	PTH, pg/ml[d]	TSH, mUI/L[d]	Obesity[e]	AHO features	Additional features	Deletion extension	Deleted Genes
1	M	8	4	206	4.36	Yes	Br/RF/SO/MR	Hyperinsulinemia	g. 57224,346_59,795,557del	*STX16, NPEPL1, GNAS-AS1, GNAS, NELFCD, CTSZ, TUBB1, SLMO2, ATP5E, ZNF831, EDN3, PHACTR3, SYCP2, PPP1R3D, CDH26*
2a[a]	F	10	0.5	600	14	Yes	Br/RF/MR	-	g.57482,794_57,488,635del	exon 7_exon 13 of *GNAS*
2b[a]	F	8	0.2	88.3	12.44	No	Br/SO	-	g.57482,794_57,488,635del	exon 7_exon 13 of *GNAS*
3	M	15	16	342	17.7	No	Br/SS/RF/MR	-	g.(57463,738_57,464100)_(57466,893_57,470666)del	exon AB_exon 1 of *GNAS*
4	M	40	0.6	312	↑	No	Br/SS/RF/SO	Ankylosing spondilitis	g.57485,647_57,485,751del	intron 12_exon 13 of *GNAS*
5	M	19	2	478	↑	Yes	SS/SO/MR	Attention deficit disorder, T2D, Evans syndrome, cirrhosis, psoriasis, asthma	g.?-56987,684_59,350,455del	*VAPB, APCDD1L, APCDD1L-AS1, STX16, NPEPL1, GNAS-AS1, GNAS, NELFCD, CTSZ, TUBB1, SLMO2, ATP5E, ZNF831, EDN3, PHACTR3, SYCP2, PPP1R3D, CDH26*
6	F	6	20	21	0.70	Yes	Br/SS	Bilateral adrenal hyperplasia	g.55926,305_57,675,815del	*MTRNR2L3, RBM38, CTCFL, PCK1, ZBP1, PMEPA1, PPP4R1L, RAB22A, VAPB, APCDD1L, APCDD1L-AS1, STX16, NPEPL1, GNAS-AS1, GNAS, NELFCD, CTSZ, TUBB1, SLMO2, ATP5E* and putatively *RAE1*at the 5'end.
7a[b]	F	1	0.5	277	27	Yes	SS/SO/RF/MR	Craniosynostosys	g.57475,484_57,481,914del	intron 2_exon 6 of *GNAS*
7b[b]	F	27		120	6	No	SS/SO/MR		g.57475,484_57,481,914del	intron 2_exon 6 of *GNAS*

Abbreviations: Br, brachydactyly, clinically evident; F, female; M, male; MR, mental retardation; RF, round face; SO, subcutaneous ossification; SS, short stature; T2D, type 2 diabetes.

[a] Patients 2a and 2b are siblings.

[b] Patient 7b is the daughter of patient 7a.

[c] Age 1 refers to the age at molecular diagnosis whereas Age 2 is the one at clinical diagnosis.

[d] PTH and TSH levels refer to the ones at clinical diagnosis. Normal values are 10–65 pg/mL for PTH and 0.2– 4.0 mUI/L for TSH (levels are for patients 4 and 5 in whom a neonatal diagnosis of primary hypothyroidism was made).

[e] Obesity was defined as body mass index > 30 kg/m^2 in adults or weight _ 97th percentile in children.

A

B

Figure 1. Schematic representations of the seven GNAS locus deletions identified in patients. A, Schematic drawings of short deletions (gray lines) and of the GNAS locus (black line). Distances of the GNAS locus are shown to scale, based on HG19. Exons are indicated as black rectangles. B, Schematic drawings of long deletions (gray lines) and a region of chromosome 20 covering the GNAS locus and the deleted genes. The scheme of chromosome 20 region is adapted from genome browser (Hg19), distances are shown to scale. For patients 2, 4, 7, (panel A) and 1 and 6, (panel B), the sequences at the breakpoints and the 5' and 3' breakpoint positions are indicated. For patients 3 (panel A) and 5 (panel B), for whom the precise deletion breakpoints were not characterized, the dotted gray line indicates the region of uncertainty. Putative breakpoint positions are also indicated.

DISCUSSION

In about 70% of cases, PHP1A/PPHP are due to haploinsufficiency caused by maternally/paternally-inherited heterozygous inactivating mutations of the *GNAS* gene[1,6]. A subset of patients not carrying mutations in *GNAS* coding exons might have a deletion removing the entire or part of the *GNAS* gene. Constitutional aberrations of the long arm of chromosome 20 are quite rare, and, to our knowledge, only 7 cases with an interstitial chromosome 20q deletion including *GNAS* have been described in the literature so far[9,10,13–15].

Here, we report 1 PPHP and 6 PHP1A patients with different submicroscopic deletions including part or the whole GNAS locus. Accordingly, we describe novel deletions in 7/112 probands (6.25%), confirming that these events represent a significant cause of PPHP/PHP1A, and that, although undetectable by standard PCR-based sequencing, they can be clearly identified by aCGH or MS-MLPA.

For a subset of PHP patients, for whom no mutations are identified by *GNAS* coding region sequencing, the presence of a deletion should be considered for a proper genetic counseling. Indeed, some patients studied for the presence of imprinting defects by methylation-quantitative techniques could be misdiagnosed as PHP1B patients, as previously indicated[10] and now confirmed by patients 1 and 5 of the present work. Unfortunately, a deep phenotypic characterization does not allow to clearly identify a priori the causative molecular defect.

Our findings suggest that some clinical features, namely mental retardation, short stature, round face, brachydactyly, subcutaneous ossifications, are common to most patients, as expected by the removal of *GNAS* Gsα-coding exons. As for the PHP1A phenotype in particular, in accordance with previously reported series showing no genotype/phenotype correlation in this disease[7,16], in this cohort there is no apparent clinical difference between patients with point mutations and those with GNAS deletions. Moreover, as described for point

mutations, intrafamilial differences in phenotypic expression can also be found in those with *GNAS* rearrangements and be not only associated to the imprinting defect (family 2 and 7 of the present report, and reference 15).

Hyperinsulinemia, namely reduced insulin sensitivity, and type 2 diabetes have been recently identified as an additional feature of adult PHP1A[17]. On the contrary, complex phenotypes (attention deficit disorder, Evans syndrome, cirrhosis, psoriasis, asthma and bilateral adrenal hyperplasia) may result from a contiguous gene deletion syndrome, as they are hardly explained by *GNAS* haploinsufficiency alone. We compared our cases with those previously described by two other groups and determined the smallest region of overlap (SRO) among deletions, in order to unveil genes contributing to common and, conversely, patient-specific clinical features[9,14] (Supplementary Figure 1). Patients with a paternal deletion suffered from pre- and post- natal severe growth retardation, dysmorphism, hypotonia and feeding difficulties, but no further similarities can be deduced as two cases, with no typical AHO phenotype, were too young and may have developed other signs over the time. As for maternal deletions, there aren't significant differences in involved genes that might explain the reported clinical differences. However, the number of patients with large deletions is too small to speculate the contribution of specific adjacent genes in determining complex phenotypes.

Single or multi-exon rearrangements in GNAS can be routinely screened by MS-MLPA or aCGH[10,18]. However, these diagnostic techniques do not provide detailed information on breakpoints location, so different deletions involving the same exon(s) cannot be distinguished, nor can insight be acquired into the developmental mechanism of these rearrangements. Therefore, we have molecularly characterized the precise deletion boundaries in 5 of the 7 probands and hypothesized the developmental mechanism of these rearrangements.

Genomic deletions can occur during the repair of DNA double strand breaks (DSB's) by several mechanisms: (i) non-replicative repair mechanisms as

non-homologous end-joining (NHEJ) or non-allelic homologous recombination (NAHR) and (ii) replicative-based repair mechanisms[19].

The major DNA-repair mechanism in humans is NHEJ, either classical with short terminal microhomologies of no more than 4 bp, or non-classical (microhomology-mediated end-joining - MMEJ). The insertion of additional bases at the breakpoint junction, as in patient 7, is a phenomenon associated to classical NHEJ, which is thus one potential mechanism for this deletion[19].

NAHR, the most common mechanism underlying disease-associated genomic rearrangements and recurrent deletions, occurs between nonallelic homologous sequences, generally repetitive elements of at least 200-bp, such as long or short interspersed nuclear elements (LINE's or SINE's, including Alu-elements) or low copy repeats (LCR's). Diverse studies demonstrated that a high content of Alu-elements results in increased frequency of gene disruption by large deletions in several human diseases, also called Alu-recombination mediated deletion (ARMD)-events[20]. We analyzed the regions deleted in our patients for the presence of any of these elements and we found repetitive elements of the same family at both breakpoints in 4 cases (patient 1, 2, 6 and 7), which could indicate NAHR as causative mechanism. Therefore, an Alu-mediated NAHR might result in deletions of patients 2, 6 and 7, while patient 1 showed LINE2 elements at proximal and distal breakpoint junctions.

Alternative mechanisms (NHEJ) appear to be involved in patient 4 deletion, as no repetitive elements were found in the proximity of the breakpoints. The most plausible cause for the deletion of patient 1 is a replicative-based repair mechanism, even though the presence of 2-bp microhomology at the breakpoint junction and deletion-associated DNA sequence motif at the proximal region could also be consistent with classical NHEJ.

Overall, although each deletion event appears to be unique, Alu sequences play a major role in determining the instability of the region. Regardless of the specific recombination mechanism, genomic architectural

features have been associated with many rearrangement breakpoints. This suggests that chromosomal rearrangements are not random events, but result from a predisposition due to the existence of a complex underlying genomic architecture that may create instability in the genome.

In conclusion, our data confirm that *GNAS* deletions must be considered as a possible and significant cause of PPHP/PHP1A. In order to avoid misdiagnosis with PHP1B and to determine the recurrence risk in the offspring, patients negative for point mutations should be further investigated for such rare genetic defects.

ACKNOWLEDGMENTS

The authors thank physicians, patients, and families who participated in the study. Euro-Pseudohypoparathyroidism network Consortium: Belgium (Kathleen Freson and Benedetta Izzi, Department of Cardiovascular Sciences, Center for Molecular and Vascular Biology, University of Leuven), France (Agnes Linglart, Caroline Silve, Stephanie Maupetit-Mehouas, and Virginie Grybek; INSERM U986, Hôpital Bicêtre, Le Kremlin Bicêtre, Paris; and Anne Barlier, Laboratory of Biochemistry and Molecular Biology, Conception Hospital, Aix-Marseille University, Marseille), Germany (Olaf Hiort, Susanne Thiele, Bettina Brix, and Ralf Werner, Division of Experimental Pediatric Endocrinology and Diabetes, Department of Pediatrics, University of Luebeck), Italy (Giovanna Mantovani and Francesca M. Elli, Department of Clinical Sciences and Community Health, University of Milan, Endocrinology and Diabetology Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan; and Luisa de Sanctis, Department of Public Health and Pediatrics, University of Turin, Reggina Margherita Children's Hospital, Turin); Spain (Guiomar Perez de Nanclares, Intza Garin, and Arrate Pereda, Molecular (Epi)Genetics Laboratory, BioAraba National Health Institute, Hospital Universitario Araba-Txagorritxu, Vitoria- Gasteiz; and Beatriz Lecumberri, Endocrinology Service, Hospital Universitario La Paz, Madrid); Turkey (Serap Turan, Pediatric Endocrinology, Marmara University School of Medicine Hospital, Istanbul), United Kingdom (Deborah J. Mackay, Wessex Regional Genetics Laboratory, Salisbury NHS Foundation Trust, Salisbury).

International clinical group for PHP: France (Professeur Patrice Rodien, Endocrinology and diabetes, Angers University, Angers), Spain [Fundación Jiménez Díaz (F. Blanco); Hospital Central de Asturias, Asturias (I. Riaño); Hospital Clínico San Carlos, Madrid (O. Pérez); Hospital General de Elda, Alicante (F. Aleixandre), Hospital de Navarra, Navarra (E. Anda, M. García, R. M. Rodríguez, and M. J. Goñi); Hospital Universitario de Alava-Txagorritxu, Álava (I. Diez-Lopez); Hospital del Mar, Barcelona (M. Bonet); Hospital General de Alicante, Alicante

(M. Zapico, P. J. López Mondejar); Hospital General de Ciudad Real, Ciudad Real (E. Palomo); Hospital Infantil Universitario del Niño Jesús, Madrid (M. T. Muñoz, O. Rubio- Cabezas, and J. Argente); Hospital Infantil Vall D'Hebron, Barcelona (D. Yeste, A. Casterás); Hospital Sant Joan de Deu, Barcelona (L. Martorell, L. Suárez, R. Cardona, G. Marti and E. Gean); Hospital Severo Ochoa, Madrid (B. García-Cuartero); Hospital Teresa de Herrera, A. Coruña (M. S. Pereira and B. Rodríguez); Hospital Universitario 12 de Octubre, Madrid (J. Sánchez del Pozo, J. Cruz-Rojo, C. Rodríguez); Hospital Universitario Príncipe de Asturias, Madrid (J. D. García Díaz); Hospital Universitario Virgen de la Arriaxaca, Murcia (E. Guillen-Navarro, C. Guillen); Hospital Universitario Virgen del Rocío, Sevilla (Gonzalez Meneses); Hospital Virgen de la Salud, Toledo (I. Luque, A. Menéndez, and A. Vicente); and Hospital Virgen del Camino, Navarra (S. Berrade and M. Oyarzabal)].

This work was supported by the Euro-Pseudohypoparathyroidism network (EuroPHP), grant from the European Society for Pediatric Endocrinology Research Unit [to AL], Attractivité Grant from Paris-Sud University 2013 [to AL], ANR EPIFEGROW (Agence nationale de la recherche) [to AL], the Italian Ministry of Health [GR-2009-1608394 to GM], recurrent funding from INSERM U986 [to AL and CS], Instituto de Salud Carlos III [PI10/0148 and PI13/00467 to GPdN]. GPdN is supported by I3SNS Program of the Spanish Ministry of Health [CP03/0064; SIVI 1395/09]. AP is funded by the Spanish Ministry of Health [FI11/00309].

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SUPPLEMENTARY INFORMATION

1. Detailed description of molecular biology techniques applied to unravel and characterize GNAS structural rearrangements in our series

1.1. GNAS locus MS-MLPA analysis

Dosage of allele segments and methylation analyses of GNAS locus were carried out by MS-MLPA using the SALSA MLPA ME031 GNAS probemix (MRC-Holland, Amsterdam, The Netherlands) and ABI3130xl and ABI3500 Genetic Analyzers (Perkin-Elmer Corp.), as previously described (S1, S2). Data analysis was performed using GeneMapper software (Applied Biosystems, Foster City, CA) and Coffalyser v9.4 (MRC-Holland, Amsterdam, The Netherlands). A probe ratio below 0.7 for different consecutive probes was considered suggestive of a heterozygous deletion.

1.2. Genome-wide SNParray

An Illumina Human660W-Quad BeadChip was run for patients #1 and #5. This array includes 657,366 markers distributed evenly across the genome, with 14,854 markers located on chromosome 20. Images were analyzed using the Chromosome Viewer tool contained in Genome Studio (Illumina, San Diego, CA, USA).

1.3. Custom CGHarray

Two independent custom aCGH have been used:

Patients #6 and #7: A custom CGH array was designed to cover by high density the GNAS locus and 15 additional genes localized on chromosome 1, 3, 6, 7, 9, 12, 16, 17, 19, 20, 22 and X (15K oligo array, Agilent Technologies, CA, USA; design ID33306). The GNAS locus (chr20: g.57414773-g.57486255) was covered by a total of 476 probes, and *STX16* gene (exon 3 and 4) by 19 probes (i.e a density of ~ one 60 mer oligonucleotide CGH probe every 150-bp). 1 000-kb flanking 3' and 5' regions at the boundary of the locus were covered by a density of ~ one probe every 4000-bp. On each slide, samples comprised DNA from one control used as reference DNA (identical on each slide), and from 7 patients

affected by one pathology putatively associated with a defect in one of the selected genes; therefore samples from non-affected patients for a specific gene served as controls for that gene. Two to four patients affected with PHP were studied on each slide. Array design was validated by analysing samples with previously identified deletion in each gene. Data were analyzed using Feature Extraction V.91.1 and CGH analysis V3.4.27.

Patients #5 and #6: Whole genome analysis was conducted using a custom chromosome 20 focused 4x44k oligonucleotide human array-CGH (AMADID 49562, Agilent Technologies, Santa Clara, CA), following manufacturer's protocol. Custom array covered chr20: g.57,200,000-g.57,500,000 with one probe each 200-bp, whole chromosome 20 with one probe each 2-kb and a backbone with one probe each 300-kb. Commercial healthy male DNA (Promega Biotech, Madison, WI) was used as hybridization control. Microarray data was extracted and visualized using Feature Extraction software v10.7 and Agilent Genomic Workbench v5.0 (Agilent Technologies). Copy number altered regions were detected using ADM-2 (set as 6) statistic provided by DNA Analytics, with a minimum number of 5 consecutive probes. Genomic build NCBI37 (Hg19) was used for the experiments.

1.4. Boundaries' delimitation

As a first step toward the delimitation of deletions, different methods for semiquantitative amplifications were used.

Semiquantitative amplification of specific sequences (about 100-bp length) surrounding the region found deleted by MS-MLPA was performed (primers available upon request). Briefly, serial dilutions of wild-type DNA were amplified by non-saturated PCR (20 cycles) together with patients DNA and, after agarose electrophoresis, were analyzed by densitometric scanning of photographs of gels with ImageJ software.

Alternatively, semiquantitative multiplex PCR of short fluorescent fragments (QMPSF) was employed. Firstly, we designed diverse PCR fragments for each flanking region. In the 5'flanking region the reverse primer was marked

with fluorescence, whilst in the 3'flanking region the marked primer was forward. These PCRs were performed vs. a known 2 copies region of the genome (as control region) to get a relative semiquantitative PCR (stopped at exponential phase). After the multiplex reaction, the DNA fragments were separated on an ABI3500 Genetic Analyzer and analyzed using GeneMapper software (Applied Biosystems).

To calculate allele dosage we used the numerical sample-to-control comparison. We calculated numerical normalized ratios (R) using the formula: $R=(\text{peak intensity}_{\text{amplicon } x \text{ sample}}/\text{peak intensity}_{\text{amplicon } x \text{ control}})/(\text{peak intensity}_{\text{amplicon ref sample}}/\text{peak intensity}_{\text{amplicon ref control}})$. An R value close to 0.5 (0.4–0.6) represented a 2-fold reduction. As a result we found regions that were located inside the deletion (PCR relative ratio~0.5) and other regions that were located outside the deletion (PCR relative ratio~1). The objective was to approach as much as possible to the deletion, for the purpose of obtaining a large PCR of the deleted allele and sequences it to identify the breakpoints. This final PCR was run with the nearest to the deletion forward unlabeled primer of the 5'falnking region (in which two copies were observed) and the nearest to the deletion unlabeled reverse of the 3'flanking region (conditions and primers available upon request).

Then, to locate breakpoints at nucleotide level, long range PCRs with primers flanking deleted sequences and a highly performing proofreading polymerase, LA Taq DNA Polymerase (Takara Bio Inc., Otsu, Japan), were performed (conditions and primers available upon request).

Finally, amplicons from deleted alleles were excised from agarose gel after electrophoretic separation, extracted with the MinElute Gel Extraction kit (Qiagen, Hilden, Germany) and sequenced.

1.5. Parental origin of deletions

When possible, deletion's parental origin was determined either by long-PCR crossing the boundaries, by microsatellite markers analysis or deduced by the methylation status obtained by MS-MLPA. Three microsatellites were genotyped by migration of the fluorescent- labelled PCR products on a 3130xl Genetic Analyzer (AppliedBiosystems), a 18AC repeat (g.58,304,592-g.58,304,928) localized at the 5' end of the GNAS locus, a 28AC repeat (g.58,439,667-g.58,440,096) within the GNAS locus, and a 19AC repeat (g.58,529,668-g.58,530,005) localized at the 3' end of the GNAS locus.

1.6. In silico analysis of breakpoint regions

Breakpoint regions and junction fragments were subjected to an extensive bioinformatic analysis to assess the involvement of the genomic architecture in the origin of the deletions as previously described (S3).

2. Detailed description of novel GNAS structural rearrangements identified in our series

2.1. Short deletions

MS-MLPA investigation of sisters #2a and #2b led to the hypothesis of a deletion encompassing, at least GNAS exons from 7 to 13. Breakpoint characterization and parent's analysis confirmed a maternally-inherited interstitial deletion of 5,842-bp at genomic location g.57,482,794_57,488,635, according to the Genome Reference Consortium Human Build 37 (GRCh37) (Figure 1A). Their mother (#2m) presented only short stature with normal serum calcium, phosphorous and PTH levels.

In patient #4, MS-MLPA revealed a heterozygous deletion within GNAS exon 13 and subsequent analysis showed a 106-bp *de novo* interstitial deletion including part of intron 12 and of exon 13 (genomic location g.57,485,642_57,485,748) (Figure 1A).

According to custom made CGH analysis, patient #7a presented a heterozygous deletion of about 6200-bp extending from intron 2 (first 5'deleted

probe at 57,475,366) to intron 5 (last 3'deleted probe at 57,481,550), and thus leading to the partial deletion of introns 2 and 5, and the entire sequence of exons 3, 4 and 5, and introns 3 and 4. MS-MLPA analysis confirmed the deletion. Fine delimitation allowed the identification of a 6,431-bp deletion ranging from locations g.57,475,484 to g.57,481,914. Within the deletion a 16-bp insertion of unknown genetic origin was identified (Figure 1A). The same genetic defect was present in the mother of the patient (#7b).

In patient #3 the deletion could not be delimited at the nucleotide level due to lack of further material, but available data allowed to determine a deletion extension range between 2,793 and 6,928-bp [g.(57,463,738_57,464,100)_(57,466,893_57,470,666)del] (Figure 1A). Parents were not available.

2.2. Long deletions

In patient #1, SNP array followed by quantitative PCR and long-PCR revealed the presence of an heterozygous maternally inherited deletion of 2.6-Mb, extending from g.57,224,346 to g.59,795,557 (Figure 1B). The deletion encompassed *STX16, NPEPL1, GNAS-AS1, GNAS, NELFCD, CTSZ, TUBB1, SLMO2, ATP5E, ZNF831, EDN3, PHACTR3, SYCP2, PPP1R3D* and *CDH26* genes. The mother was clinically asymptomatic.

A similar approach was performed for patient #5. The statistical deletion obtained after SNP-array and aCGH extended from g.56,987,684 to g.59,350,455 (size 2,362,772-bp), though data was also compatible with a deletion beginning at 56,982,790 bp (size 2,367,666-bp). Exact breakpoints however could not be determined as it couldn’t be amplified and sequenced (Figure 1B). Putatively involved genes are *VAPB, APCDD1L, APCDD1L-AS1, STX16, NPEPL1, GNAS-AS1, GNAS, NELFCD, CTSZ, TUBB1, SLMO2, ATP5E, ZNF831, EDN3, PHACTR3, SYCP2, PPP1R3D* and *CDH26*. The deletion was present in the unaffected mother and the maternal grandfather.

In patient #6 custom made CGH analysis led to the identification of a 1.7-Mb *de novo* deletion extending from g.55,926,306 to g.57,675,816 (Figure 1B).

Microsatellite markers genotyping of the proband and her parents confirmed the paternal origin of the deletion. The deletion encompassed *MTRNR2L3, RBM38, CTCFL, PCK1, ZBP1, PMEPA1, PPP4R1L, RAB22A, VAPB, APCDD1L, APCDD1L-AS1, STX16, NPEPL1, GNAS-AS1, GNAS, NELFCD, CTSZ, TUBB1, SLMO2* and *ATP5E* genes, and putatively the 3'-end of *RAE1*.

2.3. In silico analysis of breakpoint regions

In total, 2 of 8 sequence motifs were present. An immunoglobulin heavy chain class switch repeat in the proximal region of patient #1 and deletion hotspot consensus sequence, at the distal region of patient #4. The other breakpoint regions did not contain any known sequence motif.

The analysis of the AT-percentages at sequences located 125-bp down- and upstream of proximal and distal breakpoints showed AT-enriched regions (≥75%) on both breakpoints for patient #2.

The Repeat Masker track in the UCSC genome browser was used to analyze the presence of known repetitive elements intersecting breakpoints. A repetitive element was found at 4 of 5 breakpoints (80%), all of them belonging to the same class, consisting of three Alu- Alu and one LINE2-LINE2 combinations. In these cases, a Blast2 analysis was performed to determine the percentage of sequence identity between the repetitive elements. The highest percentage of sequence identity was observed between Alu elements in patient #2 (88%), while the lowest between LINE2 elements in patient #1 with only 13-bp complementarity in both LINEs.

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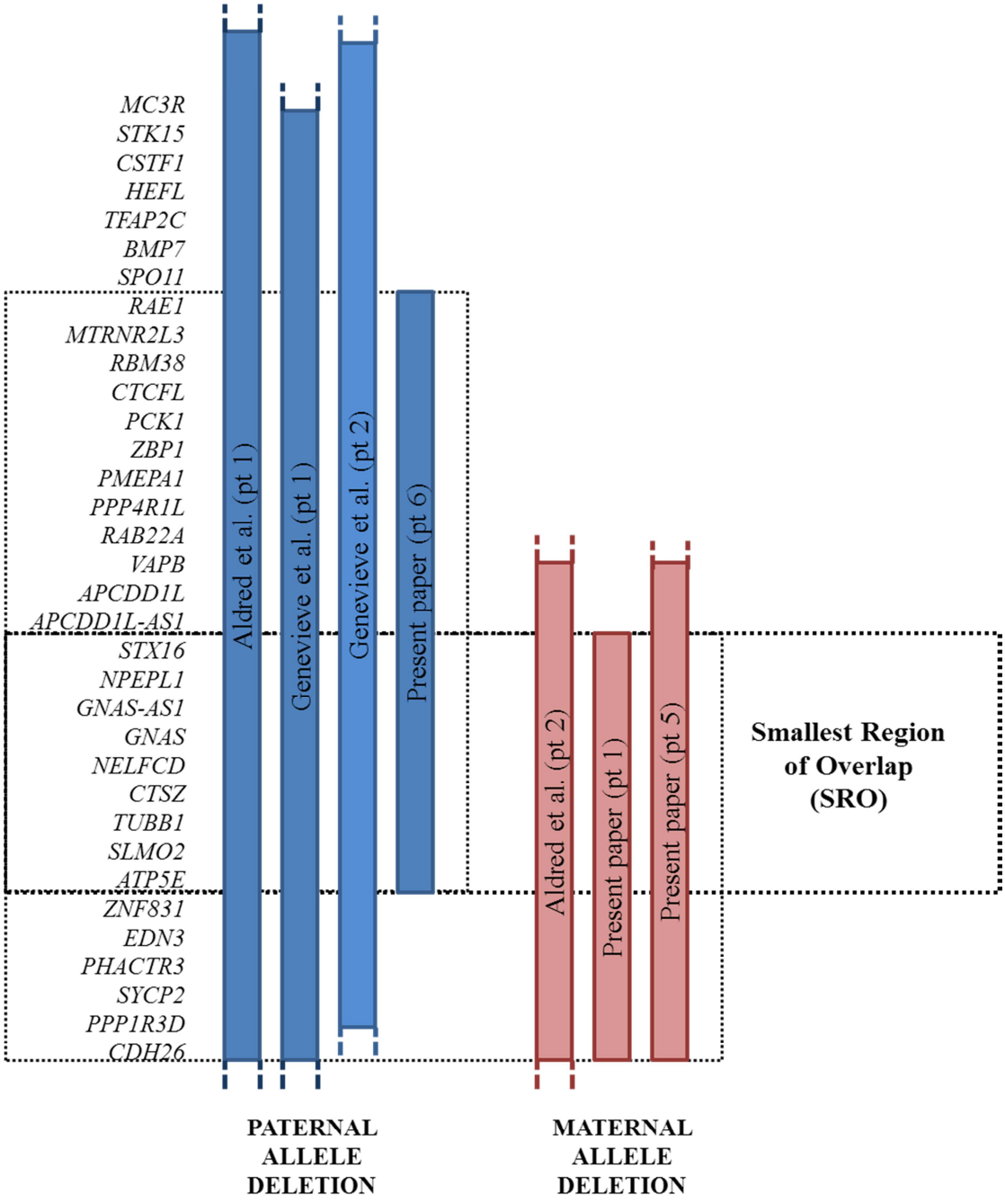
Supplementary Table 1: Brief summary of the techniques employed in the analysis of each patient.

Patient	MS-MLPA	SNP array	Custom aCGH 15k	Custom aCGH 44k	Sq-PCR	QMPSF	Long range PCR
1	✔	✔				✔	✔
2	✔				✔		✔
3	✔						
4	✔				✔		✔
5	✔	✔		✔		✔	
6			✔	✔			✔
7			✔				✔

Supplementary Table 2: Overview of the characteristic of the deletion breakpoints. The positions of the proximal and distal breakpoints or breakpoint regions were given according to the UCSC Human Genome Browser assembly from February 2009 (GRCh37/hg19). Breakpoint sequences are given, including 25 bp down-and upstream from the breakpoints or breakpoint regions if homologous sequences (highlighted in bold) were present at the breakpoint junctions. Down-and upstream borders of the deleted sequences are indicated in small characters. Alternating purine-pyrimidine sequences are underlined. Finally, DNA sequence motifs associated with deletions are marked in grey, while the motif sequence, repetitive elements identified, the identity between the repetitive elements and the plausible molecular mechanisms for each deletion can be found in the last columns. NHEJ (Non-homologous end joining). ARMD (Alu recombination-mediated deletion).

Patient	Start (hg19)	End (hg19)	Size (bp)	Breakpoint Sequences	Micro-homology	Repetitive elements (proximal region)	Sequence Motifs (proximal region)	Repetitive elements (distal region)	Sequence Motifs (distal region)	Sequence identity between repetitive elements	Putative molecular mechanism
1	57224346	59795557	2571212	Proximal: ATCACGTTCTAGCCTGGGGGAGAGC**gc**caggaaaagtaaacaggtccccc Distal: tctgacaaatatttttttaagcacttGCTATGTTCTAGGGACTGTAACAAG	2 bp (GC)	L2c	TGGGG	L2a	-	13 bp palindromic sequence	Replicative / Classical NHEJ
2a&2b	57482794	57488635	5842	Proximal: AAGGAAACTCAGAGAAAAAGAGAACaacgcagcttaaaacttttaaaatg Distal: attttttaatctgaatgcctgtaaTCCCAGCACTTTGGGAGGCCGAGGT	-	AluSg4	-	AluY	-	88%	ARMD
4	57485643	57485747	106	Proximal: TTTTGTTTTCATATGACATCAGAGG**ctg**gctgacagccgtccctggtagg Distal: tgtttgtgcccgcagaggatcagca**CTG**CCAGTGGAGATGGGCGTCACTA	3 bp (CTG)	-	-	-	TGGAGA		Replicative / Classical NHEJ
6	55926306	57675816	1749511	Proximal: AGGAAGTGGCTCCAGGGCGCACGCGcgttgtttccgcggtagtcagggca Distal: cctcaggtgatccactcacctcagcTTCCCAAAGTGCTGGAATTACAGGC	-	AluSq2	-	AluSx	-	85%	ARMD
7	57475484	57481914	6431 + 16pb insertion	Proximal: AGCAATCCCTGTATCTCGCGTCTGTcttctgtcttccttcctttgtctgt Distal: tgtaaagatatcttctgtttgttgaTGC TGCAGAAATGCACAAAGCAGTC	-	AluSx	-	AluSg4	-	84%	Classical NHEJ / ARMD

Supplementary figure 1: Schematic representation of reported deleted regions and genes in the long arm of chromosome 20. The common deleted region in patients with a rearrangement affecting the paternal allele includes genes from *RAE1* to *ATP5E*. Deletions affecting the maternal allele are smaller than paternal ones, and encompasses genes from *STX16* to *CDH26*. The smallest region of overlap (SRO) among all described multigenic deletions of 20q ranges from *STX16* to *ATP5E*.



PUBLICATION 3

Brachydactyly E: isolated or as a feature of a syndrome

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Orphanet Journal of Rare Diseases **2013; 8:141**

ABSTRACT

Brachydactyly (BD) refers to the shortening of the hands, feet or both. There are different types of BD; among them, type E (BDE) is a rare type that can present as an isolated feature or as part of more complex syndromes, such as: pseudohypoparathyroidism (PHP), hypertension with BD or Bilginturan BD (HTNB), BD with mental retardation (BDMR) or BDE with short stature, PTHLH type. Each syndrome has characteristic patterns of skeletal involvement. However, brachydactyly is not a constant feature and shows a high degree of phenotypic variability. In addition, there are other syndromes that can be misdiagnosed as brachydactyly type E, some of which will also be discussed. The objective of this review is to describe some of the syndromes in which BDE is present, focusing on clinical, biochemical and genetic characteristics as features of differential diagnoses, with the aim of establishing an algorithm for their differential diagnosis. As in our experience many of these patients are recruited at Endocrinology and/or Pediatric Endocrinology Services due to their short stature, we have focused the algorithm in those steps that could mainly help these professionals.

Brachydactyly (BD) refers to a family of limb malformations characterized by shortening of the hands, feet or both[1]. It was added to the international Nosology and Classification of Genetic Skeletal Disorders in 2001, in the group of genetically determined dysostoses[2,3]. Different types of brachydactyly can be distinguished based on anatomic grounds, the most commonly used classification being that provided by Bell[4] and modified by Temtamy & McKusick[5]. Most types are rare, except for A3 (BDA3, OMIM#112700) and D (BDD, OMIM#113200) that have a prevalence of around 2%[1]. In this review, we focus on brachydactyly type E (BDE, OMIM#113300), which is rare and can be diagnosed as an isolated finding or as part of several genetic syndromes[1,5,6].

BDE encompasses variable shortening of the metacarpals/metatarsals, frequently with involvement of the phalanges[5]. Hertzog[7] classified BDE into three distinct varieties: type E1, with shortening of metacarpal IV, sometimes associated with shortening of metatarsal IV (possible involvement of an isolated metatarsal); type E2, with shortening of metacarpals IV and V (and metatarsals) associated with shortening of the distal phalanx of the thumb; and type E3, with various combinations of short metacarpals without phalangeal involvement[8]. However, these patterns are not held to exactly in all syndromes; as well as the distal phalanx of the thumb, others phalanges are often shortened.

In this review, we describe some common syndromes in which BDE is present, outlining their main clinical, biochemical and genetic characteristics (Additional file 1: Table S1), with the aim of establishing an algorithm for the accurate diagnosis of BDE in association with other features. Even if acrodysostosis and tricho-rinho-phalangeal syndrome cannot be considered as conditions with a pure brachydactyly E, as they have peculiar radiological features, sometimes they are misdiagnosed as syndromic BDE, so we will try to define the specific characteristics that distinguish them from all the other conditions.

One of these specific features is multihormonal resistance, mainly to parathyroid hormone (PTH) and thyroid hormone (TSH). We use this feature to

classify the syndromes into two groups depending on the presence of multihormonal resistance as many of these patients are recruited at Endocrinology and/or Pediatric Endocrinology Services due to their short stature that leads to the analysis of hormonal profile.

1. Isolated brachydactyly type E: *HOXD13* type (OMIM#113300)

While BDE is commonly reported as part of a syndrome, it occasionally appears as an isolated entity[9–11]. Four heterozygous mutations in the homeobox D13 gene (*HOXD13,* 2q31.1), two missenses and two nonsenses, have been identified as causative of isolated BDE[9–12]. In two of these four cases, an overlap between BDE and BDD (shortening of the distal phalanx of the thumb) was noted in the patients[9]. Other hand malformations that have been observed in combination with BDE are syndactyly of digits III and IV, synpolydactyly of finger IV, and long distal phalanges[9]. In addition, 21 independent mutations have been identified in *HOXD13*, most of them producing synpolydactyly (SPD; OMIM#186000), or in rare cases syndactyly, and other unspecified limb malformations[11].

Regarding BDE caused by mutations in *HOXD13*, the pattern profile showed many variations between affected patients: most of them had shortening of metacarpals III and sometimes also IV and V (Figure 1A); and in the

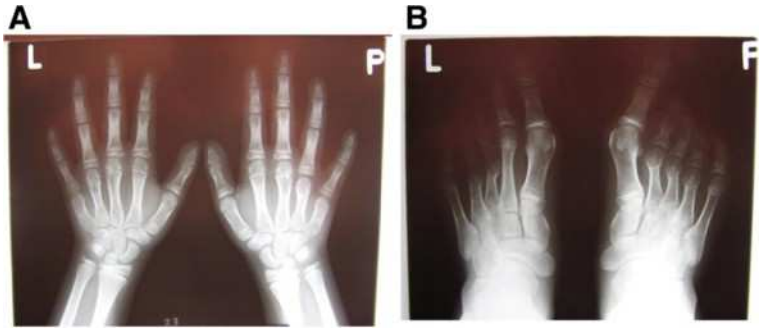

Figure 1. Radiographs of hands (A) and feet (B) of a non adult individual with heterozygous mutation in HOXD13(OMIM#113300) (courtesy of Dr. Aleksander Jamsheer). Shortening and widening of IV and V metacarpals are more evident in right; I, IV and V distal brachyphalangy, and mild clynodactyly of the distal phalanx of II and V, in left hand. In the feet, note asymmetrical shortening of the metatarsals: in right feet II, III, IV are shortened and in the left one, only III and IV. Note also, the clynodactyly of right hallux.

feet, shortening of the metatarsals IV was frequently seen, sometimes in combination with that of metatarsals I, III or V and broadening of the hallux (Figure 1B). In addition, little-finger distal phalanx hypoplasia/aplasia, lateral phalangeal duplication and/or clinodactyly of finger IV, and syndactyly of fingers III/IV were frequently observed[9,11,13]. Finally, in general, affected individuals had normal stature and no appreciable psychomotor developmental delay (Additional file 1: Table S2) [9,11,13,14].

2. Brachydactyly type E as part of syndromes

2.1. BDE with multihormonal resistance

Pseudohypoparathyroidism

Pseudohypoparathyroidism (PHP) refers to several distinct, but related, disorders in which resistance to parathyroid hormone (PTH) is the most prominent feature[15]. However, resistance to thyroid-stimulating hormone (TSH) and several other pituitary hormones that mediate their action through G-protein-coupled receptors is often present as well. As a result of PTH resistance, patients with PHP develop hypocalcaemia and hyperphosphataemia. Resistance seems to occur only in the proximal renal tubule, while the action of PTH is unimpaired in other target tissues, such as bone and the renal thick ascending limb[16–18].

BDE is present in 70-78% of individuals with PHP[19]. There are, however, great differences in hand shortening: some patients display severe shortening of all hand bones, whereas commonly in others metacarpals and distal phalanges are more impaired than other segments. In particular, Poznanski *et al.* reported that the distal phalanx of the thumb and metacarpal IV were the most affected in 75% and 65% of patients respectively[20]. On the other hand, while de Sanctis *et al.* did not observe differences in prevalence of shortening, they reported that metacarpal V and distal phalanges I and IV were the most severely affected (85.7% < −2 SDS) (Figure 2)[19]. They also noted variations in the patterns of bone shortening between subjects with the same mutation, within the same family as

well as among unrelated individuals[19]. Most publications on PHP only mention shortening of metacarpals III, IV and V or metatarsals, but it should be clarified that the shortening of these bones is usually more evident than shortening of phalanges; the latter may also occur, but is more difficult to assess because of the wide range of variation within the normal population[21].

PHP is associated with alterations in the *GNAS* locus, which maps to the telomeric end of the long arm of chromosome 20 (20q13.2-20q13.3)[22]. *GNAS* exemplifies a locus of high complexity[23]. One of its products is the α-subunit of the stimulatory heterotrimeric G protein (Gsα), a ubiquitous signalling protein that is essential for numerous different cellular responses. Gsα expression is biallelic in most tissues, but paternal Gsα expression is silenced in a few types of tissue (renal tubule, thyroid gland, hypophysis and gonads)[24–26] and this is important in the development of phenotypes associated with *GNAS* mutations. Maternal imprinting (and thus paternal expression) occurs on XLαs, the large form of Gsα, and two non-coding RNA molecules, the A/B transcript and the NESPas antisense transcript. On the other hand, paternal imprinting is seen on NESP55, a chromogranin like neuroendocrine secretory protein[27].

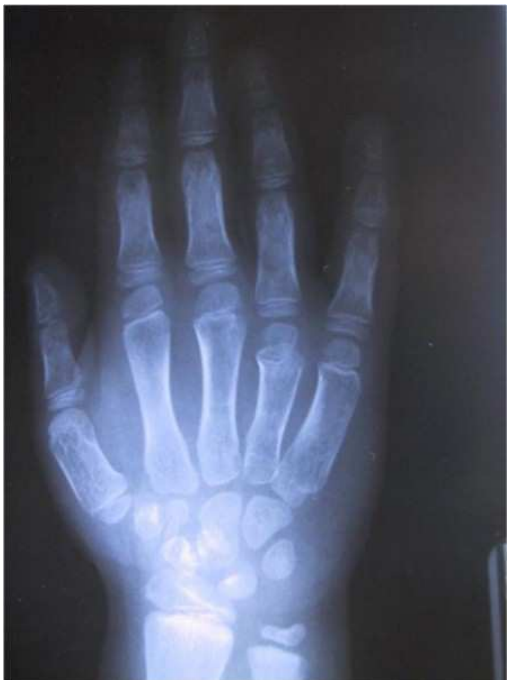

Figure 2. Hand of a patient with pseudohypoparathyroidism type Ia (OMIM#103580). Note the shortening of first metacarpal and proximal phalanx of the thumb and more severe shortening of metacarpals IV and V (courtesy of Dr. Beatriz Garcia-Cuartero).

Heterozygous inactivating mutations within Gsα-encoding *GNAS* exons, leading to diminished activity, are found in patients with PHP-Ia (OMIM#103580). These patients have not only resistance to hormones but also the Albright's hereditary osteodystrophy (AHO) phenotype (as an example, see Figure 1 at Miao et al[28]) (Additional file 1: Table S3)[27]. This phenotype includes BDE (Figure 2), small stature, obesity with a rounded face, subcutaneous calcifications, and mental retardation[29]. Patients with AHO features but no evidence of hormone resistance are said to have pseudopseudohypoparathyroidism (PPHP, OMIM#612463), and also carry heterozygous inactivating Gsα mutations. Maternal inheritance of such a mutation leads to PHP-Ia, AHO and hormone resistance, while paternal inheritance of the same mutation causes PPHP[27].

On the other hand, PHP-Ib (OMIM#603233) is another form of PHP which can follow an autosomal dominant (AD) trait (AD-PHP-Ib) or occur as a sporadic disorder. These patients present with PTH and sometimes TSH resistance, but resistance for other hormones is not found. Apart from this, patients often lack AHO features and exhibit normal Gsα activity in erythrocytes and fibroblasts. Nevertheless, there have been some reports of patients with mild AHO[30–33] and these individuals have diminished Gsa activity[34]. Generally, AD-PHP-Ib is caused by specific deletions in the syntaxin 16 gene (*STX16*) (*STX16del4-6* or *STX16del2-4*); which are associated with maternal inheritance and cause loss of methylation at *GNAS* exon A/B or 1A. Other form of AD-PHP-Ib, due to maternally inherited microdeletions of *NESP55* identified within *GNAS*, produces a loss of all maternal *GNAS* methylation imprints[35,36].

Sporadic PHP-Ib cases also display *GNAS* imprinting abnormalities that involve NESPas, XLαs and A/B. However, the genetic lesion, if any, underlying these epigenetic defects remains to be discovered and most of these cases could represent true stochastic errors in early embryonic maintenance of methylation[37–39]. Some of these patients have been shown to be affected by paternal uniparental isodisomy (pat20iUPD) involving part or the whole long arm of chromosome 20, which includes the *GNAS* locus[39–42]. A mild BDE and AHO

phenotype has been reported in some studies[30,31,43]; in most cases, only metacarpal IV and/or V shortening was seen, although mild shortening of all metacarpals and some distal phalanges has also been described[33]. Sanchez *et al.* found BDE in 60% of patients with PHP-Ib in their study (no subjects presenting shortening of distal phalanx I). In two patients, BDE was combined with Madelung-like deformities (involvement of the distal ulna and radius)[43]. On the other hand, Mantovani *et al.* did not find any correlation between the severity of the AHO phenotype and methylation defects, their patients' phenotype ranging from mild to severe AHO[33].

Acrodysostosis with multihormonal resistance (ACRDYS1, OMIM#101800)

Parathyroid hormone-related protein and G-proteins are part of the same signalling pathway affecting cartilage differentiation and growth in the metacarpals. This could explain the similarity of PTHLH-related BDE, pseudohypoparathyroidism and acrodysostosis.

Acrodysostosis is a rare skeletal dysplasia characterized by severe generalized brachydactyly of hands (Figure 3) and feet with a relatively long first thumb, dysostosis, short stature and facial abnormalities: typically a round face with maxillary and nasal hypoplasia; but sometimes also an increased mandibular angle, mandibular prognathism, epicanthal folds and hypertelorism (see Figure 3 Pt.7, 8, 11 at Linglart *et al.*[44]). Advanced skeletal maturation, spinal

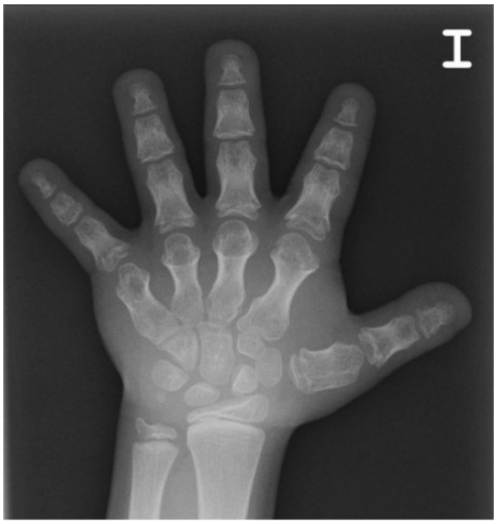

Figure 3. Hand of a patient with acrodysostosis and multihormonal resistance (OMIM#101800). Severe and generalized brachydactyly, through very short and broad tubular bones, including ulna can be observed. Metacarpals II-V are proximally pointed and cone-shaped proximal phalangeal epiphyses are prematurely fused. The general appearance of the hand is bulky and stocky (courtesy of Prof. Dr. Jesús Argente).

stenosis, obesity, mental deficiency, notably delayed speech and impaired hearing are also frequently observed (Additional file 1: Table S4)[44–51]. Although Maroteaux & Malamut defined this pathology as a new syndrome distinct from PHP/AHO in 1968[47], these two entities have been confused many times because they share many features[44]. One of these is multihormonal resistance to PTH or TSH[52], though it is not always present as there are several forms of acrodysostosis and the genes involved are different (see below)[44]. In the case of acrodysostosis with multihormonal resistance (ACRDYS1, OMIM#101800), the gene responsible is cAMP-dependent protein kinase A (*PRKAR1A*), which has been localized to 17q24.2 and is involved in the signalling pathway downstream of *GNAS*. This is the reason for resistance to PTH and other hormones, as in PHP[44,48].

The characteristic feature in this syndrome is severe BDE, characterized by shortening of metacarpals/metatarsals and phalanges II-V, phalangeal epiphyses being cone-shaped and prematurely fused, and affected bones appearing bulky and stocky (Figure 4)[44,45,48], while thumbs and halluces tend to be less affected[44,52,53]. In addition, a decreased interpedicular distance has been reported in 75% of patients. These severe brachydactyly and vertebral abnormalities have not been noted in PHP patients, so they could help us to differentiate between these two syndromes[45,52]. Finally, another type of feature that could aid the differential diagnosis is subcutaneous ossification, as this is not seen in acrodysostosis[45,52].

2.2. BDE without multihormonal resistance

2.2.1. Short stature

Bilginturan BD or hypertension with brachydactyly syndrome (HTNB, OMIM%112410)

Bilginturan *et al.* were the first to describe this syndrome in a large non-consanguineous Turkish family[54]. Subsequently, other authors have also studied this family and its new generations[55–66]. Affected members of the family had

severe hypertension (30 mm Hg difference between affected and unaffected family members) from childhood[54] and a stroke by the age of 50 years if untreated[55]. Almost all the affected children have systolic blood pressure values over the 99th percentile[66]. However, the renin-angiotensin-aldosterone system (supine and upright) and circulating catecholamines respond normally, patients were not salt sensitive, their renal function was normal, they had no retinopathy or cardiac enlargement and the increase in radial wall thickness was minimal[55,56,59]. Affected individuals also presented BDE, were on average 10 cm shorter and had lower mean birth weight than their unaffected relatives, as well as having a stocky build and rather round face (see Figures 2A & 3A at Bilginturan et al.[54]) (Additional file 1: Table S5)[54,55,66].

Brachydactyly among the affected Turkish family members was heterogeneous. In most cases, shortening of more than one metacarpal and metatarsal was present[54], digits IV and V being most frequently affected but, in some cases, shortening has been seen in all the metacarpals and metatarsals in this and other families[56,57,66,67]. Further, the shortening is not always symmetrical. All the phalanges of the hand were shortened, but the proximal and middle phalanges of the III and IV digits were relatively normal. Phalanges revealed cone-shaped epiphyses at proximal ends. All the phalanges of the toes were shortened and some patients presented symmetrical fusion between the middle and distal phalanges of toes IV and V, or only V (Figure 4). Additionally, shortening of carpal bones in the axial direction was also observed[54]. Bähring *et al.* compared the brachydactyly pattern profile of a 5.5-year-old Japanese patient, who carried a *de novo* (12)(p11.21p12.2) chromosomal deletion[68], with a 6-year-old boy from the Turkish family[57]. Both boys presented almost identical brachydactylies; type 16 cone-shaped epiphyses, particularly in the proximal interpahalangeal joints of digits II and V, brachyphalangy of digits II-IV and brachymetacarpalia of digits IV and V. The Japanese patient presented brachymesophalangy and cone-shaped epiphyses in digits II-V, and the Turkish boy only in digits II and V[57]. Intellectual disability, subcutaneous calcifications, obesity or differences in body mass index have not been observed[55,57,66], except

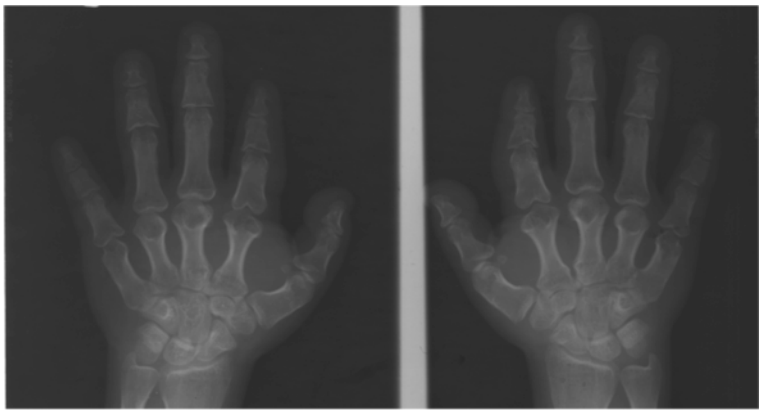

Figure 4. Hands of a patient with Bilginturan BD or HTNB (OMIM%112410). Metacarpals are short (specially II-V) with globular ends, irregular articular surfaces and cup deformity in the epiphysis of the proximal and medial phalanges. All the phalanges of the hands are also shortened, but the proximal and middle phalanges of the III and IV digits are relatively normal (courtesy of Dr. Sylvia Bähring and Dr. Okan Toka, unpublished case).

in an isolated case reported by Derbent *et al.*, where the patient presented obesity and a defect in the left renal artery[69]. It is important to highlight that brachydactyly is not appreciable until around 6 years of age and it aggravates significantly during early puberty as a result of an impaired growth spurt at the prepubertal stage. Given this, it is important to pay attention to the first signs of brachydactyly when an elevated blood pressure is detected in children (systolic blood pressure values >99th percentile)[66].

Another clinical feature in the Turkish family was the presence of aberrant posterior inferior cerebellar artery (PICA) loops only in affected members. Naraghi *et al*[70] reported 100% loop cosegregation with hypertension and brachydactyly in a group of 27 members of the Turkish family (15 affected and 12 unaffected). The prevalence of the loops was not influenced by age or sex. Further, a brief report described a case of a boy with the same syndrome not only with the PICA abnormality but also abnormal renal arteries[71]. Additional studies found that affected individuals showed less active sympathetic nerve traffic to muscle than unaffected family members and also could not buffer blood pressure-elevating effects of phenylephrine[60,65]. These findings confirmed that baroreflex blood pressure buffering was impaired in affected individuals[60,66]. The arterial baroreflex is a critical cardiovascular reflex that provides continuous buffering of acute fluctuations in arterial blood pressure due, for example, to changes in posture, exercise, and emotion[72]. PICA loops can produce

neurovascular compression of the ventrolateral medulla at the root entry zone of cranial nerves IX and X[63,70], which are very important in the baroreflex mechanism. Arterial baroreceptors are mechanoreceptors: those located in the carotid sinuses are innervated by cranial nerve IX and those in the aortic arch by cranial nerve X. When arterial pressure increases, these nerves send an input to the nucleus of the solitary tract and barosensitive neurons from here initiate a sympatho-inhibitory pathway to reduce arterial blood pressure[72].

The molecular basis for this syndrome is unknown, but several researchers have found evidence of linkage to chromosome 12p, specifically to 12p11.21-12p.12.2[55,56,73,74]. Surprisingly, in a genome-wide linkage analysis, chromosome 12p was also implicated in Chinese patients with primary hypertension (not brachydactyly)[75]. Bahring *et al.* identified a complex rearrangement involving a deletion, reinsertion and inversion of the 12p12.2-p12.1 region[64]. This region is, however, relatively gene poor, only further characterized expressed sequence tags (EST) being identified in the[64]. These authors also proposed *Kir6.1*, *SUR2*, *LSOX5* and *PDE3A*, which are within the studied segment, as candidate genes. Although, no alterations were found in these genes, a higher mRNA expression of *PDE3A* was detected in older affected family members (while the younger subjects affected showed expression values in the same range as those unaffected). On this basis, they suggested that the higher vascular expression of *PDE3A* was probably not the cause of the hypertension, but rather the result of an increase in blood pressure with age[64]. Slightly different rearrangements in this segment were found later in other families, apart from the Turkish one[65]. Finally, in this inverted sequence, a non-protein coding exon was found only to be expressed in unaffected family members; the rearrangement could be the cause for this lack of expression. This transcript was predicted to be an miRNA expressed in primary fibroblasts[65]. The fibroblasts of affected family members showed faster cell growth, but the function of this miRNA remains to be clarified[65].

More severe cases caused by deletions in the 12p11.21-12p.12.2 region were reported by Lu *et al*[74] and Nagai *et al.*[68]. One patient, with a deletion

spanning 71 annotated genes (PDE3A→BICD1), had moderate mental retardation, short stature, borderline high blood pressure (120/80 mmHg) and characteristic brachydactyly[74]. Another patient presented a more severe phenotype, mild mental retardation, short stature, high blood pressure, brachydactyly and cone-shaped epiphyses of the hands, hypoplastic hair and skin, oligodontia, a small thoracic cage and a hypoplastic pelvis[68]. The difference in the severity between the patients might be attributable to the extent of the deletion[74].

BDE with short stature, PTHLH type (OMIM#613382)

Maass *et al.* were the first to implicate the parathyroid hormone-like hormone gene (*PTHLH*), localized to 12p11.22, in BDE, after the identification of a translocation, t(8;12) (q13;p11.2) in a family with autosomal-dominant BDE and other malformations, including short extremities, dysmorphic facies, macrocephaly, prominent forehead and a depressed nasal root[76]. Despite the *PTHLH* gene not being disrupted by the translocation, it produced a downregulation of the gene and consequently of its downstream targets, *ADAMTS-7* and *ADAMTS-12*[76]. These genes are highly expressed in chondrogenically differentiated fibroblasts, so they seem to play an important role in chondrogenesis[77,78]. In a more recent publication, Maass *et al.* proposed that the downregulation of *PTHLH,* and its targets, was due to the disruption of the interaction with a regulatory element localized 24.43 Mb downstream of *PTHLH, CISTR-ACT* [79]. This element is a *cis*-regulatory element of *PTHLH* and also a *trans*-regulatory element of *SOX9* (17q)[76,80]*,* which is another essential gene in chondrogenesis whose haploinsufficiency causes skeletal malformations[81]. This regulation occurs by an lncRNA (*DA125942*), derived from the *CISTR-ACT* locus, that coordinates the expression of *SOX9* and *PTHLH* generating a loop in the chromatin[79,80]. The translocations t(8;12) (q13;p11.2) and t(4;12) (q13.2-13.3;p11.2), later identified in another family, prevent the formation of such a loop and result in overexpression of the lncRNA and misexpression of *PTHLH* and *SOX9*[79,80].

In an independent study, a deletion and three point mutations in the coding sequence of *PTHLH* were found to be responsible for BDE with short stature[82]. Further, in an isolated case of symmetrical enchondromatosis caused by a *de novo* duplication of 12p11.23 to 12p11.22 including *PTHLH*, the patient showed BDE with cone-shaped phalangeal epiphyses[83].

As in the previous group, the pattern of brachydactyly varies considerably. Several authors have reported shortening of metacarpals III, IV and V in most cases, while proximal, middle and distal phalange involvement was variable and shortening and cone-shaped epiphyses on middle and distal phalanges II and V were frequently mentioned (Figure 5)[76,82,83]. In two cases (one patient with a deletion involving another 6 genes and the second a carrier of a small duplication), metacarpals I and II were also shortened[82,83], and in the case of translocation t(8;12) mentioned above, shortening of metacarpals I , III, IV and V was observed (Maass *et al.*; IV:2 patient)[76].

Apart from BDE, other features were seen in these patients (Additional file 1: Table S6): most but not all had short stature[76,82], tooth problems were reported by Klopocki *et al.* in two out of five families[82], and those affected with translocation t(8;12) had dysmorphic facies with macrocephaly[76]. Neither hypertension nor mental retardation was mentioned, but learning difficulties were reported in one family[82].

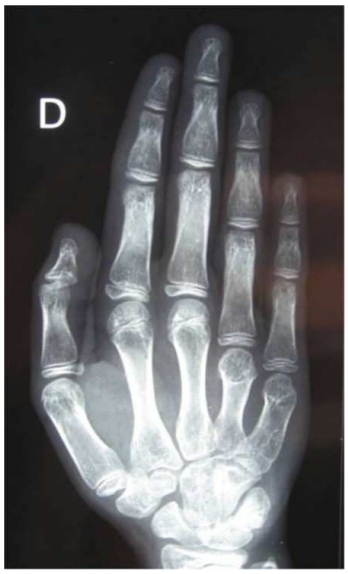

Figure 5. Right hand in a non adult individual with heterozygous mutation in PTHLH. Severe shortening of IV and V metacarpals is clearly observed (courtesy of Dr. Cécile Teinturier-Thomas, personal collection; Dr. Caroline Silve, molecular diagnosis, unpublished case).

2.2.2. AHO-like

Brachydactyly mental retardation syndrome (BDMR, OMIM#600430)

Brachydactyly mental retardation syndrome (BDMR), also referred to as Albright hereditary osteodystrophy-like syndrome, is a rare pathology characterized by variable features: short stature, obesity, developmental delay, behavioural disorders, autism spectrum disorder and craniofacial and skeletal abnormalities, like BDE (see Figure 1 at Fernandez-Rebollo *et al.*[84]) . Major malformations have also been reported, including cardiac, tracheal, gastrointestinal, genitourinary, CNS and skeletal abnormalities (Additional file 1: Table S7)[85,86].

This syndrome is caused by deletions in 2q37. Recently, the minimum critical region responsible for this syndrome has been mapped to the 200 kb which involve the histone deacetylase 4 gene (*HDAC4*), localized exactly to 2q37.3[87]. In addition, point mutations in *HDAC4* have been found in two patients with BDMR[88]. On the other hand, a patient with a deletion in 2q37.3, but not including *HDAC4*, exhibited a similar phenotype but without BDE, cardiac or other major malformations[88]. Given this, it was proposed that *HDAC4* haploinsufficiency could be causative of BDE in this syndrome[88]. A recent report, of a family case with a microdeletion involving *HDAC4* and other two genes, puts this statement in doubt, because none of the three affected patients showed BDE[87]. So far, we know that BDE is a variable feature and only reported in about a half of patients with 2q37 microdeletions[87].

Histone deacetylase 4 is involved in bone, muscle, neurological and cardiac development[89]. Felder *et al.* demonstrated that *FARP2*, *HDLBP* and *PASK* genes were downregulated in a patient with a 2q37.3 terminal deletion compared to levels in his healthy family members and controls[90]. These are good candidate genes because they are involved in neuronal and/or skeletal development[90], but point mutations in these genes which cause BDMR or other similar syndrome have not yet been reported in the bibliography, except for the case of a patient with autism associated with a *de novo MECP2* duplication who

also had a second *de novo* duplication of 2q37.3 material involving *PASK* and *HDLBP*. However, this patient did not have an AHO-like phenotype[91].

BDE is only penetrant in 50-60% of cases and the pattern profile is variable [88,92]. Frequently, metacarpals III and IV and metatarsal IV are shortened, but other metacarpals (II-V) have also been found to be affected, generally sparing metacarpal/metatarsal I[86,88,90,93–98]. Further, individuals with similar deletions or family members with the same deletion may present different phenotypes; this could be a result of incomplete penetrance of the haploinsufficiency genes, epigenetics or regulation by other genes[92].

It is worth mentioning that there is a very similar syndrome, but without BDE, known as Smith-Magenis syndrome (SMS; OMIM#182290) which is caused by deletions in 17p11.2 or mutations in the *RAI1* gene[88]. In addition, *RAI1* expression is reduced in BDMR cases[88]. Hence, when individuals are suspected of having the BDMR phenotype, but no 2q37 deletions or *HDAC4* mutations are detected, deletions at 17p11.2 and the *RAI1* gene should be analysed.

Acrodysostosis without multihormonal resistance (ACRDYS2, OMIM:#614613)

In acrodysostosis without hormonal resistance, the affected gene is the phosphodiesterase 4D, cAMP-specific (*PDE4D*) gene, localized to 5q11.2[44,46,49,99]. Clinical features (Additional file 1: Table S4) are similar to ACRDYS1 apart from PTH resistance not generally being present[44,99], though an exception was reported by Michot *et al.*[46]. Other features it has been suggested may differ in the two types are: (i) facial dysostosis, which is more evident in ACRDYS2 than in ACRDYS1; and (ii) intellectual disability, which is common in ACRDYS2, while ACRDYS1 has only been associated with some behavioural disorders and/or learning difficulties[44,46]. On the other hand, in the study of Lee *et al.* all patients (three with *PDE4D* and two with *PRKAR1A* mutations) had midface hypoplasia and there were no differences in developmental disability according to the gene affected (among those with *PDE4D* mutations, one was not affected, a second was mildly affected and a third was significantly affected; whereas both patients

with *PRKAR1A* mutations had mild developmental disabilities)[46]. Lynch *et al.* described eight patients; all of them had some degree of learning difficulties, developmental delay and intellectual disability[99]. These authors suggested that in patients with acrodysostosis associated with normal stature (only 5/15 presented short stature[46,49,99], progressive obesity (frequently developing after 6 years of age), and mild intellectual disability but no PTH resistance, *PDE4D* rather than *PRKAR1A* mutations should be suspected. BDE, stenosis of the lumbar spine, nasal hypoplasia, a flat nasal bridge and short stature are features shared by both subtypes of acrodysostosis (see Figure 3 Pat. 15, 16 at Linglart *et al.*[44]). In addition, some cases of hypogonadism have been found with defects in either gene[44,49,99].

Thus, it is difficult to distinguish acrodysostosis cases with *PDE4D* mutations from those with *PRKAR1A* mutations on the basis of clinical observation only[49]. Therefore, biochemical studies of calcium and phosphorus metabolism are essential.

Tricho-rhino-phalangeal syndrome (TRPS)

This syndrome is classified into three types: TRPS I (OMIM#190350), TRPS II (OMIM#150230) and TRPS III (OMIM#190351). Mutations in the trichorhinophalangeal syndrome I gene (*TRPS1*), localized to 8q23.3, have been found in 88% of cases of TRSP I and TRSP III[100]. Lüdecke *et al.* described TRPS III as an extreme of the clinical spectrum of TRSP I[100]. On the other hand, TRPS II is a contiguous gene syndrome involving *TRPS1* and *EXT1* genes; patients develop cartilaginous exostoses in addition to the features seen in TRPS I and III[100,101].

The striking features shared by the three types are sparse, slowly growing scalp hair, laterally sparse eyebrows, a bulbous tip of the nose (pear-shaped nose), long flat philtrum, thin upper vermillion border and protruding ears (see Figure 3 Momeni *et al.*[102]) (Additional file 1: Table S8)[100]. In radiography, the main findings are: short stature, BDE, mild metaphyseal convexity during the first year of life and premature fusion of the growth plates of the tubular bonds[100]. Additionally, hip malformations (coxa plana, coxa magna or coxa vara) have been

reported in more than 70% of cases and degenerative arthrosis appears in older individuals[100]. Patients show a retarded skeletal age until puberty, and then an accelerated skeletal age, while the mean length at birth is normal[100]. That is, the growth retardation is a progressive process that can be appreciated in metacarpophalangeal pattern profile (MCPP) analysis comparing children and adults, and comparisons of their relative height[100]. Growth hormone deficiency has been reported in some cases[103–105]. Intellectual disability has also been reported in the three types, but most commonly in TRPS II[100,101,106,107].

Regarding brachydactyly, shortening of the metacarpals has been reported in about half of patients and cone-shaped epiphyses of middle phalanges, type 12/12A (only appreciable after infancy in early childhood, before epiphyses fuse), in almost all patients, in most cases in mesophalanges II and III. Outcarving and deformation of the cones is another striking feature and this is more easily appreciated after epiphysis fusion (Figure 6)[108,109]. In addition, ivory cones were reported by Giedion (again, a feature that is only appreciable after infancy in early childhood, before epiphyses fuse). Hypoplasia of the thumb, as well as shortening of metacarpals II-V and of middle phalanges II and V is frequently seen, although shortening of all middle phalanges has been also reported[101,106,108,110], as have small feet and a short hallux[110]. Poznanski *et al.*

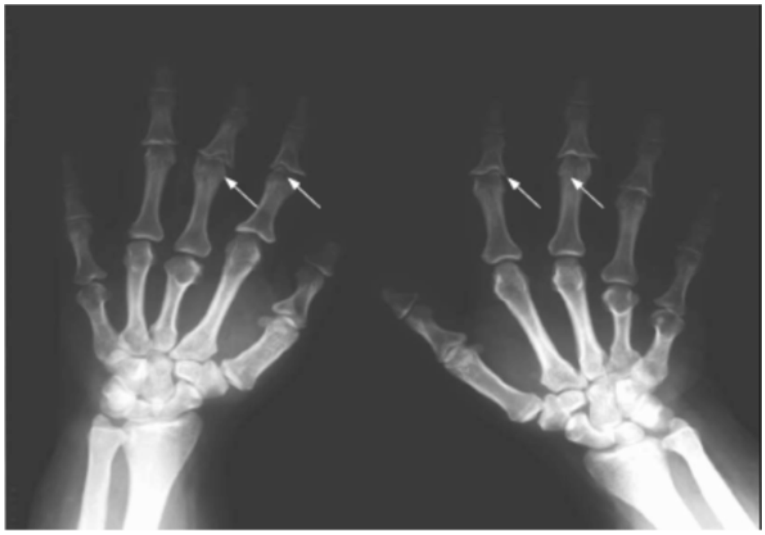

Figure 6. Hands of a patient with tricho-rhino-phalangeal syndrome I (OMIM#190350). The patient presents asymmetrical brachydactyly; metacarpals III and V are shortened on the left hand and IV and V on the right. Further, middle phalanges are shortened and cone-shaped epiphyses are shown with the typical outcarving and deformation (arrows) (courtesy of Dr. Sharona Azriel, unpublished case).

conducted MCPP analysis comparing PHP and PPHP with TRPS and found no similarity between pattern profiles in the two syndromes[20]. These data were published before the association of the syndrome with the *TRPS1* gene. More recently, Lüdecke *et al.* used MCPP analysis to compare various groups of TRPS patients; first of all, they concluded that like height differences, the brachydactyly was more marked in adults than in children. On the other hand, patients with missense mutations (TRPS III) had more severe brachydactyly than the patients with the nonsense ones (TRPS I). Given this, they classified the brachydactyly pattern with respect to the mean profiles for TRPS I and TRPS III, but these mean values were only intended for classification not to be used as a tool for diagnosis[100]. Additionally, patients with missense mutations were shorter than the nonsense carriers, but patients with gene contiguous syndrome (TRPS II) were the smallest[100]. Finally, the brachydactyly pattern is variable, as reflected in a lack of correlation between MCPPs in patients with the same recurrent mutation as well as the presence of asymmetric brachydactyly in some patients[100].

The severity of the TRPS phenotype might also depend on the size of the deletion in 8q and whether there is mosaicism for the deletion, which has been reported in four patients, two with TRPS I and two with TRPS II[101].

Turner syndrome (TS)

Turner syndrome is a chromosomal disorder with a complete or partial monosomy of X chromosome. The genetic background is variable, the most frequent karyotypes are: 45,X (~50%), karyotypes with an isochromosome of X (i(Xq) or i(Xp)), the mosaic 45,X/46XX (%30), and karyotypes containing an entire or parts of Y chromosome[111,112].

The main features are short stature (90-100%), gonadal dysgenesis, pubertal delay, primary amenorrhea (85%), estrogens insufficiency, cardiac anomalies and/or other congenital malformations like brachydactyly E (see Figure 1 at Bondy[113])[111,112,114–116]. Other frequent features are: lymphedema, congenital malformations of the urinary system (30-40%), abnormalities of the

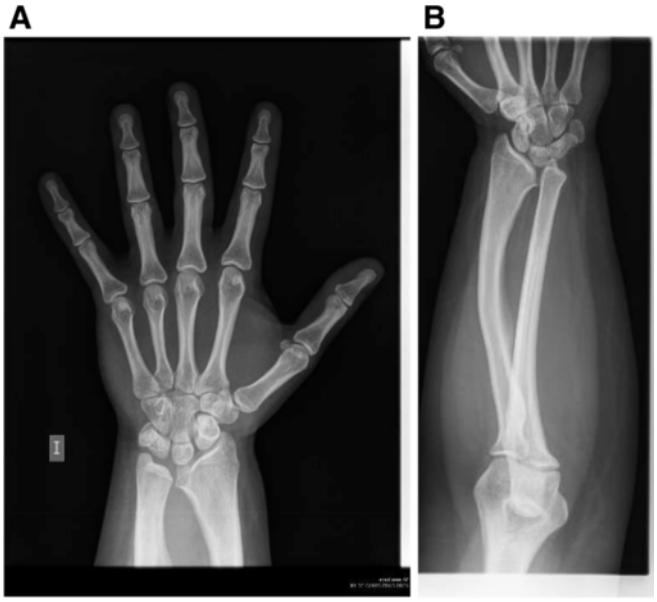

Figure7. Hand (A) and radius (B) of a patient with Turner syndrome. It can be observed the ulnar and palmar slant of the radial articular surfaces, with a triangular appearance of the distal radial epiphysis and slight shortening of the IV metacarpal; the radius is short and bowed.

external ocular appendages, (epicanthal folds, ptosis, hypertelorism, upward slanting palpebral fissures), strabismus and hypermetropia (25–35%), hearing problems and ear malformations, dysmorphic craniofacial features, abnormalities in tooth development and morphology (early eruption), melanocytic nevi, normal GH secretory pattern, altered liver enzymes¸ decreased bone mineral density, etc.[111,112,114–117] (Additional file 1: Table S9).

Regarding brachydactyly E, the more typical brachydactyly of Turner syndrome is the shortened of the IV metacarpals, but it is variable[20,114,118]. Poznanski compared the brachydactyly associated with PPHP-PHP *versus* TS and observed that the shortening of the bones are less severe in TS than in PPHP-PHP; shortening of I distal (D1) phalanx's is less frequent in TS (5% in contrast to the 75% of PPHP-PHP), so if a patient presents short fourth metacarpals and a normal D1, it is more probable that have Turner syndrome than PHP-PPHP. Finally, he mentions drum stick phalanges and thin bones in TS (Figure 7) [20].

Although TS is quite well known entity, there is often a delay in the diagnosis as over 20% of patients are diagnosed after 12 yr[115,119]. As other syndromes mentioned in this review, it is essential to be diagnosed during

infancy for proper early treatment to avoid complications linked to TS. In fact there are some publications explaining the clinical guidelines to be followed to optimize care for young woman and adults with TS[111,112,114–117].

CONCLUDING REMARKS

The BDE pattern profile can vary considerably within a syndrome, even between unrelated patients with identical mutations and among affected family members. In addition, brachydactyly is sometimes not appreciable until 6 years of age, because it is a progressive feature and tends to become more evident during early puberty.

With the aim of helping to identify the most probable genetic diagnosis to inform the clinical management of the aforementioned syndromes with BDE, we have established a diagnostic algorithm including specific differential features (Figure 8). In a multidisciplinary approach for patients with BDE, there are some very subtle but helpful clues that allow us to distinguish between some of the syndromes. (i) BDE observed in individuals with acrodysostosis can be differentiated fairly clearly because of its severity: all the bones in the hand are affected and are characteristically stocky in appearance[44,45,48]. (ii) TRPS can also be easily recognised because the MCPP is significantly different and there are also other characteristic clinical features: sparse, slowly growing scalp hair and a bulb-shaped nose are very suggestive of this syndrome[20].

Poznanski *et al.* also carried out MCPP analysis comparing isolated BDE and PHP/PPHP, but at the time these two syndromes had not been genetically well differentiated and hence the conclusions may not be reliable[20]. Indeed, patients with different pathologies could be confused if attention were to be focused on radiographic findings alone; hence, biochemical analyses should be included.

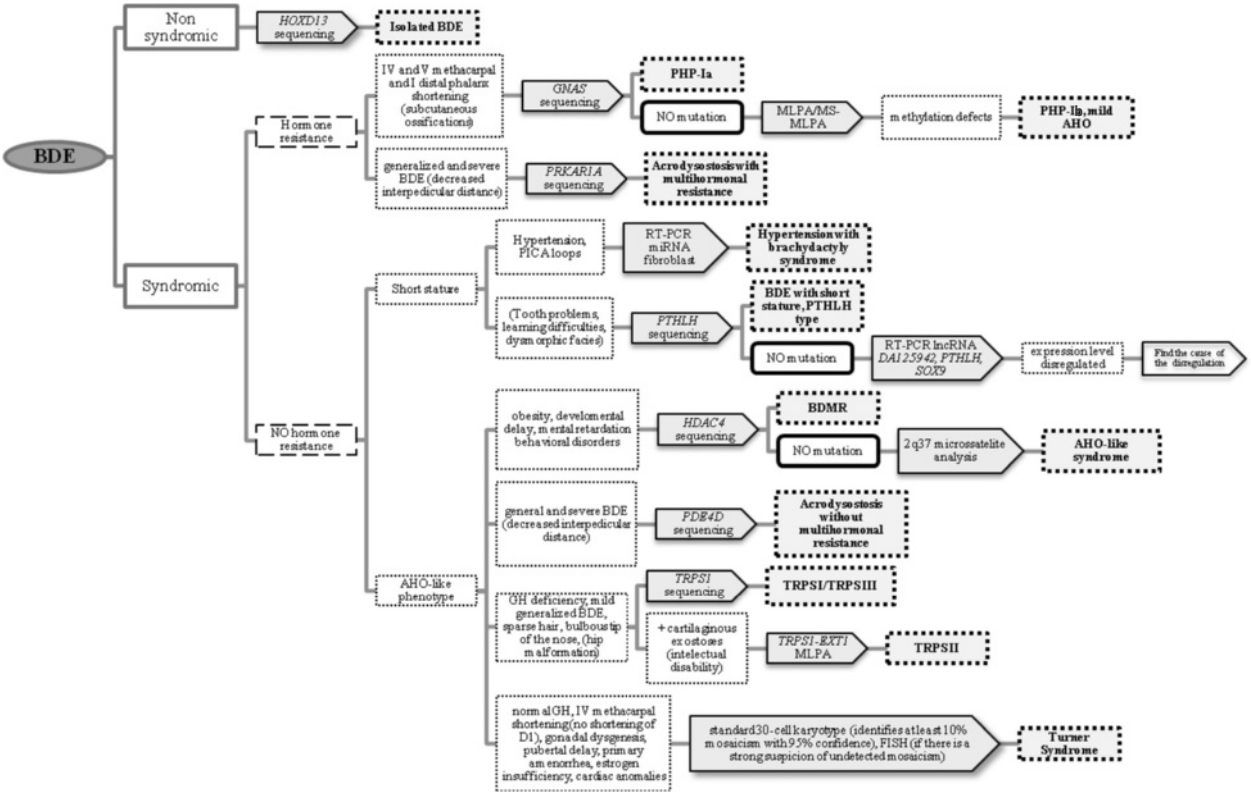

Figure 8. Proposed clinical algorithm to suggest the most probable genetic diagnosis. Features in brackets are not always present in the syndromes. BDE: Brachydactyly type E; PHP-Ia: pseudohypoparathyroidism type Ia; MLPA: Multiplex ligation-dependent probe amplification; MS-MLPA: Methylation specific-MLPA.

The other syndromes discussed have not yet been compared by MCPP analysis, but it seems that the pattern of brachydactyly does not differ appreciably between them. In such cases, when the BDE pattern is not sufficiently informative, there are other features which can help in the differentiation of each syndrome (Additional file 1: Table S1). These features are listed in the supplementary material (Additional file 1: Table S2–S9).

COMPETING INTERESTS

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

We thank all the members of the Spanish PHP group for their clinical advice and all the people that provide us with clinical radiographies to complete our review: Dr. Aleksander Jamsheer, Prof. Dr. Jesus Argente, Dr. Beatriz Garcia-Cuartero, Dr. Sylvia Bähring, Dr. Okan Toka, Dr. Cécile Teinturier-Thomas, Dr. Caroline Silve and Dr. Sharona Azriel.

This work was partially supported by the Carlos III Health Institute (PI10/0148). Mrs Arrate Pereda is funded by the Spanish Ministry of Health (FI11/00309), Dr Intza Garin by a Spanish fund for health research (FIS) programme (I3SNSCA10/01056), Dr Elena Beristain by a grant from the Spanish Ministry of Science and Innovation (MICINN; PTA2009-2147), and Dr Guiomar Perez de Nanclares in part by the I3SNS Programme of the Spanish Ministry of Health (CP03/0064; SIVI 1395/09).

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SUPPLEMENTARY INFORMATION

Supplementary Table 1: Summary of description of each syndrome. ACRDYS: acrodysostosis; BDE: brachydactyly type E; BDMR: Brachydactyly mental retardation syndrome; PHP: pseudohypoparathyrodism; TRPS: Tricho-rhino-phalangeal syndrome.

Syndrome	omim	Gene	Localization	Resistance	Brachydactyly pattern	Other features
ACRDYS1	**OMIM#101800**	PRKAR1A	17q24.2	PTH, TSH	Metacarpal and phalanges II-V of hands and foot	Short stature, obesity, nasal hypoplasia, flat nasal bridge, advanced skeletal maturation, spinal stenosis, hearing loss and mental retardation, hypogonadism
ACRDYS2	**OMIM:#614613**	PDE4D	5q11.2	NO		
BDE with short stature, PTHLH type	**OMIM#613382**	PTHLH and DA125942 lncRNA	12p11.22	NO	Metacarpals: III, IV, V Phalanges: middle and distal involvement is variable. Middle and distal phalanges shortening of digits II and V and cone-shaped epiphyses is common	Short stature, tooth problems, (dysmophic facies, learning difficulties)
BDMR	**OMIM#600430**	2q37/HDAC4	2q37.3	NO	Metacarpals: III, IV, sometimes II-V Metatarsal: IV sparing metacarpal/metatarsal I	Short stature, obesity, rounded face, mild-moderate mental retardation

Hypertension with BDE	**OMIM%112410**	miRNA (12p12.2-p12.1)	12p11.21-12p.12.2	NO	Metacarpals: IV, V Phalanges: all but the proximal and middle phalanges of the III and IV digits relatively normal. Type 16 cone-shaped epiphyses, particularly in the proximal interpahalangeal joints of the II and V digits.	Hypertension, 10 cm on average shorter than unaffected family members, lower birth weight mean, stocky build, rather round face
Isolated BDE	**OMIM#113300**	HOXD13	2q31.1	NO	Metacarpals: III or/and IV; Metatarsals: IV; sometimes I, III or V. Broadening of the hallux. Little finger distal phalanx hypoplasia/aplasia, finger IV: lateral phalangeal duplication and/or clinodactyly, and fingers III/IV syndactyly	Generally, normal stature, normal psychomotor development
PHP	OMIM**#103580**	GNAS	20q13.2-20q13.3	PTH, TSH…	Metacarpals: III, IV, V Phalanges: I distal	Short stature, obesity, rounded face, subcutaneous calcifications, mental retardation
TRPS I/III	OMIM**#190350** OMIM**#190351**	TRPS1	8q23.3	NO	Hypoplasia of the thumb Metacarpals: II-V Phalanges: II, V middle. Sometimes shortening of all middle phalanges Type 12/12A cone-shaped epiphyses in middle phalanges, in most cases in mesophalanges II and III.	Short stature, sparse, slowly growing scalp hair, laterally sparse eyebrows, a bulbous tip of the nose, long flat philtrum, upper vermillion border and protruding ears, hip malformations, retarded skeletal age until puberty, and then an accelerated skeletal age
TRPS II	OMIM**#150230**	TRPS1-EXT1		NO		TRPSI/III features + cartilaginous exostoses
Turner Syndrome		X0		NO	Metacarpals: IV	Short stature, gonadal dygenesis, pubertal delay, primary amenorrhea, estrogens insufficiency, cardiac anomalies

Supplementary Table 2. Summary of the phenotype associated with isolated BDE *HOXD13* type[9–12].

Radiological findings

BDE

Digit V, distal phalamgeal hypoplasia/aplasia

Lateral phalangeal duplication and/or clinodactyly of finger IV

III/IV finger syndactyly

IV/V toe syndactyly with partial or complete digit duplication

Supplementary Table 3. Summary of the phenotype associated with PHP-Ia[27,29].

Clinical features
Rounded face
Short stature
Central obesity
Variable degrees of mental retardation (adult 27% and the pediatric population 64%)
Resistance to hormones PTH, TSH, gonadotropins, and GHRH (variable severity)
Hypocalcaemia, hyperphosphataemia, and elevated circulating PTH
Hypogonadism, delayed or incomplete sexual maturation, slightly hypoestrogenic, and/or infertility (particularly females)
Amenorrhea or oligomenorrhoea
Prolactin deficiency
Radiological findings
BDE
Subcutaneous ossifications

Supplementary Table 4. Summary of the phenotype of acrodysostosis[46,52,99].

Clinical features

Prenatal onset of skeletal dysplasia

Short stature

Broad face and widely-spaced eyes

Maxillonasal hypoplasia, flattening of nasal ridge, small and upturned nostrils

Prominent mandible

Hypoplastic ear helices, prominent ears

Peripheral dysostosis

Mental retardation/delayed development (77-80%)

Recurrent otitis media and hearing loss (30–67%)

Epicanthic folds (39%)

Hypogonadism (cryptorquidism, irregular menses, small hypoplastic genitalia) (29%)

Dental abnormalities (delayed tooth eruption/hypodontia) (26%)

Renal abnormalities (3%)

Limitation of extension of elbows

Enthesopathy

Obesity

Scoliosis

Heterochromia of iris

Eczema, sleep apnoea, rhinitis

Radiological findings

Severe hypoplasia of the skull

Thickened calvarium

BDE affecting all metacarpals/metatarsals and phalanges of digits II-V

Cone-shaped epiphyses with early epiphyseal fusion in hands and feet

Hyperplasia of the metatarsal and phalanges of the hallux

Decreased interpedicular distance, loss of widening in cephalocaudal direction

Increased mandibular angle (68–81%)

Supplementary Table 5. Summary of the phenotype associated with Bilginturan BD or hypertension with brachydactyly syndrome[54–66]

Clinical features

Severe hypertension (30 mm Hg difference between affected and unaffected family members)

Stroke

Aberrant posterior inferior cerebellar artery (PICA) loops (100%)

Short stature

Lower mean birth weight

Stocky build

Rather round face

Renal arteries abnormalities (one case)

Radiological findings

BDE

Cone-shaped epiphyses

Supplementary Table 6. Summary of the phenotype associated with BDE with short stature, PTHLH type[76,82].

Clinical features

Short stature

Short extremities

Dysmorphic facies

Macrocephaly

Prominent forehead

Depressed nasal root

Tooth anomalies

Radiological findings

BDE

Cone-shaped epiphyses

Supplementary Table 7. Summary of the phenotype associated with Brachydactyly mental retardation syndrome[86,88,90,92].

Clinical features

Sparse hair (20%); sparse, arched eyebrows

Rounded face or full cheeks; flat mid-face; microcephaly (10%)

Deep set eyes, up-slanting palpebrae

Short nose, deficient, notched nares, low-set columella

Short philtrum, hypoplastic Cupid´s bow of upper lip

Pinna anomalies (minor)

Inverted nipples

Major malformations (30%): Cardiac (20%), tracheal, gastrointestinal (11%), genitourinary common (11%)

CNS, skeletal less common

AHO-like phenotype; obesity, short stature (23%)

Hypotonia; connective tissue phenotype, joint laxity, umbilical and inguinal hernias (29%)

Mild-moderate mental retardation, speech delay

Autism (24%), behaviour disorders, seizures (35%), epileptic (25%), episodes of depression, hyperactivity

Eczema, asthma, recurrent infections, Wilms tumour, cystic kidneys

Radiological findings

BDE in hands and feet (50-60%)

Supplementary Table 8. Summary of the phenotype associated with TRPS[100,108].

Clinical features
Sparse, slowly growing scalp hair
Medially thick, laterally thin and sparse arched eyebrows
Bulbous tip of the nose with a broad and prominent nasal root
Long flat philtrum
Upper vermillion border
Protruding ears
Seizures
Web-shaped neck
Mild metaphyseal convexity
Premature fusion of the growth plates of the tubular bonds
Hip malformations (70%); coxa plana, coxa magna or coxa vara
Osteonecrosis of the femoral head
Cardiopathies
Renal diseases
Intellectual disability
Radiological findings
Hypoplasia of the thumb, shortening of II-V metacarpals and middle phalanges
Nonsymmetric brachydactyly
Type 12/12A midphalangeal cones
Clinodactyly of digits III, IV
Finger nail abnormalities
Small feet and short hallux
Triangular distal ulnar epiphyses
Sella turcica bridge
Cartilaginous exostoses (TRPSIII)

Supplementary Table 9. Summary of the phenotype associated with Turner syndrome[111,112,114–117].

Clinical features

Short stature (>50%)

Normal GH secretory pattern

Prominent ears (>50%)

Retrognatia (>50%)

Narrow palate (>50%)

Cubitus valgus (25-50%)

Ptosis (25-50%)

Strabismus (25-50%)

Epicanthal folds (10-25%)

Hypertelorism

Upward slanting palpebral fissures

Abnormalities in tooth development and morphology

Early eruption of the secondary teeth

Scoliosis (10-25%)

Kyphosis (10-25%)

Pectus excavatum (10-25%)

Flat feet (10-25%)

Genu valgum (<10%)

Madelung deformity (<10%)

Patellar dislocation (<10%)

Growth failure (>50%)

Chronic otitis media (>50%)

Low BMD (>50%)

Fractures (>50%)

Feeding problems (25-50%)

Sensonneural hearing loss (25-50%)

Opstructive sleep apnea (10-25%)

Articulation problems (10-25%)

Hyperacusis (<10%)

Low posterior hairline (>50%)

Lymphedema (>50%)

Nail dysplasia (>50%)

Webbed neck (25-50%)

Single palmar crease (10-25%)

Inverted nipples (10-25%)

Infertility (>50%)

Gonadal failure (>50%)

Delayed puberty (>50%)

Learning disability (>50%)

Renal malformation (25-50%)

Hypertension (25-50%)

Multiple nevi (25-50%)

Hypothyroidism (10-25%)

Cardiac problems (10-25%)

Diabetes mellitus (10-25%)

Celiac disease (<10%)

Inflammatory bowel disease (<10%)

Von Wilebrand's disease (<10%)

JRA (<10%)

Pilomatrixoma (<10%)

Liver enzymes (glutamyl transferase, alanine amino transferase, aspartate amino transferase, and alkaline phosphatise) commonly raised

Radiological findings

BDE (short IV methacarpals) (25-50%).

PUBLICATION 4

PRKAR1A and *PDE4D* mutations cause acrodysostosis but two distinct syndromes with or without GPCR-signalling hormone resistance

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Journal of Clinical Endocrinology & Metabolism **2012, 97(12):E2328 –E2338**

ABSTRACT

Context: Acrodysostosis is a rare skeletal dysplasia that is associated with multiple resistance to G protein-coupled receptor (GPCR) signalling hormones in a subset of patients. Acrodysostosis is genetically heterogeneous because it results from heterozygous mutations in *PRKAR1A* or *PDE4D*, two key actors in the mutation GPCR-cAMP-protein kinase A pathway.

Objective: Our objective was to identify the phenotypic features that distinguish the two genotypes causing acrodysostosis.

Patients and Methods: Sixteen unrelated patients with acrodysostosis underwent a candidate gene approach and were investigated for phenotypic features.

Results: All patients had heterozygous *de novo* mutations. Fourteen patients carried a *PRKAR1A* (*PRKAR1A* patients), five each a novel *PRKAR1A* mutation (p.Q285R, p.G289E, p.A328V, p.R335L, or p.Q372X), nine the reported *PRKAR1A* p.R368X mutation; two patients harboured a mutation in *PDE4D* (*PDE4D* patients) (one novel mutation, p.A227S; one reported, p.E590A). All *PRKAR1A*, but none of the *PDE4D* mutated patients were resistant to PTH and TSH. Two *PRKAR1A* patients each with a novel mutation presented a specific pattern of brachydactyly. One *PDE4D* patient presented with acroskyphodysplasia. Additional phenotypic differences included mental retardation in *PDE4D* patients. In addition, we report the presence of pigmented skin lesions in *PRKAR1A* and *PDE4D* patients, a feature not yet described in the acrodysostosis entity.

Conclusions: All *PRKAR1A* and *PDE4D* patients present similar bone dysplasia characterizing acrodysostosis. Phenotypic differences, including the presence of resistance to GPCR-cAMP signalling hormones in *PRKAR1A* but not *PDE4D* patients, indicate phenotype-genotype correlations and highlight the specific contributions of *PRKAR1A* and *PDE4D* in cAMP signalling in different tissues.

INTRODUCCTION

Acrodysostosis, once called syndrome of pug-nose and peripheral dysostosis[1] or syndrome of peripheral dysostosis, nasal hypoplasia, and mental retardation[2] and also known as Maroteaux-Malamut syndrome[3], refers to a group of rare skeletal dysplasias that share characteristic features including severe brachydactyly, facial dysostosis and nasal hypoplasia. In the early 1970s, it was considered as a new clinical and radiological entity by Robinow *et al.*[4] and Giedion[1]. The first reported cases of acrodysostosis were all sporadic[3–5]. Subsequently, observations of familial acrodysostosis with apparent autosomal dominant inheritance were reported[6–10]. However, these familial occurrences were based on phenotypic criteria and genetic inheritance of acrodysostosis has not yet been reported.

The literature describing acrodysostosis cases is confusing because upon review, some reported patients may have had other phenotypically related diseases, such as pseudohypoparathyroidism type 1A (PHP1A, MIM103580) or pseudopseudohypoparathyroidism (PPHP, MIM 612463), that share some features with acrodysostosis[11–13]. In addition, it has recently become clear that several phenotypic forms of acrodysostosis exist. A prominent question has been whether patients display or not abnormal mineral metabolism associated with resistance to PTH and/or resistance to other hormones that bind G protein coupled receptors (GPCRs) linked to Gsα, as observed in PHP1A[14].

In three unrelated patients affected with acrodysostosis with hormonal resistance (ADOHR) (OMIM 101800), we have recently identified the same heterozygous nonsense mutation p.Arg368X in the *PRKAR1A* gene encoding the cAMP-dependent protein kinase type 1A regulatory subunit protein (*PRKAR1A*)[15]. This mutation leads to a gain of function of this subunit and thus to a constitutive loss-of-function in protein kinase A (PKA), the most common effector system downstream of cAMP[16–18]. This explains both the skeletal phenotype and the multihormonal resistance. This finding led to 1) the identification of the ADOHR syndrome, 2) the first description of a group of acrodysostosis based on genetic

analysis, and 3) the hypothesis of other genes in the cAMP signalling pathway as potential candidates for acrodysostosis genetics. Accordingly, mutations affecting *PDE4D* encoding phosphodiesterase (PDE) 4D, a class IV cAMP specific PDE that hydrolyzes cAMP[19], were recently reported in a subset of acrodysostostic patients (patients with acrodysostosis and *PDE4D* mutation referred to as ADOP4 in the present manuscript)[20,21].

The current study reports the largest series of patients with acrodysostosis, allowing us to draw some phenotype-genotype correlations at the clinical, hormonal, and radiological levels. Fourteen patients harboured a *PRKAR1A* mutation, and two had a *PDE4D* mutation. All *PRKAR1A* and *PDE4D* patients presented a similar dysostosis characterizing acrodysostosis. Three patients (two each with a novel *PRKAR1A* mutation, one with a *PDE4D* mutation) presented with a distinct skeletal feature. Although all *PRKAR1A* patients were resistant to PTH and TSH, we found no evidence of hormonal resistance in *PDE4D* patients.

PATIENTS AND METHODS

Patients

Sixteen patients with acrodysostosis were included in this study. Fourteen were Western Europeans living in Belgium (one), France (four), Germany (three), Greece (one), Italy (one), Spain (three), and New Caledonia (one). One patient was from a mixed descent (Western European and West African) and one patient from Polynesian descent, both living in France. Thirteen had not been reported before, whereas three (patients 1–3) were previously reported by our group[15].

The patients were initially referred to the hospitals for the diagnosis of skeletal dysplasia at ages ranging from 2–24 yr. None of the patients' families were related or lived in the same region, and all cases were sporadic. Informed written consent was obtained from the patients and patients' parents (including for use of photographs of patients' faces) and controls for all studies.

Biochemical analysis

All biochemical measurements were performed at the referring centers using standard clinical laboratory procedures for electrolyte and hormone concentrations in blood and urine. Uri nary cAMP was measured using the cAMP Biotrak enzyme immunoassay system (Amersham, Courtaboeuf, France) as described[15] for all patients except for two patients (6 and 10; for these patients, cAMP was measured using a RIA from Innovation Beyond Limits, Germany). Thyroid peroxidase antibody tests were performed in all patients and were negative.

Responses to PTH infusion

PTH infusion tests were previously reported for patients 1–3 and six controls[15]. The PTH infusion test was performed on patient 16 as previously described[15].

Sequence analysis

Genomic DNA and RNA were extracted from peripheral blood mononuclear cells using standard procedures. Intronic and, when required, exonic primers were used to amplify the exons and intron-exon junctions for *PRKAR1A (*NM_002734) (12 exons)[15] and *PDE4D* (NM_001104631) (17 exons) (primers available on request). PCR products were analyzed by direct nucleotide sequence analysis.

RESULTS

The results are presented for the 16 patients including three reported in our initial publication[15]. All patients (seven males, nine females) had characteristic features of acrodysostosis including severe brachydactyly affecting metacarpals, metatarsals, and phalanges, with facial dysostosis and nasal hypoplasia[1,4,22–24] (see below). One patient (patient 16) was diagnosed as acroskyphodysplasia. The patients' ages at the time of the study ranged from 3–26 yr (Tables 1 and 2).

TABLE 1. Clinical and radiological acrodysostosis features of the patients

Feature	Patients															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Sex	M	F	M	F	F	F	M	F	M	M	M	F	M	F	F	F
PRKAR1A mutation	R368X	R368X	R368X	R368X	R368X	R368X	R368X	R368X	R368X	R335L	A328V	Q372X	G289E	Q285R		
PDE4D mutation															E590A	A227S
Clinical features																
Birth weight [g or (SD)]	SGA	2400	2100	1850	1780	2640	(—2.0)	(—1.9)	(—1.8)	1810	ND	2200	2530	2400	1845	3300
Birth length [cm or (SD)]	SGA	45	49	43	40	(—2.0)	(—2.0)	(—2.2)	(—1.6)	43	ND	42	46	(—0.5)	50	51
Age at study (yr)	24.0	16.0	11.0	26.0	13.0	4.5	11.0	8.6	12.6	5.0	24.0	20.4	14.0	3.0	7.0	14.0
Height (SD)[a]	**—4.0**	**—3.0**	—2.5	**—3.5**	—3.5	—2.5	—1.0	—2.5	—1.5	—3.2	**—2.9**	**—4.6**	—1.0	—1.8	0.4	**—3.0**
Facial dysostosis																
Broad face with widely spaced eyes	Yes	Yes	Yes	Yes	Yes	ND	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Maxillonasal hypoplasia (note 1)[b]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Clinical peripheral dysostosis																
Small and broad hands and feet	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Stubby digits (note 2)[c]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Note 3[d]	Yes	Yes	Yes	Yes	Yes
Big toe enlargement	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Radiological findings																
Skull																
Severe hypoplasia	Yes	Yes	Yes	Yes	ND	No	Yes	Yes	Yes	ND	No	No	No	Yes	No	No
Thickened calvarium	Yes	Yes	Yes	Yes	ND	No	Yes	Yes	Yes	ND	No	Yes	ND	Yes	Yes	ND
Hands																
Severe brachydactyly (note 4)[e]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Note 3[d]	Yes	Yes + digit 1	Yes	Yes	Yes
Cone-shaped epiphyses with early epiphyseal fusion	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes (note 5)[f]	Note 6[g]	Yes	Yes	Yes	Yes	Yes
Advanced skeletal maturation	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	ND	Yes	Yes	Yes	Yes	Yes
Feet																
Similar to the hands (note 7)[h]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Note 8[i]	Note 9[j]	Yes	Yes	Yes	Yes
Axial skeleton																
Widening lumbar interpediculate distance (note 10)[k]	No	No	No	No	ND	ND	ND	ND	ND	Yes	No	No	No	ND	Yes	Note 11[l]

F, Female; M, male; ND, not documented; SGA, small for gestational age.

[a]Values in bold represent final height.

[b]Note 1: Maxillonasal hypoplasia with flattening of nasal ridge associated with small and upturned nostrils. See also Figure 3 and Supplemental Figure 5.

[c]Note 2: Stubby digits with shortening of the terminal phalanges and nails. Unless otherwise indicated, hand digits 1–5 and foot digits 2–5 are similarly and symmetrically affected.

[d]Note 3: Brachymetacarpia affecting mostly the fourth and fifth digits. See also note 8 and Figure 4.

[e]Note 4: Severe brachydactyly affecting all metacarpals and phalanges of digits 2–5.

[f]Note 5: Also at the elbow.

[g]Note 6: Likely at the middle phalanges, but evaluation in adulthood.

[h]Note 7: Similar to the hands except for hyperplasia of the metarsal and phalanges of the big toe.

[i]Note 8: Brachymetatarsia affecting mostly the third to fifth digits. See also Figure 4.

[j]Note 9: brachymetatarsia affecting mostly the third and fourth digits. See also Figure 4.

[k]Note 10: widening occurring in the cephalocaudal direction.

[l]Note 11: acroskyphodysplasia present.

TABLE 2. Additional clinical, biochemical, and radiological features in the patients with acrodysostosis

	Patients															
Feature	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Sex	M	F	M	F	F	F	M	F	M	M	M	F	M	F	F	F
PRKAR1A mutation	R368X	R368X	R368X	R368X	R368X	R368X	R368X	R368X	R368X	R335L	A328V	Q372X	G289E	Q285R		
PDE4D mutation															E590A	A227S
Clinical features																
Age at study (yr)	24.0	16.0	11.0	26.0	13.0	4.5	11.0	8.6	12.6	5.0	24.0	20.4	14.0	3.0	7.0	14.0
BMI SD	1.8	2.3	1.0	0.6	1.5	—1.0	3.3	0.2	1.3	ND	1.0	3.2	0.3	0.9	2.2	8.7
Cognitive functions: BD/MR[a]	+/—	+/—	+/—	—/—	+/—	—/—	+/—	—/—	+/—	+/—	ND	+/slight MR	—/—	—/—	—/+	—/+
Pigmented skin spots	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	No	No	No	No	No	Yes	No
Epicanthic folds[a]	No	No	ND	Yes	Yes	Yes	Yes	ND	ND	Yes	ND	Yes	Yes	ND	Yes	Yes
Endocrine features																
Cryptorchidism[a]	Bilat	NA	Bilat	NA	NA	NA	No	NA	No	No	No	NA	No	NA	NA	NA
Tanner stage (at study)		B5	G1	B5	B3	B3		B1	G2			B5	G4	B1	B1	B5
Age at menarche (yr)	ND	12.5	NA	13–14	NA	NA	ND	NA	NA	ND	ND	10.5	NA	NA	NA	11.3
Menstrual cycles[a]		Reg[b]	NA	Irreg	NA	NA		NA	NA			Irreg	NA	NA	NA	Reg
LH (IU/liter) (<9 yr, 0.01–0.5; >9 yr, 0.5–5.0)[c]	NA	3.1	NA	5.4	1.9	<0.5	NA	0.01	NA	NA	NA	13.0	NA	ND	<0.5	8.7
FSH (IU/liter) (<9 yr, 0.01–0.5; >9 yr, 0.8–4.4)[c]	NA	8.8	NA	11.0	4.8	1.3	NA	1.6	NA	NA	NA	8.8	NA	ND	<0.7	8.0
25-Hydroxyvitamin D[a] (ng/liter) (10–40)	18	36	28	32	ND	25	22	ND	ND	37	ND	11	12	20	22	18
Treatment																
Vitamin D analog	No	No	Yes	ND	Yes	Yes	ND	Yes	Yes	ND	ND	ND	ND	No	ND	No
L-T_4	Yes	Yes	Yes	ND	ND	Yes	ND	Yes	Yes	ND	ND	Yes	ND	Yes	ND	No
Other treatments	No	rhGH	rhGH	ND	ND	rhGH	ND	rhGH	ND	ND	ND	Metf	ND	ND	ND	No

LH and FSH values are shown only for female patients. BD, Behavioral Disorder; Bilat, bilateral; BMI, body mass index; F, female; Irreg, irregular; M, male; Metf, metformin; MR, mental retardation; NA, not appropriate; ND, not documented; Reg, regular; rhGH, recombinant human GH.

[a]Features previously reported in patients affected with acrodysostosis. For reviews, see Refs. 1, 4, 22, and 24.

[b]Mother of an unaffected child.

[c]Normal ranges for age intervals in females are shown in parentheses.

Screening for *PRKAR1A* and *PDE4D* mutations

De novo missense mutations of *PRKAR1A* were identified in 14 patients, i.e. 11 in addition to the three previously reported[15]. We found that six of 11 patients carried the recurrent heterozygous mutation p.R368X (c.1101C>T) in exon 11 of *PRKAR1A*. Each of the 5 other patients carried a novel variant, two variants localized in *PRKAR1A* exon 9 (c.854A>G/p.Q285R; c.866G>A/p.G289E) and three in exon 11 (c.983C>T/ p.A328V; c.1004G>T R335L; c.1114C>T, p.Q372X) (Table 1). The remaining two patients had no mutations in *PRKAR1A*. In one patient (patient 16), we identified a novel heterozygous missense variant in *PDE4D*, c.679G>T/p.A227S. The second patient (patient 15) carried the recently reported c.1769A>C /p.E590A change[20] also in *PDE4D* in the heterozygous state. No patient presenting these clinical and radiological criteria was negative for these two genes in our series. All *PRKAR1A* and *PDE4D* mutations were absent in the parents and/or sib lings, as well as in 200 controls, and in available databases

(Single Nucleotide Polymorphism performance database/ National Center for Biotechnology Information, 1000 genomes, exon variant servers, Human Genome Mutation Database, *PRKAR1A* mutation database) (data not shown). The total number of sequences that have been analyzed in control subjects and patients with Carney syndrome is difficult to determine, but several hundred ethnically diverse families have been evaluated. In our laboratory, about 300 index cases suspected to have Carney complex or isolated primary pigmented nodular adrenocortical disease (PPNAD), myxoma, or lentiginosis were analyzed for the *PRKAR1A* gene, and none presented the nucleotidic variation presented here. In addition, 100 Western European and 100 West African controls were tested and did not present these variations. cAMP-dependent protein kinase type 1A regulatory subunit protein (*PRKAR1A*) proteins carrying each of the five novel *PRKAR1A* mutations identified in this study could be ex pressed. Each of these mutant proteins demonstrated a defect in PKA activation characterized by a reduced sensitivity to cAMP similar to that described previously for the p.R368X mutation in *PRKAR1A*[15] (Silve, C., and E. Clauser, unpublished data). A similar impairment in the response of PKA to cAMP was recently described for *PRKAR1A* carrying the p.T239A mutation[25]. Throughout the manuscript, the aminoacid substitutions in *PDE4D* identified uniquely in patients with acrodysostosis are described as mutations, although the functional impact of these substitutions has not been assessed experimentally.

Biochemical parameters and hormone values

All 14 patients carrying a *PRKAR1A* mutation were resistant to PTH, as indicated by increased circulating PTH levels ranging from 70 –296 pg/ml (160 ± 16, mean ±SEM, n = 14) (Figure 1A) (higher than the upper limit range of PTH levels), in the presence of low (one patient) or normal (13 patients) serum calcium (Figure 1B) or normal (four patients) or increased (10 patients) serum phosphate (Figure 1C) and in the absence of vitamin D deficiency or renal insufficiency. Resistance to TSH was documented by increased TSH in 12 of 14 patients with normal or low T_4 levels (Figure 1D). Two patients had normal TSH levels (Figure 1D). The two *PDE4D*-mutated patients had normal serum calcium

and T_4 levels (Figures 1B and D) but, in contrast to *PRKAR1A*-mutated patients, normal serum phosphate, PTH, and TSH (Figures. 1A, C, and D).

cAMP studies

We measured urinary cAMP levels, a marker of PTH effects on the renal proximal tubule, in eight of 14 *PRKAR1A* patients and in two of two *PDE4D* patients and plasma cAMP in five of 14 *PRKAR1A* patients and one of two *PDE4D* patients. The *PRKAR1A* patients had increased (seven of eight) or high-normal (one of eight) urinary cAMP concentrations (Figure 2A). *PRKAR1A* patients had increased (four of five) or high normal (one of five) plasma cAMP concentrations. In contrast, basal urinary levels of cAMP were normal in the two *PDE4D* patients (Figure 2A), and plasma cAMP level was normal in the studied *PDE4D* patient (Figure 2B).

A sharp rise in urinary cAMP after infusion of recombinant PTH, similar to that observed in controls, was observed in the patient with a *PDE4D* mutation (Figure 2C). *PRKAR1A* patients also had a sharp rise in urinary cAMP after infusion of recombinant PTH (Figure 2C), as previously reported[15].

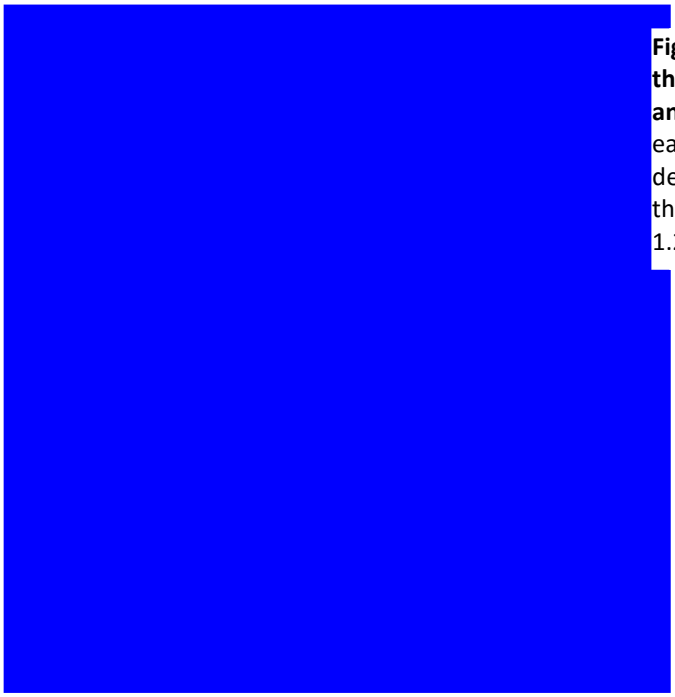

Figure 1. Biochemical data measured in the patients affected with acrodysostosis and *PRKAR1A* or *PDE4D* mutations. For each parameter, the normal range is delimited by the horizontal lines. *, For this value in panel C, normal range was 1.28 –1.92 mmol/liter.

Features of acrodysostosis

Dysostosis

Most patients (11 of 14 *PRKAR1A* and one of two *PDE4D* patients) were born small for gestational age (Table 1). All 16 patients showed typical clinical and x-ray features of the facial and peripheral dysostosis characterizing acrodysostosis[1,4,22] (Table 1, Figures 3 and 4, and Supplemental Figure 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals. org). In all patients evaluated during childhood or adolescence except two, bone age was advanced. The six patients who had reached their final height (patients 1, 2, 4, 11, 12, and 16) had severe short stature (mean -3.5 SD ranging from -2.9 to -4.6). There were, however, a few differences in the skeletal phenotype when comparing patients with ADOHR to patients with ADOP4 or when comparing individuals with ADOHR: 1) the maxillonasal hypoplasia with flattening of nasal ridge was more severe in the two patients carrying the *PDE4D* mutations compared with patients with *PRKAR1A* mutations (Figure 3 and Supplemental Figure 1); 2) a similarly severe and symmetric defect of all tubular bones of hands and feet (except for the big toe)

Figure 2. cAMP levels measured in the patients affected with acrodysostosis and *PRKAR1A* or *PDE4D* mutations. A and B, Basal urinary (A) and plasma (B) levels. Urinary cAMP under baseline conditions was elevated in the eight investigated *PRKAR1A* patients and within the normal range in the two *PDE4D* patients. C, Response to PTH infusion of patients with acrodysostosis with *PRKAR1A* or *PDE4D* mutations and control subjects. Subjects received a bolus iv injection of 40 µg recombinant human PTH1-34 as described[15]. Urinary cAMP, expressed as micromoles per millimole creatinine, was measured at the indicated times in the three *PRKAR1A* patients, one *PDE4D* patient, and six control subjects. All patients, including the *PDE4D* patient, showed a brisk rise in urinary cAMP after PTH infusion.

present in all *PRKAR1A* and *PDE4D* patients except two (patients 11 and 12), who each carry a novel *PRKAR1A* mutation (Table 1, Figure 4, and Supplemental Figure 1); 3) all *PRKAR1A* and *PDE4D* patients presented coneshaped epiphyses of the hands and feet (Table 1 and Figure 4), and patient 10 who had a novel *PRKAR1A* mutation also had early fusion of the elbow (not shown); 4) loss of widening of the lumbar interpediculate distance in the cephalocaudal direction frequently observed in *PRKAR1A* and not present in the two *PDE4D* patients (Table 1). Patient 16 carrying a *PDE4D* mutation was originally diagnosed as acroskyphodysplasia (Table 1).

Additional clinical, biochemical, and radiological features.

Although both patients with a *PDE4D* mutation presented with mental retardation, none of the *PRKAR1A* patients did. However, eight of the *PRKAR1A* patients showed some behavioural disorders and/or difficulties at school. Body mass index was low in three patients, normal or high-normal in eight patients, and increased in six, including one *PDE4D* patient. Nine patients exhibited pigmented skin lesions, including one *PDE4D* patient (Table 2 and Supplemental Figure 2). Epicanthic folds were common among the *PRKAR1A* patients as well as in the two *PDE4D* patients (Table 2).

Figure 3. Front (A–E) and side (F–J) pictures of the face of patients with *PRKAR1A* (R1A) or *PDE4D* mutation. Note the facial dysostosis in all patients and the more severe maxilla-nasal hypoplasia in patients 15 and 16 harboring a *PDE4D* mutation (D, E, I, and J).

Figure 4. Pictures (A, B, E, F, I, J, M, N, Q, and R) and x-rays (C, D, G, H, K, L, O, P, and S) of the hands and feet of patients with *PRKAR1A* (R1A) or *PDE4D* mutations. All patients present a shortening of metacarpals, metatarsals, and phalanges characterizing BDE. Note the shortening of all bones except the big toe and the bulky and stocky aspect of all affected bones in patients 4, 15, and 16. Standard x-rays show the short and broad metacarpals, metatarsals, and phalanges, the cone-shaped epiphyses, and advanced carpal and tarsal maturation. In patient 11, the brachydactyly affects more severely the fourth and fifth digits of the hands (E and G) and third, fourth, and fifth digits of the feet (F and H). In patient 12, the brachymetacarpia affects all digits of the hands (I and K) but only the third and fourth digits of the feet (J and L).

Among the seven male *PRKAR1A* patients (the two *PDE4D* patients were female), bilateral cryptorchidism was present in two and absent in five. Tanner stage in female *PRKAR1A* and *PDE4D* patients were in accordance with age; onset of menarche was within the normal range, with regular cycles in two patients and irregular in two patients. The three adult female patients had slightly increased LH and/or FSH levels (Table 2). Patient 3 gave birth to an unaffected child.

Other developmental defects observed each in only one patient are summarized below and comprise right renal and bilateral vas deferens agenesis (patient 1, reported in Ref. 15), scoliosis (patients 6, 10, and 15), cervical and

flattened lumbar lordosis (patient 6), bilateral 2-3 toe syndactyly (patient 7), dysplasia of both hips and dental defects (patient 8), increased size of the jaw with severe malocclusion (patient 9), postexertional malaise and re current pain at rest of the lower limbs for the last 5 yr (patient 11), delayed tooth eruption/hypodontia) (patients 12, 13 and 16), and recurrent pain of the lower limbs, knees, hands, and back (patient 12).

DISCUSSION

Among 16 patients having acrodysostosis, 14 harboured a *PRKAR1A* mutation and two a *PDE4D* mutation. Although all 16 *PRKAR1A* and *PDE4D* patients presented acrodysostosis features, only the 14 patients harboring a *PRKAR1A* mutation expressed resistance to PTH and TSH.

The patients present a general shortening of metacarpals and metatarsals and/or phalanges, characterizing brachydactyly type E (BDE)[26]. A major characteristic of the BDE observed in acrodysostosis is the severity of the bone defect with advanced skeletal maturation of tubular bones of the hands and feet[1,4]. These bones follow a particular pattern of development, because their shape and growth depend on endochondral ossification, a process highly regulated by ligand-receptor interactions between PTHrP/PTH receptor type 1 (PTH1R)[27,28]. Activation of PTHR1 facilitates the continuous proliferation of chondrocytes in the growth plate and postpones their programmed differentiation into hypertrophic chondrocytes[29]. Conversely, inhibition of this pathway accelerates the normal differentiation process of growth plate chondrocytes and leads to advanced skeletal maturation, as observed in Blomstrand chondrodysplasia, caused by PTHR1R loss-of-function mutations[29]. PTH1R belongs to the group B of the GPCR families and signals mainly through the Gsα-cAMP-PKA pathway[30]. Thus, we propose that most of the skeletal abnormalities in acrodysostosis are likely to result from the impairment in PKA activation by PTHrP due to *PRKAR1A* or *PDE4D* mutations. In this context, loss-of-

function mutations in *PTHLH*, the gene coding PTHrP, have been identified in autosomal-dominant BDE with short stature[31,32]. Because the disease affects the ligand PTHrP, resistance to hormones are not expected and has not been reported.

PTH1R mediates not only the pleiotropic paracrine effects of PTHrP but also the endocrine actions of PTH in kidney and bone through the activation of the Gsα-cAMP-PKA signalling pathway shared by numerous agonists[30]. In ADOHR, the genetic defect that affects PRKAR1A and PKA activity, the most common downstream effector of cAMP, results in a broad phenotype of GPCR-Gsα-cAMP PKA resistance, causing the skeletal dysostosis and hormonal resistance. This phenotype is broadly reminiscent of PHP1A[14,33], where maternally inherited loss-of-function in *GNAS*, the postreceptor regulator of the GPCR-Gsα-cAMP pathway, results in resistance to PTH and TSH and in a somewhat comparable skeletal dysplasia (in PHP1A, the BDE is variable, usually limited to the fourth and fifth metacarpals, phalangeal shortening is generally mild or absent, and the bone dysplasia is usually not present at birth or in early childhood)[14,26].

As previously reported, basal urinary cAMP levels were increased in ADOHR patients, consistent with a defect in the PTH-GPCR-Gsα-cAMP pathway downstream of adenylyl cyclase[15]. In contrast, patients with the *PDE4D* mutation do not demonstrate obvious alterations in cAMP production, as would be expected if renal PTH hormonal resistance was present. Indeed, basal and PTH-stimulated urinary cAMP values, a key marker of PTH effects on the renal proximal tubule[33], are within the normal range, results in agreement with the normal values for serum calcium, phosphate, and PTH in these patients. However, the presence of bone dysostosis, which is similar to that observed in ADOHR patients, indicates that there is a defect in the cAMP signalling pathway in skeletal tissues.

Thus, although both *PRKAR1A* and *PDE4D* defects affect the GPCR-Gsα-cAMP-PKA signalling pathway, their functional consequences were different in

the current series of patients. This is consistent with two recent studies. Michot *et al.*[21] reported in their *PRKAR1A*-mutated patients phenotypes of PTH and TSH resistance that are in agreement with our original[15] and current observations. It is interesting that these authors found that four of five of their PDE4 patients were not resistant to PTH and TSH and that the sole *PDE4D*-mutated patient said to be possibly PTH resistant could have simple vitamin D deficiency, which would explain his normal serum phosphate, low 25-OH-cholecalciferol, and high serum PTH. It is also possible that there is some phenotypic variability in the biochemical and endocrine consequences of the *PDE4D* mutations. Evaluation of additional patients affected with *PDE4D* mutations will be required to confirm these findings. Lee *et al.*[20] did not provide information about the PTH resistance in their patients and reported congenital hypothyroidism in one of two *PRKAR1A*- and one of three *PDE4D*-mutated patients. Current evidence suggests that responses to LH, FSH, calcitonin, GHRH and epinephrine (present work)[15] are altered in some patients with ADOHR, but we have no data yet in ADOP4.

In ADOP4 (i.e. patients carrying a *PDE4D* mutation), the genetic defect affects PDE4D, a member of the PDE families that hydrolyze cAMP, and contributes to the specificity and to the temporal and spatial compartmentalization of the cAMP-PKA signalling[19]. Although our series comprises only two *PDE4D* patients, the role of *PDE4D* mutation in acrodysostosis is supported by the two recent works by Michot *et al.*[21] and Lee *et al.*[20]. In humans, the PDE4D family consists of at least nine N-terminal splice variant isoforms arising from one subfamily gene[34]. Little is known about the specific expression, function, and regulation of these isoforms in human tissues, such as the kidney and the thyroid. In the thyroid gland, an increase in total PDE activity associated in part with an increase in *PDE4D* mRNAs has been shown in autonomous thyroid adenomas bearing mutant *TSHR* or Gsα genes[35]. Possible explanations for the phenotype observed in ADOP4 patients are that the affected PDE4D isoforms are not similarly expressed in different tissues, explaining for instance the absence of PTH resistance in the renal proximal tubule but the presence of PTHrP resistance in the growth plate and the bone

phenotype. This has yet to be proven, because other alternatives are possible, such as compensation by other PDE4D isoforms or PDE that would specifically correct the deficiency of the affected PDE4D isoforms in some tissues such as the kidney and the thyroid. Alternatively, *PDE4D* may be expressed in both renal proximal tubule and bone cells but not involved in the renal response to PTH. These results highlight the specific contributions of PRKAR1A and PDE4D in cAMP signalling in different tissues.

Half of our patients with *PRKAR1A* mutations and ADOHR and one patient with *PDE4D* mutations and ADOP4 presented pigmented skin lesions, a feature not reported before. The presence of pigmented skin lesions may not be unexpected because cAMP is a key second messenger produced by melanocytes and is closely associated with melanocyte differentiation[36,37]. Most lesions were reminiscent of the café au-lait spots observed in McCune-Albright syndrome[38] and the Carney complex[39], two diseases associated with constitutive activation of the PKA-mediated pathway downstream of the melanocortin 1 receptor (*MC1R*). Although the genetic defects causing McCune-Albright syndrome and the Carney complex is known (heterozygous gain of function of Gsa and haploinsufficiency of the *PRKAR1A* gene, respectively), the precise molecular mechanisms of the pigmented skin lesions in McCune-Albright syndrome (café-au-lait spots) and Carney complex (café-au-lait spots and lentigines) are not elucidated. The current observation of pigmented skin lesions in two genetic disorders with constitutive inhibition of the PKA-mediated pathway casts additional complexity on the pathogenesis of the skin lesions.

In addition to hormonal resistance, two additional phenotypic differences were observed in the current *PRKAR1A*- and *PDE4D*-mutated patients. First, although both diseases present with a very similar acrodysostosis, differences are observed for maxillonasal hypoplasia, a canonical feature for the diagnosis of acrodysostosis (more severe in ADOP4) and for the lumbar interpediculate distance widening (frequently absent in ADOHR). Furthermore, one patient with *PDE4D* mutation had acroskyphodysplasia, suggesting that patients having this skeletal dysplasia should be screened for mutations in *PDE4D*.

The second difference, although we deal with a small series of patients, is the mental retardation observed in our two patients with *PDE4D* mutations, as reported by Michot *et al.*[21] in *PDE4D* patients. Lee *et al.*[20] report developmental disability, described as mild in two *PRKAR1A* and one *PDE4D* patient and significant in one *PDE4D* patient. In contrast, the patients with *PRKAR1A* mutations and ADOHR presented no mental retardation (our series)[21], although eight of 14 presented some behavioral disorders. Mild to moderate mental retardation is common in patients with PHP1A and Gsa deficiency[14]. Taken together, these observations illustrate the key role of the GPCR-Gsa-cAMP-PKA signalling pathway and its specific effectors for the development of cognitive functions.

It is now clear that R368X is a recurrent mutation as initially postulated[15], because it is observed in nine of 14 *PRKAR1A* patients of our series and in four of five *PRKAR1A* patients of Michot *et al.*[21]. The current study allowed the identification of five new missense or nonsense novel mutations in the *PRKAR1A* gene. All of them affect a conserved residue in the cAMP binding domain B as the previously identified R368X. In our series, the skeletal phenotype in patients carrying the R368X is quite uniform, whereas two patients carrying novel mutations displayed some specific differences in the skeletal dysplasia, raising the possibility of a genotype-phenotype correlation. The ADOHR phenotype can now be said to be consistently associated with GPCR hormone resistance[15,21].

In a patient with ADOP4, glycine 289 is mutated to glutamate (G289E). A mutation changing glycine 289 to tryptophan (G289W) has been identified in Carney complex. Similarly, other mutations identified in our manuscript are close to mutations previously reported in patients with Carney complex. Point mutations have been known to cause changes in the tridimensional structure of the protein, and these changes, which can alter protein function, depend on the properties of the substituted amino acid. Previous examples of different substitutions affecting the same amino acid but causing opposite effects on protein function have been described in the literature[40–42].

The current study identified one previously reported *PDE4D* mutation (E590A)[20] and one novel *PDE4D* mutation (A227S) adjacent to two previously reported mutations (F226S[21] and Q228E[20]), compatible with this amino acid stretch having a role in the *PDE4D* structure/function. The mutations identified affect long and short isoforms of *PDE4D*, but the understanding of their effects on biochemical, biological, and skeletal phenotypes will require additional studies in more patients and in vitro.

In conclusion, although these results do not exclude a defect in other genes causing acrodysostosis with or without hormonal resistance, the current series of observations in patients with acrodysostosis syndrome confirms the dismemberment of this entity into two different genetic and phenotypic syndromes that can be called ADOHR, due to *PRKAR1A* defects, and ADOP4, due to *PDE4D* defects. The existence of GPCR-hormone resistance is typical of the ADOHR syndrome.

ACKNOWLEDGMENTS

We thank the patients affected with acrodysostosis and the mem- bers of their families for participating in the study.

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G.P.d.N. is cofunded by the Instituto de Salud Carlos III I3SNS Program (CP03/0064; SIVI 1395/09). A.P. is supported by Fondo de Investigación Sanitaria (FIS) program (FI11/00309). This work was supported by Grant PI10/0148 from the Instituto de Salud Carlos III (to G.P.d.N.), a grant from the Italian Ministry of Health to G.M. (GR-2009-1608394), grants from FIS (PI10/0747) (to J.A.), Centro de Investigación Biomédica en Red (CIBER) de la Fisiopatología de la Obesidad y Nutrición (CIBERobn) (CB03/06) (to J.A.) and Fundación Endocrinología y Nutrición. O.R.-C. is supported by a Miguel Servet program from FIS (CP11/00263), Instituto de Salud Carlos III, Madrid, Spain.

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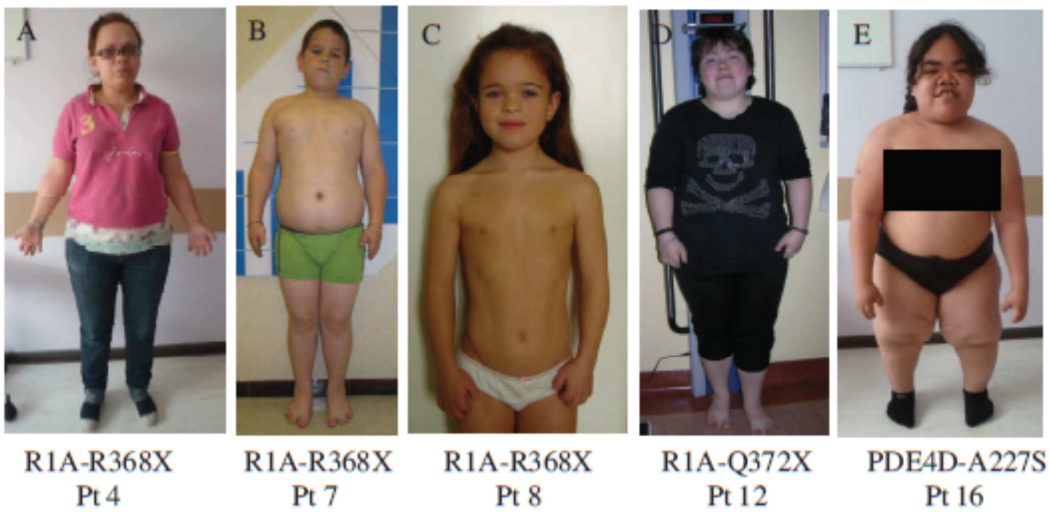
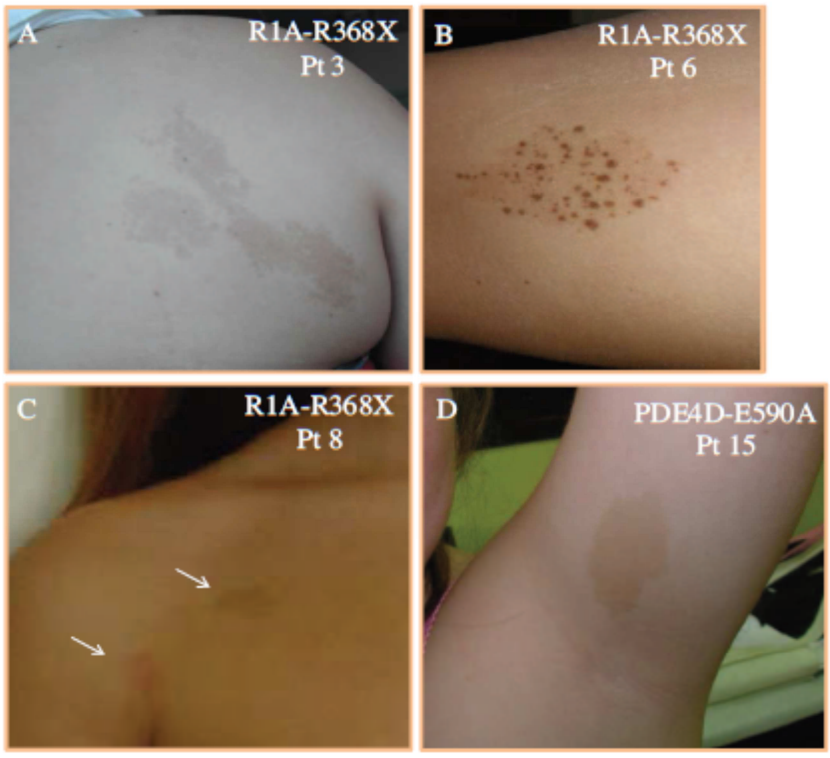
SUPPLEMENTARY INFORMATION



Supplemental figure 1. Pictures of patients affected with a heterozygous *PRKAR1A* (R1A) (panels A to D) or *PDE4D* (panel E) mutation illustrating the characteristic features including the stocky appearance, and the facial and peripheral dysostosis with severe brachydactyly.



Supplemental figure 2. Examples of pigmentary skin lesions associated with the R368X-*PRKAR1A* mutation (patient 3: panel A, patient 6: panel B, patient 8: panel C) and a *PDE4D* mutation (patient 15, panel D). Note the café-au-lait spot appearance in patients 3, 8 and 15.

PUBLICATION 5

Gain of function of *PDE4D* mutations identified in acrodysostosis type 2 explains the similarity between the features shown in both types of acrodysostosis

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Manuscript in preparation

ABSTRACT

Mutations in *PRKAR1A* and *PDE4D* genes have been recently associated with two types of acrodysostosis with similar skeletal dysplasia manifestations, but they differ in the presence of hormonal resistance (ACRDYS1) or absence of hormonal resistance (ACRDYS2), respectively. Functional studies of *PRKAR1A* mutations indicated that the mutated regulatory subunit impairs the protein kinase A (PKA) response to stimulation by cAMP, leading to a downregulation of the pathway. In current study, preliminary functional studies of *PDE4D* mutations have indicated that analised four mutated PDE4D3 forms have an increased hydrolysis activity in compare to the WT form. The increased cAMP hydrolysis is predicted to produce a lower activation of PKA, with explains the similarities of the skeletal abnormalities observed in both types of acrodysostosis.

INTRODUCTION

Acrodysostosis refers to a heterogeneous group of rare skeletal dysplasia that share characteristics features, including severe brachydactyly, facial dysostosis and nasal hypoplasia[1–8]. The coincidences at clinical and radiological levels between acrodysostosis and pseudohypopharathyroidism, as well as, the variability of clinical manifestations within acrodysostosic patients, have occasioned confusions between these syndromes over a long time[8]. Recently, at least two types of acrodysostosis have been identified: i) acrodysostosis type 1 (ACRDYS1) caused by gain-of-function mutations in the gene encoding the cyclic AMP (cAMP)–dependent regulatory subunit of protein kinase A (*PRKAR1A*)[3] and ii) acrodysostosis type 2 (ACRDYS2) caused by mutations in gene encoding cAMP-specific phosphodiesterase 4D (*PDE4D*)[4,5]. Both genes are involved in cAMP-mediated G protein-coupled receptor (GPCR) signalling cascade, but their functional consequences seem to be fairly different in the reported patients[4,5,9]. According to the skeletal lesions, both manifest with an overlapping severe skeletal dysplasia, facial dysostosis being more severe in ACRDYS2. Although, the main difference lies in the lack of hormonal resistance in most of ACRDYS2 patients[4,6,7,9]. In addition, developmental disability seems to be more severe in ACRDYS2. In fact, generally ACRDYS1 patients, exhibit only behavioural disorders[4–7,9].

Functional studies of *PRKAR1A* mutations causative of ACRDYS1 have indicated that they lead to a gain-of-function in regulatory subunit of PKA to hold the catalytic subunit in an inactive conformation, caused by an impairment in the ability of regulatory subunit to dissociate from catalytic subunit because of the insensitivity to bind to cAMP[3,8,10] leading to a decrease in PKA activity. Because *PDE4D* mutations are translated into similar manifestations one would expect that these alterations produce a downregulation of the pathway as happen with *PRKAR1A* mutations. So the aim of this study was to elucidate the effect of four *PDE4D* mutations, which were identified in four different acrodysostosic patients, to understand the origin of the downregulation[9].

METHODS

The methods are summarised in the Supplementary Appendix.

RESULTS

CHO cells were transfected to express WT, p.S190A, p.E590A, p.A227S or p.L312V forms of PDE4D3, tagged with the epitope HA. The expression of WT and mutant proteins was determined by immunoblotting using anti-HA epitope antibody. Of note, endogenous PDE4 activity was measured in CHO cells to determine the total activity due to the recombinant PDE4D3, but the endogenous PDE4 activity resulted insignificant (data not shown).

The increase in the PDE4D3 activity by 8-Br-cAMP treatment caused a shift in the mobility of the WT-PDE4D3, which is blocked by H89.

It is known that increased intracellular cAMP levels increase cellular PDE4 activity[11,12]. So firstly, in attempt to study the expression of recombinant WT-PDE4D3 in CHO cells they were stimulated with increased concentrations of 8-Bromoadenosine-3', 5'-cyclic monophosphate (8-Br-cAMP). In absence of 8-Br-cAMP, PDE4D3 migrated as a single band of approximately 93-kDa. However, in cells treated with increasing concentrations of 8-Br-cAMP, a double band was observed (one of 93-kDa and the other of 98-kDa). Curiously, the appearance of the 98-kDa species with the increasing concentrations of 8-Br-cAMP, was associated with a decrease in the intensity of the 93-kDa band (Figure 1). It has been previously suggested[13] that PDE activation is associated with phosphorylation mediated by PKA, which leads to a shift in electrophoretic mobility of the PDE4D3 protein. Indeed, in the current study it was confirmed that a PKA specific inhibitor H89, blocks PDE4D3 phosphorylation, as it could be appreciated by the decrease in the intensity of the 98-kDa band (Figure 1).

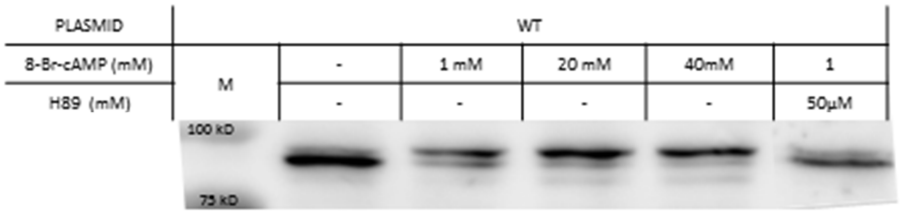

Figure 1. Expression of recombinant WT-PDE4D3 analysed by Western blotting in transfected CHO cells. Cells were stimulated with different concentration of 8-Br-cAMP as indicated in each well, additionally cells of the last well were treated with H89, PKA specific inhibitor, 1h before the stimulation. All the lysates were then subjected to SDS-PAGE and detected by anti-HA epitope antibody. The data shown are representative of at least three separate.

The shift in the mobility of the PDE4D3 mutants is also observed in basal conditions and it is not blocked by H89.

Then, PDE expression was measured in WT-, S190A, E590A, A227S and L312V PDE4D3 recombinant CHO cells with 8-Br-cAMP. As observed in the previous experiment, WT-PDE4D3 in basal conditions was expressed as the 93-kDa single band, but the four mutants displayed a double band in basal as well as in stimulated conditions (Figure 2). The shift in mobility that mutants species exhibited seemed not to be dependent of PKA activity, because when cells were pretreated with H89 inhibitor it had no effect on the expression of the both bands (data not shown).

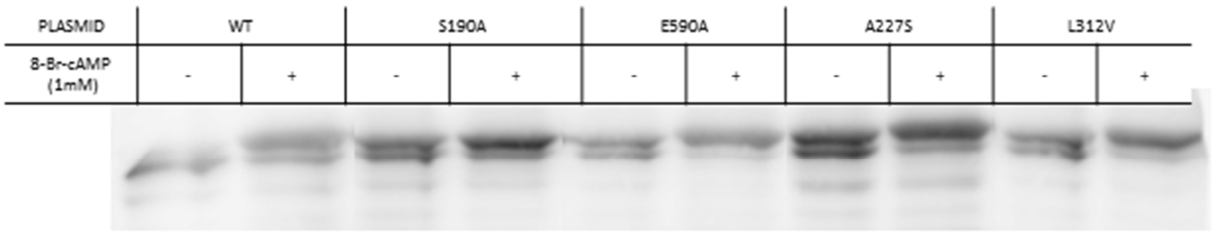

Figure 2. Expression of recombinant WT-PDE4D3 and the mutants proteins, in transfected CHO cells. Cells lysates were prepared from transfrected CHO cells. Cells were stimulated with 1mM of 8-Br-cAMP. Proteins were electrophoresed into 12% to SDS-PAGE. This blot is representative of at least three separate experiments using different transfections.

Dephosphorylation reverses the shift in the mobility of immunoprecipitated WT as well as of mutants PDE4D3.

PDE4D3 protein was immunoprecipitated (IP) from the lysates obtained from recombinant cells of WT and four mutants, in both, basal and stimulated conditions. After IP, each immunoprecipitate was treated with alkaline phosphatase. For proteins treated with alkaline phosphatase, anti-HA showed only a single band for all mutant PDE4D3, opposite to protein immunoprecipitates not treated with alkaline phosphatase. As previously observed, WT-PDE4D3 in basal conditions showed a single band (Figure 3). Therefore, this experiment suggested that the upper band observed in the immunoblot of each mutant protein corresponded to a phosphorylated form of PDE4D3 and the lower band to the non-phosphorylated forms.

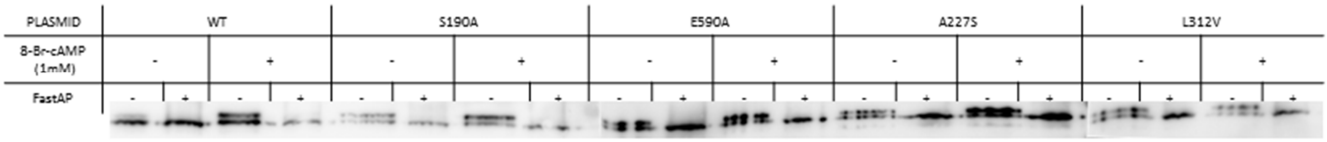

Figure 3. Immunoprecitades of WT-PDE4D3 and the mutants proteins treated with alkaline phosphatase. Cells were stimulated with 8-Br-cAMP 1 mM as indicated in the second line. After harvesting, the expressed recombinant PDE4D3 was immunoprecipitated in each case. Then, immunoprecipitates were dephosphorylated with alkalina phosphatase as indicated in the third line. Finally, immunoprecipitates electrophoresed into 12% SDS-PAGE and they were detected by anti-HA epitope antibody. This blot is representative of at least three separate experiments using different transfections.

PDE4D3 mutant proteins showed an increased 8-Br-cAMP hydrolysis activity in compare to WT-PDE4D3.

Intracellular accumulation of 8-Br-cAMP was monitored by BRET sensor CAMYEL[14]. BRET experiments showed that the decrease in the BRET signal was significantly more drastic in cells transfected with WT-PDE4D3 than in the cells transfected with a mutant protein. As BRET signal is inversely proportional to the accumulation of 8-Br-cAMP, results indicated that in cells transfected with WT-PDE4D3, the accumulation of the analogous is higher than in mutants (Figure 4), demonstrating a lower PDE activity.

On the other hand, when PKA activity was inhibited by H89, the BRET signal reduction further increased in cells transfected to express WT-PDE4D3, while cells transfected with a mutant protein showed a similar BRET signal reduction with or without inhibitor (Figure 4). Thus, this experiment indicated again, that PKA activity does not regulate activity of mutants PDE4D3.

Previous results, coincide with the cAMP-PDE hydrolysis activity assay, which indicated that mutants have a significantly ($p<0.05$) increased cAMP hydrolysis activity, in comparison to WT (Figure 4).

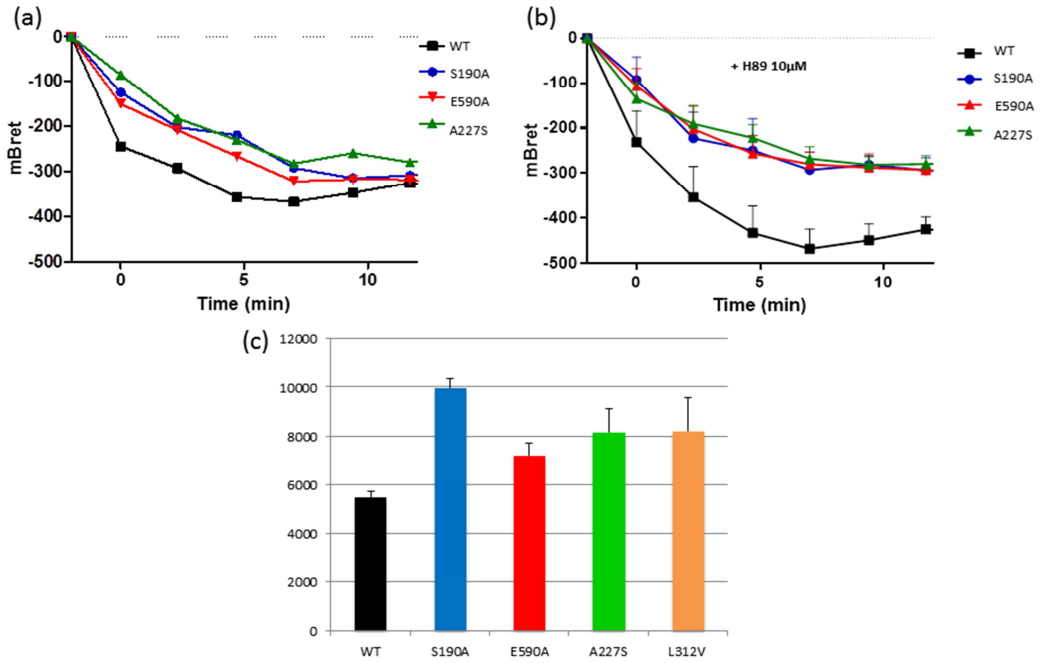

Figure 4.: Measuring of PDE4D3 hydrolysis activity. (a) BRET signal is inversely proportional to the 8-Br-cAMP concentration; so in this case the signal decrease indicate the accumulation of 8-Br-cAMP in time-course, for WT-PDE4D3 and the three of four studied mutants proteins. (b) Cells were treated with H89 PKA specific inhibitor 1h before the challenged with 8-Br-cAMP. (c) WT-PDE4D3 and the four mutants hydrolysis activity was measured in cell lysates, by cAMP activity assay. The results shown are representative of at least three separate experiments using different transfections.

DISCUSSION

Cyclic nucleotide phosphodiesterase enzymes (PDEs) are responsible for the hydrolysation of second messenger cAMP and cGMP in mammalians to control the magnitude and duration of the cAMP response. Eleven families of PDEs have been described encoded by 21 genes; the classification is based on the kinetic and pharmacologic properties, as well as substrate specificity and sequence homology[15]. The largest family is the PDE4 one; comprising four genes, *PDE4A* to *PDE4D*, which are differenced by their selectivity for cAMP over cGMP. Particularly, PDE4D is specific for cAMP hydrolysis[15]. The alternative splicing, the use of alternate promoters and transcription start sites give rise to the expression of several isoforms of each PDE4[16]. Depending on the presence or absence of highly conserved N-terminal domains, these isoforms are classified into long (provided by UCR1 and UCR2 regulatory regions), short (lack UCR1) or super short forms (lack UCR1, but with a truncated UCR2). The presence of UCR1 and UCR2 domains confers to the long forms the property to be activated by PKA phosphorylation[17,18], as well as to behave as dimers, while the forms which lack one of these domains are regulated at transcriptional level and act as monomers[19].

When intracellular cAMP concentration is increased, PKA phosphorylates the specific motive localized at UCR1 domain of long PDE4D forms, which leads to the activation of the protein and produces an increase in the hydrolytic activity[20]. This phosphorylation motive namely RRES*F, is presented in long isoforms of all sub-families of PDE4 genes. But particularly, PDE4D3 isoform apart from the Ser54, additionally harbours another target at Ser13 for PKA phosphorylation[21]. Previous mutagenesis studies of these two phosphorylation sites suggested that the PKA-mediated mobility shift of PDE4D3, also observed in current experiments, requires phosphorylation at both, Ser13 and Ser54. But, curiously, these mutagenesis experiments indicated that the activation of the enzyme is entirely attributed to phosphorylation of Ser54, because the removal of Ser13 seems to have no effect on the activation of the enzyme[11,13,21].

In the present study, four mutations identified in *PDE4D* as causative of acrodysostosis ACRDYS2 in four patients (patients' clinical data who harboured E590A and A227S, were previously published[9]) have been chosen to characterize the functional defect resulting from the mutations. S190A and E590A are localized in the UCR1 domain. S190A (S190 corresponds to the consensus phosphorylation site Ser54) mutation alters the consensus phosphorylation site S54 (Ser is substituted by an Ala aminoacid and consequently is unable to be phosphorylated). A227S is localized at the UCR2 domain and L312V at the catalytic domain. Expression experiments, carried out by transient transfection in CHO cells with WT-PDE4D3 and with mutants, indicated, that mutants showed an increased phosphorylation status in basal conditions, inferred from the appreciated shift in the mobility[11,13,21] of all PDE4D3 mutants in basal conditions. In fact, when protein immunoprecipitates were dephosphorylated with alkaline phosphatase treatment, the mutant proteins displayed as a single band of the low MW, as observed for the WT-PDE4D3 in basal conditions. Interestingly, S190A whose phosphorylation at Ser54 is blocked by a substitution to Ala, also dysplayed as a doble band in western blotting analysis, as the other three mutants. This could indicate: i) the shift in the mobility that shows the PDE4D3 isoform cannot be attributed only to the phosphorylation of both, Ser13 and Ser54 residues, ii) apart from these two phosphorylation targets, others additional phosphorylation sites may be present in PDE4D3,. This second point was discussed by Sette *et al.*[13]. These authors observed that the double mutant for Ser13 and Ser54 was still phosphorylated by PKA and proposed others possible consensus sites to be phosphorylated. However they observed that among the potential phosphorylation sites studied, only mutation in Ser54 to Ala abolished the PKA-dependent activation of the enzyme; since the specific activity appreciated by this mutant was comparable with or lower than that of the wild type enzyme, in basal as well as in PKA stimulation conditions[13]. In contrast, PDE activity assay as well as BRET experiments performed in the current study indicated that the four studied mutants presented an increased hydrolyzation activity in basal conditions, in comparison with WT. Of note, the hydrolyzation activity of mutants seems not to be further increased by the increase of

8-Br-cAMP, neither blocked by PKA inhibition with H89 specific inhibitor. This suggests that the increased phosphorylation observed in the studied mutants may not be dependent of PKA phosphorylation, and therefore it could be induced by other regulatory proteins which phosphorylate PDE4D3 at sites distinct from PKA consensus sites. Actually, this idea is supported by a recent finding in cardiac myocytes, which implicate CaMKII (Ca2+/calmodulin-dependent kinase II) protein in the activation of PDE4D by phosphorylation in distinct sites from PKA sites, which produces a synergic effect on the mobility shift[22].

In summary, current findings indicate that mutations in *PDE4D* causing ACRDYS2 are gain-of-function mutations leading to increased cAMP hydrolytic activity. This enhanced cAMP hydrolysis, is predicted to downregulated the PKA activation and subsequently, the downstream p-CREB signalling pathway implicated in the acrodysostosis skeletal dysplasia observed in ACRDYS1 patients[3,10,23,24]. Interestingly, very recently, mutations with similar gain-of-function property, have been identified in the gene encoding PDE3A; a phosphodiesterase with a prominent role in the heart, as a cause of brachydactyly with hypertension syndrome (HTNB; OMIM#112410)[25]. On the other hand, the lack of the hormone resistance observed in ACRDYS2 patients, could not be explained by these preliminary results about the functional studies of ACRDYS2 *PDE4D* mutations. But very likely the diversity of PDE isoenzymes and their tissue specific expression explain the milder effect on hormone resistance in these patients[12,26,27]. Regarding to the mental retardation, new finding about *CCD21A* (Coiled-coil and C2 domain-containing 1A) mutations associated with non-syndromic intellectual disability, have discovered the implication of this protein in the attenuation of the phosphorylation of PDE4D[28]. Therefore, this finding would explain why this feature is preferentially observed in ACRDYS2 patients.

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SUPPLEMENTARY INFORMATION

METHODS

PDE4D3 expression plasmids and site-directed mutagenesis

cDNA encoding WT cAMP-specific human phosphodiesterase PDE4D3 was provided by M. Houslay, and subcloned into the expression vector of pcDNA.3.1 (Invitrogen) with a C-terminal hemagglutinin (HA) epitope as described before[1]. After complete sequencing of the cDNA insert, the p.S190A, p.E590A, p.A227S and p.L312V mutation identified in the patients were introduced into the WT-PDE4D3 cDNA construct as described before[1,2].

Cell culture and transient transfection

Experiments were performed in CHO cells. Some experiments were also carried out in HEK293, to confirm that the results were not conditioned by cell-type. CHO cells and HEK293 cells were cultured as described before[3] in Ham's F12 Nutrient Mixture (Gibco-Life Technologies, California, EE.UU) and DMEM (Gibco-Life Technologies, California, EE.UU), respectively, containing 10% foetal bovine serum, 100 U/ml penicillin G and 100 µg/ml streptomycin. Transfection of CHO and HEK293 cells were performed in 24-well plates. In each well 80,000 CHO cells or 130,000 HEK293 cells were cultured in a volume of 500 µl of medium. Cells were transfected using X-tremeGENE DNA Transfection Reagents (Roche, Basel, Switzerland) with 300 ng DNA pcDNA.3.1 construct encoding WT-, S190A-, E590A-, A227S- or L312V-PDE4D3 according to the manufacturer's instructions. All studies were performed 48 h after transfection.

PDE4D3 activity determination assays

48 h post-transfection cells were washed twice with PBS at room temperature. Cells were stimulated with 1mM 8-Br-cAMP (8-Bromoadenosine-3', 5'-cyclic monophosphate, BioLog, Life Science Institute, Bremen, Germany) and incubated 20 min at room temperature. The effect of the inhibition of the PKA dependent phosphorylation, was studied by adding 10µM of H89 (Sigma-Aldrich, 10 µM) was PKA specific inhibitor, 1h before the treatment with 8-Br-

cAMP. Cells were lysed following different procedures depending on the next experiment.

Preparation of cell extracts for immunoprecipitation

Cells were lysed in ice (lysis buffer without EDTA: 50 mM-Tris pH 7.5, 150 mM NaCl, 0,5% Igepal/N40, 10 µl per ml of Aprotinin and Leupeptin before use) and shacked during 10 min. Then cells were scraped, transferred to a tube (10 wells in each tube) and kept in ice 10 min. Finally, tubes were centrifuged 15 min at 14,000 rpm at 4ºC and transferred the supernatant to a new tube. Samples were stored at -80ºC.

Immunoprecipitation of PDE4D3

For immunoprecipitation, samples were incubated with 1 µg anti-HA High Affinity antibody (Roche, Basel, Switzerland) on ice for 1 h. and immunoprecipitated by G-sepharose for 1 h at 4ºC. The immune complexes were washed twice with lysis buffer without EDTA (50 mM-Tris pH 7.5, 150 mM NaCl, 0,5% Igepal/N40, 10 µl per ml of Aprotinin and Leupeptin before use).

Dephosphorylation to analyse the change in the mobility of the PDE4D3

15 µl of 1x lysis buffer[3] was added to the immunoprecipitated sample in which dephosphorylation was not going to be performed (control sample for dephosphorylation) and stored at -20ºC until Western blot. Dephosphorylation was carried out using 10 U of FastAP (Thermosensitive Alkaline Phosphatase, Thermo Scientific, Life Technologies, California, EE.UU) and 2 µl of FastAP buffer. This was incubated for 1 h at 37ºC after which a further 5 U of FastAP were added and incubated again for a further 30 min at 37ºC. The reaction was then stopped by addition of 2 µl 5x Laemmli buffer[3]. Samples were boiled at 95ºC, centrifuged 1 min at 14,000 rpm and the supernatant was loaded in 12% SDS-PAGE. Then, the immunoblot was performed with HA antibody as described above.

PDE4D3 protein expression analysis

Samples were preboiled resuspended in 1X Laemmli buffer[3]. Protein samples were separated by a 12% SDS-PAGE and transferred to nitrocellulose membranes. Then SDS-PAGE was transferred to Immobilon-P membranes (Millipore). Blots were blocked for 1h at room temperature with Odysee® blocking buffer (LI-COR Biosciences), and incubated overnight at 4ºC with primary antibody anti-HA (1/500 dilution, Santa Cruz Biotechnology, INC.). Blots were then washed with TBST using the SNAP i.d. system (Millipore), incubated with a goat anti-rabbit horseradish peroxidase conjugated secondary antibody (1/1000) and visualised using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Thermo Scientific). Bands present on film exposed to the blots were visualized using the LAS-4000mini (Fujifilm, Minato, Tokyo, Japan) and quantified using the Multi-Gauge V3 program (Fujifilm). Blots shown are representative of at least three individual experiments.

cAMP-PDE activity assay

The PDE activity was assayed as described[1].

BRET Measurement

Intracellular accumulation of 8-Br-cAMP was monitored by BRET sensor CAMYEL[4] to measure and compare the hydrolysis ability of WT-PDE4D3 and mentioned four mutants.

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PUBLICATION 6

Pseudohypoparathyroidism *versus* tricho-rhino-phalangeal syndrome: patient reclassification

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Journal of Pediatric Endocrinology and Metabolism **2014; 27(11-12):1089–1094.**

ABSTRACT

Objectives: Given that tricho-rhino-phalangeal syndrome (TRPS) and pseudohypoparathyroidism/pseudopseudohypoparathyroidism (PHP/PPHP) are very rare monogenic disorders that share some features (distinctive facies, short stature, brachydactyly and, in some patients, intellectual disability) that lead to their misdiagnosis in some cases, our objective was to identify clinical, biochemical or radiological signs that could help to distinguish these two syndromes.

Methods and results: We report on two cases, which were referred to the Endocrinology and Pediatric Endocrinology Services, for obesity. Clinical evaluation initially suggested the diagnosis of PHP-Ia (phenotype suggestive of Albright hereditary osteodystrophy, AHO, with PTH resistance) and PPHP (phenotype resembling AHO, without PTH resistance), but (epi)genetic analysis of the GNAS locus ruled out the suspected diagnosis. Further clinical re-evaluation prompted us to suspect TRPS, and this was confirmed genetically.

Conclusion: TRPS was mistakenly identified as PHP/PPHP because of the coexistence of obesity and brachydactyly, with PTH resistance in one of the cases. Specific traits such as sparse scalp hair and a pear-shaped nose, present in both cases, can be considered pathognomonic signs of TRPS, which could help us to reach a correct diagnosis.

INTRODUCTION

Tricho-rhino-phalangeal syndrome (TRPS-I, OMIM #190350 and TRPS-III, OMIM #190351) and pseudohypoparathyroidism/ pseudopseudohypoparathyroidism (PHP-Ia, OMIM #103580; PPHP, OMIM #612463) can be clinically confused because they have an overlapping phenotype with rare or subtle dysmorphic features, short stature, brachydactyly and intellectual disability[1-4]. Typical facial dysmorphic traits described in TRPS (sparse, slowly growing scalp hair, laterally sparse eyebrows, a bulbous tip of the nose [pear-shaped nose], and long flat philtrum)[2] can be very mild thus complicating the clinical diagnosis. We report on a family and an isolated case initially diagnosed as PHP-Ia/PPHP in which we later identified mutation in the *TRPS1* gene, highlighting both the features that can be shared by the two syndromes and those that finally helped us to reach the correct diagnosis and confirm it with the appropriate genetic study.

SUBJECTS AND METHODS

Patient 1

The proband, a 31-year-old woman, was referred to the Endocrinology Department for morbid obesity. She had a personal history of obesity from her first pregnancy at the age of 18, with a progressive increase in weight. She did not have any other clinical complications but occasionally took pain relief medication. The patient had menarche at age 12, and her menstrual cycles were irregular with oligomenorrhea, without presenting amenorrhea. She had had four uncomplicated pregnancies which resulted in normal deliveries, and none of her children had fetal macrosomia. The proband's mother and brother, who were short in stature, were both affected by polyarthrosis. The patient did not show any symptoms suggestive of hormonal dysfunction. She tended to have a high calorie diet and her ability to exercise was limited by arthralgias of both hips and knees. She had learning difficulties during childhood. Her height was

141.5 cm (-4 SD) and body mass index (BMI) 42.7 kg/m^2. On physical examination, she was observed to have facial features including a round face, sparse hair, a thin upper lip and a prominent lower lip, a pear-shaped nose, tooth hypoplasia and stubby fingers and toes (Figure 1 A-C). Her round face, brachydactyly, shortening of III-V metacarpals and middle phalange together with her very short stature suggested Albright's hereditary osteodystrophy (AHO) phenotype. A bone X-ray survey and thorough biochemical and hormonal tests were performed. Her karyotype was normal (46, XX). However, repeated blood tests showed normal levels of calcium and phosphorus with PTH levels above the normal limit (70-110 pg/ml, reference range: 10-40 pg/ml) and normal 25-OH-vitamin D levels (72.8 ng/ml). Urine calcium was also normal (100-130 mg/24 hours) in repeated measurements. Basal pituitary function was normal except for low IGF-1 levels, below the age-adjusted normal level (53 ng/ml), which were confirmed in several measurements. The possibility of

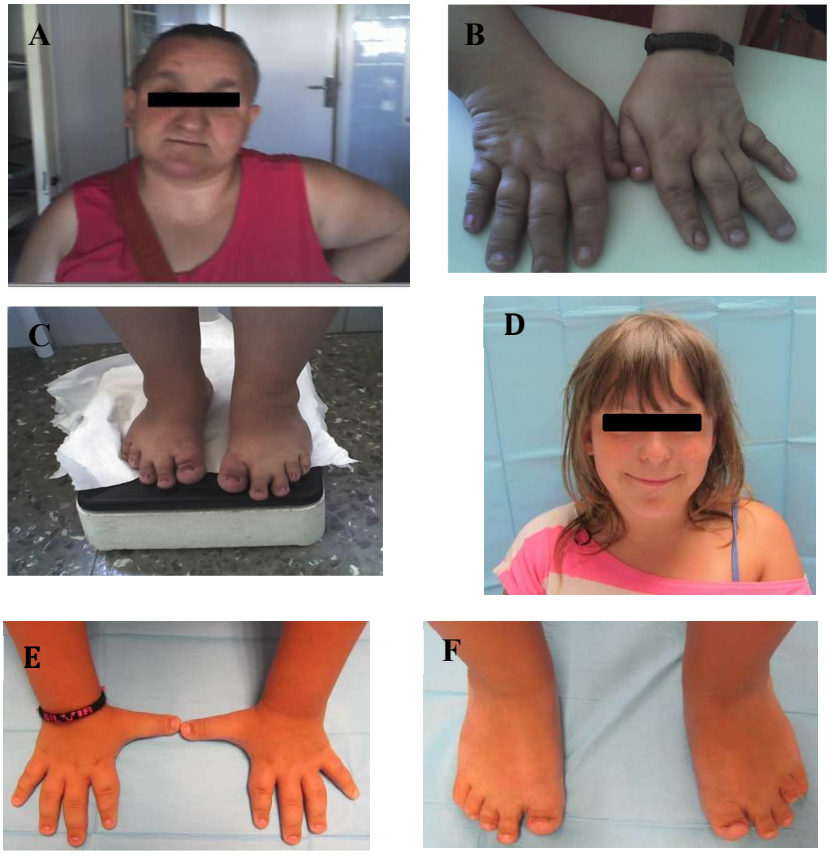

Figure 1. Phenotypic appearance of the patient 1 (A), and patient 2 (D) revealing round face, sparse hair, thin lips, and pear-shaped nose; (B and E) Hands and (C and F) feet with stubby fingers and toes, and brachydactyly.

isolated GH deficiency was further examined and confirmed by the results of two GH provocation tests: glucagon test (GH peak< 0.7 ng/ml), and an insulin hypoglycemia test (GH peak of 0.1 ng/ml with hypoglycemia of 40 mg/dl). Hand X-rays revealed cone-shaped epiphyses of all middle and I-IV proximal phalanges of both hands and shortening of metacarpals IV-V of the right hand and III and V of the left one (Figure 2A). A lumbar spine X-ray revealed sacralization of the L5 vertebra and bilateral last-rib hypoplasia. Knee X-rays showed marked hypoplasia of the medial tibial condyles and unilateral hypertrophy of internal femoral condyle.

Two of her children had normal stature, normal X-ray surveys and no dysmorphic traits (Figures 2B, C). However, her 13-year-old daughter had height in the 10th percentile, brachydactyly, clynodactyly, and mild dysmorphic traits (wide mouth, sparse hair, long philtrum and bulbous tip of the nose), though no learning difficulties. Similar to in her mother, hand X-rays showed cone-shaped epiphyses of all middle and I-IV proximal phalanges of both hands and shortening of the metacarpals, III-V of the right hand and III and V of the left hand (Figure 2C). No alterations were identified in the calciotropic axis (calcium, phosphorus, and PTH) and the IGF-1 level was normal.

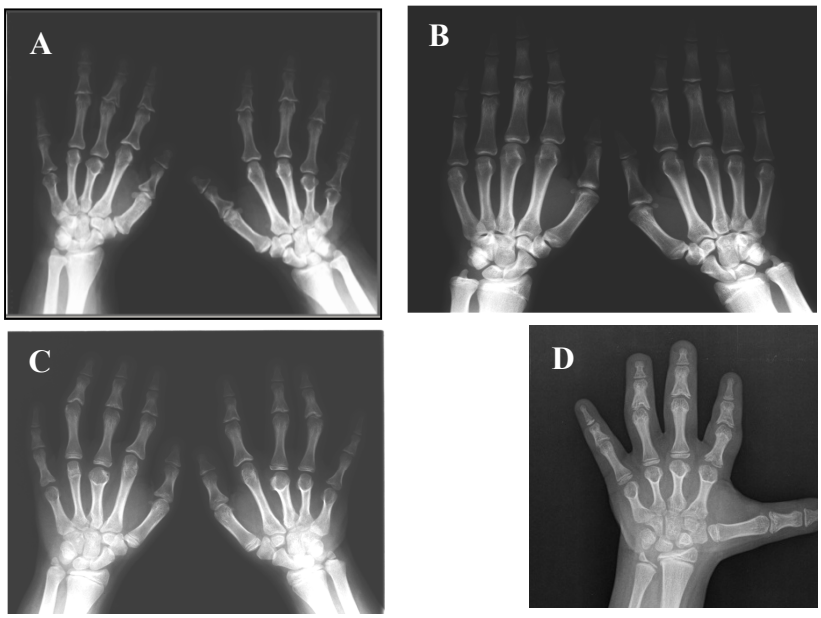

Figure 2. Hand X-rays of (A) patient 1 showing cone-shaped epiphyses and outcarving cones of middle and terminal phalanges of both hands, shortening of metacarpals and proximal and middle phalanges; (B) the unaffected son and (C) the affected daughter. (D) Left hand X-ray of patient 2 also revealing cone-shaped epiphyses and outcarving cones of middle and terminal phalanges, shortening of metacarpals and proximal and middle phalanges.

The proband's mother and brother had a similar phenotype but further medical evaluations and photographs were not available.

Patient 2

An 11- year-old girl was referred to the pediatric endocrinologist due to overweight and a family history of obesity and type 2 diabetes. She was born at term by normal delivery with a weight of 2600 g (p6) and length of 49 cm (p39). She had a normal psychomotor development although she has been attending speech therapy since 5 years of age. The onset of pubertal signs started at 8½ years of age. Anthropometric measurements included: height of 139.8 cm (p18), and weight of 50.2 kg (p79), yielding a BMI of 25.6 kg/m2 (+1.96 SD). She had sparse hair, laterally sparse eyebrows, long flat philtrum, and thin upper vermillion border, a pear-shaped nose, protruding ears (Figure 1D), shortened fingers (Figure 1E) and toes and flat feet (Figure 1F). Her karyotype was normal (46,XX). Thorough biochemical and hormonal analyses (blood count, hepatic, renal and lipid profiles, serum levels of calcium, phosphorus, PTH, IGF-I, TSH, FSH and LH) found completely normal results.

Interestingly, hand X-rays showed cone-shaped epiphyses of middle and proximal II-IV phalanges and I proximal phalanx and shortening of the II-V metacarpals (Figure 2D), initially suggesting a diagnosis of PPHP.

Molecular studies

Genetic analyses were performed after informed consent of the patient and/or parents (in the case of minors). Genomic DNA was extracted from peripheral blood mononuclear cells using QIAamp DNA mini kit (QIAGEN, Düren, Germany). Firstly, the GNAS locus was analyzed, including sequencing of *GNAS* 13 coding exons and evaluation of methylation, as previously described[5]. No abnormalities were identified (data not shown). Then intronic and, when required, exonic primers were used to amplify the coding exons and intron-exon junctions of *TRPS1* (ENST00000395715) (primers available on request). PCR products were analyzed by direct nucleotide sequence analysis using standard methods on an ABI3500 (Applied Biosystems, Foster City, CA, USA).

RESULTS

Patient 1 and her daughter were clinically diagnosed with PHP-Ia because of the recurrent PTH resistance (only in the index) and phenotype characteristics of AHO. The molecular study did not reveal genetic or epigenetic alterations in the GNAS locus. After clinical revaluation, mild dysmorphic traits including sparse hair and bulbous nose tip suggested TRPS. Direct sequencing of the *TRPS1* gene revealed an adenine deletion (c.2830delA) in exon 7, which leads to a frameshift mutation (p.Arg944Glyfs*3) (Figure 3A). This mutation has not been previously reported but the cosegregation within the family, the *in silico* studies and the absence of the mutation in 100 healthy controls suggest pathogenicity of the mutation.

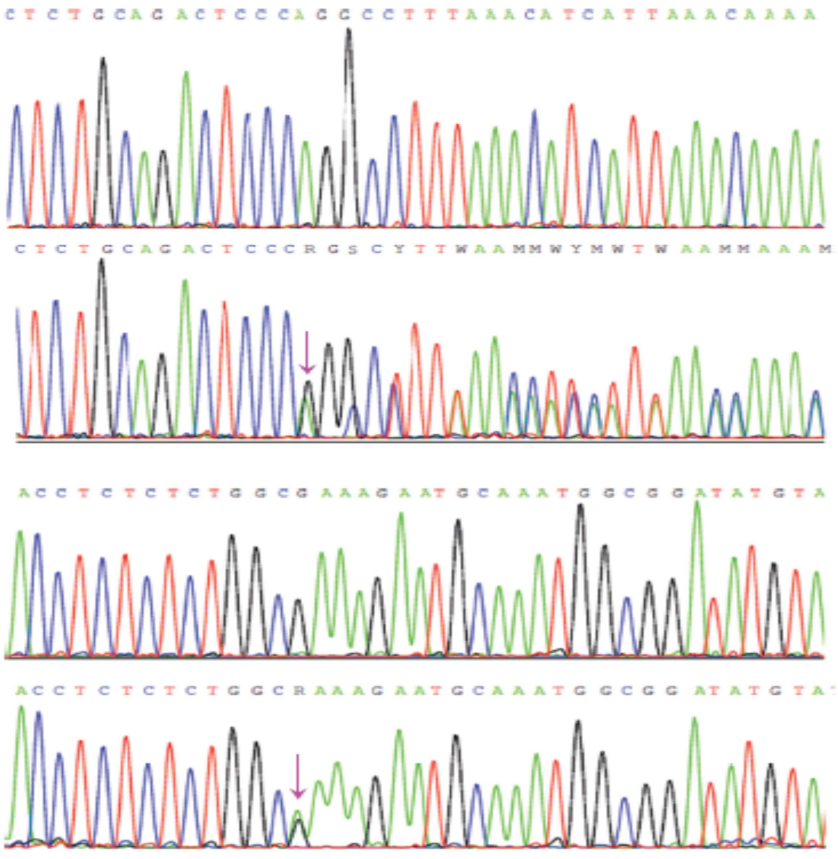

Figure 3 (A) Electropherogram of direct sequencing of *TRPS1*, exon 7. The lower panel corresponds to the sequence with the mutation (red arrow) found in patient 1 and her daughter, and the upper panel to the wild type. (B) Electropherogram of direct sequencing of *TRPS1*, exon 6. The lower panel corresponds to the sequence with the mutation (red arrow) found in patient 2, and the upper panel to the wild type.

On the other hand, patient 2 was initially given the diagnosis of possible PPHP due to the combination of language delay, obesity, short stature and brachydactyly without PTH resistance. After negative (epi)genetic results at the GNAS locus, *TRPS1* analysis identified a *de novo* mutation in exon 6, p.Arg921Gln (Figure 3B), which is considered a recurrent mutation (previously reported as p.Arg908Gln[2,6]).

DISCUSSION

AHO, described more than 70 years ago by Albright et al.[7], is a clinical entity that encompasses heterogeneous clinical findings including obesity, short stature, variable degrees of mental retardation, brachymetarcarpy and brachymetatarsy, as well as subcutaneous calcifications. It is one of the few monogenic hereditary obesity and mental retardation syndromes. Its prevalence is estimated to be approximately 0.79/100,000 (according to the Orphanet Report Series, November 2008).

This syndrome is often associated with pseudohypoparathyroidism (PHP-Ia, OMIM #103580)[7], a state of endocrine resistance to peptide hormones eliciting their signalling through the Gsα pathway. The current classification distinguishes between several entities of PHP with and without AHO, and AHO without endocrine abnormalities (pseudopseudohypoparathyroidism = PPHP, OMIM #612463)[1], all of them mainly caused by (epi)genetic alterations within or upstream of the GNAS locus. However, in a large subset of patients it is not possible to make a definitive diagnosis with a conclusive correlation between clinical, biochemical, and molecular genetic findings. In fact, the AHO phenotype is not specific to PHP or PPHP, as it is also present in other syndromes as Brachydactyly Mental Retardation syndrome (BDMR or AHO-like syndrome, OMIM #600430) and can even be misdiagnosed as other entities such as acrodysostosis (ACRDYS1, OMIM #101800; ACRDYS2, OMIM #614613)[8-10].

TRPS is a rare syndrome, the phenotype of which could also be easily confused with the AHO phenotype given the presence of brachydactyly and short stature. Even though obesity is among the AHO signs, but not those of TRPS, the patients reported in this article were referred to the endocrinologist for obesity (or overweight with a family history of obesity associated with type 2 diabetes). It is possible that obesity or overweight in the patients reported in the present work might have acted as a confusing factor, while they are attributable to the current obesity pandemic. Other clinical characteristics suggestive of an AHO phenotype such as a round face, shortening of metacarpals and short stature were also present. Besides, hormonal analysis of patient 1 revealed elevated serum PTH levels with normal phosphorus and calcium values. This normocalcemic hyperparathyroidism was not secondary to a 25-OH-vitamin D deficiency/insufficiency, and it was deduced that there was resistance to PTH. Though uncommon, these biochemical characteristics have already been described in PHP-Ia patients with *GNAS* loss-of-function mutations[5,11,12]. On the other hand, isolated GH deficiency was also observed in this patient and resistance to growth hormone-releasing hormone has been described in PHP-Ia patients[13]. However (epi)genetic analysis of the GNAS locus obtained normal results which have been described in nearly 30% of suspected cases of PHP/PPHP[14]. Clinical re-evaluation of our patients revealed some mild but specific features that prompted us to suspect the possibility of TRPS such as a pear-shaped nose, sparse hair and tooth hypoplasia. The genetic study of *TRPS1* revealed patient 1 and her affected daughter carried a not previously described nonsense mutation (c.2830delA) that cosegregates within the family with the phenotype. Patient 2 presents a previously described recurrent *de novo* mutation (Arg921Gln)[2,6]. Based on our findings we propose exhaustive clinical re-evaluation of each PHP/PPHP patient without (epi)genetic alterations at the GNAS locus, to proactively look for specific phenotypic features that might redirect the genetic study towards TRPS.

This is not the first time that TRPS and PHP/PPHP have been misdiagnosed[15,16]. Even though resistance to PTH is a typical feature of PHP-Ia, it

has already been reported in a patient with clinical characteristics of TRPS[16]. The observations described 30 years ago in that 14-year-old boy could make more sense nowadays looking at our TRPS case (patient 1), because, as stated before, partial resistance to PTH without associated hypocalcemia has also been found in some PHP-Ia patients[5,11,12].

The correct diagnosis of these patients leads to adequate follow-up and genetic counselling. In this case, these patients should not be treated and monitored in the same way as in PHP/PPHP[14] and they should receive genetic counselling appropriate for an autosomal dominant disease, without imprinting.

With the present report we would like to highlight the main differences that could help to distinguish the syndromes, in particular the sparse scalp hair and bulbous tip of the nose, both exclusive to and distinctive of TRPS[17]. Regarding radiological findings, although they are not highly specific for PHP/PPHP (apart from shortening of the IV and V metacarpals and distal phalanx of the thumb[18,19]), patients with TRPS present cone-shaped epiphyses at the phalanges, outcarving and deformation of the cones, more easily appreciated after epiphysis fusion[20,21]. These features have not been observed in patients with PHP/PPHP[19,22].

Moreover, the very common skeletal deformities that are typically found in patients with TRPS may also be misdiagnosed with a rheumatic process even in young adult patients[23]. Despite it not being the case, the hip and knee arthralgias of patient 1, together with her morbid obesity and the polyarthrosis of her mother and brother, might also have distracted her physicians from the correct diagnosis. Therefore, while managing and controlling the global epidemic of obesity and in order to achieve the best possible long-term outcome for our patients and their families, clinicians should maintain a general vision of the whole clinical picture of each obese/overweight patient and still be able to identify the presence of distinctive phenotypic features of syndromes like those exclusive to TRPS we have described in this report.

ACKNOWLEDGEMENTS

This work was partially supported by the Carlos III Health Institute (PI10/0148, PI13/00467 to GPdN). Mrs Arrate Pereda is funded by the Spanish Ministry of Health (FI11/00309), Dr Intza Garin by a Spanish fund for health research (FIS) programme (I3SNSCA10/01056) and Dr Guiomar Perez de Nanclares in part by the I3SNS Programme of the Spanish Ministry of Health (CP03/0064; SIVI 1395/09).

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PUBLICATION 7

Report of two novel mutations in *PTHLH* associated with brachydactyly type E and literature review

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Accepted by *American Journal of Medical Genetics, Part A*

ABSTRACT

Autosomal-dominant brachydactyly type E (BDE) is a congenital limb malformation characterized by small hands and feet as a result of shortened metacarpals and metatarsals. Alterations which lead to haploinsufficiency of PTHLH, the gene coding for parathyroid hormone related protein (PTHrP), have been identified as a cause of BDE in seven families. Here we report three patients affected with BDE, caused by PTHLH mutations expected to result in haploinsufficiency, and discuss our data compared to published cases.

INTRODUCTION

Brachydactyly (BD) refers to a group of limb malformations characterized by short fingers and toes. Autosomal-dominant brachydactyly type E (BDE) encompasses variable shortening of the metacarpals/metatarsals, with frequent involvement of the phalanges[1,2]. Brachydactyly type E can be isolated or syndromic and associated with other abnormalities as well as conditions such as Turner syndrome, pseudohypoparathyroidism, Bilginturan BD, Brachydactyly mental retardation syndrome, and BDE with short stature[3,4]. In some patients, the genetic cause of BDE remains unknown[3]. Haploinsufficiency of *PTHLH*, the gene coding for parathyroid hormone-related protein (PTHrP), has been identified as a cause of BDE, commonly associated with short stature[5–7].

The PTHrP protein is a polyhormone with multiple biologically active domains conferring different actions as an autocrine, paracrine, endocrine and intracrine hormone[8]. It has many biological actions during fetal growth and development and throughout life[9]. Its actions include: cartilage, mammary gland and dental development; involvement in gestation, reproduction and lactation; smooth muscle relaxation, central nervous system activity, and effect on the skin and hair follicles[9,10]. Haploinsufficiency of *PTHLH* causes a dysregulation leading to premature differentiation of chondrocytes without undergoing sufficient cell division to attain a normal bone size[11,12].

Here we report a family in which the daughter and her mother presented with BDE and short stature, a sporadic patient with mild BDE, and we compared both patients to previously reported patients.

CLINICAL REPORTS

This work was approved on January 3, 2013 by the Basque Clinical Research Ethics Committee (CEIC-E, Vitoria-Gasteiz, Spain; protocol number PI2012105).

Patient 1

The girl was born at 38 weeks of gestation with a birth weight of 2560 g (-2 SD) and length of 49 cm (-1 SD). She was referred to our clinic at 11.5 years of age for mild short stature [height=135.5 cm (-1.5 SD)]. Her growth rate was normal during the first four years of her life, following which it diminished to -1.5 SD. Her Tanner stage was P2 B1 at 11.5 years old. She had shortening of the fourth and fifth metacarpals and fourth metatarsal (Figure 1A-C). Premature fusion of the epiphyses was observed in the shortened bones (Figure 1B). She also showed shortening of the limbs, affecting primarily the forearms and the lower legs, with a slightly reduced arm span (ratio of arm-span to height of 0.97). Skeletal survey showed apparently low bone mineral density; however, DEXA scans were not performed. She had no missing teeth but she had several dental malpositions. Blood tests for PTH and TSH hormonal resistance were normal. At the age of 12.5 years, pubic hair was at P4 and axillary hair was noted; however, there was very little breast development (Figure 1D). In contrast, pelvic ultrasound showed an uterine length of 54 mm with thickening of endometrial mucosa and serum estradiol was 162 pmol/L, consistent with pubertal stage III.

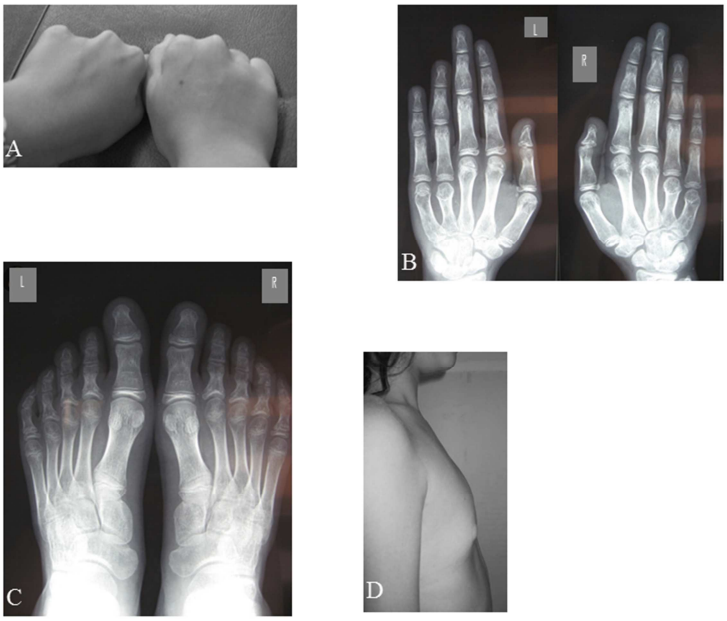

Figure 1. Patient 1. A: Hands: Note the retraction of the fourth and fifth knuckles due to short metacarpals. **B:** Hand X-rays: note the bilateral shortening of the fourth and fifth metacarpals and the first distal phalanges. Shortened metacarpals show premature fusion of epiphyses. **C:** Feet X-rays: mild shortening of the fourth metatarsal. **D:** No breast development at the age of 12.8 in spite of uterine length of 54 mm and serum estradiol levels of 162 pmol/L.

She reached menarche at 13.5 years, but her breast tissue was small (maximal chest circumference at 17.5 years: 78 cm).

Patient 2

The mother of patient 1 was 147 cm (- 3 SD) tall with short forearms and legs. Similar to Patient 1, her forearms and lower legs were primarily affected. Her arm span was reduced and arm span to height ratio 0.95. Hand X-rays showed short third, fourth and, fifth metacarpals, all the distal phalanges (Figures 2A and B) and the third and fourth metatarsals (Figure 2C). In addition, cone-shaped epiphyses were noted on hand and feet X-rays. She also had masses of the gums (Figure 2D). There was no oligodontia. She had small breasts but could breastfeed. Bone X-rays showed apparently low bone mineral density as observed in her daughter. Further investigations (phospho-calcium metabolism, PTH, TSH) were normal.

Both mother and daughter had apparently normal intellectual development and no reported learning difficulties. Segregation of the syndrome suggested an autosomal dominant mode of inheritance.

Figure 2. Patient 2. A: Hands: note the retraction of the third to fifth knuckles due to short metacarpals. **B**: Hand X-rays showing bilateral shortening of the third to fifth metacarpals and on X-rays all distal phalanges. The shortened metacarpals have cone-shaped epiphyses. **C:** Feet X-rays: shortened third and fourth metatarsals bilaterally. **D:** Gingival mass.

Patient 3

The girl was referred for pediatric endocrine consultation at 11.5 years old because of short fourth metacarpals (Figure 3A) and metatarsals (Figure 3B). Bone age was 13.5 years [13] (Figure 2A). Growth rate was above the mean until 12 years of age after which the velocity decreased, as predicted by the almost 2 years advanced bone age observed when she was 11.8 years old (Figures 2D-F). Predicted final height was 156 cm (-1 SD) with target height 164 cm. Breast development was III-IV, pubic hair was stage IV and her axillary hair was at Tanner III. Menarche occurred at 12.3 years.

Investigations showed normal values for phosphorus, calcium, PTH, and TSH. Dental development and general development were normal, and her family history was negative for brachydactyly.

Figure 3. Patient 3. A: Hands. **B:** Hand X-rays at age 11y10m showing a bone age of 13y6m**. C:** Photograph of feet showing bilateral mild shortening of the fourth metatarsal**. D:** Hand X-rays at age 13y2m showing a bone age of 14y6m**. E:** Hand X-rays at age 13y9m showing a bone age of 15y**.** Note mild BDE with isolated shortening of bilateral fourth metacarpals and premature fusion of corresponding epiphyses. **F:** Growth chart: note that the growth velocity decreased prematurely around 12 years and that final height is virtually attained at 13y.

MOLECULAR ANALYSIS

DNA studies

Genomic DNA was extracted from peripheral blood mononuclear cells using a QIAamp DNA Mini Kit (QIAGEN, Düren, Germany), according to the manufacturer's instructions. *PTHLH* coding exons and exon-intron junctions of all transcripts were amplified (primers available on request), and analyzed by direct nucleotide sequence analysis using standard methods on an ABI 3500 genetic analyzer (Applied Biosystems, Foster City, CA, USA) as described[14]. We employed the reference sequences NM_198965.1 for mutation description according to HGVS nomenclature.

RNA studies

RNA was extracted from peripheral blood mononuclear cells, using PAXgene Blood RNA Kit (QIAGEN, Düren, Germany) according to the manufacturer's instructions. Exonic primers were designed to amplify transcripts of the three isoforms of PTHrP protein. An intronic primer was designed to detect intron retention for intron V in mutant transcripts. Primer annealing conditions were set up using a commercial mRNA mix (qPCR Human Reference Total RNA, Clontech Laboratories, Mountain View, CA). Reverse transcription polymerase chain reaction (RT-PCR) was performed in parallel with total RNA extracted from patients and with commercial mRNA mix (as a positive control for the technique and primers) under established conditions.

Splice site *in silico* analysis

The Human Splicing Finder v2.4.1 online tool[15] was used to analyze the effect of mutations.

RESULTS

PTHLH sequencing showed distinct and novel alterations in the two families. Patients 1 and 2 had a c.47_101+73del128 heterozygous deletion, which comprises bases 47 to 101 of exon V and 73 bases of intron V (total deletion: 128 bp) (Figure 4). Patient 3 had a c.101+3delAAGT heterozygous *de novo* deletion (parents were non carriers). This alteration deletes four nucleotides from position 3 to 6 of intron V (Figure 4). Both mutations are localized in the first coding exon of the protein (the promoter consists of exons I, II, III and IV) shared by all PTHrP isoforms (Figure 4) and have not been previously reported in the literature or in available databases.

The c.47_101+73del128 defect removes the canonical donor site of exon V. Consequently, new potential splice sites localized before or after the deletion breakpoint are predicted to be used. These aberrant splicings are expected to produce transcripts with premature stop codon 5' to the last 50 nucleotides of the penultimate exon[16], which are predicted to be degraded by nonsense-mediated mRNA decay.

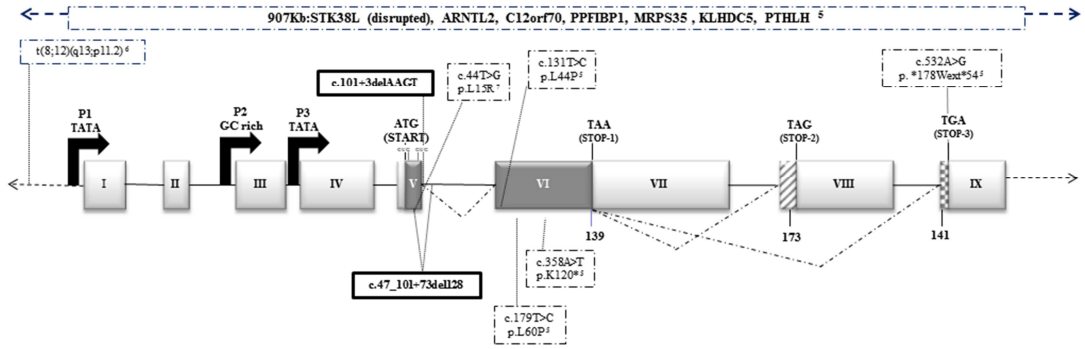

Figure 4. Representation of the structural organization of the *PTHLH* gene. Gene expression is regulated by three promoters (black arrows). The gene consists of nine exons (coding in gray boxes, and both promoter untranslated exons and untranslated 3' regions of exons VII, VIII and IX non-coding exons in white boxes) with alternative splicing generating three isoforms with different C-terminal region (in gray the common 139 amino-acid (aa) sequence for all transcripts [exon V and VI], in dashed gray the splicing for 173 aa isoform [exon VIII] and dotted gray for the 141 aa isoform [exon IX]). Numbers below the exons specify the amino acid position corresponding to the stop codons for each isoform (139 aa, 173 aa, and 141 aa, respectively); the alternative non-AUG initiations codons are indicated in small gray letters. Apart from the two intragenic mutations reported here (flagged in bold outline boxes), seven distinct mutations that lead to *PTHLH* haploinsufficiency have previously been reported (flagged in dotted outline boxes): a translocation with breakpoints upstream of the gene; a chromosomal microdeletion encompassing six genes including *PTHLH*; three missense; one nonsense, and one non-stop change.

The second deletion (c.101+3delAAGT) is predicted to cause a moderate decrease in the consensus sequence value of the natural donor splice site (WT 84.38; Mut 60.23; Var. % 28.62). As a result, novel additional potential donor sites with a higher consensus value could be created. Use of these sites is predicted to lead to aberrant transcripts with a premature stop codon, as in the first family.

RNA was analyzed by RT-PCR to confirm the presence/absence of mutant transcripts. However, while we were able to amplify and sequence RT-PCR products from commercial control RNA, no amplification was observed for total RNA extracted from patients or controls. RNA quality was confirmed by independent amplification of 18S (data not shown).

Table 1: Summary of features shown in reported patients.

Report	Klopocki et al., 2010					Maass et al., 2010	Wang , et al 2015	Present Report		
	Family 1	Family 2	Family 3	Family 4	Family 5	Family report	Family report	Patient 1 (Index)	Patient 2 (Mother)	Patient 3
Gene defect	Microdeletion [*STK38L* (disrupted), *ARNTL2, C12orf70, PPFIBP1, MRPS35, KLHDC5,* ***PTHLH***]	c.179T>C/p.L60P	c.131T>C/p.L44P	c.532A>G/p.*178Wext*54	c.358A>T/p.K120*	t(8;12)(q13;p11.2)	c.44T> G/p.L15R	c.47_101+73del128		c.101+3delAAGT
Mutated sequence	Complete deletion	Exon VI	Exon VI	Exon IX	Exon VI	Upstream of the gene	Exon V	Exon V		Exon V
Affected isoforms	139 aa, 141 aa, 173 aa	139 aa, 141 aa, 173 aa	139 aa, 141 aa, 173 aa	141 aa	139 aa, 141 aa, 173 aa	?	139 aa, 141 aa, 173 aa	139 aa, 141 aa, 173 aa		139 aa, 141 aa, 173 aa
Predicted effect of mutation on protein	Deletion of entire protein	N-terminal (critical aa for binding to ECD of PTHR1)	N-terminal (region binding to PTHR1 7-TM domain)	Elongation of C-terminal	Mid-region (importin β recognition side)	Downregulated expression	Signal peptide (loss of cleavage site)	NMD		NMD
Number of affected patients in the family	8 studied individuals, (at least 3F and 1M)*	1	1	3 (1M; 2F)	3 (2F;1M)	4 (3M;1F)	7(5F; 2M)	2		1
Age	ND	41y	9y2m	14y (index), adults (1M; 1F)	At least 2F adults	ND (at least 3 adults)	ND (at least 4 adults)	11y6m	Adult	11y6m
Sex	F (index)	F (index)	F (index)	F (index)	F (index)	F (index)	M	F	F	F
BDE	MC/MT: 4th and 5th most frequently and more severely; small MP and DP, in severely affected patients	MC: 3rd-5th; MP: 2nd and 5th; DP: 1st	Cone-shaped and premature fusion of epiphyses. MC:3rd-5th; MP: 2nd and 5th; DP: 1st	Abnormal metacarpal epiphyses prematurely fused to the metaphyses. 1st small nail; MC: 3rd and 5th (index and grandfather); mother only MC: 3rd	MC: 3rd-5th	Cone-shaped epiphyses. MC: 3rd-5th; variable involvement of PP, MP, DP	Abnormal epiphyses. MC/MT: 1st-5th; PP: 2nd and 5th; MP: 2nd-5th; DP: 1st-4th	MC: 4th and 5th; MT:4th; DP: 1st	MC: 3rd-5th; MT: 3rd and 4th; DP: 1st-5th	MC: 4th; MT: 4th
Short stature	6/8 (-2.0 to -2.8 SD<3rd centile)	-2.97 SD < 3rd centile	Normal (+0.6 SD)	2/3 (-3.6 and -2.4 SD<3rd centile)	3/3	In the range of lower normal (10-97th centile)	7/7 (>−2 SD)	-1.5 SD	-3 SD	+0.5 SD at 12y, but predicted final height: −1 SD.
Arm span/height (mean normal value: 1.01-1.02)	ND	ND	ND	ND	ND	0.88-0.95	Normal	0.97	0.95	Normal
Dental abnormalities	No	No	Abnormal primary and secondary dentition	No	2/3 showed oligodontia	ND	No	Dental malpositions	Gingival masses	Normal
Apparent Intellectual disability	Learning difficulties	No	No	No	No	No	No	No	No	No
Others						Macrocephaly, prominent forehead, depressed nasal root	Round face	Apparently low bone mineral density		-

F: female; M: male; MC: metacarpals; MT: metatarsals; PP: proximal phalanges; MP: middle phalanges; DP: distal phalanges; ND: no data; NMD: nonsense-mediated decay; ECD: extracellular domain; 7-TM: 7-transmembrane helical domain. *Even if the authors showed a pedigree of 27 patients with BDE, clinical data of only eight of them was described (sex was not indicated in all of them).

DISCUSSION

In 2010, Klopocki et al.[5] reported a syndrome which they named "BDE with short stature, PTHLH type" (OMIM#613382), which consists of BDE and, in most patients, short stature associated with *PTHLH* gene mutations. Delayed dental eruption and/or oligodontia were an additional, but not an obligatory, feature. Further reports have proposed *PTHLH* as a candidate gene for patients with BDE and short stature of unknown origin[5–7,17,18].

Interestingly, PTHrP, coded by *PTHLH* gene, binds to PTHR1, a G protein-coupled receptor (GPCR), which activates the Gsα-cAMP-PKA-PDE4D signaling pathway. Mutations in genes coding for proteins in this pathway lead to pseudohypoparathyroidism and pseudopseudohypoparathyroidism (caused by *GNAS* alterations) and acrodysostosis (caused by *PRKAR1A* or *PDE4D* mutations), all associated with BDE[19,20].

The patients reported here had two distinct and novel *PTHLH* intragenic deletions. These were predicted to cause a splicing defect and PTHrP haploinsufficiency for two main reasons: (i) they are expected to lead to a truncated protein; (ii) one mutation co-segregated within the family (in Patient 3 it was a *de novo*), although we could not confirm the reduction in *PTHLH* mRNA (due to the low and most likely illegitimate expression of the transcripts in blood)[18].

Review of previously reported patients indicated that all reported patients with *PTHLH* mutations had brachydactyly with intrafamilial variability in regard to the digits affected. Of the 27 reported individuals, 19 had short stature (below -2 SD or at the lower range of normal) and three had dental anomalies (Table I).

The patients reported here had short stature (in patient 2) and brachydactyly (all three patients), features associated with the reportedly predicted function of the *PTHLH* gene[9,10] and the previously reported patients[5–7,17,18]. The described BDE pattern usually involves shortening of the third to fifth

metacarpals and first distal phalanges (as in patient 2) but other patterns of shortening have also been reported[5–7]. In this report, the phenotype of patient 3 is mild (isolated shortening of the fourth metacarpals and metatarsals). A possible explanation for this mild presentation could be the effect of the splicing defect. Indeed, as indicated in the results, even if the mutant allele presents novel potential donor splice sites with increased consensus splicing value, the natural site only presents a moderately decreased consensus splicing value and is likely partially used, as previously reported[21–25]. Thus, the haploinsufficiency in this patient would be partial. In contrast, in patient 1, affected by a more marked phenotype for BDE with height and dental alterations, the splicing defect is expected to cause complete haploinsufficiency[26].

The complexity and widespread actions of PTHrP could be responsible for the intrafamilial and interfamilial phenotypic variability. In fact, in all reported patients, even if the mutations segregated with the disease and had complete penetrance of the BDE phenotype, other manifestations in the affected individuals were still variable[5–7], as observed in the patients reported here. Most affected individuals had short stature or final height within the lower range of normality[5–7]; an adult average height of 150 cm and 162 cm in females and males, respectively, was reported by Klopocki et al.[5]. Growth of affected patients stopped prematurely[5] due to early closure of the growth plate[27]. Patient 1 was within the lower normal range for her age and her mother (Patient 2) had short stature. In addition, both patients had short forearms and lower legs and a slight reduction in arm span to height ratio (even if in the normal range), as reported[6]. Although Patient 3 had normal stature for her chronologic age up to age 12, a premature arrest in growth at age 13 was observed. In fact, her predicted final height was 156 cm, 8 cm less than her target height. This sudden growth arrest was associated with an advanced bone age that leads to a premature fusion of epiphyses of the phalanges and metacarpals, which result in abnormal cone-shaped epiphyses and brachydactyly[5–7].

Another physical feature that should be examined in *PTHLH* mutation carriers is the development of mammary glands. *Pthlh* gene-manipulated mice

have ectodermal dysplastic phenotypes[9,12,28]. PTHrP-null mice, which had been rescued from neonatal death by targeting PTHrP expression to chondrocytes, lack mammary epithelial ducts due to a failure of the initial round of branching growth required for transforming the mammary bud into the primary duct system[28,29]. In humans, complete inactivation of *PTHR1* causing Blomstrand's lethal dysplasia, results in mammary gland defects[30]. Neither the index patient reported by Maas et al.[6] nor the family reported by Wang et al.[7] had abnormal breast development, while this point is not discussed in the patients reported by Klopocki et al.[5]. In the family reported here, the mother had small breasts, although she could breastfeed her children, while the daughter had small breast size, although uterine size and shape reflected correct estrogenic effect. This indicates that PTHrP haploinsufficiency could lead to a delayed or poor development of mammary gland also in humans, albeit with variable expressivity.

In conclusion, this is the fourth report on *PTHLH* mutations leading to BDE with/without short stature and the first to report a patient with signs suggestive of breast developmental delay, suggesting that the spectrum of symptoms associated with this syndrome is broader than previously reported.

CONFLICT OF INTEREST

None of the authors has a conflict of interest to declare.

ACKNOWLEDGEMENTS

The authors thank the individuals and their families who took part in this study. This work was partially supported by the Carlos III Health Institute (PI10/0148 and PI13/00467 to GPdN) within the *Plan Estatal de I+D+I 2013-2016,* and by FEDER (European Regional Development Fund), and continued funding from INSERM and Assistance Publique - Hôpitaux de Paris (CTT, AL, and CS). Mrs Arrate Pereda was funded by the Spanish Ministry of Health (FI11/00309), and Dr Guiomar Perez de Nanclares was in part funded by the I3SNS Program of the Spanish Ministry of Health (CP03/0064; SIVI 1395/09).

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PUBLICATION 8

Manifestaciones clínicas causadas por alteraciones en la vía de señalización de la proteína G

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In *IV Symposium Nacional de Endocrinología Pediátrica*. Yturriaga R, Argente J, Garagorri JM (Eds.). Salerno: Momento Médico, 2014: 49-64.

El hipoparatiroidismo, el pseudohipoparatiroidismo, la acrodisóstosis, la condrodisplasia de Blomstrand y la braquidactilia tipo E aislada asociada a talla baja son entidades clínicas que pueden ser confundidas unas con otras al presentar manifestaciones clínicas y fisiopatológicos comunes. El objetivo de la presente revisión es mostrar las características comunes y específicas de cada una de estas enfermedades de cara a poder dar unas pequeñas guías que faciliten el correcto diagnóstico clínico.

BASES FISIOPATOLÓGICAS

La PTH, principal hormona reguladora del calcio sérico, actúa en los riñones y los huesos como órganos diana a través de su receptor PTHR1 acoplado a proteína Gsα[1]. La secreción de PTH de la glándula paratiroidea está estrictamente regulada y aumenta en respuesta a niveles bajos de calcio en suero[2].

Con el fin de entender las similitudes de estas cinco enfermedades, conviene recordar que el mecanismo de acción de la PTH se basa en su unión a un receptor de membrana (PTHR1) que está acoplado a una proteína G (Figura 1)[3]. La proteína G estimuladora (Gs) es miembro de la familia de proteínas G, y está constituida por la subunidad α específica que se une al nucleótido guanina del grupo GTP/GDP e interacciona con los receptores y efectores específicos, y las subunidades β y γ que forman el complejo necesario para la activación de Gsα por los receptores[4,5]. La función de la proteína Gs es trasmitir señales desde los receptores de la superficie celular hasta los efectores intracelulares (la adenilato ciclasa en el caso de la PTH) que generan segundos mensajeros (AMPc). El AMPc activa a la proteinkinasa A (PKA) al unirse a sus subunidades reguladoras. A continuación, las subunidades catalíticas se liberan y fosforilan diferentes proteínas en sus residuos de serina/treonina[6]. Las fosfodiesterasas actúan como reguladores negativos al unirse al AMPc[7].

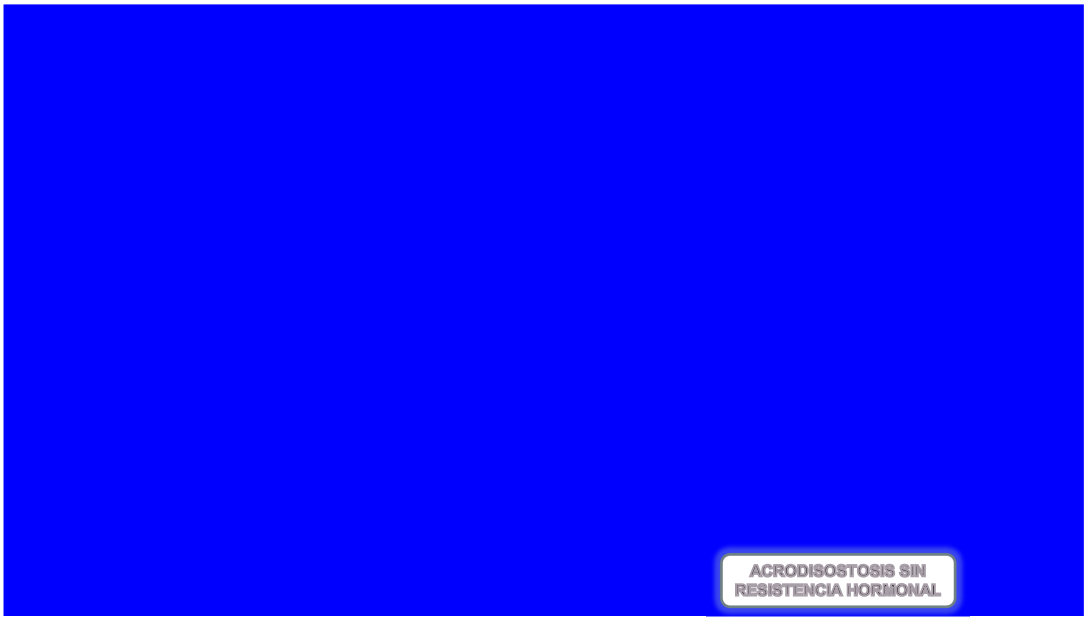

Figura 1. Esquema del mecanismo de acción de hormonas mediado por receptores acoplados a proteína Gs. La unión de la hormona a su receptor provoca la activación de la subunidad α de la proteína Gs, que interaccionará con la adenilato ciclasa, produciendo la síntesis de AMPc, que funcionará como segundo mensajero activando a la proteína kinasa A, que mediante la activación de nuevas proteínas transmitirá la información procedente del estímulo hormonal. Mutaciones inactivantes a distintos niveles de esta vía de señalización se asocian con diferentes enfermedades genéticas.

Esta vía de señalización Gsα/AMPc/PKA está presente en casi todos los tipos celulares y media multitud de funciones biológicas. En el sistema endocrino, son muchas las hormonas que usan receptores acoplados a la proteína Gsα y adenilato ciclasa para generar AMPc, por lo que una alteración en algún punto de esa vía puede provocar no solamente resistencia a la PTH sino también a la TSH, LH, FSH, GHRH, ADH, glucagón, ACTH y calcitonina, entre otras.

El hipoparatiroidismo, el pseudohipoparatiroidismo, la acrodisóstosis, la condrodisplasia de Blomstrand y la braquidactilia tipo E aislada asociada a talla baja están causados por mutaciones inactivantes en distintas proteínas de esta vía metabólica.

HIPOPARATIROIDISMO

El hipoparatiroidismo se caracteriza por hipocalcemia e hiperfosfatemia en presencia de valores bajos o inapropiadamente normales de PTH. Las causas del hipoparatiroidismo pueden ser (i) dificultad para sintetizar o secretar PTH, (ii) destrucción del tejido paratiroideo y (iii) resistencia periférica a la PTH.

Puede presentarse como manifestación de un síndrome, como en el síndrome diGeorge (causado por deleciones en el 22q11)(OMIM#192430), en el síndrome de displasia renal-sordera-hipoparatiroidismo (causado por mutaciones en *GATA3*) (OMIM#146255), el síndrome Kenny-Caffey (OMIM#244460) y Sanjad-Sakati (OMIM#241410) (ambos causados por mutaciones de pérdida de función autosómicas recesivas en *TBCE*); o de forma aislada, causado por alteraciones en el gen codificante de la *PTH* (11p15), *GCMB2* (glial cells missing-2 gene, 6p24.2) o en el receptor sensor del calcio, *CaSR* (3q21.1). Sin embargo, la mayoría de los casos de hipoparatiroidismo se desconoce la causa genética[8].

BRAQUIDACTILIA TIPO E

Fueron Mass y colaboradores los primeros en implicar al gen *PTHLH (parathyroid hormone-like hormone gene)*, localizado en 12p11.22, en la braquidactilia de tipo E (BDE) asociada a talla baja (OMIM#613382) al identificar una traslocación t(8;12) (q13;p11.2) en una familia con BDE autosómica dominante en presencia de extremidades cortas, facies dismórfica, macrocefalia, frente prominente y raíz nasal deprimida[9]. Recientemente también se han descrito mutaciones inactivantes a nivel de este gen[10].

PTHLH codifica para la proteína relacionada con la PTH (PTHrP) que, debido a su gran homología con la PTH puede usar su mismo receptor y activarlo[11]. Estudios en ratón muestran que la unión de PTHrP a PTHR1 facilita la proliferación continua de condrocitos en la placa de crecimiento y retrasa su

diferenciación en condrocitos hipertróficos. Por otro lado, la falta de PTHrP acelera el proceso de diferenciación de los condrocitos en la placa de crecimiento, lo que conlleva un mayor aumento de su apoptosis y su reemplazo prematuro por osteoblastos[1,12]. Esto podría explicar la talla baja final de los pacientes con mutaciones en PTHLH que, además, pueden presentar un acortamiento de la envergadura en relación al tronco[9]. Koplocki *et al.* observaron que la velocidad de crecimiento de estos pacientes se frena de golpe y de forma prematura, lo que explica su talla final disminuida[10]. Los huesos principalmente afectados son los metacaparpos, metatarsos y falanges, todos con una única placa de crecimiento.

OSTEOCONDRODISPLASIA DE BLOMSTRAND

La osteocondrodisplasia de Blomstrand (BOCD, OMIM#215045) es una enfermedad autosómica recesiva causada por mutaciones homozigotas o en heterozigosis compuesta en el gen *PTHR1*.

El gen *PTHR1* codifica para el receptor de la PTH y del péptido asociado a la PTH (PTHrP). Este receptor juega un importante papel en la regulación del metabolismo fosfo-cálcico a nivel fetal y en la vida adulta al mediar las acciones de la PTH sobre sus órganos diana. Algunos estudios han demostrado también que pudiera participar en el desarrollo fetal debido a la función de PTHrP sobre el desarrollo del cartílago y el hueso[13,14].

Clínicamente la osteocondrodisplasia de Blomstrand se caracteriza por una maduración adelantada y una osificación prematura de todos los elementos esqueléticos. Además presenta manifestaciones extraesqueléticas como polihidramnios, hidropsia fetal, pulmones hipoplásicos, anomalías dentales, coartación de la aorta y ausencia de desarrollo mamario[15–18].

Algunos autores proponen que la BOCD puede subdividirse en la tipo I, de manifestación más severa y caracterizada por huesos extremadamente cortos y malformados, y la tipo II, de menor severidad[17]. Hoogendam y colaboradores

proponen que estas diferencias en la gravedad de los síntomas pueden deberse al tipo de alteración identificada, siendo las que conllevan una inactivación completa de la proteína responsables de la subtipo BOCD-I, mientras que aquellas mutaciones que mantienen una actividad residual se asociarían con BOCD-II[19].

PSEUDOHIPOPARATIROIDISMO TIPO I

El Pseudohipoparatiroidismo (PHP) comprende un grupo heterogéneo de enfermedades endocrinológicas que se caracterizan por la existencia de hipocalcemia, hiperfosfatemia y resistencia tisular a la PTH. En este desorden endocrino, según parece, la resistencia a la PTH ocurre sólo en el túbulo renal proximal, mientras que las acciones de la PTH se mantienen intactas en otros tejidos diana, como el hueso y el túbulo grueso ascendente. El nivel de fosfato sérico suele ser elevado debido a la incapacidad de la PTH para inhibir la reabsorción de fosfatos en el túbulo renal proximal y debido a la acción intacta de resorción de la PTH en el hueso, la cual conduce a la movilización de fosfato y calcio. En pacientes con PHP la concentración de PTH en suero es elevada, indicando que el defecto es la resistencia del órgano diana en lugar de deficiencia de PTH. Debido a las funciones intactas de la PTH, en algunos pacientes con PHP, una elevada concentración de PTH puede mantener dentro de los rangos de normalidad el nivel de calcio sérico durante períodos prolongados de tiempo. Sin embargo, la mayoría de estos pacientes presentan, en algún momento de sus vidas, hipocalcemia asociada con manifestaciones clínicas, como espasmos musculares o convulsiones, y requieren el tratamiento con suplementos de calcio oral y de 1,25 dihidroxivitamina D[4–6].

Clásicamente, los pacientes con PHP-I se han subclasificado en función de la presencia (PHP-Ia, OMIM#103580, y PHP-Ic, OMIM#612462) o ausencia (PHP-Ib, OMIM#603233) del fenotipo de Osteodistrofia Hereditaria de Albright (Tabla 1A). Intentaremos resumir los principales avances que ha habido en los últimos años comparándolos con la clasificación más clásica.

Tabla 1A. Clasificación clásica de los distintos subtipos de pseudohipoparatiroidismo (PHP) de tipo I.

	FENOTIPO	RESIST. HORMONAL	Ca S	P S	AMPc PTHex	ACTIVIDAD Gsα
PHP IA	AHO	PTH TSH Gn	↓	↑	↓	↓ 50%
PHP IB	NORMAL	PTH TSH	↓	↑	↓	NORMAL
PHP IC	AHO	PTH TSH Gn	↓	↑	↓	NORMAL

AHO: Osteodistrofia Hereditaria de Albright; CaS: calcio sérico; PS: fosfato sérico; PTHex: administración exógena de PTH.

Osteodistrofia hereditaria de Albright

La Osteodistrofia hereditaria de Albright (AHO, *Albright's Hereditary Osteodistrophy*) fue descrita por Albright en 1942 como una entidad clínica caracterizada por braquidactilia, cara redondeada, baja estatura, obesidad central, osificaciones subcutáneas y grado variable de retraso mental[20].

En concreto, la braquidactilia se describe como acortamiento del III, IV y V metacarpianos y I falange distal. Sin embargo, este diagnóstico no siempre es sencillo debido a que, en población general, el acortamiento de los dedos de la mano es una manifestación inespecífica. Resulta, por tanto, imprescindible la realización de una radiografía de la mano para poder medir los metacarpianos y las distintas falanges y establecer el Z-score de cada hueso para construir el perfil metacarpofalángeo[21,22].

Aunque el retraso mental en sus diferentes grados formó parte de la descripción inicial del síndrome, varias líneas de trabajo señalan una gran discrepancia en la frecuencia y severidad de este síndrome entre la población adulta con AHO/PHP-Ia (27%) y la pediátrica (64%)[23,24]. Es necesaria una evaluación sistemática con test estandarizados para poder evaluar la frecuencia y severidad de este retraso mental en poblaciones más amplias de pacientes.

En muchos pacientes con AHO, durante su exploración física, es posible observar la presencia de nódulos calcificados. Se trata de verdaderas osificaciones heterotópicas intramembranosas, normalmente limitadas a los tejidos subcutáneos, de número y tamaño variable. En algunos pacientes, pueden progresar a tejidos más profundos, como ocurre en la heteroplasia ósea progresiva (POH, OMIM#166350)

En cuanto a la obesidad, a pesar de haber sido descrita como una característica del fenotipo de AHO, se ha descrito recientemente que se trata de un signo más propio del PHP-Ia que del AHO[25].

Diez años más tarde de la descripción del fenotipo de AHO, Albright y colaboradores observaron que este fenotipo podía ocurrir no sólo asociado a resistencia a la PTH, sino también en su ausencia, y nombraron a esta nueva manifestación como pseudopseudohipoparatiroidismo[26]. El PPHP puede encontrarse en familias con PHP-Ia o de forma aislada.

Mutaciones inactivantes: Pseudohipoparatiroidismo tipo Ia y Ic

Los pacientes con PHP-Ia y –Ic presentan resistencia a otras hormonas que, al igual que la PTH, realizan su función a través de receptores ligados a proteína G, como puede ser la TSH, gonadotrofinas y GHRH. El grado de resistencia a estas hormonas presenta diferente severidad entre distintos pacientes y varía a lo largo del tiempo[4,5,27,28].

Así, la manifestación clínica más evidente suele ser la resistencia renal a la PTH, que en la mayoría de los casos se presenta como hipocalcemia, hiperfosfatemia y valores elevados de PTH circulante, a pesar de que existen algunos pacientes que se mantienen normocalcémicos a pesar de la resistencia a PTH[5,27]. La resistencia a la PTH se desarrolla en los primeros años de vida, con la hiperfosfatemia y elevación de PTH precediendo a la hipocalcemia. A diferencia de los pacientes con hipoparatiroidismo, los pacientes con PHP no suelen desarrollar hipercalciuria, sino que suelen mantener una función renal normal.

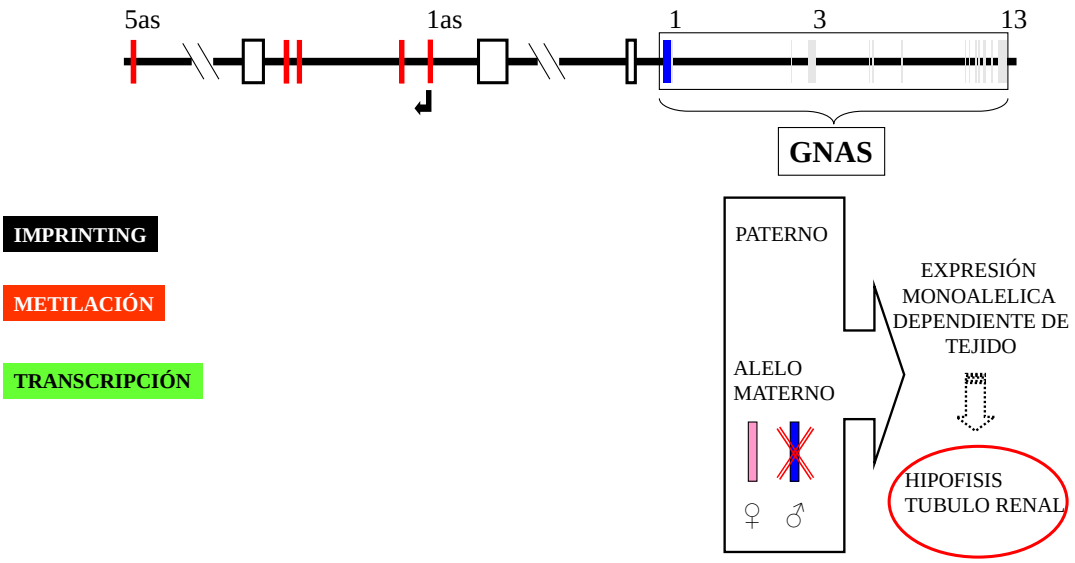

Figura 2. Patrón de impronta del gen *GNAS*. Los transcritos de Gsα se expresan bialélicamente excepto en algunos tejidos como el túbulo renal proximal, tiroideas, gónadas e hipófisis. *Se desconoce el mecanismo de impronta del exón 1 de *GNAS*.

La resistencia a la TSH suele manifestarse en la infancia o adolescencia[5], aunque se han descrito casos donde el hipotiroidismo ha sido detectado en el screening neonatal[29]. La resistencia es generalmente leve, con valores de TSH mínimamente elevados con valores de hormona tiroidea entre normales y ligeramente bajos.

Los paciente con PHP-Ia, en concreto las mujeres, presentan evidencia clínica de hipogonadismo, manifestado como maduración sexual retrasada o incompleta, amenorrea u oligomenorrea, y/o infertilidad[30].

La deficiencia de GH causada por resistencia a GHRH ha sido descrita en largas series de pacientes con PHP-Ia, este defecto parece ser más frecuente en adultos que en niños[31,32].

En 1990 se describió la primera mutación inactivante en heterozigosis en el gen que codifica para la subunidad α de la proteína G estimuladora (Gsα), el gen *GNAS*, como causa del PHP-Ia[33]. Este gen está localizado en el brazo largo del cromosoma 20 (20q13) y consta de 13 exones codificantes. Se han descrito mutaciones a lo largo de todo el gen, por lo general específicas de cada familia, si bien existe un *hot-spot* en el exón 7 consistente en la deleción de cuatro nucleótidos (para revisión de las mutaciones, consultar Elli *et al.*[34]).

Estudios en familias donde coexistían pacientes con PHP y PPHP permitieron identificar que, los hijos de mujeres portadoras presentan PHP-Ia, mientras que los hijos de varones portadores presentan PPHP. Esto es debido al fenómeno de impronta (del inglés, *imprinting*). En concreto, el gen *GNAS* presenta expresión materna (impronta paterna) en determinados tejidos como son túbulo renal, hipófisis y tiroides. En el resto de los tejidos, la expresión es bialélica (Figura 2). Por tanto, cuando la madre es portadora de la mutación y la transmite a la descendencia, a nivel de túbulo renal, hipófisis y tiroides no existe copia funcional, generándose la resistencia hormonal; resistencia que no aparece en el caso de padre portador, dado que la expresión en esos tejidos no se ve afectada. La haploinsuficiencia en el resto de los tejidos, bien por mutación paterna o materna, sería la responsable de la aparición del fenotipo de AHO (Figura 3). Por tanto, de cara al consejo genético hemos de tener en cuenta que las mutaciones en *GNAS* desde un progenitor portador pueden ser transmitidas en el 50% de los casos. Si la mutación es transmitida por un varón a su descendencia, los hijos no presentarán alteraciones hormonales, es decir, presentarán un cuadro de pseudopseudohipoparatiroidismo. Sin embargo, si es una mujer la que transmite la mutación, la descendencia presentará pseudohipoparatiroidismo (es decir, fenotipo de Albright en presencia de alteraciones hormonales como PTH, TSH y gonadotropinas).

Los pacientes con PHP-Ia y PHP-Ic son clínicamente idénticos, con presencia de fenotipo AHO y resistencia multihormonal[35]. Sin embargo, los pacientes con PHP-Ia muestran un descenso parcial (alrededor del 50%) de la actividad de proteína Gsα en las membranas de varios tipos celulares (eritrocitos, fibroblastos, plaquetas) debido a una disminución en los niveles de RNA y/o proteína[36,37], mientras que este defecto está ausente en los pacientes con PHP-Ic, aunque parece poder explicarse por las limitaciones del ensayo más que por diferencias biológicas reales[38]. De hecho, los pacientes con PHP-Ic suelen presentar mutaciones en el exón 13 del gen *GNAS*, exón que codifica para el dominio de unión de Gsα al receptor transmembrana acoplado a proteínas G[35,38]

Alteraciones en la impronta: Pseudohipoparatiroidismo tipo Ib

Clásicamente, con PHP-Ib nos referimos a la presencia de resistencia a la PTH en ausencia de otras alteraciones hormonales, ausencia de fenotipo de AHO y actividad normal de Gsα[4]. Posteriormente, en amplias series de pacientes se describió la resistencia a la TSH[39,40], mientras que la acción de la GH parece conservada[40].

Normalmente se presenta de forma esporádica, aunque en algunas ocasiones se presenta de forma familiar con un patrón autosómico dominante (AD-PHP-Ib). No se han observado diferencias clínicas entre las formas familiares y las esporádicas[41].

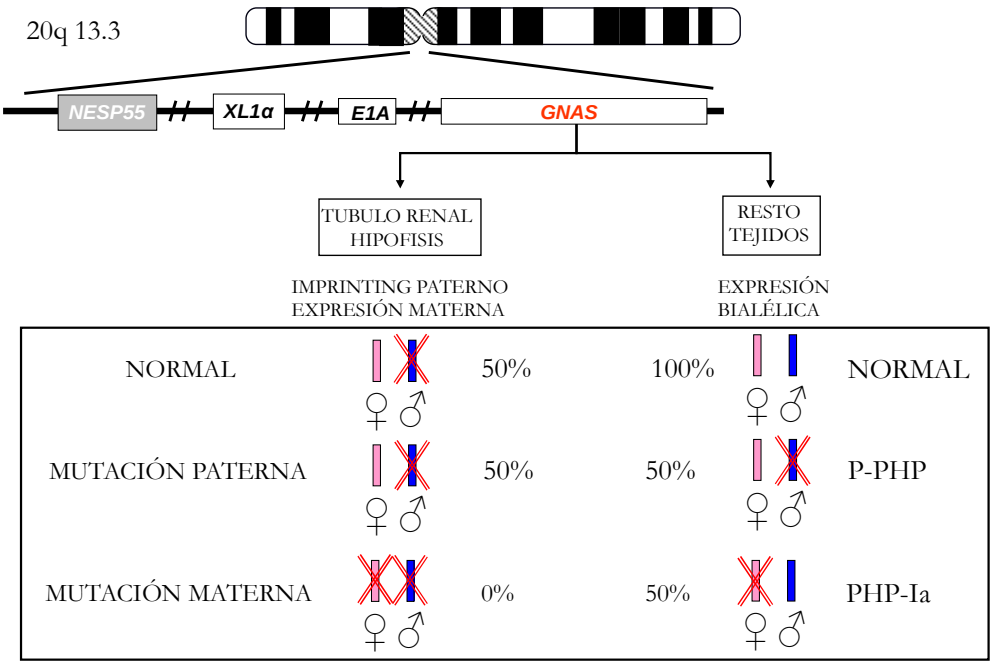

Figura 3. El gen de la proteína Gsα procedente del padre está sometido al fenómeno de *imprinting* en el túbulo renal proximal, tiroideas, gónadas e hipófisis y no se expresa. En el resto de tejidos la expresión es bialélica. Cuando el alelo de la madre presenta una mutación, en túbulo renal, tiroides, gónadas e hipófisis, no se expresa ningún alelo (0%), ya que el alelo paterno está inactivado por el efecto del *imprinting*, y en el resto de los tejidos como no está inactivado el alelo paterno (es expresión bialélica) se produce el 50% de la actividad. Desde un punto de vista clínico conllevará la aparición de pseudohipoparatiroidismo en la descendencia. Si es el alelo paterno el mutado, no habrá resistencia hormonal, ya que en hipófisis, tiroides, túbulo renal, etc es el alelo mutado el que está inactivo (se mantiene el 50% de la actividad correspondiente al alelo materno) y y en el resto de los tejidos, en los que debiera haber expresión bialélica un alelo está alterado por la mutación (expresión del 50%) dando un fenotipo AHO característica de pseudopseudohipoparatiroidismo

Desde el punto de vista genético, el PHP-Ib se ha visto asociado a alteraciones en la impronta del locus GNAS. En el locus GNAS, además del gen *GNAS* que, como hemos visto codifica para la subunidad α estimuladora de la proteína G, existen otros tres exones "uno" alternativo que se enlazan con los exones 2-13 del gen *GNAS*. Son los exones *NESP55* (proteína neuroendocrina 55, *neuroendocrine protein 55*), *XLαs* (forma extralarga de la proteína Gsα) y exón *A/B* (transcrito no codificante). Además de éstos existe un transcrito antisentido llamado *NESPas* o *AS* (Figura 4). Cada uno de estos transcritos está sometido al fenómeno de impronta, así *NESPas*, *XLαs* y el exón *A/B* presentan *imprinting* materno, mientras que *NESP55* presenta *imprinting* paterno (para revisión, consultar Bastepe[42]).

La base (epi)genética subyacente al PHP-Ib se ha elucidado en los últimos años, y es diferente en los casos familiares (AD-PHP-Ib) y en los esporádicos (sporPHP-Ib)[43,44]. Así, la mayoría de los pacientes con AD-PHP-Ib presentan pérdida en la metilación del exón A/B exclusivamente[45]. Distintos estudios han observado que los pacientes con AD-PHP-Ib, portadores de la pérdida de metilación aislada para el exón A/B, presentan, asimismo una microdeleción de 3,2kb o 4,3kb en el gen *STX16*[46,47] o del exón *NESP55*[48]. En familias con AD-PHP-Ib y una alteración del patrón de metilación incluyendo al resto de los promotores (o exones alternativos) se han encontrado otra serie de deleciones que incluyen al exón *NESP55* y a dos de los exones del promotor antisentido *NESPas*[49,50].

Por su parte los pacientes con sporPHP-Ib presentan alteración en la metilación en todos los promotores sin deleciones identificadas hasta la fecha[44]. En algunos casos, esta alteración de la metilación se ha visto asociada a disomía paterna del cromosoma 20[51–53]. La disomía uniparental (*Uniparental Disomy* o UPD, en inglés) se refiere a la situación en la que las dos copias de un cromosoma provienen del mismo progenitor.

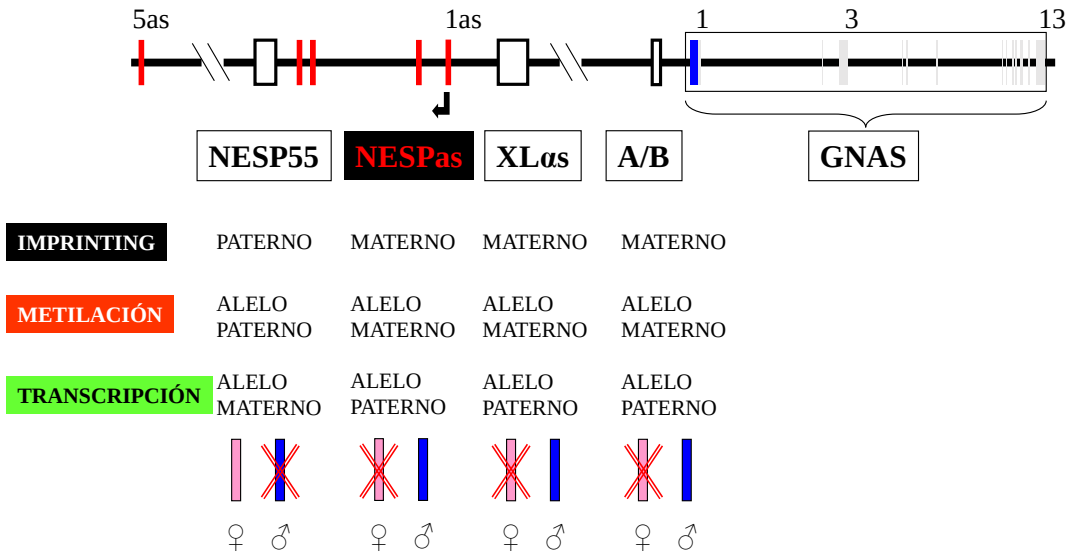

Figura 4. Patrones de impronta del *locus* GNAS. El transcrito NESP55 tiene imprinting paterno, mientras que los exones XLαs, A/B y NESPas presentan imprinting materno en todos los tejidos.

A lo largo de los últimos años, otros *loci* con impronta materna se han venido asociando al síndrome de hipometilación materna; en estos casos, el fenotipo predominante se ve modificado con nuevos síntomas propios de otras enfermedades de impronta, en función de los *loci* y tejidos afectados, alimentando la hipótesis del mosaicismo epigenético. En la búsqueda de factores que afecten a la impronta en las etapas iniciales del desarrollo, bien durante la implantación de las marcas en línea germinal, bien durante los procesos de mantenimiento de dichas marcas tras la fertilización, nuevos estudios se están centrando en genes susceptibles de intervenir en dichos procesos. Así, se ha encontrado que individuos con diabetes neonatal transitoria (TNDM) y alteración en la metilación en otros *loci* además de 6q24, presentan mutaciones en el gen *ZFP57*, implicado en el mantenimiento de la metilación del ADN durante las primeras etapas multicelulares del desarrollo. Otros estudios han encontrado mutaciones en línea germinal en el gen *NLRP2*, con un papel en el establecimiento de la impronta en etapas tempranas del desarrollo, en pacientes con BWS e hipometilación en *PEG1* (para revisión, consultar Enggermann[54]). Trabajos muy recientes de nuestro grupo, han mostrado que el síndrome de hipometilación está presente en algunos pacientes con PHP, pero este defecto multilocus no está causado por alteraciones en los genes reguladores descritos hasta la fecha[55–57].

Los importantes avances realizados en el conocimiento del PHP-Ib y los mecanismos (epi)genéticos subyacentes han permitido una reclasificación de estos pacientes al identificarse, por una parte, pacientes con PHP-Ib y fenotipo AHO que presentaban trastornos en el patrón de metilación[58–61] y, por otra parte, un descenso en la actividad Gsα en los pacientes con PHP-Ib causado por alteraciones epigenéticas[62] (Tabla 1B).

De cara al consejo genético, la enfermedad se presenta sólo cuando la microdeleción (bien en *STX16*, bien en *NESP55/NESPas*) es heredada por línea materna. Al heredarse la microdeleción, la descendencia presenta pérdida en la metilación, que es la causa de la aparición de la enfermedad. El cuadro clínico es más leve, no presentándose fenotipo de Albright (o si existe es muy leve), y sólo se han encontrado alteraciones hormonales a nivel de PTH y TSH. Si la microdeleción es heredada del padre, no tendrá consecuencias en esa generación, y sólo aparecerá pérdida en la metilación (y por tanto cuadro de pseudohipoparatirodismo) cuando sea transmitida por una mujer en cualquiera de las generaciones posteriores.

En los casos asociados a disomía paterna, la probabilidad de transmitir la enfermedad es la misma que en la población general.

Tabla 1B. Si bien la clasificación definitiva del pseudohipoparatiroidismo de tipo I se encuentra actualmente en revisión, los avances realizados durante los últimos años nos han permitido identificar algunas variaciones (resaltadas en rojo) respecto a la clasificación clásica.

	FENOTIPO	RESIST. HORMONAL	Ca S	P S	AMPc PTHex	ACTIVIDAD Gsα
PHP IA	AHO	PTH TSH Gn	↓	↑	↓	↓ 50%
PHP IB	NORMAL Mild-AHO	PTH TSH	↓	↑	↓	↓ 30%
PHP IC ¿?	AHO	PTH TSH Gn	↓	↑	↓	NORMAL ¿?

AHO: Osteodistrofia Hereditaria de Albright, mild-AHO: forma leve de fenotipo AHO, CaS: calcio sérico, PS: fosfato sérico, PTHex: administración exógena de PTH.

En algunos casos, en los que hay alteración del patrón de metilación pero no se detectan microdeleciones asociadas ni se identifica ninguna otra causa genética subyacente, no podemos precisar el riesgo de transmisión de la enfermedad. Por lo cual sólo podemos decir que la enfermedad, en el individuo estudiado, es causada por la alteración en el patrón de metilación, pero el riesgo en las generaciones siguientes es incierto.

ACRODISÓSTOSIS

La acrodisóstosis es un grupo de displasias esqueléticas muy poco comunes que comparten características clínicas y radiológicas como estatura baja, disostosis facial, hipoplasia nasal, y braquidactilia generalizada y severa. El fallo en el crecimiento es progresivo, apreciándose, sobre todo, en la edad adulta. El aspecto de la cara suele ser ancho, con hipoplasia maxilonasal y aplanamiento del puente nasal. Además, el tamaño del cráneo está reducido y hay un engrosamiento de la calota.

En cuanto a la braquidactilia, ésta es muy severa y generalizada, tanto en las manos como en los pies, excepto por el dedo gordo del pie que suele ser relativamente más grande. Este acortamiento es simétrico. Las radiografías muestran epífisis en forma de cono y una maduración avanzada de los huesos. Otra importante característica radiológica es la disminución de la distancia interpedicular en la columna lumbar.

También se han descrito otras características pero que no se presentan en todos los pacientes como: retraso mental o retraso en el desarrollo (77-80%), otitis recurrente y pérdida de audición (30-67%), hipogonadismo (29%), pliegue del epicanto (39%), anomalías dentales (retraso en la erupción dental, hipodontia) (26%), anomalías renales (3%), limitación en la extensión del codo, entesopatías, obesidad, escoliosis, heterocromía del iris, lesiones pigmentadas de la piel, aumento del ángulo mandibular (68-81%) y estenosis espinal. Es importante mencionar que en la acrodisóstosis no se han observado

osificaciones heterotópicas que sí aparecen en la mayoría de los casos de PHP-Ia[63].

El PHP y la acrodisóstosis se han confundido durante mucho tiempo a pesar de que en 1968 Maroteaux & Malamut las describieron como dos entidades diferentes basándose en su clínica[64]. La razón de esta confusión es la similitud que tienen las dos patologías a nivel clínico: fenotipo AHO o similar, braquidactilia, retraso mental, en ocasiones, y resistencia multihormonal (PTH y TSH preferentemente)[63,65]. La resistencia multihormonal no aparece siempre en la acrodisóstosis, ya que existen, al menos dos tipos de acrodisóstosis, siendo precisamente ésta la característica diferenciadora[63].

Así, por una parte hablaremos de la acrodisóstosis con resistencia multihormonal (ACRDYS1, OMIM#101800 o ADOHR), que está causada por mutaciones inactivantes en heterozigosis en el gen *PRKAR1A,* que codifica para la subunidad alfa de la proteina quinasa A o PKA dependiente de cAMP, también localizada en la vía de la proteína G. La primera mutación en *PRKAR1A* asociada a ACRDYS1, R368X (mutación recurrente), fue descrita recientemente por Linglart *et al.* en tres pacientes[65], y posteriormente grupos independientes o series más amplias del mismo grupo han confirmado la presencia de esta mutación en más casos independientes, así como identificado otras mutaciones nuevas[66–69].

Se han realizado varios estudios funcionales de las mutaciones descritas en el *PRKAR1A* y se ha deducido que las mutaciones en la subunidad reguladora (*PRKAR1A*) de la PKA, hacen que ésta tenga una menor afinidad por el cAMP. Como consecuencia, la disociación de la subunidad catalítica está reducida y esto produce una menor activación de las proteínas que se sitúan aguas abajo de la cascada, encargadas de la activación de otros genes. Resumiendo, la enfermedad está causada principalmente por una disminución en la actividad de la PKA[63,65].

Mutaciones en este gen también originan el síndrome de Complejo de Carney, un síndrome tumorogénico que cursa con displasia adrenal micronodular pigmentada, mixoma cardiaco, lentiginosis y en ocasiones con osteocondromixomas, pero no presentan anormalías metabólicas como en la

ACRDYS1. Los estudios funcionales de estas mutaciones parecen indicar que el defecto subyacente de la enfermedad, al contrario que en la ACRDYS1, es el aumento en la actividad de la PKA, pero todavía no está muy bien definido[63].

Por otra parte, podemos diferenciar la acrodisóstosis sin resistencia hormonal (ACRDYS2, OMIM#6146139), cuyo gen causante ha sido descrito recientemente, el gen *PDE4D* (fosfodiesterasa 4 D)[63,67,68,70]. En este caso los pacientes no tienen resistencia hormonal, al contrario que en la ACRDYS1 y en el PHP, a excepción de un caso descrito por Michot *et al.*,[67] que sí presentaba resistencia. En algunos casos la resistencia también puede ser secundaria a un nivel bajo de vitamina D[71].

En el caso de la ACRDYS2, no se conoce bien la causa subyacente de la patología[63,66]. La PDE4D hidroliza el cAMP, regulando los niveles del mismo y la señalización cAMP-PKA[66,68]. En este caso se esperaría que hubiese un aumento en el nivel de cAMP y de su respuesta, pero en cambio, se ha propuesto que la inactivación de la PDE4D produce una desensibilización por el cAMP en la vía, que como consecuencia origina un fenotipo similar al de la acrodisóstosis de tipo PRKAR1A y del PHP[68]. Se han descrito al menos 9 isoformas para esta proteína, pero no se sabe mucho sobre su expresión, función y regulación en los tejidos humanos. Se plantea que estas isoformas no se expresan igualmente en los diferentes tejidos, de ahí la ausencia de resistencia a la PTH en el túbulo renal proximal, pero sí la presencia de resistencia a la PTHrP en la línea de crecimiento y como consecuencia la aparición del fenotipo esquelético[66]. También se proponen otros planteamientos como que ocurra una compensación entre las diferentes isoformas en algunos tejidos o que a pesar de que el PDE4D se exprese en el túbulo renal proximal y en células óseas, no actúe en la respuesta renal a la PTH[66].

Los dos tipos de acrodisóstosis comparten la mayoría de las características descritas anteriormente, aunque la disostosis facial y la hipoplasia maxilonasal suelen ser más evidentes en la ACRDYS2, la disminución de la distancia interpedicular es poco frecuente en la ACRDYS1 y el retraso mental sólo

se ha descrito en la ACRDYS2, a pesar de que los pacientes con ACRDYS1 también tengan cierta dificultad en el aprendizaje[66,67].

Como se ha dicho anteriormente la acrodisóstosis comparte varias características con el PHP como son el fenotipo AHO, la braquidactilia y el retraso mental, por eso pueden ser confundidos[63,65]. Para realizar una comparación adecuada es necesario diferenciar por una parte el PHP *vs.* ACRDYS1 y por otra parte el PHP *vs*. ACRDYS2. Los dos tipos de acrodisóstosis cursan con una braquidactilia muy severa y generalizada, en cambio en el PHP suele observarse principalmente acortamiento del IV y V metacarpianos y I falange distal. Por eso, ante un caso con fenotipo AHO, sin resistencia hormonal y braquidactilia severa deberá sospecharse de ACRDYS2[63,66–68,70]. Por otra parte, en un caso de AHO, con resistencia hormonal, disostosis facial no tan marcada y braquidactilia severa, se sospecharía ACRDYS1[63,65,72]. Finalmente, otra característica importante a tener en cuenta son las osificaciones subcutáneas, que nunca aparecen en la acrodisóstosis y sí en el PHP[63]

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6.GENERAL DISCUSSION

This doctoral dissertation integrates two main studies: first, the screening study for structural alteration in *GNAS* genes in a series of patients with clinical suspicious of PHP1A or PPHP/POH; second, reclassification of patients manifesting brachydactyly type E (BDE), in whom GNAS locus defects (mutations and epimutations) were previously discarded, to propose a candidate gene as responsible of the pathology. The following discussion seeks integration between the findings of these two studies and in the context of the data available in the literature. Finally, an updated version of the initially proposed diagnosis algorithm is included, as a result of the knowledge acquired with these studies and new finding published about genes implicated in syndromes with BDE.

1. Screening for structural alteration in *GNAS* gene

Since firsts germline mutations in *GNAS* gene, associated to AHO, were described in 1990[1,2] more than 200 mutations have been described throughout this gene (www.lovd.nl/GNAS). Generally, mutations are specific of the family, thought, a hot spot has been described within exon 7, consisting in a deletion of 4 nucleotides (c.564_568delGACT/p.D189Mfs*14)[3,4]. Moreover, other recurrent mutations have been detected[5]. In the series of patients of the current study, 27 new families that were positive for *GNAS* point mutations were detected. Hot spot mutation within exon 7 was detected in 8 families (29.6%). In addition, 10 novel (not previously described) mutations were identified. The pathogenicity of these alterations was confirmed by bioinformatics prediction software and/or by the determination of the cosegregation of the mutation with the pathology in the family.

Most of the mutations were *de novo*, as previously described[5] and just in five cases it was confirmed to be maternally inherited. All of the carrier mothers were clinically diagnosed as PPHP after the genetic test, quite frequent in this pathology as its AHO phenotype is frequently mildly manifested and it is particularly difficult to detect[6]. Probably that is why, we only detected 2 isolated cases of PPHP and one of POH without family history within the current series of

patients. As we appreciated, short stature is the main reason for consultation in these cases[7] and they frequently undergo IUGR (publication 1). Generally, patients with heterozygous *GNAS* mutations use to show certain degree of low birth weight, but when the mutation is located on the paternal allele it is more pronounced than with mutations on maternal allele[8]. Furthermore, recent studies have showed that in patients who carry mutation within paternal *GNAS* exons 2-13, as we identified three patients, IUGR is more pronounced than in those who carry the mutation in exon 1. These data indicate that apart from paternally derived Gαs, other paternally derived GNAS transcript, most likely Xlαs, play an important role in foetal growth and development and also in placental development[8–10].

The presence of BDE facilitates the correct clinical diagnosis, especially in PPHP and POH, when other features are rather inappreciable because of mild manifestation of AHO phenotype. Actually BDE is the most specific feature of AHO[6,11]. But it should be taken in account that brachydactyly is a progressive trait that may not be observed in early childhood, generally it is not appreciated until the age of 6 years[7], as we observed in current series of patients (publication 1).

About POH, it is a rare manifestation of paternally inherited Gsα mutations[12], which presents with cutaneous ossification, that progress to involve subcutaneous and deep connective tissues[13]. Generally, patients have no other morphological manifestations of AHO or any evidence of hypocalcaemia, pseudohypoparathyroidism, hypothyroidism, or hormone resistance, but some cases with limited AHO features have been reported, nevertheless obesity seems to be totally absent[14]. The mutation pattern does not predict a specific disorder (PPHP or POH) nor the variability in the manifestation of the phenotype[14]. In fact, the POH patient reported in our study (publication 1) harboured the recurrent mutation within exon 7, which has been linked to PPHP and POH, as well as to PHP1A when maternally transmitted. The reason why some patients develop POH is unclear[12–15]. A recent study observed that the POH lesions frequently follow dermomyotomes and appear toward only one side of the body (sidedness)[16]. Accordingly, introduction of a dominant negative Gsα mutant to

chick embryo somite induced a POH-like phenotype that showed exclusive sidedness[16]. In addition, mouse models showed that the total ablation of *Gnas* in multiple mesenchymal tissues causes progressive heterotopic ossification through the activation of Hedgehog signalling[17]. So this findings provide some clarification about POH, suggesting that the underlying cause may be a second-hit postzygotic mutations affecting tissues in patients with inherited heterozygous inactivating Gsα mutations[16].

1.1. Novel molecular defects in GNAS locus as cause of PHP1A/PPHP

The detection rate of Gsα-coding mutations in PHP1A patients is of approximately 70%[6,18] (and personal data). This suggests the existence of other unknown causes or molecular defects that remains out of the scope of our screening methods. Some studies have demonstrated that chromosomal rearrangements in 20q may also cause PHP1A[19], as occurs in PHP1B[20–29]. Therefore, we included MS-MLPA as routine method in the screening of GNAS abnormalities in patients with PHP1A/PPHP phenotype and no *GNAS* point mutations. This study revealed the presence of 4 heterozygous microdeletions in 20q, in the current series of patients, and other 3 different microdeletions in the series of patients of collaborating laboratories (6 with PHP1A and 1 with PPHP). Three gross microdeletions involving whole GNAS locus, and four intragenic microdeletions were reported. Examining the clinical data of patients, no significant clinical differences were appreciated between patients with point mutations and those reported with *GNAS* microdeletions (publication 2). So, these findings indicated that *GNAS* deletions must be considered as a possible and relatively significant cause of PHP1A/PPHP.

2. Other syndromes associated with BDE which could be misdiagnosed as PHP

PHP involves a heterogenic group of rare disorders associated with AHO phenotype, which includes, small stature, rounded face, subcutaneous ossifications, mental retardation and BDE[30,31]. Excepting subcutaneous ossifications, these features are rather unspecific, because they appear in other disorders, such as AHO-like syndrome[32] or acrodysostosis[33–35]. In addition, resistance to PTH, which is a specific characteristic of the PHP1 subtype, is also a feature of acrodysostosis type ACRDYS1[36,37]. Less frequently, the misdiagnosis with other entities has been observed because of the presence of BDE combined with short stature and obesity, typically associated also with other dysmorphic features and sometimes also with hormonal imbalances[38,39] (and personal experience). Brachydactyly frequently coexists with short stature in these syndromes because both are the result of a premature closure of the growth plate caused by a deregulation of different genes implicated in bone development[40]. In addition, obesity or overweight could act as confusing factor, while it is attributable to the current obesity pandemic.

The discovery of other molecular mechanisms causative of PHP/PPHP (publication 2), or of new genes implicated in syndromes with a similar phenotype to AHO, has been very helpful for establishing a correct genetic diagnosis for these patients who had been masked under “AHO similar phenotype” with an unknown genetic cause (personal data) and also, to better describe the characteristic features of these less common syndromes. In our and previously reported experiences[7] the BDE is the most specific and objective feature. For this reason, it was used as inclusion criteria in this project. Thus we developed a diagnosis algorithm (publication 3), based on the BDE pattern in each syndrome and main specific characteristics of syndromes associated with BDE, to guide the genetic diagnosis of patients with BDE. BDE was initially described as variable shortening of the metacarpals/metatarsals with more or less normal length of phalanges[41]. We have considered that the most common

entities mentioned in the bibliography manifesting with BDE, apart from PHP/PPHP, are: hypertension and brachydactyly syndrome (HTNB, OMIM#112410), brachydactyly-mental retardation syndrome (BDMR, OMIM#600430), BDE with short stature, PTHLH type (OMIM#613382) and Turner syndrome (TS)[42]. Even if the brachydactyly manifested in acrodysostosis (ACRDYS1, OMIM#101800; ACRDYS2, OMIM#614613) or tricho-rhino-phalangeal syndrome (TRPS-I, OMIM#190350; TRPS-II, OMIM#150230; TRPS-III, OMIM#190351) cannot be considered as a pure brachydactyly E (because apart from I distal phalanx others phalanges are also involved) the similar pattern may lead to be misdiagnosed as syndromic BDE (publication 3). All these entities were included in a common branch in the algorithm, classified as syndromic BDE, as they manifest with other features apart from BDE. In a separated branch, isolated BDE, HOXD13 type (OMIM#113300) was included.

As a result of the reclassification of the patients, guided by our proposed algorithm, half of the patients (11/22) were resolved. In addition, new knowledge was acquired about these pathologies and the characteristic features detailed below.

2.1. Acrodysostosis

Acrodysostosis refers to a heterogeneous group of rare skeletal dysplasia that share characteristics features, including severe brachydactyly, facial dysostosis and nasal hypoplasia[43–45]. It was classified as a different entity by Robinow *et al.* in 1971[44], but until recently, when mutations in *PRKAR1A* and *PDE4D* genes were identified as causative of the disease[36,46,47], it was quite confusing to diagnose these patients. Each gene is associated to a different type of acrodysostosis; *PRKAR1A* is associated to type 1, manifesting always with hormone resistance (PTH as well as TSH)[36]; while *PDE4D* to type 2, usually without hormone resistance[46,48] (some exceptional patients have been reported with mild PTH resistance, though no data about vitamin D values were indicated[46,49]).

In the current series, we identified 4 patients with PTH/TSH resistance and typical characteristics of acrodysostosis, such as severe and generalised brachydactyly, facial and peripheral dysostosis, short stature, decreased interpedicular distance and behavioural disorders[36,43,44]. Accordingly, *PRKAR1A* gene was studied revealing a positive result in the four patients: three of them harboured the recurrent mutation initially described by Linglart *et al.*[36] and the fourth one, showed a novel mutation. We considered important to contrast compared clinical data of these patients with the data of other members of the EuroPHP consortium, to elaborate a more complete description of genotype-phenotype correlations at clinical, hormonal and radiological level, about both types of acrodysostosis (publication 4). As result of this collaborative work, we observed that both types of acrodysostosis share the generalized and severe brachydactyly and facial and peripheral dysostosis (typically, more severe in ACRDYS2), advanced bone age, severe short stature and pigmented skin lesions. On the other side, PTH/TSH resistance (which is showed in ACRDYS1 but not in ACRDYS2), decreased interpedicular distance (more specific of ACRDYS2 type) and mental retardation (observed in ACRDYS2 patients but not in ACRDYS1 patients as they only presented some behavioural disorders and/or difficulties at school) are the main differential characteristics (publication 4).

Of note, in contrast with the rest of the syndromes reflected in the algorithm, in which brachydactyly is usually not marked until the age of 6 years[7,50], it is appreciated that in acrodysostosis the shortening and cone-shaped epiphyses are manifested since early childhood[46] (publication 4).

We also identified an additional patient with clinical characteristics suggestive of acrodysostosis and mental retardation, who harboured a mutation in *PDE4D*. Initially, *PRKAR1A* gene was studied in this patient because she showed PTH resistance. But in a second biochemical analysis, low levels of vitamin D suggested that the PTH increased level could be secondary to vitamin D deficiency[51]. As soon as patient was treated with vitamin D, PTH levels normalised. Therefore, vitamin D level is an important parameter that should be taken into account before deciding a patient presents a real PTH resistance.

The functional studies of four of the described mutations found in *PDE4D* gene associated to ACRDYS2 (one of the studied mutations was identified in the present series) indicated that they have a gain-of-function character. This finding explains the phenotypic parallelism between ACRDYS1 and ACRDYS2. Actually, in the type 1, the PKA signalling is downregulated because an impairment of the regulatory subunit to bind cAMP and thus dissociate from catalytic subunit to activate the downstream pathway[36,52]. The gain-of-function mutations in *PDE4D*, parallel this situation, because the increased hydrolysis of cAMP causes a lower activation of the PKA regulatory subunit (publication 5).

2.2. Tricho-rhino-phalangeal syndrome

TRPS is a rare autosomal dominant malformation syndrome that classically has been classified in three types: TRPS-I (OMIM#190350), TRPS-II (OMIM#150230) and TRPS-III (OMIM#190351). Typical features common to all three types are: slowly growing scalp hair, laterally sparse eyebrows, a bulbous tip of the nose, protruding ears, thin upper lip, brachydactyly involving mostly metacarpals and mesophalanges and short stature[53]. TRPS-I and TRPS-III are variants of a single disease associated both with mutations in *TRPS1* gene[54]. Type III is considered when patients show the severe end of the clinical spectrum, with very short stature and very severe brachydactyly[53]. Generally, missense mutations in *TRPS1*, seem to cause a more severe manifestation of the syndrome because of the dominant negative effect of the protein (those classified as TRPS-III), than nonsense mutations or splice-site mutations, which cause pure haploinsufficiency (those classified as TRPS-I)[53]. On the other hand, TRPS-II is as contiguous gene syndrome associated with deletions involving *TRPS1-EXT1* genes. These patients have multiple cartilaginous exostoses in addition to the features mentioned before, but with a more severe manifestation[55]. Mental retardation has been described in many patients with TRPS-II as well as in two patients with TRPS-I with cytogenetically visible deletion in 8q24[56,57]. In contrast, patients with TRPS-I and a submicroscopic deletion or a *TRPS1* point mutation, usually show normal intelligence, excepting some patients with mild mental retardation reported by Lüdecke et al[53].

Given the presence of brachydactyly and short stature, TRPS could be confused with the AHO phenotype, even more if obesity (or overweight) and/or PTH resistance[39] and other hormone imbalance (GH deficiency, has been also reported in some TRPS cases[58–62]) appears in the clinical profile, as showed in the two published cases (publication 6) of the current series of patients. This is not the first time that PHP and TRPS syndromes are confused in the literature[39]. Our publication defines the most characteristic and illustrative features of TRPS syndrome in compare to PHP, and thereby we have been able to identify other two patients with TRPS who were misdiagnosed as presenting AHO phenotype, because they showed severe brachydactyly and short stature.

Keeping in mind all identified cases, in our opinion, the most characteristic and illustrative features of TRPS syndrome are: bulbous tip of the nose (or pear-shaped nose), thin upper lip, brachydactyly involving the phalanges and with the typical outcarving of the phalangeal epiphyses, and sparse, slowly growing scalp hair (not easily appreciable in some cases).

2.3. Brachydactyly type E with short stature, PTHLH type

Alterations which lead to the haploinsufficiency of PTHLH, the gene coding for parathyroid hormone related protein (PTHrP) were identified as a cause of autosomal-dominant BDE in 6 families[63–65]. Klopocki *et al.*[63] named this syndrome as “BDE with short stature, *PTHLH* type” (OMIM#613382), because it is almost always associated with short stature[63,64,66]. As we have appreciated in one patient (case 2, publication 7), the short stature could not be manifested until middle childhood. In this case, the patient had shown a normal stature for her age, but she presented advanced bone age, consequently, she underwent an early stop in growing. In addition, the progenitors’ target height should be taken in account to consider when the patients show a height in the lower range of normality. Some patients also presented delayed tooth eruption and/or oligodontia but it is not an obligatory feature[63] (publication 7).

Overall, the current study has helped to find the genetic origin of 11/22 patients with BDE or similar pattern who were misdiagnosis as PHP/PPHP. As we have performed for the 11 resolved cases, the remaining cases wre also classifyed an studied by the candidate gene approach guided by proposed algorithm. However, we have not found any genetic alteration in the studied candidate genes. So even if a great number of genes have been identified as the causative of BDE in recent years some of the BDE cases remain unexplained[67].

However, if we look back to some of these negative cases, in many of them, hand radiographies or clinical photographs were not available (Table 4: PHP0068, PHP1008, PHP1014, PHP1020), which often provide key data to suggest other candidate genes. For example, in patients PHP1074 and PHP1096 after discard the candidate genes associated to the disorder proposed by the clinician, we reviewed the radiographies and we appreciated that the pattern of the brachydactyly in both cases was more suggestive of brachydactyly type A (BDA) than type E, because they show shortening of the mesophalanges (BDA) rather than of the metacarpals (BDE). Patient PHP1074 shows shortening of at least II to V mesophalanges, similar to BDA1. Genes candidate for these types of brachydactyly are *IHH*[68] or *GDF5*[69]. Consequently, we proposed these loci for the next genetic studies. Patient PHP1096 shows a brachydactyly similar to BDA4, with shortening of mesophalanges II and V. To date, the only candidate gene proposed for this type of brachydactyly is *HOXD13*[70], which has already been studied. Therefore, we are rewiring the clinical data to try to find new candidate genes.

Below, an updated version of the initially proposed diagnosis algorithm (publication 3) is included (Figure 6.1.), as the result of the knowledge acquired with these studies and new finding published about genes implicated in syndromes with BDE. These are the proposed changes:

a) Microdeletions involving GNAS locus, have been included as additional genetic origin of PHP1A/PPHP (publication 2).

b) Very recently, implication of *PDE3A* has been identified as genetic origin of the hypertension with brachydactyly syndrome[71].

c) Mental retardation has been included as a characteristic feature of ACRDYS2 (publication 4)[45,48,49].

d) Outcarving of the phalangeal epiphyses has been added to TRPS because it is a very iformative finding of this syndrome (publication 6).

e) *HDAC4* mutations and 2q37 microdeletions are both causative of BDMR syndrome, also denominated AHO-like syndrome.

f) GH deficiency has been eliminated from the characteristics of TRPS, because we considered it as a less frequent sign, since we only detected in one case of the four described in the current series of patients (publication 6) and very few cases have been reported in the literature[58–62].

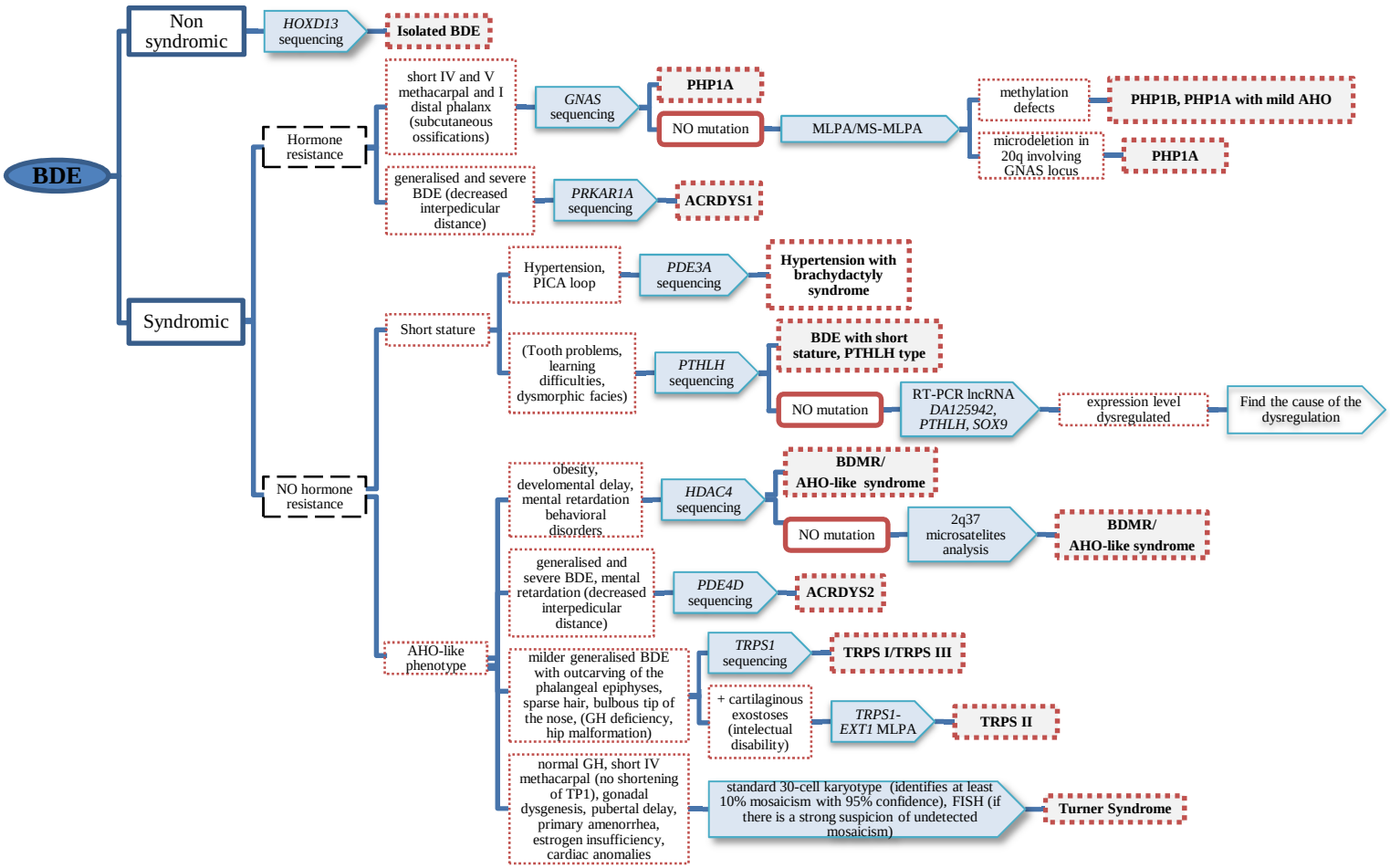

Figure 6.1. Proposed new clinical algorithm to suggest the most probable genetic diagnosis. Features in brackets are not obligatory findings in these syndromes. BDE: Brachydactyly type E; PHP1A: Pseudohypoparathyroidism type 1A; MLPA: Multiplex ligation-dependent probe amplification; MS-MLPA: Methylation specific-MLPA.

3. One pathway for several related diseases: PTH1R–Gsα–cAMP–PKA signalling pathway

The reason for the similarity between all mentioned diseases arises from the pathway in which they converge, the PTH1R-Gsα-cAMP-PKA signalling pathway[72,73] (publication 8). On the other hand, genetic alterations in different levels of this pathway, which in theory would have a similar effect on downstream genes, produce syndromes with rather differences in the severity of the clinical manifestations at the skeletal morphogenesis or at endocrine aspects. Possibly, these differences are the result of the tissue specific expression of each gene[72].

If we look back over the pathway function, first ligand (PTH or PTHrP) joins to PTHR1 receptor, α-subunit (Gsα) of the multimeric protein Gsα exchanges GDP for GTP and dissociates from the β and γ subunits[74]. Then Gsα further activates the adenylyl cyclase, which catalyses the transformation of ATP into the second messenger cAMP. cAMP binds to the regulatory subunit of tetrameric holoenzyme PKA, which produces the dissociation from catalytic subunit. Subsequently, the PKA catalytic subunit phosphorylates a broad spectrum of proteins on serine/threonine residues, among which is the transcription factor CREB (cAMP response element-binding protein). At this point, phosphodiesterases (such as PDE4D or PDE3A), act as negative regulators of the pathway by hydrolysing cAMP[75].

Therefore, if we place the mentioned syndromes in the pathway, in the highest point of the pathway would be PTHrP protein. Since its homology with PTH hormone, they both bind to the same receptor PTHR1[76]. Interestingly, even both ligands share the same receptor, any *PTHLH* mutation has been associated with abnormal regulation of calcium and phosphorus metabolism[67]. As mentioned before, PTHrP is encoded by *PTHLH* gene (OMIM+168470). Haploinsufficiency of this gene originated by heterozygous point mutations[63,66] (publication 7), as well as by deletions involving this gene[63] or rearrangements that downregulate its expression[64], cause BDE with short stature, *PTHLH* type.

Even its widespread expression in both foetuses and adults tissues, including epithelia (skin, mammary gland and teeth), mesenchymal tissues, endocrine glands, and the central nervous system[77–80], the haploinsufficiency of this protein seems to cause only BDE with short stature (or in the range of lower normal), and occasionally teeth problems[63,64,66]. The patient 2 of the publication 7 is the first case reported with apparently breast hypoplasia. However, very little is known about PTHrP protein's role in multiple other tissues in which it is expressed, apart from the growth plate[81]. The growth plate consists of columns of proliferating and differentiating chondrocytes that progressively enlarge to prehypertrophic and then hypertrophic chondrocytes. In the growth plate, PTHrP works with Indian Hedgehog protein (IHH) and others collaborating genes to determine the size and shape of the skeleton. PTHrP is secreted primarily by immature chondrocytes at the top of the columns[81]. With the initiation of the differentiation pathway, prehypertrophic chondrocytes express Phtr1 receptor and Ihh. Ihh is secreted by prehypertrophic chondrocytes inducing the secretion of PTHrP, which binds to Pthr1 in late proliferating cells and blocks the differentiation of these cells to hypertrophic chondrocyte. When the prehypertrophic chondrocytes have fully differentiated into hypertrophic, they turn off Ihh expresion[81,82]. So as was expected, *PTHLH* haploinsufficiency caused a deregulation in this feedback resulting in premature differentiation of chondrocytes which should continue proliferating until reach the correct size and shape of the bone[67,82]. Probably a biallelic PTHrP expression is needed for normal pre- and post-natal bone development and growth[67], but the remaining tissues on which it acts are not as sensitive as skeleton to the change in protein expression or another protein supplies this haploinsufficiency. Only one missense mutation has been functionally tested, indicating a loss-of-function effect[63]. The characteristics of the remaining described mutations, and the similarity in the manifestations they produce, indicate that usually this syndrome is caused by loss-of-function mutations.

Then, there would be PHP syndrome caused by genetic or epigenetic alteration in the GNAS locus, where Gsα protein is encoded. Gsα is ubiquitously

expressed and apart from PTHR1 receptor, it couples a large number of other GPCR, through TSH, LH, FSH, GHRH, ADH, glucagon, ACTH, calcitonin, neurohormones and paracrine and autocrine factors act[83]. Thus, it mediates a wide spectrum of biological functions and probably that is why PHP syndrome manifests with such a wide spectrum of symptoms, including skeletal alterations and multihormonal resistance.

Downregulation of the pathway can also occur at the level in which cAMP produces the activation of the pathway. PKA is a heterotetrameric enzyme comprising two regulatory and two catalytic subunits[84]. ACRDYS1 appears when *PRKAR1A*, the gene encoding the regulatory subunits, is mutated. As mentioned before, mutations have a dominant negative effect in PKA, associated with a decreased responsiveness to cAMP, thus catalytic subunit is partially inactivated and CREB phosphorylation is reduced[36,52]. The mutations in ACRDYS2 are predicted to produce the same effect in CREB phosphorylation, but in this case, the mutations present an activating effect in PDE4D phosphodiesterase producing an increased hydrolysis of cAMP, translated in a lower activation of PKA (publication 5). In both types of acrodysostosis, the result of the downregulation in skeletal dysplasia is almost the same, being more severe in type 2. If we make a comparison between acrodysostosis and PHP or BDE with short stature, we can appreciate that the skeletal dysplasia is much more severe in acrodysostosis, indicating a higher effect over the PTHrP action in the bone. In fact, skeletal manifestations, such as brachydactyly, are appreciated earlier than in PHP or BDE with short stature (publication 4). On the other hand, PHP is the syndrome that manifests with more severe endocrine effect in relation to hormonal resistance. While PHP is characterized by hypocalcaemia and hyperphosphatemia in addition to multihormonal resistance (at least PTH and TSH, but sometimes also other hormones which act through GPCR)[85,86], ACRDYS1 is manifested generally with normocalcaemia and normophosphataemia and only with PTH and TSH resistance. On the contrary, ACRDYS2 lacks general hormone resistance which reflects the low expression of PDE4D in endocrine tissues[87].

In addition, recently, another phosphodiesterase, PDE3A, has been associated with the hypertension and BDE syndrome. Mutations reported in patients reflected the same effect as mutations in *PDE4D*, in relation to protein function. However, skeletal manifestations are milder than in acrodysostosis. Studies in mouse indicated that PDE3A is involved in chondrogenesis and the activating function of the protein seems to produce a *PTHLH* downregulation as observed in chondrogenically induced fibroblasts from affected individuals[71].

Histone deacetylase 4 (encoded by the gene *HDAC4*, OMIM*605314) and zinc finger transcription factor TRPS1 (encoded by the gene *TRPS1*, OMIM*604386) associated with Brachydactyly mental retardation syndrome[88] and Tricho-rhino-phalangeal syndrome[54], respectively, seem to be also connected with the pathway. HDAC4 acts as a corepressor for DNA-binding transcription factors. TRPS1 encodes a zinc finger transcription factor that acts as a repressor of GATA-mediated transcription[54,89]. Growth plate chondrocytes undergo a tightly regulated process of differentiation that results in the longitudinal growth of the bones. Abnormalities of the growth plate may lead to short stature and skeletal deformities[90,91]. Chondrocytes originating within the periarticular resting zone of the growth plate differentiate through a series of intermediate phenotypes (proliferative, prehypertrophic, and hypertrophic chondrocytes) before reaching a terminally differentiated hypertrophic state. The balance between proliferation and differentiation is an important regulatory step controlled by multiple signalling molecules, including PTHrP, HDAC4 and TRPS1[92]. PTHrP signalling regulates the differentiation of proliferative to hypertrophic chondrocytes in the growth plate during the late foetal and perinatal stages. The length of the proliferative zone of the growth plate is determined by a combination of the stimulatory effect of Ihh on periarticular chondrocyte differentiation and the inhibitory effect of PTHrP on hypertrophic differentiation[92]. Recently, PTHrP has also been shown to modulate chondrocyte differentiation through PTH1R by regulating the movement of HDAC4 into the nucleus, which regulates the activity of a network of transcription factors such as Zfp521, MEF2, and Runx2[81,93,94]. HDAC4 regulates chondrocyte hypertrophy and

endochondral bone formation by interacting with and inhibiting the activity of Runx2, a transcription factor necessary for chondrocyte hypertrophy[88,94]. On the other hand, Trps1 is required to maintain the normal organization of chondrocytes in the growth plate. Trps1 regulates the hypertrophic differentiation of chondrocytes by interacting with the Ihh/PTHrP feedback loop. Interestingly, Trps1 binds to the Pthlh promoter suppressing its transcription, so regulates negatively the Pthrp expression[92].

Throughout this section only alterations leading to the syndromes described over this thesis have been mentioned. However, several alterations in this pathway, which result in different types of clinical manifestations, have also been described (publication 8) such as hypoparathyroidism[95] (OMIM#146200), Jansen's Metaphyseal Chondrodysplasia[96] (JMC, OMIM#156400), Blomstrand' disease[97] (BLC, OMIM#215045), Eiken Familial Skeletal Dysplasia[98] (OMIM#600002), Ollier's disease or Enchondromatosis[99] (OMIM%166000), primary failure of tooth eruption (PFE)[100] (OMIM#125350), Carney complex[101] (OMIM#160980), etc.

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7.CONCLUSIONS

CONCLUSIONS

- ✓ Apart from point mutations, *GNAS* deletions must be considered as a possible cause of PPHP/PHP1A.

- ✓ Brachydactyly type E is the most relevant feature in these syndromes, and the association of this with other manifestations allow establishing a diagnosis algorithm to guide the most probable genetic diagnosis.

- ✓ Despite lack of family history of PHP, in patients with brachydactyly type E, short stature and intrauterine growth restriction, PPHP should be suspected; or POH if, in addition, heterotopic intramembranous ossifications are observed.

- ✓ When PTH resistance is observed together with severe and generalized brachydactyly, acrodysostosis type ACRDYS1 should be suspected. On the other hand, if this type of brachydactyly is manifested without PTH resistance but together with mental retardation, acrodysostosis type ACRDYS2 should be suspected.

- ✓ ACRDYS2 is caused by activating mutations in *PDE4D*.

- ✓ The most specific characteristics of tricho-rhino-phalangeal syndrome are; brachydactyly, with the involving of the phalanges and the characteristic outcarving of the phalangeal epiphyses; sparse, slowly growing scalp hair; a bulbous tip of the nose (or pear-shaped nose) and thin upper lip.

- ✓ In Brachydactyly type E with short stature, PTHLH type syndrome, patients show BDE with short stature, but the short stature could not be appreciated until middle childhood, since this is the result of premature closure of a growth plate. Target height should be taken in account.

- ✓ The genetic cause of some of the BDE cases remains unexplained.

8.RESUMEN Y CONCLUSIONES

RESUMEN

El pseudohipoparatiroidismo (PHP) comprende un grupo heterogéneo de enfermedades endocrinológicas que se caracterizan por la existencia de hipocalcemia, hiperfosfatemia y resistencia tisular a la PTH asociadas (PH1A) o no (PHP1B) al fenotipo de AHO. El fenotipo AHO se describe clásicamente como la presencia de cara redondeada, cuello corto, obesidad, calcificaciones subcutáneas, braquidactilia tipo E (BDE) bilateral (en manos y pies) y/o retraso mental. Datos más actuales sugieren que la obesidad y el retraso mental son más propios del subtipo PHP1A.

Desde el punto de vista genético, el PHP está causado por alteraciones en el gen *GNAS* o en la región 5' de este locus complejo. El locus GNAS y los diferentes transcritos para los que codifican (proteínas Gsα, XLαs, NESP55 y los transcritos A/B y NESP-AS/GNAS-AS) están sometidos a imprinting o impronta genética. En concreto, XLαs, A/B y GNAS-AS presentan impronta materna, es decir, sólo el alelo paterno es el funcional. NESP55, por el contrario, presenta impronta paterna. En cuanto al gen GNAS, que codifica para Gsα, si bien no se ha observado que esté sometido a impronta, sí se ha observado una expresión mayoritaria del alelo materno en determinados tejidos como hipófisis, túbulo renal proximal, gónadas y tiroides e, incluso, ligeramente aumentada en aquellos que presentan expresión bialélica.

Esta herencia basada en el fenómeno de impronta es la responsable de que distintas alteraciones en el mismo locus e, incluso, de que la misma alteración genética dentro de la misma familia originen diferentes manifestaciones clínicas.

Así, los pacientes con PHP1A, normalmente, portan mutaciones inactivantes en heterozigosis en uno de los 13 exones de GNAS. Mutaciones de GNAS en línea germinal también se encuentran en los pacientes con pseudopseudohipoparatiroidismo (PPHP), que presentan AHO sin resistencia hormonal. Ambos tipos de PHP se encuentran en las mismas familias: el PHP1A es el fenotipo si la mutación es heredada de la madre, mientras que el PPHP es el

fenotipo si la misma mutación es heredada del padre. En ocasiones, las mutaciones heredadas del padre se manifiestan con el fenotipo POH (heteroplasia ósea progresiva), asociado a osificaciones subcutáneas que progresan a tejidos más profundos que las observadas en el PHP1A o PPHP. Por el momento no se han identificado las causas responsables de la evolución a PPHP o POH cuando el paciente presenta una mutación en el alelo paterno.

Por su parte, el PHP1B se subdivide desde el punto de vista (epi)genético en la forma familiar autosómica dominante (AD-PHP1B) y en la forma esporádica (spor-PHP1B). La mayoría de los AD-PHP1B presentan una pérdida de metilación aislada en A/B generalmente asociada a deleciones en *STX16* en el alelo materno. Por su parte, la forma esporádica de PHP1B presenta una pérdida de metilación completa en GNAS-AS, XLαs y A/B, pero en este caso no se han encontrado otras alteraciones en *cis* o *trans* que expliquen esta pérdida de metilación. En un pequeño porcentaje de pacientes con spor-PHP1B ha sido posible identificar disomía uniparental paterna como responsable de la enfermedad.

El pseudohipoparatiroidismo presenta grandes problemas en su diagnóstico debido, principalmente, a que el PHP es un cuadro clínico muy poco frecuente, encuadrado en el grupo de enfermedades raras y, por otra parte, a que las manifestaciones del fenotipo AHO son en su mayoría inespecíficas y muy variables. De hecho, la característica más específica de este fenotipo es la braquidactilia tipo E, que en este caso se manifiesta principalmente con acortamiento del cuarto y quinto metacarpianos y la falange distal del dedo pulgar.

La BDE, en general, se define como acortamiento variable de los metacarpianos que puede ir unida además con acortamiento de las falanges. Puede presentarse de forma aislada o formando parte de varios síndromes. Los síndromes asociados a la BDE en muchas ocasiones presentan, además, características del fenotipo AHO e incluso resistencia a la PTH, como ocurre en la

acrodisostosis. La coincidencia en estas características conlleva la posibilidad de diagnosticar de forma errónea a los pacientes, con las consiguientes implicaciones en el tratamiento, seguimiento y consejo genético. Consideramos imprescindible elaborar algoritmos y recomendaciones que permitan identificar características distintivas de las diferentes patologías coincidentes, para poder orientar en el correcto diagnóstico y es, principalmente a este objetivo al que se han dedicado los esfuerzos y el trabajo de la presente tesis.

De hecho, de los 53 pacientes recibidos para estudio genético del gen GNAS por sospecha clínica de PHP1A/PPHP, 27 presentaron mutaciones puntuales en dicho gen asociadas a estas patologías (24 casos asociada a PHP1A, 2 a PPHP y 1 a POH). Curiosamente, los tres pacientes con alteración en el alelo paterno se diagnosticaron de forma aislada, sin historia familiar de PHP1A, hecho bastante infrecuente dado que las manifestaciones clínicas del PPHP/POH generalmente son muy sutiles. Es muy probable que existan casos infradiagnosticados de estas patologías por la costumbre de buscarlos, principalmente, en las madres portadoras de pacientes con PHP1A. De hecho, en esta serie, las cinco madres portadoras de alteración genética identificadas durante el estudio familiar fueron diagnosticadas clínicamente de PPHP a posteriori del estudio genético. Nuestra experiencia sugiere que el PPHP/POH, en caso de ausencia familiar, debiera sospecharse en pacientes con talla baja y braquidactilia tipo E que hayan presentado retraso del crecimiento intrauterino. Es importante recalcar que es posible no detectar esta braquidactilia hasta los seis años de vida del paciente.

En las enfermedades causadas por haploinsuficiencia, además de mutaciones puntuales inactivantes, también es posible identificar variaciones en el número de copia. Por ello, en los 26 pacientes con PHP1A/PPHP y estudio negativo para mutaciones puntuales en el gen GNAS, se estudió el número de copias del gen *GNAS*. Gracias a ello, se identificó una pérdida completa del locus GNAS en tres pacientes y una deleción intragénica en otro. Por tanto, parece que las variaciones en el número de copia en *GNAS*, en concreto deleciones, también son responsables del PHP1A/PPHP. Dado que las manifestaciones clínicas son

similares entre los pacientes con mutación puntual o con deleción, recomendamos incluir el estudio del número de copias del gen GNAS en aquellos pacientes con sospecha de PHP1A/PPHP.

Como se ha mencionado, las características fenotípicas de AHO pueden ser difíciles de identificar. Dado que en 22 de los pacientes no habíamos encontrado la causa (epi)genética responsable de la enfermedad, se revisó de nuevo los datos clínicos recibidos. En la mayoría de ellos, la BDE (o un patrón similar) junto con la estatura baja y la obesidad habían sido las características principales de sospecha clínica. Revisando en la literatura los síndromes más comunes asociados a BDE (o con un patrón similar a ésta) identificamos la braquidactilia E aislada, la braquidactilia E con estatura baja de tipo PTHLH, la acrodisostosis tipo 1 y tipo 2 (ACRDYS1 y ACRDYS), la hipertensión con braquidactilia (HTNB), el síndrome trico-rino-falángico (TRPS), el síndrome de braquidactilia con retraso mental (BDMR) y finalmente el síndrome de Turner (TS). Cada uno de ellos está asociados con determinadas manifestaciones específicas, lo que nos permitió la elaboración de un algoritmo de diagnóstico para guiar el abordaje clínico de estos pacientes y de esta forma facilitar la clasificación de éstos, basado en su patrón de braquidactilia y otras características presentes en estos síndromes. Finalmente se sugería el gen candidato más probable en cada caso.

Gracias a esta revisión y al algoritmo propuesto se consiguió diagnosticar genéticamente a la mitad de los pacientes que quedaban sin diagnóstico. En primer lugar, en tres pacientes con resistencia a la PTH y braquidactilia muy severa y generalizada se identificó la mutación causante en el gen *PRAKR1A* asociado a la acrodisostosis tipo 1. En otra paciente, con características fenotípicas similares pero sin resistencia hormonal y con retraso mental, se identificó una mutación en el gen *PDE4D*, asociada a la acrodisostosis tipo 2 (ACRDYS2). Dado que el efecto de las mutaciones en PDE4D sobre el funcionamiento de la proteína y su papel en la cascada de señalización no es muy

conocido, se realizaron estudios funcionales para determinar el efecto de las mutaciones en *PDE4D* causantes de la ACRDYS2, y se constató que las mutaciones tienen un carácter activante. En el caso de PDE4D, la proteína mutante hidroliza mayores niveles de cAMP, produciendo una desregulación en la vía de señalización de la PKA coincidente con el efecto que producen las mutaciones en *PRKAR1A*.

Por otro lado en otros cuatro pacientes con características propias del fenotipo del TRPS-I (principalmente epífisis en forma de cono, nariz bulbosa, pelo ralo y escaso), fue posible identificar la mutación patológica en el gen *TRPS1*, asociado a este síndrome. Finalmente en dos pacientes se encontraron mutaciones en el gen *PTHLH*, asociadas a la BDE con estatura baja de tipo PTHLH. Si bien una de las pacientes consultó por talla baja, la otra presentaba una altura adecuada para su edad cronológica. El estudio detallado de la radiografía de ambas permitió observar que, además de la braquidactilia, presentaban una edad ósea avanzada, característica a tener en cuenta para sospechar esta patología.

No es casualidad que todos estos síndromes presenten manifestaciones clínicas similares; la razón de ello es que los genes asociados a estas patologías se localizan en la vía de señalización PTHR1-Gsα-cAMP-PKA y producen efectos similares sobre la vía causando así patologías con manifestaciones clínicas comunes pero de severidad variable dependiendo del nivel de la vía que esté afectado.

Respecto a los 11 pacientes sin causa genética identificada para explicar su enfermedad, para algunos de ellos no hemos tenido acceso a las radiografías de las manos, lo que nos han impedido definir algún gen candidato; en algunos otros hemos observado que la braquidactilia que presentaban no era de tipo E, sino probablemente tipo A (es necesario abrir nuevas líneas de actuación para

dar respuesta a estos casos); y, finalmente, es posible que aún no se hayan identificado todas las causas genéticas responsables de estas enfermedades.

CONCLUSIONES

- ✓ A parte de las mutaciones puntuales las microdeleciones en *GNAS* también deben de tenerse en cuenta como causa genética de PPHP/PHP1A.
- ✓ La braquidactilia tipo E es el rasgo más orientativo en estos síndrome; y la asociación de ésta con otros rasgos permite establecer un algoritmo de diagnóstico que guie hacía el diagnóstico genético más probable en cada caso.
- ✓ A pesar de la ausencia de historia familiar de PHP, en pacientes con braquidactilia tipo E, estatura baja y retraso del crecimiento intrauterino, debe sospecharse de PPHP; o de POH si además de estas características aparecen osificaciones heterotópicas intramembranosas, que progresan a tejidos más profundos.
- ✓ Ante la presencia de resistencia a la PTH acompañada de braquidactilia severa y generalizada desde edades muy tempranas (incluso antes de los 6 años), se debe sospechar de acrodisostosis de tipo ACRDYS1. Por el contrario, si este tipo de braquidactilia se presente en ausencia de resistencia y acompañada de retraso mental, el diagnóstico más probable es ACRDYS2.
- ✓ La ACRDYS2 está causada por mutaciones activantes en *PDE4D*.
- ✓ Los rasgos más específicos del síndrome trico-rino-falángico son; la braquidactilia, que se manifiesta junto con el acortamiento de las falanges y la característica deformación en cono de las epífisis de las mismas; el pelo ralo y escaso; la nariz bulbosa (o nariz en forma de pera) y el labio superior fino.

- ✓ En el síndrome de braquidactilia tipo E con estatura baja de tipo *PTHLH*, los pacientes presentan BDE con estatura baja, pero ésta puede no apreciarse hasta la edad adulta, ya que es resultado del cierre prematuro de la placa de crecimiento. La estatura diana debe tenerse en cuenta.

- ✓ El origen genético de muchos de los casos de braquidactilia tipo E sigue siendo desconocido.