



## Genetic analysis of adults heterozygous for *ALPL* mutations

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### Abstract

Hypophosphatasia (HPP) is a rare inherited metabolic bone disease due to a deficiency of the tissue nonspecific alkaline phosphatase isoenzyme (TNSALP) encoded by the *ALPL* gene. Patients have consistently low serum alkaline phosphatase (AP), so that this parameter is a good hallmark of the disease. Adult HPP is heterogeneous, and some patients present only mild nonpathognomonic symptoms which are also common in the general population such as joint pain, osteomalacia and osteopenia, chondrocalcinosis, arthropathy and musculoskeletal pain. Adult HPP may be recessively or dominantly inherited; the latter case is assumed to be due to the dominant negative effect (DNE) of missense mutations derived from the functional homodimeric structure of TNSALP. However, there is no biological argument excluding the possibility of other causes of dominant HPP. Rheumatologists and endocrinologists are increasingly solicited for patients with low AP and nonpathognomonic symptoms of HPP. Many of these patients are heterozygous for an *ALPL* mutation and a challenging question is to determine if these symptoms, which are also common in the general population, are attributable to their heterozygous *ALPL* mutation or not. In an attempt to address this question, we reviewed a cohort of 61 adult patients heterozygous for an *ALPL* mutation. Mutations were distinguished according to their statistical likelihood to show a DNE. One-half of the patients carried mutations predicted with no DNE and were slightly less severely affected by the age of onset, serum AP activity and history of fractures. We hypothesized that these mutations result in another mechanism of dominance or are recessive alleles. To identify other genetic factors that could trigger the disease phenotype in heterozygotes for potential recessive mutations, we examined the next-generation sequencing results of 32 of these patients for a panel of 12 genes involved in the differential diagnosis of HPP or candidate modifier genes of HPP. The heterozygous genotype G/C of the COL1A2 coding SNP rs42524 c.1645C > G (p.Pro549Ala) was associated with the severity of the phenotype in patients carrying mutations with a DNE whereas the homozygous genotype G/G was over-represented in patients carrying mutations without a DNE, suggesting a possible role of this variant in the disease phenotype. These preliminary results support COL1A2 as a modifier gene of HPP and suggest that a significant proportion of adult heterozygotes for *ALPL* mutations may have unspecific symptoms not attributable to their heterozygosity.

**Keywords** Adult hypophosphatasia · Dominant inheritance · Dominant negative effect · Modifier gene

### Abbreviations

ALPL Alkaline phosphatase liver-type  
AP Alkaline phosphatase  
DNE Dominant negative effect  
HPP Hypophosphatasia  
NGS Next-generation sequencing

PB Prenatal benign  
TNSALP Tissue nonspecific alkaline phosphatase

### Introduction

Hypophosphatasia (HPP) is a rare inherited metabolic bone disease due to a deficiency of the tissue nonspecific alkaline phosphatase isoenzyme (TNSALP) encoded by the *ALPL* gene. Although its clinical spectrum is a continuum, HPP

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has been divided into six clinical subtypes that may significantly overlap [1]. Perinatal HPP is the most severe form and is almost always lethal, presenting with hypoplastic lungs, extensive hypomineralization, rachitic bone deformities and disturbances of calcium and phosphate metabolism. In contrast, the prenatal benign (PB) form is characterized by prenatal symptoms that spontaneously improve, resulting in non-lethal HPP [2]. Clinical signs of the infantile form appear during the first 6 months of life and include rickets, premature craniosynostosis, irritability, seizures and nephrocalcinosis due to hypercalciuria. This form is lethal in approximately 50% of the cases. Childhood HPP is heterogeneous. It occurs after 6 months of age and is characterized by delayed walking, a waddling gait, and rickets that may cause short stature. Premature loss of primary teeth leads to the diagnosis [3]. Adult HPP is also heterogeneous, with patients presenting recurrent stress fractures and low mineral density during middle age and patients presenting only mild nonpathognomonic symptoms such as joint pain, osteomalacia and osteopenia, chondrocalcinosis, arthropathy and musculoskeletal pain [4–6]. Odontohypophosphatasia (odontoHPP) is characterized by dental manifestations of HPP not associated with abnormalities of the skeletal system. Patients with severe or moderate HPP have consistently low serum alkaline phosphatase (AP), so that this parameter is a good hallmark of the disease [7]. A treatment with enzyme replacement therapy has been shown to be efficient [8] and is now available for pediatric-onset HPP.

TNSALP is a central actor of bone mineralization [9]. The enzyme catalyzes the hydrolysis of various phospho-monoesters among which are inorganic pyrophosphate, pyridoxal-5'-phosphate and nucleotides. This catalytic activity depends on a homodimeric configuration from which the dominant negative effect (DNE) of some *ALPL* mutations likely derive. Five functional domains have been identified in the three-dimensional (3D) structure of TNSALP modeled from the crystal structure of human placental AP [10–12]. The active site is the center of catalytic activity. The calcium site is highly conserved in vertebrates, but its function remains to be elucidated. The homodimer interface is crucial because dimerization is indispensable for allostery. The crown domain is a key factor of uncompetitive inhibition, heat stability and allosteric behavior and, together with the N-terminal alpha helix, contributes to stabilization of the dimeric structure. The crown domain has also been shown to bind collagen [13, 14].

Severe forms of HPP (perinatal and infantile) are almost always recessively inherited, while less severe forms (childhood, adult, odonto and PB) may be dominantly or recessively inherited [15–17]. The more severe the disease, the more often it is subject to recessive inheritance [15, 18]. Both dominant and recessive forms are due to loss-of-function mutations in the *ALPL* gene.

The prevalence of severe HPP was estimated at 1/100 000 in Canada based on pediatric registries [19], and more recently at 1/300 000 in Europe based on molecular diagnosis [20]. This corresponds to carrier frequencies of 1:160 and 1:270, respectively. Because heterozygotes can express the disease, mild HPP is expected to be more frequent, with its actual frequency depending on the proportion of dominant mutations and their penetrance [20]. The actual proportion of persons with repeatedly low AP in the population is unknown. According to recent reports it might range from 1/1544 [21] to 1/81 [22], and 50–84% of them could harbor pathogenic *ALPL* mutations [23, 24]. Even with a low penetrance, these mutations may account for the high incidence of mild forms of HPP. However, symptoms such as musculoskeletal pain, dental problems such as carries, osteomalacia and osteopenia or symptoms resembling osteoporosis are very common in the general adult population including heterozygotes for a recessive *ALPL* mutation. More simply, low serum AP levels indicate an *ALPL* mutation [21, 23, 25, 26], but an *ALPL* mutation does not necessarily indicate HPP. Rheumatologists and endocrinologists are increasingly solicited for patients with low AP and nonpathognomonic symptoms of HPP. Many of these patients are heterozygous for an *ALPL* mutation and a challenging question is to determine if these symptoms, which are also common in the general population, are attributable to their heterozygous *ALPL* mutation or not.

In the attempt to address this question, we studied a cohort of 61 adult patients heterozygous for an *ALPL* mutation. To distinguish heterozygotes for dominant and recessive mutations, respectively, we classified the *ALPL* mutations according to their statistical likelihood to show a DNE and identified potential recessive *ALPL* mutations. To identify other genetic factors that could trigger the HPP phenotype in heterozygotes for a recessive mutation, we examined the next-generation sequencing (NGS) results of 32 of these patients for a panel of 12 genes involved in the differential diagnosis of HPP or candidate modifier genes of HPP. A coding SNP in *COL1A2* was found to be associated with markers of severity.

## Materials and methods

### Patients and *ALPL* gene sequencing

With the exception of patient 51 who was studied at the Universitätsklinikum Freiburg, Germany, all patients were tested for diagnostic purposes in our laboratory and signed a consent form. All the patients were unrelated excepted patients 8 (daughter) and 9 (mother). Seventy-two percent of the 61 patients were ascertained in France, while the others were from European countries, the USA or Australia. Eighty

percent of the patients were female. They presented various features ranging from recurrent fractures and bone fragility to mild and nonpathognomonic symptoms such as musculoskeletal pain, diffuse pain, osteomalacia and osteopenia or symptoms resembling osteoporosis, calcifications and chondrocalcinosis. Patients were included in the study if they were found to carry only one heterozygous mutation after the entire coding sequence of the *ALPL* gene and intron/exon borders were analyzed by Sanger methodology or NGS. Mutations detected by NGS were confirmed by Sanger methodology. The patients were also screened for large deletions by either semi-quantitative polymerase chain reaction (PCR), quantitative PCR, arrayCGH or by NGS with the software CovCop [27]. Although none of these methodologies detect deletions exhaustively, we considered that these patients did not carry a large heterozygous deletion.

### Classification of mutations

Mutations were classified according to their localization in TNSALP. The 3D structure was previously modeled from the crystal structure of the human placental AP [10, 11]. It allows the localization of residues affected by mutations in five crucial domains (active site, homodimer interface, crown domain, calcium site, N-terminal alpha helix) or in other regions of the protein. The model was visualized using the open source software PyMol version 0.98 (Delano Scientific LLC).

### DNE of mutations

Mutations were assumed to have a DNE if they were previously shown to have this effect by coexpression studies, i.e., the residual enzymatic activity of cotransfected cells is <50% of wild-type (WT) activity, the value expected in the recessive model [15, 28–35]. The cut-off dominant effect/no dominant effect was set to 45% of WT activity for mutations with no residual activity. For mutations with residual activity, the cut-off was  $0.90(100 + \text{activity})/2$  expressed as % of WT. For instance, a mutation with 25% of residual activity will have a DNE if the residual activity of cotransfected cells is less than the cut-off  $0.9(100 + 25)/2 = 56.25\%$  (instead of 45% for a mutation with no residual activity). Insertions/deletions, splice site and nonsense mutations were assumed to have no DNE.

### SNP genotyping

The *COLIA2* SNP rs42524 (chr7:94043239; Hg19) [c.1645C > G (p.Pro549Ala)] was genotyped partly by NGS and partly by allelic discrimination with real-time PCR using a Taqman probe (ThermoFisher).

### Statistical calculations

Statistical calculations were performed using the open access calculator <http://www.socscistatistics.com>. Differences between distributions were assessed by chi-squared tests, comparison of means by *t* tests, and comparisons of proportions by *Z* tests.

## Results

### Mutations with a DNE

Among the 334 mutations reported in the *ALPL* gene mutation database ([http://www.sesep.uvsq.fr/03\\_hypo\\_mutations.php](http://www.sesep.uvsq.fr/03_hypo_mutations.php), accessed on March 31, 2017), 46 missense mutations were tested for a DNE by various investigators [15, 28–42], and the results were listed in the database. We localized each tested mutation on the 3D model and found that 97% (29/30) of the mutations with a reported DNE were localized in the crown domain, the active site or its vicinity, or in the homodimer interface. In contrast, only 37% (6/16) of the mutations with no DNE were localized in these regions ( $p < 10^{-5}$ , chi-squared test) that harbor 44% of all missense mutations listed in the database. This corroborates our previous results reporting that most of the amino acids affected by dominant mutations were clustered in these particular domains of the protein [15].

### Statistical classification of mutations in adult patients heterozygous for an *ALPL* mutation

We localized where the 42 missense mutations that were found in the patients occurred in the protein (Table 1). The mutations were then divided into three classes. Class 1 includes missense mutations localized in the homodimer interface, the crown domain or the active site. This class was predicted to contain most of the missense mutations with a DNE but also possibly mutations with no DNE. Class 2 includes missense mutations localized in other regions and is statistically composed of mutations with no detectable DNE. These mutations may be recessive with the phenotype triggered by another factor in combination with the *ALPL* mutation, or may be responsible for another mechanism of dominance such as haploinsufficiency, or alternatively may constitute DNE not detectable by activity measurement [43]. Class 3 includes non-missense mutations, as well as the missense mutation c.791A > G (p.Lys264Arg), for which the major effect is to lead to the complete skipping of exon 7 [44]. These mutations are assumed to have no DNE. We found that only 27 (44%) of the patients carried a mutation of class 1, suggesting that, according to our statistical classification, over half (56%) of the patients carry an *ALPL*

**Table 1** Mutations found in the 61 patients heterozygous for an *ALPL* mutation

Patient	Mutation ( <i>ALPL</i> )	3D	Class	DB	Gender	Age at onset of symptoms (years)	AP	Fractures	rs42524 ( <i>COL1A2</i> )
1	p.A114T	ASV	1	ND	M	63	74	No	C/C
2	p.S445P	CD	1	Yes	F	35	55	Yes	G/C
3	p.G426S	CD	1	ND	F	29	72	No	G/C
4	p.E476A	HI	1	Yes	F	17	ND	Yes	G/C
5	p.A176T	ASV	1	Yes	F	39	ND	No	G/C
6	p.A114T	ASV	1	ND	F	36	62	Yes	G/C
7	p.E452K	ASV	1	Yes	F	19	64	No	G/C
8	p.R184W	AS	1	Yes	F	27	45	No	G/C
9	p.R184W	AS	1	Yes	F	42	37	Yes	G/C
10	p.N417S	CD	1	Yes	F	42	ND	No	G/C
11	p.E476K	HI	1	ND	F	36	37	Yes	G/C
12	p.R391C	HI	1	No	F	16	ND	Yes	G/C
13	p.E476K	HI	1	ND	F	32	52	Yes	G/C
14	p.A443V	CD	1	No	F	28	45	No	G/C
15	p.N417S	CD	1	Yes	F	Nd	36	Yes	G/C
16	p.R184Q	AS	1	Yes	F	54	46	No	G/C
17	p.A443V	CD	1	No	F	55	40*	Yes	G/G
18	p.T68M	HI	1	ND	F	75	48	Yes	G/G
19	p.R391H	HI	1	Yes	F	16	ND	Yes	G/G
20	p.R391H	HI	1	Yes	F	48	35	No	G/G
21	p.A176T	ASV	1	Yes	F	36	47	No	G/G
22	p.N417S	CD	1	Yes	F	40	47	Yes	G/G
23	p.A468V	HI	1	ND	F	70	87	No	G/G
24	p.R272H	HI	1	ND	M	69	68	No	G/G
25	p.A114V	NS	1	ND	F	70	*	Yes	G/G
26	p.R391H	HI	1	Yes	M	36	90	Yes	G/G
27	p.R184Q	AS	1	ND	M	51	50	No	G/G
28	p.G162S	NS	2	ND	F	51	ND	ND	C/C
29	p.A116T	NS	2	Yes	F	55	ND	Yes	G/C
30	p.T148I	NS	2	ND	F	47	55	Yes	G/C
31	p.E191K	NS	2	No	F	45	68	No	G/C
32	p.I490F	NS	2	No	F	34	ND	Yes	G/C
33	p.E191K	NS	2	No	F	46	71*	Yes	G/C
34	p.I215V	NS	2	ND	M	58	ND	Yes	G/C
35	p.G491R	NS	2	ND	F	72	68	No	G/C
36	p.E476K	NS	2	ND	F	65	56	No	G/G
37	p.R136H	NS	2	No	F	76	62	No	G/G
38	p.R223Q	CD	2	ND	F	42	42	No	G/G
39	p.T148I	NS	2	ND	M	43	74	No	G/G
40	p.Y117C	NS	2	ND	F	16	57	No	G/G
41	p.V461I	NS	2	ND	F	66	ND	No	G/G
42	p.R136C	NS	2	ND	M	26	67	Yes	G/G
43	p.R136C	NS	2	ND	F	22	68	Yes	G/G
44	p.G200D	NS	2	ND	F	59	86	Yes	G/G
45	p.R136H	NS	2	No	F	77	ND	No	G/G
46	p.R136H	NS	2	No	F	60	65	No	G/G
47	p.V461I	NS	2	ND	F	40	80	No	G/G
48	p.D156Y	NS	2	ND	F	25	57	No	G/G

**Table 1** (continued)

Patient	Mutation ( <i>ALPL</i> )	3D	Class	DB	Gender	Age at onset of symptoms (years)	AP	Fractures	rs42524 ( <i>COLIA2</i> )
49	p.H180N	NS	2	ND	F	36	85	Yes	G/G
50	p.T366N	NS	2	ND	F	34	49	No	G/G
51	p.A40 V	NS	2	No	F	23	91	No	G/G
52	c.648 + 1G > A	NA	3	NA	M	61	34*	No	C/C
53	c.302_310 del	NA	3	ND	M	46	65	Yes	G/G
54	c.187_205 del	NA	3	NA	F	55	82	Yes	G/G
55	p.W270X	NA	3	ND	F	38	80	No	G/G
56	c.997 + 2T > A	NA	3	NA	F	64	92	Yes	G/G
57	p.K264R	NA	3	NA	M	74	65	No	G/G
58	c.648 + 1G > A	NA	3	NA	F	18	50	No	G/G
59	c.1412_1429del	NA	3	NA	F	51	ND	Yes	G/G
60	c.814delC	NA	3	NA	M	43	ND	No	G/G
61	p.K264R	NA	3	NA	M	58	47	No	G/G

Serum alkaline phosphatase (AP) is given as % of the lower limit of the normal range. When repeated measures were taken, the mean is provided

The last column gives the genotypes at the SNP locus rs42524 (chr7:94043239; Hg19) [c.1645C > G (p.Pro549Ala)]

3D localization (3D), ASV active site vicinity (< 6 Å), AS active site, CD crown domain, HI homodimer interface, NA not applicable, NS not specific

DB indicates whether the mutation experimentally showed a dominant negative effect (yes) or not (no)

\* Indicates a reported history of treatment with bisphosphonates

ND not done (not tested), NA not applicable

mutation that does not show a DNE. In contrast to the adult patients, the heterozygous patients with other clinical forms in our cohort were preferentially carrying class 1 mutations, from 76% in odontoHPP ( $n = 45$ ) to 83% in childhood HPP ( $n = 18$ ), and 100% in the PB form ( $n = 6$ ) (not shown). When considering three parameters reflecting the severity of the phenotype (age of first symptoms, serum AP activity and history of fractures) we found that patients with class 1 mutations seem to be slightly more severely affected. The differences were statistically significant for serum AP and borderline for the age of diagnosis (Table 2). Among 22 informative cases, familial history was documented in 70% of class 1 and in 50% of classes 2 or 3 patients.

### NGS analysis

We examined the NGS results of 32 patients tested for a panel of 12 genes involved in HPP (*ALPL* and promoter region), in the differential diagnosis of HPP (*COLIA1*, *COLIA2*, *FGFR3* and *SOX9*) or candidates as modifier genes of HPP (*ANKH*, *ENPP1*, *PHOSPHO1*, *PTH1R*, *PTH2R*, *SPPI* and *TNFRSF11A*) [45]. Among the 280 variants detected in these genes, three revealed statistically significant heterogeneity in their distribution depending on the class of mutation: rs2277632 in *COLIA1* ( $p = 1.1 \cdot 10^{-2}$ ),

rs42524 in *COLIA2* ( $p = 5.9 \cdot 10^{-3}$ ) and rs42518 in *COLIA2* ( $p = 7.2 \cdot 10^{-3}$ ); the two latter variants were in strong linkage disequilibrium, and we finally focused on the variant rs42524 because it is coding and because it had the most significant  $p$  value. Genotyping of rs42524 was extended to other patients by allelic discrimination using a Taqman<sup>®</sup> probe, allowing confirmation that this variant statistically differed in its distributions in class 1 and classes 2 + 3 (chi-squared = 7.81,  $p = 5.2 \cdot 10^{-3}$ , Table 3). Fifty-six percent of patients with class 1 were found to carry the heterozygous genotype G/C, whereas only 21% of patients of class 2 or 3 carry this genotype. The latter were found to be under-represented when compared with a sample of 109 unrelated individuals from the general French population (Table 3). Thus, there is a relationship between the genotype at this locus and the class of *ALPL* mutation. As with class 1 mutations (Fig. 1a), the heterozygous genotype G/C is statistically associated with a more severe phenotype (Fig. 1b), suggesting that it could contribute to aggravation of the phenotype. The sample size was too small in some categories (especially G/C classes 2 and 3) to perform statistical analysis on split data; however, the age of onset was significantly lower in patients with G/C in class 1 than in classes 2 and 3 ( $32.3 \pm 10.6$  years vs  $51.0 \pm 12.1$ , respectively,  $p = 8.7 \cdot 10^{-4}$ ), whereas there was no significant difference between

**Table 2** Distribution according to age at onset of symptoms (years), serum AP and fractures in the three classes of patients

<i>N</i>		Class 1	Class 2	Class 3	Total
		27	24	10	61
Gender	M	4	3	5	12
	F	23	21	5	49
Age of onset of symptoms ± SD	Age	41.5 ± 17.0	46.6 ± 17.6	50.8 ± 15.7	45.4 ± 17.5
	<i>N</i>	26	24	10	60
AP ± SD	%	54.1 ± 16.3	66.7 ± 13.1	64.3 ± 19.8	60.7 ± 16.6
	<i>N</i>	21	18	8	47
Fractures	%	51.8	39.1	40	44.8
	<i>N</i>	27	23	10	60

***P* values**Age of first symptoms (*t* test)Class 1 vs class 2: *p* = 0.16Class 1 vs class 3: *p* = 0.08Class 2 vs class 3: *p* = 0.17Class 1 vs classes 2 + 3: *p* = 0.08AP (*t* test)Class 1 vs class 2: *p* =  $6.3 \times 10^{-3}$ \*\*Class 1 vs class 3: *p* = 0.08Class 2 vs class 3: *p* = 0.36Class 1 vs classes 2 + 3: *p* =  $6.6 \times 10^{-3}$ \*\*Fractures (*z* score test)Class 1 vs class 2: *p* = 0.18Class 1 vs class 3: *p* = 0.26Class 2 vs class 3: *p* = 0.48Class 1 vs classes 2 + 3: *p* = 0.17

AP levels are expressed as % of the lower limit of the normal range. Fractures: proportion (%) of patients presenting at least one fracture not attributable to a trauma during their clinical course

\*Statistically significant *p* < 0.05; \*\*Statistically significant *p* < 0.01

the G/C and G/G genotypes in classes 2 and 3 and G/G in class 1 ( $51.0 \pm 12.1$ ,  $51.4 \pm 18.0$  and  $46.3 \pm 18.6$ , respectively) (Fig. 1c). The same was observed for AP level where GC class 1 patients have significantly lower AP than others ( $50.1 \pm 12$  vs  $64.5 \pm 16$ ; *p* =  $4.6 \times 10^{-3}$ ). This suggests that the possible aggravating role of G/C is conditioned on mutations with a DNE. Regarding the history of fractures, GC class 2 and 3 seem to be the most severe combination; however, the number of informative cases was very low in this category (*n* = 7). Finally, our results suggest that the rs42524 *COL1A2* SNP could be a modifier gene of HPP at least in adults heterozygous for *ALPL* mutations.

## Discussion

Classically, autosomal dominant inheritance may be due to negative interactions between WT and mutated proteins (DNE), addition of a new property of the protein (gain of function), or inability to produce enough protein with the

WT allele (haploinsufficiency). Similar mechanisms are also found at the RNA level. Due to the homodimeric structure of functional TNSALP [9], dominant HPP is believed to result from the DNE of loss-of-function mutations, and this has been experimentally confirmed for many mutations. However, there is no biological argument excluding the possibility of other causes of dominant HPP. Here, we separated the *ALPL* mutations detected in adult heterozygotes in three groups according to their molecular consequence and their localization, and we found that only one-half of the patients carry a mutation with a predicted DNE. We hypothesized that class 2 and class 3 mutations had no DNE. This was expected for mutations c.187\_205del and p.L6X resulting in early termination of translation, experimentally established for five missense mutations previously tested with site-directed mutagenesis (p.R136H, p.E191K, p.G249V, p.V431A, p.I490F), and predicted by 3D modeling for the remaining missense mutations. With respect to localization and molecular effect, the variety of class 2 and class 3 mutations does not support a mechanism of gain of function,

**Table 3** Distribution of rs42524 genotypes in the three classes of adult patients

	Class 1	Class 2	Class 3	Classes 2 + 3	Total	General population
<i>COL1A2</i> rs42524						
G/G	11 (0.41)	16 (0.67)	9 (0.90)	25 (0.74)	36 (0.59)	53 (0.49)
G/C	15 (0.56)	7 (0.29)	0	7 (0.21)	22 (0.36)	46 (0.42)
C/C	1 (0.04)	1 (0.04)	1 (0.10)	2 (0.06)	3 (0.05)	10 (0.09)
Total	27	24	10	34	61	109

*P* values (genotypes G/G and G/C were considered)

Class 1 vs classes 2 + 3:  $p = 5.2 \cdot 10^{-3}$ \*\*

Class 1 vs general population:  $p = 0.31$

Class 2 vs general population:  $p = 0.16$

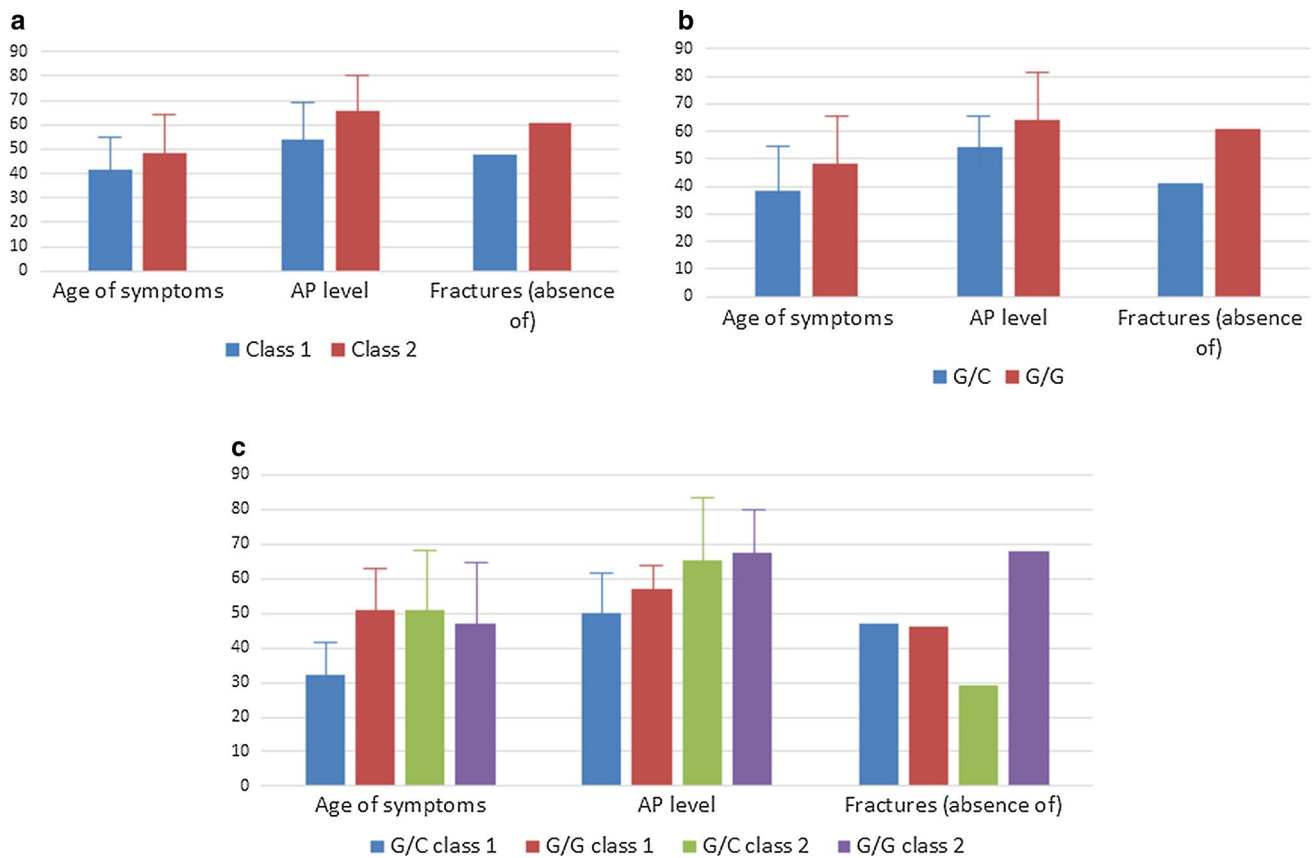
Classes 2 + 3 vs general population:  $p = 1.4 \cdot 10^{-2}$ \*

The general population consisted of 109 unrelated persons from the French population. For this sample, the frequencies were in Hardy–Weinberg equilibrium ( $p = 0.97$ , chi-squared test)

\* Statistically significant  $p < 0.05$ ; \*\* Statistically significant  $p < 0.01$

although we do not exclude a particular mutation that could exhibit such a mechanism. Overall, however, we hypothesize that class 2 and class 3 mutations might not result in gain of function or in DNE defined here as the negative interaction between the WT and the mutated TNSALP monomers, which is experimentally detectable by the inhibition of WT AP activity. More likely, these mutations result in another mechanism of dominance. Most of the class 2 and 3 patients carry G/G suggesting an excess of this genotype or a lack of the G/C genotype when compared to the general population or to other patients with dominant HPP (childhood, ontoHPP and PB). All together, these data suggest that adults heterozygous for *ALPL* mutations may express a disease phenotype resulting from *ALPL* class 1 mutations, aggravated by *COL1A2* G/C, or resulting from *ALPL* class 2 mutations, associated with *COL1A2* G/G. Some patients with class 2 mutations and carrying *COL1A2* G/C could be not symptomatic and therefore not ascertained, explaining their under-representation, or alternatively G/G could trigger the affected phenotype. An interesting example is patient 35 who was referred to us for hypercalcemia and low serum AP. She was found to carry the heterozygous class 2 mutation p.G491R and the *COL1A2* G/C genotype. Finally the patient was diagnosed with primary hyperparathyroidism due to parathyroid hyperplasia which explained her hypercalcemia. In this scheme, the G/C genotype aggravates the DNE of class 1 mutations but has a neutral effect in patients with class 2 mutations. These results could marginally explain some cases of weak genotype–phenotype correlation in adult HPP [5, 46–51]. We are aware that these proposals, based on very preliminary results, remain speculative until the study of larger cohorts and/or coexpression of *COL1A2* and TNSALP experiments will be performed.

A scaffold of fibrillar collagen type 1 is needed in the extracellular space to allow elongation of mineralization, and TNSALP is known to bind to collagen [52, 53]. In addition mutations in *COL1A2* are responsible for osteogenesis imperfecta, the major differential diagnosis of HPP regardless of the clinical form considered [45, 54, 55]. The aggravating effect reported here was found only in patients with class 1 mutations. This suggests that the negative interaction between WT and mutated TNSALPs, i.e., the DNE, is potentiated by the collagen scaffold issued from the G/C genotype, whereas the collagen scaffold from the reference genotype G/G does not negatively interact with TNSALPs. Previous studies reported associations of this SNP with various phenotypes including low bone mineral density in the Swedish population [56] and neovascular macular degeneration [57] and hypertensive intracerebral hemorrhage [58] in Japanese and Chinese populations, respectively. In these studies, the G/C genotype was determined to be at risk. It has been suggested that this effect on the phenotype was due to disadvantageous protein interactions in G/C subjects



**Fig. 1** Comparison of classes of mutations and rs42524 genotypes for three markers of phenotypic severity—age of onset (years), serum AP (% of the lower limit of the normal range), and absence of fractures (% of patients). The latter corresponds to the proportion of patients

with no reported history of no traumatic fractures. **a** Classes of mutation, **b** *COLIA2* rs42524 genotypes G/G and G/C, **c** classes of mutation and *COLIA2* rs45524 genotypes taken together

during the formation of collagen helices [56]. The TNSALP-collagen binding site corresponds to the crown domain [13, 14, 52, 59], a region belonging to the area with class 1 mutations, making such a potentiating effect consistent. Class 1 mutations affecting the crown domain seem to be more severe than other class 1 mutations for AP (48.1 vs 56.1, not significant), but there is no difference for age (38.7 vs 41.2) or fractures (0.50 vs 0.47). Studies of TNSALP-COL1A2 interactions in patient-derived fibroblasts harboring various genotypes could provide an experimental confirmation of these results.

The patients studied here are at the mildest extremity of the HPP clinical spectrum. In this category, the disease and the normal phenotypes overlap, and it cannot be excluded that a portion of these patients are genuine heterozygotes for a recessive mutation not responsible for the symptoms. We noticed that the proportion of adult HPP patients referred to our laboratory who were heterozygous for a class 2 mutation increased from 25 to 50% before and after 2010, respectively, reflecting that although low AP is not well recognized by many clinicians [60], there is an increasing

awareness among specialists regarding this warning sign. *ALPL* mutation testing is being increasingly requested, confirming the need to manage mutation findings by thorough clinical examination to distinguish HPP patients and simple carriers. Genotyping the rs42524 SNP as well could be useful to correlate the phenotype with the genotype.

In conclusion we found here that the rs42524 *COLIA2* SNP modulates the phenotype of patients heterozygous for *ALPL* mutations. These findings and their resulting hypotheses remain very speculative and must be confirmed by testing more patients, and by coexpression experiments in order to confirm the hypotheses presented here.

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### Compliance with ethical standards

**Conflicts of interest statement** EM received honoraria from Alexion. CR received honoraria from Alexion and research grants from Ultragenix



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