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Article

Screening for Hypophosphatasia in Adult Patients at a Maximum Care Provider—Retrospective Analyses over Fifteen Years

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Abstract: **Introduction:** Persistent hypophosphatasia (pHPE) can be an expression of hypophosphatasia (HPP), a rare disorder of bone metabolism resulting from *ALPL* gene pathogenic variants. The aim of this study was to identify the role of auxiliary general biomarkers to verify low ALP serum activity as an alert parameter for an *ALPL* gene pathogenic variants. **Material:** A retrospective analysis of adult patients with at least one reduced ALP value of less than 21 U/L was performed. 88 patients had a temporary HPE (tHPE group). 12 out of 20 patients with persistent HPE agreed to a genetic analysis. 11 patients had a pathogenic *ALPL* gene variant (*ALPL* group). Hemoglobin [HB], alanine aminotransferase [ALT], aspartate aminotransferase [AST], gamma-glutamyl transferase [GGT], calcium, phosphate, thyrotropin [TSH], albumin, total protein, and C-reactive protein [CRP] levels) represented basic biomarkers. A statistical comparison of general biomarkers was performed between both groups. **Results:** The mean HB, ALP, AST, calcium, albumin, and total protein levels were lower in the tHPE group ($p < 0.01$), and CRP was increased in the tHPE group ($p < 0.01$). **Conclusion:** HB, AST, calcium, albumin, total protein, and CRP can be used as auxiliary general biomarkers to verify low ALP activities with respect to detecting pathogenic *ALPL* gene variants and possible HPP. A combination of low ALP and general biomarkers within their physiological range was found to be an alert constellation for pathogenic *ALPL* gene variants and possible clinically manifested HPP, and the clinical unit should be informed.

Keywords: alkaline phosphatase; hypophosphatasia; hypophosphatasemia; *ALPL* gene; mutation

Introduction

Hypophosphatasia is a rare inherited disorder characterized by defective bone mineralization and a deficiency of tissue-nonspecific alkaline phosphatase (TNSALP) activity [1–5]. The diagnosis of hypophosphatasia can be challenging due to its unspecific clinical presentation, with musculoskeletal pain being a common symptom in adult patients [6–9]. Low serum alkaline phosphatase activity is often the first indicator leading to a suspicion of hypophosphatasia [10]. Adult patients with hypophosphatasia require comprehensive management that includes assessing bone and joint complications, chronic pain, and mood disorders [11,12]. Diagnostic delays are common in hypophosphatasia, emphasizing the need for increased awareness and timely identification of the

condition [13]. Atypical femoral fractures have been reported in adult patients with hypophosphatasia, highlighting the importance of considering this condition in cases of unusual fractures, especially during bisphosphonate exposure [14,15]. Furthermore, the use of asfotase alfa has shown success in treating adult patients with childhood-onset hypophosphatasia, indicating the potential for enzyme replacement therapy in managing this condition [16–18]. In this paper, we present a retrospective analysis spanning fifteen years focusing on screening for hypophosphatasia in adult patients at a maximum care provider. By examining the clinical, biochemical, and genetic spectrum of hypophosphatasia in adults, we aim to contribute to the understanding and management of this rare metabolic disorder. In particular, efforts will be made to identify the challenges of laboratory diagnostic criteria using broad-available laboratory parameters.

Materials and Methods

Patients and Study Groups

This retrospective study was approved by the local ethics committee (no. 508/16-ek). All laboratory data of adult patients (older than 18 years) of the University Hospital Leipzig (UHL) for the years 2000–2015 were retrospectively analysed to identify patients with ALP serum activity ≤ 21 U/L. The study cut-off of 21 U/L was based on reports from adult patients with confirmed clinical manifestations of severe HPP [14]. Hemoglobin (HB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), calcium, phosphate, thyrotropin (TSH), albumin, total protein and C-reactive protein (CRP) levels were defined as general biomarkers and measured at baseline and at follow-up.

All ALP levels available at the UHL were analysed. The reference range was 35–105 U/L for healthy women and 40–130 U/L for healthy men [19,20]. 204 patients with at least one measured ALP serum activity < 21 U/L were selected [18]. In cases of multiple ALP serum activities, the lowest was used as the inclusion value. After analysis of the medical history, 134 (66%) of the 204 patients had physiological ALP levels as defined by tHPE. 46 of them had no measurement of other common biomarkers and were therefore excluded. In 88 of them, at least one of the general biomarkers was measured simultaneously. They were included in the **temporary hypophosphatasemia group** (N = 88, **tHPE group**).

Twenty of the remaining 70 patients with only decreased ALP levels were contacted and agreed to participate in a follow-up examination, including a medical history and a follow-up laboratory test (flowchart). Eighteen of these showed a decrease in ALP at follow-up and have been further investigated. Two had ALP levels within reference ranges at follow-up and were excluded.

Methods

Blood samples were taken between 8:00 and 10:00 am after a 12-hour fast. Two S-Monovettes® 2.7 ml K3EDTA and one S-Monovette® 4.7 ml serum (Sarstedt AG & Co. KG, Postfach 1220, D-51588 Nümbrecht) were used. All biomarkers were measured at the Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics (ILM), UHL, using commercially certified biochemical assays. The determination of ALP activity in serum was performed using the “Alkaline Phosphatase according to IFCC Gen.2 ALP2” kit (Roche Diagnostics Deutschland GmbH) on a cobas® c501 instrument using the release of p-nitrophenol after cleavage of p-nitrophenyl phosphate by the enzyme. The product can be quantified by extinction.

PLP levels were measured using the Vitamin B6 in Serum/Plasma HPLC kit (Chromsystems Instruments & Chemical, Gräfelfing, Germany). After protein precipitation, vitamin B6 was derivatised and separated by HPLC. Quantitation was performed by fluorescence (415 nm).

The Ostase® BAP EIA assay (Immunodiagnostic Systems Holdings, UK) was used to quantify BALP. BALP was purified by immunosorbent assay (EIA) using biotin-linked monoclonal antibodies and quantified using the same enzyme assay described for ALP.

The genetic analysis was performed at the Institute of Human Genetics at the UHL by targeted enrichment of the gene target regions of the human exome plus adjacent intronic regions (+/- 10 bp)

using the TruSight®One enrichment kit (Illumina Inc., San Diego, California, USA). More than 95% of the target regions were covered at least twenty-fold. Sequencing was performed on a NextSeq550 sequencing instrument (Illumina Inc., San Diego, California, USA) using 150 base pair paired-end reads. Varvis software (Limbus Medical Technologies GmbH, Rostock, Germany) was used for bioinformatic analysis.

Statistics

Statistical analysis was performed using SPSS (version 24, IBM) for Windows (Microsoft). Independent samples with normal distribution were calculated using the two-tailed t-test, and frequency differences were calculated using the chi-squared test.

Results

Comparison of tHPE-Group and ALPL-Group

Within and between the tHPE and ALPL groups, the sex and age distributions were statistically comparable (**Table 1**; $p > 0.05$). The frequencies of pathological findings for the general biomarkers were equally distributed between the sexes in both the tHPE and ALPL groups ($p > 0.05$). Therefore, sex-specific analysis was not performed. Pathological values for HB, ALT, AST, creatine, calcium, albumin, total protein and CRP occurred significantly more frequent in the tHPE group than in the ALPL group ($p \leq 0.05$; **Table 2**). The mean levels of HB, ALP, AST, calcium, albumin and total protein were lower in the tHPE group than in the ALPL group ($p < 0.05$; **Table 2**). However, analysis of serum concentrations of CRP showed significantly higher values in the tHPE group ($p \leq 0.05$). No significant differences were found between the tHPE and ALPL groups for creatinine, GGT and TSH levels ($p > 0.05$; **Table 2**).

Table 1. Descriptive statistics and overview of diagnoses at the time of inclusion ALP values (lowest detected ALP value), ALPL alkaline phosphatase gene, tHPE temporary hypophosphatasemia, (1) chi-test 1. 00, (2) t-test $p=0.286$; (3) t-test $p=0.297$; (4) U-test $p=0.931$; *=9.1; CCI Craniocerebral Injury, ICH Intracranial Haemorrhage.

	tHPE-group (N=88)	ALPL-group (N=11)
Sex distribution ⁽¹⁾	female 46 : male 42	female 6 : male 5
mean age		
all ⁽²⁾	57.0 (SD 19.7)	51.6 (SD 14.7)
female	54.9 (SD 20.4) ⁽³⁾	54.2 (SD 15.3) ⁽⁴⁾
male	59.3 (SD 19.0) ⁽³⁾	48.4 (SD 15.0) ⁽⁴⁾
main diagnosis at the time of determination of the inclusion ALP	N (%)	N (%)
blood loss anaemia		
gastrointestinal haemorrhage	2 2.3	2 18.2
traumatic acute haemorrhage	12 13.6	

postoperative haemorrhage	9	10.1		
cancer-induced chronic anaemia	3	3.4		
acute myocardial ischemia	2	2.3		
neurological/neurosurgical diseases				
CCI without ICH	3	3.4		
CCI with ICH	1	1.1		
epileptic incident	2	2.3		
oligoastrocytoma	1	1.1		
cerebral stroke	4	4.5	1	*
optic nerve sheath meningioma			1	*
multiple system atrophy cerebellar variant	1	1.1		
systemic infections				
sepsis caused by various factors	9	9.1		
pneumonia	4	4.5		
gastrointestinal diseases				
liver insufficiency	9	10.3	1	*
acute abdomen by various diseases	5	5.4		
acute biliary pancreatitis	1	1.1		
colitis ulcerosa			1	*
haematological diseases				
multiple myeloma	6	6.5		
leukemia (several forms)	6	6.5		
non-Hodgkin's lymphoma	2	2.3		
drug-induced agranulocytosis (cytostatica)	2	2.3		
rheumatological disease				
systemic lupus erythematosus	2	2.3		
(plasmapheresis)				
psoriatic arthritis			2	18.2
arthralgias with unclear cause				

ovarian hyperstimulation syndrome	1	1.1	
chorioretinitis	1	1.1	
acute renal failure			1 *
thoracal vertebral body fracture			1 *
dermatitis herpetiformis (Duhring)			1 *

Table 2. Differences of common biomarkers between patients with pathogenic variants in the ALPL gene (ALPL group) and patients with temporary hypophosphatasemia (tHPE group).

	group	N	mean	SD	N (%) within reference range	p
alkaline phosphatase (U/L)	ALPL	11	22.57	6.71	11 (100%)	0.005
	tHPE	88	15.25	5.49	88 (100%)	
aspartate amino- transferase (µkat/L)	ALPL	10	0.52	0.35	9 (90%)	0.004
	tHPE	71	1.85	3.68		
gamma-glutamyl transferase (µkat/L)	ALAP	10	1.60	2.78	7 (70%)	0.231
	tHPE	69	0.93	1.43	42 (61%)	
haemoglobin (mmol/L)	ALPL	11	8.46	0.85	10 (90%)	0.000
	tHPE	66	5.84	2.16	60 (91%)	
creatinine (µmol/L)	ALPL	9	70.33	36.52	8 (89%)	0.081
	tHPE	82	101.83	113.58	71 (86%)	
calcium (mmol/L)	ALPL	9	2.41	0.04	9 (100%)	0.001
	tHPE	62	1.73	0.61	49 (62%)	
thyrotropin (mU/L)	ALPL	10	1.72	1.00	9 (90%)	0.159
	tHPE	34	1.17	1.23	29 (85%)	
C-reactive protein (mg/L)	ALPL	11	1.47	1.60	11 (100%)	0.039
	tHPE	66	39.27	59.50	59 (89%)	
albumin (g/L)	ALPL	11	46.05	3.06	11 (100%)	0.000

	tHPE	51	22.17	13.48	50 (98%)	
total protein (g/L)	ALPL	11	72.05	3.28	11 (100%)	0.000
	tHPE	56	43.66	22.13	54 (96%)	

In contrast to the ALPL group, all patients in the tHPE group had known additional diseases independent of HPP that are typical of tHPE, such as severe anaemia, sepsis, and gastrointestinal, haematological, and rheumatological diseases (**Table 1**).

Patients of the ALPL-Group

All patients in the ALPL group (N = 11) exhibited a heterozygous pathological variant in the ALPL gene. Eight distinct pathological variants were identified, each associated with pathological values in the HPP-specific laboratory. Patient characteristics are detailed in Table 3. Furthermore, all patients in the ALPL group exhibited clinical symptoms consistent with suspected HPP (100% recurrent musculoskeletal complaints, 27% premature caries, and a 9% prevalence of premature loss of primary teeth). At the start of the study, none of the patients in the ALPL group had a known history of HPP or permanent ALP reduction.

Table 3. Overview of the pathological gene variants detected in the ALPL gene, along with the corresponding measured follow-up AP, BAP, and PLP serum levels observed in patients with hypophosphatasia (HPP).

gene	frequency	gender	Follow-up-AP		BAP		PLP-level (reverence value 35–110 nmol/L) nmol/L
			µkat/L	% of the lower reference value	reduced	value µg/L	
c.119C>T; p.(Ala40Val) Exon 3	1	M	0,41	61,2	Ja	4,7	141
c.1250A>G; p.(Asn417Ser) Exon 11	4	W	0,25	43,1	ja	3,1	144
		M	0,23	34,2	ja	4,3	241
		W	0,32	55,2	ja	4,5	198
		M	0,43	64,2	nein	6,7	308
c.1171del; p.(ARG391Valfs*12) Exon 10	1	M	0,34	50,7	Ja	4,6	112
c.1331A>G; p.(Gln444ARG) Exon 12	1	M	0,26	38,8	Ja	3,0	258
c.406C>T; p.(ARG136Cys) Exon 5	1	W	0,45	77,6	nein	5,9	100

c.341C>T; p.(Ala114Val) Exon 5	1	W	0,52	89,7	nein	5,4	128
c.1190-2A>T	1	W	0,56	96,6	Ja	3,8	366
c.297G>A ; p.(=)	1	W	0,31	53,5	Ja	4,0	177

In the ALPL group, HB, ALT, calcium, albumin, total protein and CRP were within reference ranges in all follow-up laboratory data. Three patients (27%) had elevated GGT levels, two (18%) had elevated phosphate levels, one (9%) had decreased phosphate levels, one (9%) had elevated TSH or AST levels and one (9%) had elevated creatinine levels at follow-up. All patients in this group had decreased BALP levels and/or increased PLP levels.

Discussion

A persistent reduction in alkaline phosphatase (ALP) activity level is a hallmark feature of hypophosphatasia (HPP) [12]. This is a crucial factor in differentiating HPP from other diseases, such as rickets [19]. In clinical practice, it is essential to pay close attention to borderline or marginally reduced ALP values, as these may be indicative of hypophosphatasia and could otherwise be overlooked [9].

The causes of low ALP are numerous and vary according to the temporality of hypophosphatasemia (tHPE) [3,8]. The prevalence of hypophosphatasemia of any cause is reported to be 1.18% to 8.46% [8,20]. In the authors opinion, first, it is essential that the assessment of a reduced ALP level takes into account other serum parameters that could represent differential diagnoses. This study shows that HB, AST, calcium, albumin, total protein and CRP were significantly more often in the physiological range in patients carrying a heterozygous ALPL pathogenic variant than in patients with tHPE. Thus, if the serum activity of ALP is decreased and these other values are within the reference range, a pathogenic variant in the ALPL gene is highly suspected in these cases, which may be a sign of possible HPP. On the other hand, if serum ALP activity is decreased and the other values are in the pathological range, a differential diagnosis other than HPP should be considered. The results presented here are consistent with the laboratory and clinical findings reported by Larid et al. and represent a valuable contribution to this existing body of knowledge [8,12].

To our knowledge, this is the first study to present general biomarkers that can be used for the first-line assessment of reduced ALP activity in relation to possible HPP-causing ALPL gene pathogenic variants in adults.

Differential Diagnosis of HPE

The results of the present study align with those of previous research, which suggests that a single reduction in ALP activity does not necessarily indicate the presence of hypophosphatemia (HPP). The proportion of patients with transient hypophosphatasemia (tHPE) was shown to be between 66.7% and 72.4% which is consistent with the demonstrated 66% [8,21,22]. There are numerous reasons for the transient HPE study conducted by Lum et al. (1995) revealed that in 47% of patients with tHPE, other medical conditions accounted for the observed decline in ALP values [8,23]. These included cardiac surgery and cardiopulmonary bypass (26.5%), malnutrition (12.0%), magnesium deficiency (4.8%), hypothyroidism (2.4%) and severe anemia (1.2%). A total of 53% of cases exhibited no identifiable cause for tHPE. In contrast to the present study, no patients in the study by Lum et al. exhibited clinically overt HPP [23]. McKiernan et al. (2014) identified numerous conditions that may potentially cause tHPE, including anaemia, major surgery, multisystem failure, tumours, sepsis, disseminated intravascular coagulopathy, gastrointestinal bleeding, severe caloric restriction, and blood transfusion [3]. It is important to note that these non-physiological conditions

are usually reflected by the biomarkers investigated in the present study, as detailed in Table 2. Therefore, it is essential to consider these factors when assessing reduced ALP levels [3].

Furthermore, McKiernan et al. (2014) showed that tHPE is associated with a more severe reduction in ALP to 10 U/L and increased mortality compared to patients with HPP [3]. According to recent literature, mean ALP levels in adult patients with confirmed HPP ranged from 27.8 U/L to 28.5 U/L, which is comparable to the mean ALP level of 22 U/L in the ALPL group studied [12]. However, it should be noted that a massively reduced ALP may also indicate a homozygous form of HPP or a childhood form diagnosed in adulthood [7]. It has also been suggested that ALP reduction may occur in up to 43% of patients receiving bisphosphonate therapy [24]. Therefore, the significantly lower ALP levels in the tHPE group are consistent with literature data and do not automatically imply HPP.

Offered Diagnostic Approach for a Suspected ALPL Gene Mutation

In the case of persistently low ALP values, HPP-specific diagnostics with BALP, PLP and PEA are usually recommended [18]. In summary, HPP-specific diagnostics are expensive, time-consuming, and not available universally. Considering the presented results, it can be argued that the reduction in alkaline phosphatase activity should be assessed in conjunction with a number of additional biomarkers. Of particular interest here are biomarkers that enable a differential diagnosis to be made. These include the levels of haemoglobin, aspartate aminotransferase (AST), calcium, albumin and total serum protein, as well as the levels of C-reactive protein (CRP).

First, in patients where a reduced alkaline phosphatase (ALP) has been identified alongside other general parameters within the reference range, a specific hypophosphatasia (HPP) diagnosis can be recommended. Second, in patients with one or more general parameters in the pathological range in addition to reduced ALP, further investigation of the treatable differential diagnoses (Table 1) is strongly recommended. Following the targeted treatment of the differential diagnoses, a re-measurement of the biomarkers is recommended. In the event, that the general biomarkers normalize concurrently with persistently low ALP levels, a pathogenic variant of the ALPL gene remains a probable explanation. In both situations, it is recommended that an extended HPP-specific diagnostic work-up be performed, including BALP and PLP levels [25]. A diagnosis of HPP can be made in the presence of additional clinical symptoms that are characteristic of the HPP. In rare cases, a pathogenic variant of the ALPL gene can coexist with pathological values of the general biomarkers presented. In such cases, genetic analysis is recommended according to the literature. However, if the patient presents with clinical and laboratory findings consistent with a diagnosis of HPP, it is not recommended that genetic analysis be conducted as a routine procedure [5,26,27].

Limitations of the Study

It should be noted that this study is not without limitations. Primarily, the retrospective design of the study may have introduced discrepancies in the number of observations for the various parameters, potentially affecting the validity of our conclusions. The precise number of patients who had their alkaline phosphatase levels measured at the UHL during the specified period could not be ascertained. During the inclusion period, software modifications were implemented, creating challenges in accessing historical data. The present study identified 10,464 cases in which at least one ALP determination was performed. Of these, 217 exhibited an ALP serum activity level of ≤ 21 U/L (0.2%). This information would have been interesting to estimate the frequency of low levels and how often they were ignored. Another drawback, despite the significant differences, was the number of patients who agreed to be followed up. While this was still relatively high in the tHPT group (88), only 11 patients in the ALPL group were available for complete data collection. Furthermore, the selected screening cut-off of 21 U/L has the potential to result in an underdiagnosis of patients exhibiting milder reductions in ALP activity due to a pathogenic variant of the ALPL gene. The results suggest that the prevalence of moderate forms of genetic HPP in adults is likely to be higher than previously assumed, highlighting the necessity for greater attention to be paid to the value of ALP [8].

It should be noted that the presented algorithm cannot be applied unreservedly to forms of HPP resulting from homozygous or compound heterozygous ALPL variants. The patients presented with an oligosymptomatic mild form of HPP in the presence of a heterozygous mutation, a finding that is consistent with data in the literature [26,28,29]. Given the genetic heterogeneity of the disease, a genetically based nosology of HPP, as suggested by Mornet et al., may be a useful approach [26]. Nevertheless, the clinical relevance of such an entity also remains to be seen in the context of patient-specific, individualized therapy, which should be the subject of further investigation. The validity of the presented algorithm for this purpose must be corroborated by prospective studies, ideally including a comprehensive genetic diagnosis for tHPE.

Conclusion

The results of this unicentric retrospective study indicate that oligosymptomatic HPP remains undiagnosed in adults. A heterozygous pathogenic ALPL gene variant is associated with persistently low ALP activity and mostly reference-value routine biomarkers (HB, AST, calcium, albumin, total protein and CRP). Persistently low ALP activity, particularly in the context of unexplained musculoskeletal pain, should raise suspicion of hypophosphatasia and prompt further diagnostic investigations.

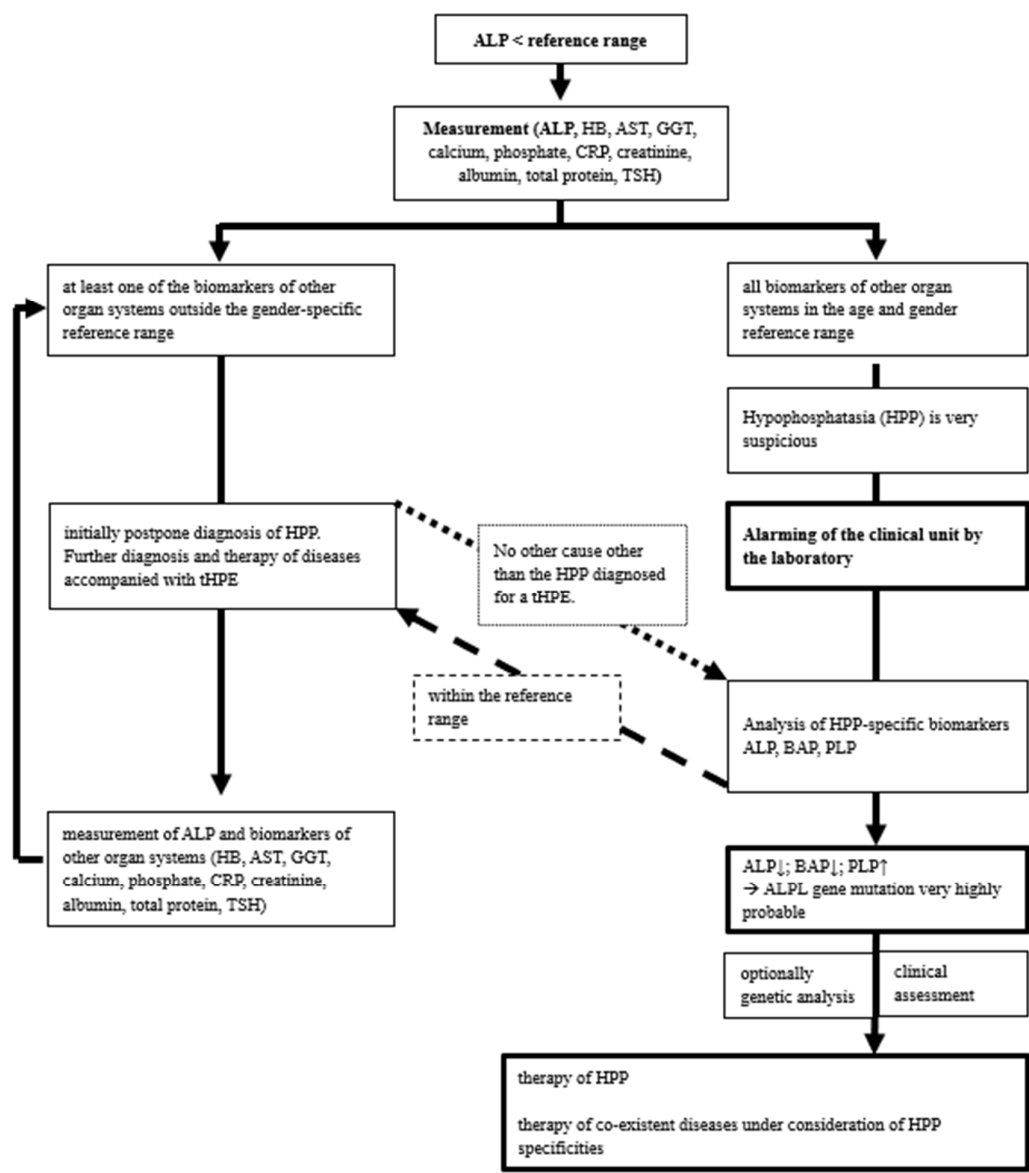
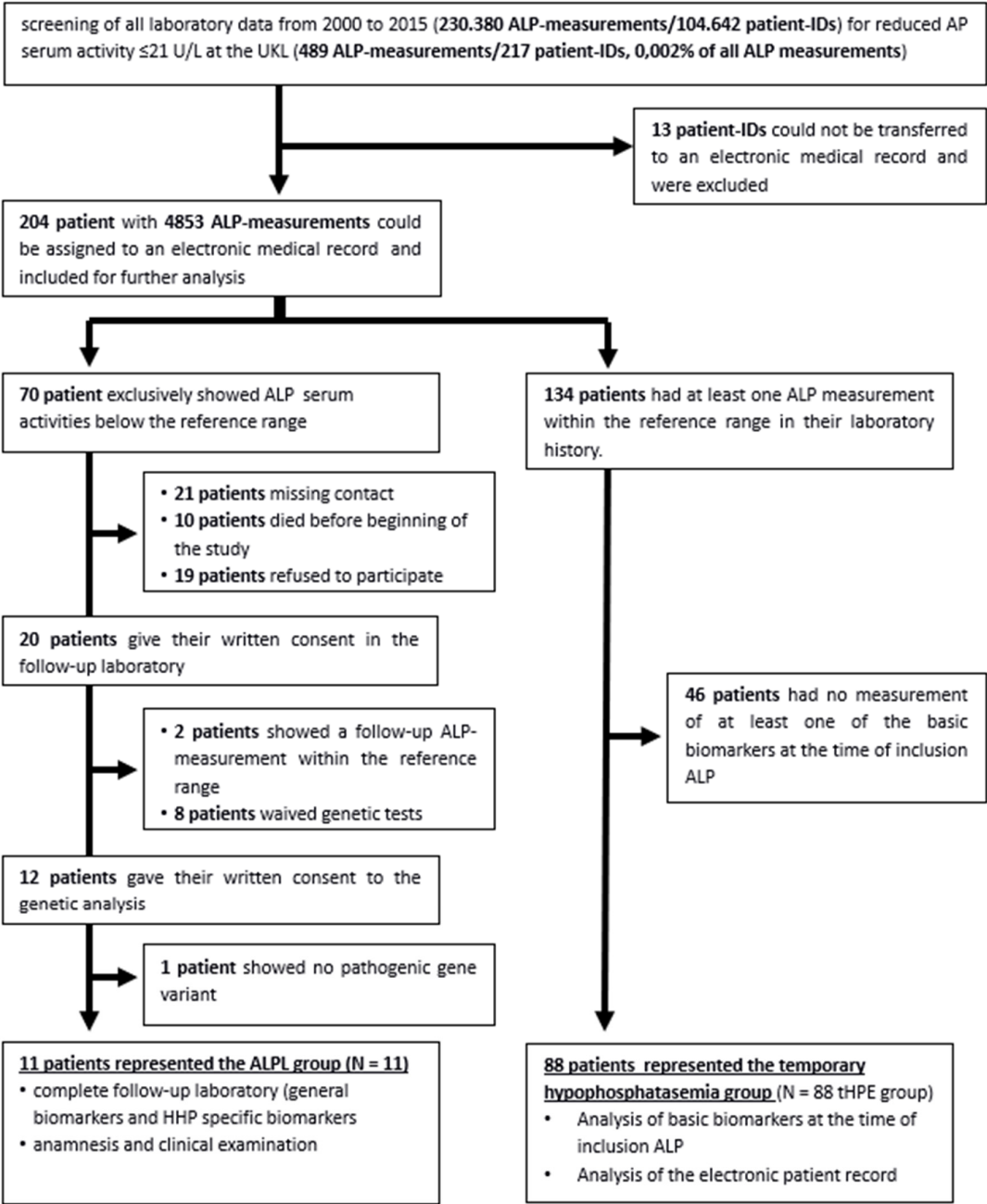


Figure 1. Overview of our recommended laboratory algorithm for the evaluation of a single low serum alkaline phosphatase (ALP) activity (hypophosphatasemia, HPE) in the setting of a pathogenic variant of the ALPL gene and possible differential diagnosis of temporary hypophosphatasemia (tHPE).



Flowchart: Flow chart of the assessment of the patients and the resulting study groups, UHL = University Hospital of Leipzig.

Author Contributions: Robert Hennings, MD contributed significantly to the idea, planning and data collection of this paper. Furthermore, he contributed significantly to the preparation of the manuscript, literature research, analysis and interpretation of the data. Johannes (Lemke JR) Lemke and Diana-Gabriela (Le Duc D) Le Duc made a significant contribution to the genetic analysis of the present work. Joachim (Thiery J) Thiery and Anke (Tönjes A) Tönjes contributed to the realisation and final proof of the manuscript. Jürgen (Kratzsch J) Kratzsch*; Prof. Dr. rer. nat. contributed significantly to the idea, planning and data collection of this paper. Furthermore, he

contributed significantly to the preparation of the manuscript and final proof of the manuscript. Andreas (Roth A) Roth*; Prof. Dr. med. contributed significantly to the idea and planning of this study. Furthermore, he contributed significantly to the preparation of the manuscript, analysis and interpretation of the data as well as final proof of the manuscript. * Both authors contributed equally to this work and should be considered equal senior authors in sense of shared senior authorship.

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