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Tissue non-specific alkaline phosphatase activity and mineralization capacity of bi-allelic mutations from severe perinatal and asymptomatic hypophosphatasia phenotypes: Results from an *in vitro* mutagenesis model

Suma Uday[a,b], Tomohiro Matsumura[c], Vrinda Saraff[a], Shiho Saito[c], Hideo Orimo[c], Wolfgang Högler[b,d,*]

[a] Department of Endocrinology and Diabetes, Birmingham Women's and Children's Hospital, Birmingham, UK
[b] Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, UK
[c] Department of Biochemistry and Molecular Biology, Graduate School of Medicine, Nippon Medical School, Tokyo, Japan
[d] Department of Paediatrics and Adolescent Medicine, Johannes Kepler University, Linz, Austria

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ABSTRACT

Background: Hypophosphatasia (HPP) is an inherited metabolic bone disease characterized by reduced mineralization due to mutations in the tissue non-specific alkaline phosphatase (*ALPL*) gene. HPP is clinically variable with extensive allelic heterogeneity in the *ALPL* gene. We report the findings of *in vitro* functional studies following site-directed mutagenesis in bi-allelic mutations causing extreme clinical phenotypes; severe perinatal and asymptomatic HPP.

Aims: Elucidate genotype-phenotype correlation using *in vitro* functional studies and 3 dimensional (3D) ALP modelling.

Methods: Clinical, biochemical and radiological features were recorded in two children with extreme HPP phenotypes: Subject 1 (S1): Perinatal HPP with compound heterozygous mutations (c.110T > C; c.532T > C); Subject 2 (S2): asymptomatic with homozygous missense mutation (c.715G > T).

Plasmids created for mutants 1 c.110T > C (L37P), 2 c.532T > C (Y178H) and 3 c.715G > T (D239Y) using *in vitro* mutagenesis were transfected into human osteosarcoma (U_2OS) cells and compared to wildtype (WT) and mock cDNA. ALP activity was measured using enzyme kinetics with *p*-nitrophenylphosphate. Mineral deposition was evaluated photometrically with Alizarin Red S staining after culture with mineralization medium. Western blot analysis was performed to identify the mature type protein expression (80 kDa). Mutations were located on a 3D ALP model. Co-transfection was performed to identify dominant negative effect of the mutants.

Results: **Phenotype**: S1, had typical perinatal HPP phenotype at birth; extremely under-mineralized bones and pulmonary hypoplasia. S2, diagnosed incidentally by laboratory tests at 4 years, had normal growth, development, dentition and radiology. All S2's siblings (3 homozygous, 1 heterozygous) were asymptomatic. All subjects had typical biochemical features of HPP (low ALP, high serum pyridoxal-5′-phosphate), except the heterozygous sibling (normal ALP).

Functional assay: Mutants 1 and 2 demonstrated negligible ALP activity and mineralization was 7.9% and 9.3% of WT, respectively. Mutant 3 demonstrated about 50% ALP activity and 15.5% mineralization of WT. On Western blot analysis, mutants 1 and 2 were detected as faint bands indicating reduced expression and mutant 3 was expressed as mature form protein with 50% of WT expression. Mutant 1 was located near the Glycosylphosphatidylinositol anchor, 2 at the core structure of the ALP protein and 3 at the periphery of the protein structure. Co-transfection did not reveal a dominant negative effect in any of the mutants.

Conclusion: Our findings expand the current knowledge of functional effect of individual mutations and the importance of their location in the ALP structure.

* Corresponding author at: Department of Paediatrics and Adolescent Medicine, Kepler University Hospital, Krankenhausstrasse 26-30, 4020 Linz, Austria.
E-mail address: Wolfgang.hoegler@jku.at (W. Högler).

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1. Introduction

Hypophosphatasia (HPP) is an inherited metabolic bone disease characterized by defective bone and dental mineralization presenting with typical radiological and biochemical features [1]. The hallmark of HPP is a low serum alkaline phosphatase (ALP) [2] secondary to mutations in the tissue non-specific alkaline phosphatase (*ALPL*) gene [3].

Tissue non-specific alkaline phosphatase (ALPL) plays an important role in mineralization by hydrolyzing inorganic pyrophosphate (PPi) to inorganic phosphate (Pi) which is essential for hydroxyapatite crystal formation. The formation of hydroxyapatite crystals within matrix vesicles that bud from the surface membrane of hypertrophic chondrocytes, osteoblasts, and odontoblasts is the first step in mineralization. This is followed by propagation of hydroxyapatite into the extracellular matrix and its deposition between collagen fibrils. Extracellular PPi, inhibits hydroxyapatite formation. PPi, pyridoxal phosphate (PLP), and phosphoethanolamine (PEA) are thought to be the physiologic substrates of ALPL, which accumulate in the event of deficiency as seen in hypophosphatasia [2].

The *ALPL* gene is located on chromosome 1p36-34 [4], consists of 12 exons distributed over 50 kb [5] and is subject to very strong allelic heterogeneity. To date, at least 389 distinct mutations in the *ALPL* gene causing HPP have been identified and the majority are missense mutations [6]. HPP is associated with an extremely variable clinical and skeletal phenotype [7,8] with variable expressivity, even in patients sharing the same genotype [9]. The currently recognized forms reflect this spectrum, ranging from very severe, early-onset disease (perinatal, infantile), to milder forms (adult and odontohypophosphatasia) [10]. Whilst severe forms of HPP are almost always recessively inherited, mild and moderate forms can be inherited in an autosomal dominant or recessive manner. The study of the *ALPL* mutations by various experimental approaches including site-directed mutagenesis, 3D modelling and immunofluorescence have partially helped inform the genetic aspects of HPP [11]. In moderately to severely affected patients a good correlation has been observed between disease severity and *in vitro* enzymatic activity of the mutant protein measured after site-directed mutagenesis [12].

We have recently described a clinically asymptomatic child with a novel homozygous *ALPL* mutation who only has the typical biochemical HPP features [13]. The extreme variability of the phenotype of patients with bi-allelic *ALPL* mutations remains currently unexplained. As yet, only a handful of asymptomatic individuals have been reported [6,14,15] but no studies have assessed *in vitro* enzymatic activity in these individuals or determined the mutation sites using 3D modelling. It is crucial to investigate genotype-phenotype correlations to be able to predict clinical outcome and inform genetic counselling.

2. Aims

Elucidate genotype-phenotype correlation using *in vitro* functional studies and 3D ALP modelling of *ALPL* mutations from extreme HPP phenotypes.

3. Materials and methods

3.1. Subjects

Two subjects with extremes of disease severity form a single paediatric tertiary centre HPP clinic were identified and recruited. Subject 1 (S1) had perinatal HPP and Subject 2 (S2) had asymptomatic HPP which was incidentally identified on a retrospective laboratory data review which we have previously reported [13]. Clinical data were also collected on siblings of S2 (Table 1).

Clinical data were collected from patient notes and clinical database on: clinical history, presenting features, anthropometry, biochemistry [serum ALP activity, pyridoxal phosphate (PLP), urinary phosphoethanolamine (PEA), serum calcium and phosphate, serum 25-hydroxy vitamin D], radiology [signs of rickets on radiographs, bone density on dual energy X-ray absorptiometry (DXA), vertebral morphometry on lateral vertebral assessment (LVA) on DXA] and genetic mutations.

3.2. Clinical laboratory assays

Serum ALP activities were determined using a dye-based assay, which measures the enzyme activity by monitoring the rate of hydrolysis of *p*-nitrophenylphosphate to *p*-nitrophenol at 410/480 nm in the presence of magnesium on an Olympus AU640 (Beckman Coulter, High Wycombe, United Kingdom) analyzer. Urinary PEA was measured by amino acid quantification by ion exchange high performance liquid chromatography on a Biochrom30 + amino acid analyzer (Biochrom Ltd., Cambridge, United Kingdom). Plasma PLP was measured by an external laboratory using high performance liquid chromatography following derivatization with fluorometric detection using a kit (Chromsystems, Munich, Germany).

3.3. In vitro laboratory assays

1. Construction of expression plasmids: novel plasmids for each mutation (Table 1) were created using an *in vitro* mutagenesis kit. ALP cDNA was inserted into the expression vector pcDNA3 (Invitrogen, Carlsbad, CA, USA) as described elsewhere [16]. To create mutant ALP expression vectors, site-directed mutagenesis was performed using a Unique Site Elimination Mutagenesis Kit (Amersham-Pharmacia Biotech, Uppsala, Sweden) as described elsewhere [16].
2. Expression in the cultured cells: the human osteoblastic osteosarcoma cell line U_2OS, which has osteoblastic features but lacks ALP activity, was transfected with the ALP expression plasmid. Mock cells were transfected with only the vector (pcDNA3) and wild type (WT) cells were transfected with wild type ALP expression vector (pcDNA3-ALP). Standardised culture conditions were employed, using D-MEM culture medium. The U_2OS cells were precultured for 24 hours in a 6 well plate (used for ALP activity and western blot assay), or a 48 well plate (used for mineralization assay). Each 2.5 μg (for ALP activity assay) or 0.5 μg (for mineralization assay) of plasmids per well were used for the transfection of cells, and the transfection was performed using Lipofectamine 2000 (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. For enzyme activity and blotting assay, after 48 hours, transfected cells were homogenised with Tris-buffered saline (pH 7.4) containing 0.1% Triton-X100 and 0.1 mM PMSF (phenylmethylsulfonyl fluoride). The homogenate were centrifuged at 15,000*g*, then the supernatant of the cell homogenate were prepared for assay. Protein concentration of cell extract was determined using Bradford assay kit (Thermo Fisher Scientific, USA). For the mineralization assay, after 30 hours, transfected cells were cultured with mineralization medium (D-MEM containing 10 mM of beta-glycerophosphate) for 5 days, and the cells were stained with Alizarin Red S.
3. Enzyme assay was used to measure ALP activity as described elsewhere [17]. The artificial substrate (1 mM *p*-nitrophenylphosphate) added to assay mixture, and its product (*p*-nitrophenol) was measured using spectrophotometer by the increasing velocity at the absorbance of 405 nm. The assays were performed in 0.1 M 2-amino-2-methyl-1,3-propanediol-HCl buffer (pH 10.0) containing 5 mM $MgCl_2$ at 30 °C and ALP activity was expressed as *p*-nitrophenol production, in nmol/min/mg protein.
4. Mineralization ability of the mutant protein was measured by culturing cells in mineralization medium for 5 days and then evaluating mineralization using Alizarin Red S staining. After solubilization with cetylpyridinium chloride, optical density of purple colour was measured by the absorbance at 570 nm using methods we have previously described [17]. Mineralization was expressed as absorbance per well (OD 570/well) of culture plate.

Table 1
Disease severity, inheritance, mutation and plasmid of affected individuals.

	Clinical severity	Mutation description	Base change	Amino acid change	Plasmid
Subject 1	Severe perinatal HPP	Compound heterozygous	c.110T > C, exon 3 (Mutation 1)	Maternal p.Leu37Pro	L37P (Mutant 1)
			c.532T > C, exon 6 (Mutation 2)	Paternal p.Tyr178His	Y178H (Mutant 2)
Subject 2	Asymptomatic	Homozygous	c.715G > T, exon 7 (Mutation 3)	p.Asp239Tyr	D239Y (Mutant 3)

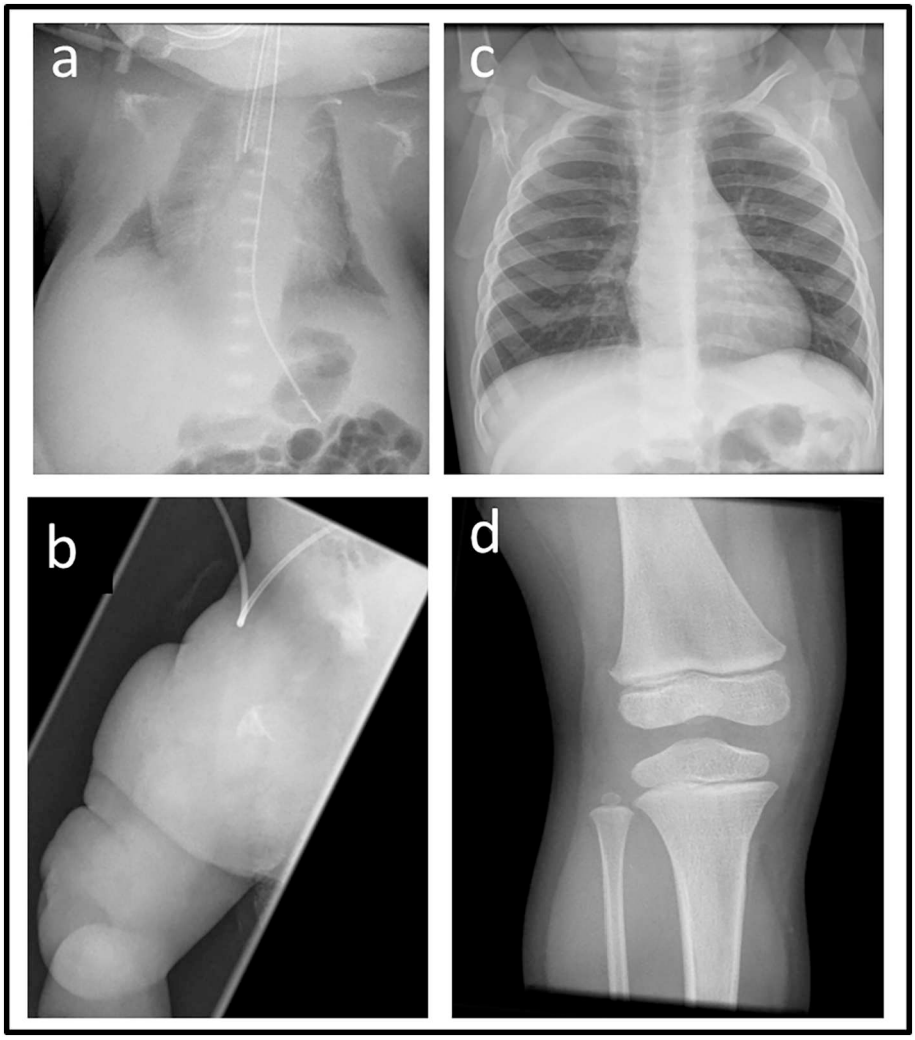


Fig. 1. Chest and knee radiographs of subject 1 (a and b) with severe perinatal HPP demonstrate severely undermineralized bones and a small bell shaped chest with pulmonary hypoplasia. In contract, the radiographs of the asymptomatic subject 2 (c and d) are normal.

5. Western blot analysis was performed to identify the mature ALP (complex type glycosylation) protein which appears as 80 kDa. ALP is glycosylated during the processing in the endoplasmic reticulum and the Golgi apparatus. First, the enzyme is attached with many mannose residues (high-mannose type) at an asparagine residue, then arrangement occurs and eventually the complex type sugar chain is completed (two sialic acids are added). Because *N*-glycosidase F cleaves the bond between the asparagine residue and *N*-acetylglucosamine, SDS-PAGE (sodium dodecyl sulphate - polyacrylamide gel electrophoresis) reveals a native size of ALP (55 kDa) after the digestion. Before the digestion SDS-PAGE reveals some of the processing status, because the high-mannose type (66 kDa) has lower molecular weight compared with the complex type (80 kDa). Some mutations influence processing of sugar moieties [18,19].
6. Localisation of mutation on 3D ALP structure: Mutations were located using a 3D structure of human ALP obtained using a simulation model based on human placental ALP and rat intestinal ALP [20].
7. Co-transfection of WT and each mutant at the ratio of 1:1 was performed to explore if one of the mutated proteins could affect the WT enzyme in a dominant-negative way resulting in an activity reduction of > 50% of the unaffected protein.

3.4. Statistics

Values for ALP activity and mineralization are reported as mean ± standard deviation. ALP activity was assessed on four independent experiments performed in duplicates and mineralization on three independent

Table 2
Clinical, biochemical and radiological characteristics of all subjects.

	Subject 1	Subject 2	Sibling 1	Sibling 2	Sibling 3	Sibling 4
Genetics						
ALPL mutation	c.110T > C c.532T > C	c.715G > T	c.715G > T	c.715G > T	c.715G > T	c.715G > T
Mutation description	Compound heterozygous	Homozygous	Homozygous	Homozygous	Heterozygous	Homozygous
Patient demographics						
Age at diagnosis (years)	Antenatal	4	8	7	5	Birth
Gender	Male	Female	Female	Female	Female	Female
Ethnic origin	Chinese	British Pakistani	British Pakistani	British Pakistani	British Pakistani	British Pakistani
History						
Presentation	Perinatal HPP	Asymptomatic	Asymptomatic	Asymptomatic	Asymptomatic	Asymptomatic
Dental health	NA	Good	Good	Good	Dental caries	NA
Gestation (weeks)	40	40	40	41^{+1}	38	37
Birth weight (Kg)	3.26	3.12	2.62	3.12	3.4	2.32
Examination						
Height/length centile for age	< 0.4th	9–25th	9–25th	50–75th	2nd–4th	2nd–9th
Weight centile for age	< 0.4th	25th–50th	91st–98th	50th	50th–75th	25th–50th
Biochemical profile						
Serum ALP IU/L (NR age adjusted)	< 5 (63–294)	92 (250–1000)	90 (105–315)	62 (105–420)	216 (105–357)	97 (80–330)
Serum adj. calcium (2.2–2.7 mmol/L)	2.62	2.4	2.42	2.31	2.41	2.59
Serum phosphate (0.9–1.8 mmol/L)	2.7	1.78	1.61	1.68	1.90	1.81
25 OH vitamin D (> 50 nmol/L)	45	30.4	49.4	46.6	51.4	40.5
PTH (13–29 ng/L)	16	53	20	32	36	63
Serum PLP (40–100 nmol/L)	240	204	362	193	199	523
Urinary PEA (< 20 μmol/mmol creatinine)	121	49	12	184	17	Not done
Radiographic features						
X-ray knee (Fig. 1)	Extremely undermineralized	No rickets	No rickets	No rickets	No rickets	Not done
Lumbar spine BMAD z score (0 ± 2)	NA	0.4	3.1	1.1	1.3	NA
Lateral vertebral assessment	NA	Normal	Normal	Normal	Normal	Not done

experiments performed in replicates of six. The differences in average ALP activity and mineralization between WT and mutants were determined using Kruskal-Wallis test in SPSS statistical software, version 22.0.

3.5. Ethics

The study was approved by North West-Greater Manchester South Research Ethics Committee (REC ref: 18/NW/0295) and Health Research Authority, United Kingdom. Written informed consent was obtained from parents of participants.

4. Results

4.1. Clinical characteristics

4.1.1. S1

Male infant born to non-consanguineous parents of Chinese origin at term by emergency caesarean section. Skeletal dysplasia was diagnosed antenatally due to small chest and short limbs. However, he tested negative for achondroplasia and thanatophoric dysplasia.

At birth, he had bradycardia and required intubation and ventilation. A small, bell shaped chest and short limbs were noted, as per antenatal scans. Perinatal HPP was suspected following an undetectable ALP and severely undermineralized bones and pulmonary hypoplasia on radiographs (Fig. 1). He also experienced seizures. The diagnosis was confirmed by detecting elevated PLP and urinary PEA (121 μmol/mmol creatinine) concentrations and compound heterozygous genetic mutations c.[110T > C (;) 532T > C], p.[Leu37Pro (;) Tyr178His)] inherited from the mother and father, respectively. Both parents had normal ALP concentrations (IU/L) [mother: 40 (NR 25–105) and father: 44 (NR 21–84)].

4.1.2. S2

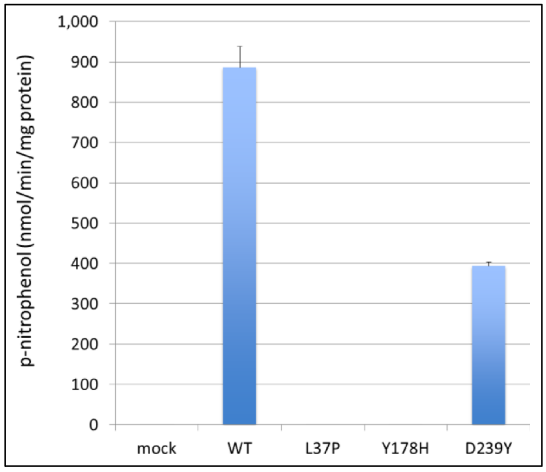
The index case, female aged 4 years, was identified incidentally during a retrospective audit of laboratory data on ALP which we have previously described [13]. Laboratory data at a single tertiary centre was reviewed over an 8-year period to identify children younger than 16 years of age with low ALP activity (< 100 U/L). Study-positive patients (n = 18) were contacted for repeat sampling and those with persistently low ALP (n = 4) had serum PLP and urinary PEA measured. Two individuals, including the asymptomatic child reported here, with elevated serum PLP and urinary PEA had genetically confirmed *ALPL* gene mutations [13]. The girl was asymptomatic at identification with no significant past medical history apart from a slight delay in walking (18 months) with initial waddling gait. Dentition was normal with no history of early tooth loss. She was born to consanguineous (first cousins) parents of Pakistani origin. There was no significant family history. Clinical examination and radiographs (Fig. 1) did not show any signs of HPP. Diagnosis was confirmed by elevated concentrations of serum PLP and urinary PEA (49 μmol/mmol creatinine), followed by genetic confirmation of a homozygous mutation [c.715G > T, p.Asp239Tyr] in exon 7 of the *ALPL* gene (Table 2).

Family investigations identified both parents to be heterozygous for the same mutation with no clinical evidence of disease and normal serum ALP concentrations (IU/L) [mother: 45 (NR 25–105) and father 61 (NR 21–84)]. Three siblings (sibling 1 aged 8 years, sibling 2 aged 7 years and sibling 4 aged 4 months) were homozygous and one sibling (sibling 3 aged 5 years) was heterozygous for the same mutation. To date, all 4 siblings remain clinically asymptomatic. None of the affected individuals have early dental loss. The clinical, biochemical and radiological features of siblings are presented in Table 2.

4.2. Laboratory results

ALP activity was negligible in mutants 1 (average 0.06% of WT) and 2 (average 0.07% of WT) and about 50% of WT in mutant 3 (Fig. 2). The results of individual experiments are presented in a table in Fig. 2. The differences in ALP activity between WT and the mutants were statistically significant ($p < 0.01$).

Mineralization ability in mutant 1 was 7.8%, mutant 2 was 9.3%



	WT	L37P	Y178H	D239Y
Transfection 1-1 (N=2)	1054.22	0.3486	0.5822	405.21
Transfection 1-2 (N=2)	1143.69	0.6972	0.3882	415.20
Transfection 2-1 (N=2)	887.19	0.9497	0.4040	376.98
Transfection 2-2 (N=2)	883.79	0.7597	0.4040	378.31
Transfection 3-1 (N=2)	720.08	0.5464	0.9877	426.82
Transfection 3-2 (N=2)	722.72	0.3643	1.1852	434.65
Transfection 4-1 (N=2)	836.76	0.4975	0.7763	407.69
Transfection 4-2 (N=2)	842.25	0.4975	0.5822	421.05
Average	886.337	0.583	0.664	408.239
Standard deviation	52.310	0.073	0.105	7.486
P value (WT vs mutant)		*P<0.01*	*P<0.01*	*P<0.01*

Fig. 2. Enzyme activity of the three ALP mutant proteins expressed as the enzyme product *p*-nitrophenol production (nmol/min/mg protein) and as a percentage compared to wild type (WT, 100%) and mock (0%). L37P (average 0.06% of WT) and Y178H (average 0.07% of WT) demonstrated negligible ALP activity *in vitro*. D239Y demonstrate 40–50% of WT activity. The table shows results from four independent experiments. Differences in ALP activity between WT and mutants were significant ($p < 0.01$).

and mutant 3 was 15.5% of WT (Fig. 3). The results are presented in the table in Fig. 3 as mean ± SD of all experiments. The results of individual experiments are presented in Supplementary Table 1. The differences in mineralization ability between WT and the mutants were statistically significant ($p < 0.01$).

On western blot analysis, the expression of mutants 1 and 2 was very low and detected only as a faint band at the mature ALP (80 kDa) band. Mutant 3 ALP was expressed as a mature form protein (80 kDa), and the expression level was decreased to about 50% of WT (Fig. 4).

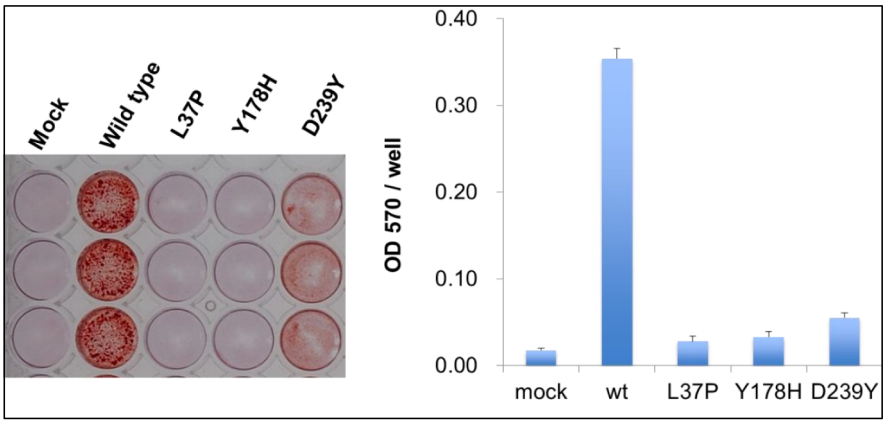
On 3D modelling, mutant 1 was located near the Glycosylphosphatidylinositol (GPI) anchor, mutant 2 at the core structure of the ALP protein and mutant 3 at the periphery of the protein structure (Fig. 5).

Co-transfection did not reveal a dominant negative effect in any of the mutants.

5. Discussion

Here we report enzyme activity and mineralization capability derived from mutations associated with extreme HPP phenotypes and expand the knowledge on genotype-phenotype correlations. S1 had severe perinatal HPP from compound heterozygous mutations c.110T > C and c.532T > C with mutant proteins L37P and Y178H located close to the GPI anchor and the active site, respectively. These mutant proteins demonstrated negligible ALP activity and substantially reduced mineralization ability *in vitro*. In contrast, S2 was asymptomatic despite a homozygous mutation c.715G > T. Our study demonstrates that the respective mutant D239Y was located at the periphery of the ALP structure and associated with about 50% of WT ALP activity and 15.51% of WT mineralization ability *in vitro*.

All three mutations are listed on the *ALPL* gene mutation database [6]. The maternally inherited mutation (c.110T > C) has been reported previously in a homozygous patient with perinatal HPP [6]. The paternally inherited mutation (c.532T > C) has been reported, as a compound heterozygous mutation [c532T > C; c.1559delT], to cause discordant foetal phenotype in two siblings (perinatal lethal and perinatal HPP with mild thoracic hypoplasia and metaphyseal mineralization defect), with 0% ALP enzyme activity *in vitro* [21]. S2, reported by us previously, was asymptomatic and diagnosed incidentally, therefore there is a possibility that c.715G > T may be a variant. However, the subject demonstrates biochemical evidence of HPP and we have previously confirmed the pathogenicity of the mutation by *in silico* analysis [13]. In addition, c.715G > T has not been reported as a variant in any of the known population or sequence databases [22]. The above features and the findings of the *in vitro* studies support the pathogenicity of c.715G > T [22].



	Mock	WT	L37P	Y178H	D239Y
Mean OD570/ well	0.017	0.353	0.027	0.033	0.054
SD	0.001	0.011	0.006	0.006	0.005
% of WT	5.01	100.00	7.86	9.34	15.51
p value (WT vs mutant)			*p<0.01*	*p<0.01*	*p<0.01*

Fig. 3. Mineralization after 5 days of culture. The left panel shows representative Alizarin Red S stained cell cultures for each mutant where only D239Y demonstrated some visible mineralization. The right panel and table express this mineralization in mean ± SD units/mg of the protein concentration expressed as absorbance per well (OD 570/well) of culture plate determined in the cells cultured in parallel. All ALP mutants showed significantly reduced ($p < 0.01$) mineralization compared to WT. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

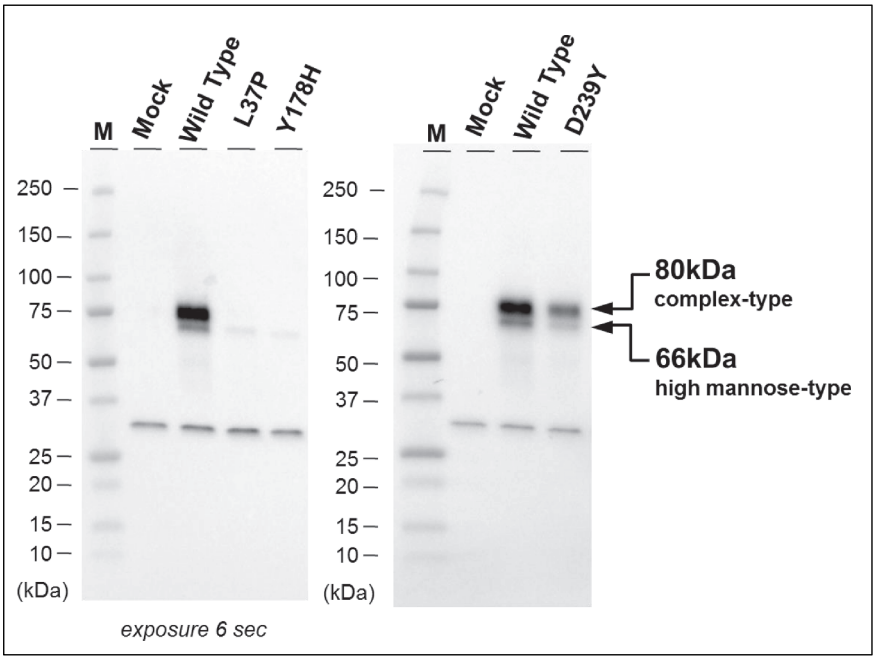


Fig. 4. Western blot analysis demonstrates reduced expression of mutant ALP proteins compared to WT. Mature ALP is expressed at 80KDa and the premature form is expressed at 66Kda. Mutants 1 (L37P) and 2 (Y178H) proteins are hardly detectable suggesting a significant reduction in expression. Mutant 3 (D239Y) is detected as a faint band at 80KDa and 66KDa suggesting reduced expression (approximately 50% of WT).

High phenotypic variability in patients with the same compound heterozygous genotypes has been reported [9,21,23,24] which limits prediction of HPP severity in future affected offsprings. The variability in clinical expression indicates that other genetic, epigenetic or environmental factors may play a role in ALP activity and its mineralization ability [9]. However, in S2 and all affected siblings the phenotype remains asymptomatic and demonstrates normal dentition which probably indicates that the c.715G > T homozygous mutation demonstrates no intrafamilial variability.

The laboratory markers (serum ALP and PLP) in S1 and S2 were correlated to the clinical severity (Table 2), as reported in the literature [25]. Sibling 3 of S2 had normal serum ALP, in line with the heterozygous carrier status. DXA scans did not reveal a low bone mineral density.

The ALP enzymatic activities of the mutants were in proportion to the laboratory ALP values of the patients. Negligible ALP activity in mutants 1 and 2 resulted in reduced *in vitro* mineralization, in keeping with the severe undermineralization seen on radiographs in S1. In mutant 3 the ALP activity was approximately 50% of WT and *in vitro* mineralization was approximately twice as much as that of mutant 1 or 2 but still substantially lower than WT. In mutant 3 the residual ALP activity was comparable with those reported for mutations associated

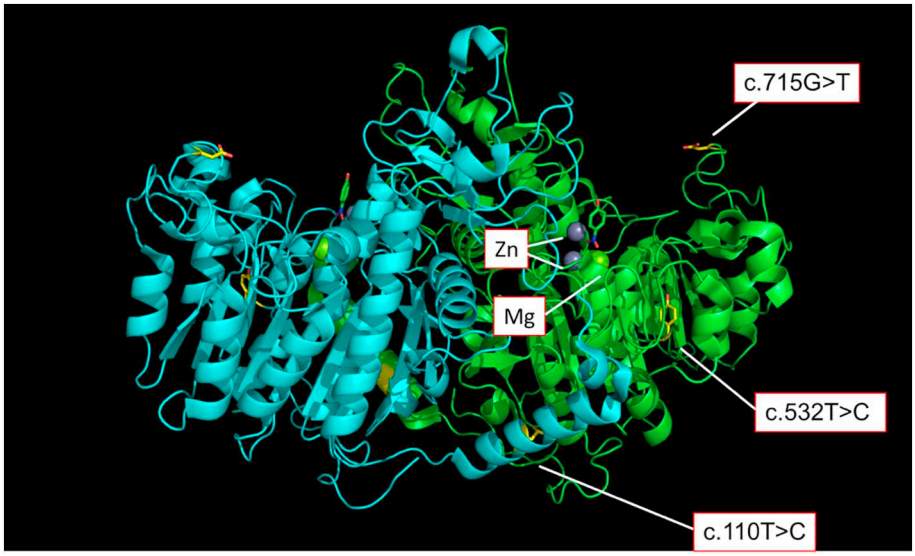


Fig. 5. A 3 dimensional (3D) human ALP structure obtained using a simulation model based on the human placental ALP. The C.110T > C (L37P) mutation is located near the Glycosylphosphatidylinositol anchor and c.532T > C (Y178H) at the core structure of the ALP protein. The C.715G > T (D239Y) mutation is located at the periphery.

with adult HPP [6]. This leads us to speculate that mineralization may be slow but near-sufficient *in vivo*. Despite the reduced ALP activity and mineralization there are no radiological features of HPP to date in S2 (at 4 years of follow up) or siblings. Whether they are likely to develop adult onset HPP or whether enzyme activity and mineralization ability *in vivo* are greater than *in vitro* remains unknown.

The human functional ALP enzyme is assumed to exist as a homodimer [20,26]. The molecular weight of each monomer is ~80 kDa and is linked to the outer membrane of the cells *via* a GPI anchor [26]. After the ALP peptide is synthesized as a native protein with a molecular weight of 66 kDa, carbohydrate chains are added as N-linked sugar chains in the endoplasmic reticulum, and the modified protein is then processed in the Golgi apparatus and is eventually localized on the outer membrane *via* a GPI anchor [20]. To express enzymatic activity, ALP needs two Zn^{2+}, one Mg^{2+}, and one Ca^{2+} ion as cofactors [26].

Mutations located in the active site and its vicinity, the homodimer interface, the crown domain, and the calcium-binding domain result in severe phenotypes, whereas mutations in the active site valley result in less severe phenotype [11,12,20]. The close proximity of mutation 1 to the GPI anchoring site may render the protein unstable preventing anchoring to the cell membrane. Mutation 2 was located close to the core structure of the ALP protein, suggesting the protein structure to be unstable. Hence the severe phenotypes and low enzyme function associated with mutations 1 and 2. In contrast, mutation 3 was located at the periphery of the protein structure and we speculate that this does not affect the stability of the protein and therefore the function is not significantly affected, hence the asymptomatic phenotype.

There are however certain limitations inherent in the functional tests of ALP activity used in this study. Although prediction of the degree of clinical severity of new mutations could be very useful, testing each mutation by *in vitro* functional tests is laborious and time consuming. Moreover, other factors, genetic and epigenetic, environmental or acquired, and polymorphism may also affect mineralization ability *in vivo*, thus limiting accurate prediction from *in vitro* models.

6. Conclusions

The study adds new evidence to the literature on genotype-phenotype correlations in HPP and expands the knowledge on the functional effect of individual mutations from children with extreme phenotypes. Our findings stress the importance of the location of the mutation in the large human ALP enzyme structure. In contrast to the high intrafamilial variability reported with compound heterozygous mutations, the homozygous mutations showed no variability enabling prediction of phenotype in future offsprings and genetic counselling.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2019.05.031.

Declaration of Competing Interest

Prof Högler has been an investigator in Alexion-sponsored clinical trials, is a board member of the global HPP registry and has received honoraria and consulting fees from Alexion. Other authors have no COI to declare.

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Credit author statement

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