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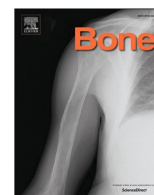

Full Length Article

Urine phosphoethanolamine is a specific biomarker for hypophosphatasia in adults

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ABSTRACT

Objectives: We investigated the utility of urine phosphoethanolamine (PEA) as a marker to aid in diagnosing and/or confirming hypophosphatasia (HPP) in adults and for monitoring patients on enzyme replacement therapy (ERT).

Methods: Data was collected from seventy-eight adults who were referred to the Vanderbilt Program for Metabolic Bone Disease for evaluation of a possible or confirmatory HPP diagnosis between July 2014 through December 2019. Fifty-nine patients were diagnosed with HPP and nineteen were excluded from a diagnosis of HPP. The urine PEA results of those patients with a confirmed diagnosis of HPP and those patients with a diagnosis of HPP excluded were captured and compared to other laboratory and clinical parameters consistent with HPP, including alkaline phosphatase (ALP) activity, plasma pyridoxal 5′-phosphate (PLP), the presence of musculoskeletal abnormalities, and genetic testing for pathogenic mutations in *ALPL*.

Results: Initial urine PEA values in patients in our HPP cohort and not on ERT were significantly higher (median = 150.0 nmol/mg creatinine, IQR = 82.0–202.0) compared patients in our HPP negative group (median 18.0 nmol/mg creatinine, IQR = 14.0–30.0, $p < 0.0001$) and higher than patients on ERT (median 65.0 nmol/mg creatinine, IQR = 45.3–79.8). Patients who began ERT had a decline in urine PEA levels after treatment with a mean decrease of 68.1 %. Plasma ALP levels were significantly lower in the group of patients with HPP and not on ERT group (median = 24.0 U/L, IQR = 15.0–29.50) compared to the patients without HPP (median = 45.50 U/L, IQR = 34.0–62.0;) and plasma PLP levels were significantly higher in the HPP non-ERT group (median = 284.0 nmol/L, IQR = 141.0–469.4) compared to the patients without HPP (median = 97.5 nmol/L, IQR = 43.7–206.0;). The area under the curve (AUC) of urine PEA, ALP, and PLP to distinguish between HPP and non-HPP patients is 0.968, 0.927 and 0.781, respectively, in our cohort. Urine PEA had 100 % specificity (95 % CI of 83.2 % to 100.0 %) for diagnosing HPP at a value >53.50 nmol/mg creatinine with a sensitivity of 88.4 %; 95% CI 75.5 to 94.9 %. ALP had a 100 % specificity (95 % CI of 82.4 % to 100.0 %) for diagnosing HPP at a value <30.5 U/L with a sensitivity of 77.2 %; (95%CI 64.8 to 86.2 %). PLP had a 100 % specificity (95 % CI of 81.6 % to 100.0 %) for diagnosing HPP at a value >436 nmol/L with a sensitivity of 26.9 %; (95%CI 16.8 to 40.3 %). The most common pathogenic or likely pathogenic mutations in our cohort were c.1250A>G (p.Asn417Ser), c.1133A>T (p.Asp378Val), c.881A>C (p.Asp294Ala), c.1171C>T (p.Arg391Cys), and c.571G>A, (p.Glu191Lys).

Conclusions: Urine PEA is a promising diagnostic and confirmatory marker for HPP in patients undergoing investigation for HPP. Urine PEA also has potential use as a marker to monitor ERT compliance. Future studies are necessary to evaluate the association between PEA levels and clinical outcomes.

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Table 1
Patient characteristics.

	HPP negative		HPP group	
	N (% of group)	Median age (range)	N (% of group)	Median age (range)
Total	19 (100)	47 (20 to 68)	59 (100)	46 (19 to 81)
Female	14 (73.7)	46.5 (20 to 68)	45 (76.2)	49 (19 to 81)
Male	5 (26.3)	60 (25 to 65)	14 (23.7)	38 (25 to 72)
Race and ethnicity				
Non-Hispanic Black or AA	1 (5.3)		0	
Unknown	4 (21.1)		7 (11.9)	
Non-Hispanic White	12 (63.2)		51 (86.4)	
Hispanic White	1 (5.3)		1 (1.7)	
Unknown White	1 (5.3)		0	
Signs and symptoms				
Significant bone problems[a]	12 (66.2)		50 (84.7)	
Significant dental problems	10 (52.6)		46 (80.0)	
Significant muscle problems[b]	10 (52.6)		41 (69.5)	
Fatigue	9 (47.4)		43 (72.9)	
Family history of bone disease	9 (47.4)		46 (78.0)	
Molecular characteristics				
Genetic testing performed	15 (78.9)		56 (94.9)	
Pathogenic or likely pathogenic mutation	0 (0.0)		49 (83.1)	
Variant of unknown significance (VUS)	0 (0.0)		6 (10.2)	
Conflicting interpretations	0 (0.0)		1 (1.7)	
No mutation detected	15(78.9)		0	

Demographics of HPP negative and HPP positive groups shown as well as their reported signs and symptoms. Absolute values are given in front and the percent of the group (either HPP negative or positive) are reported in parentheses.

[a] Bone problems include osteoporosis, osteopenia, fractures, and deformities.

[b] Muscle problems include muscle weakness and/or muscle pain.

1. Background

Hypophosphatasia (HPP) is a rare, inherited metabolic bone disorder caused by loss-of-function mutations in the *ALPL* gene that encodes the tissue-nonspecific alkaline phosphatase (TNSALP), an isoenzyme critical for the mineralization of bone and teeth [1,2]. There are currently >400 known pathogenic mutations [3]. Deficiency of TNSALP results in impaired mineralization of the bones and/or teeth and other multi-systemic complications. Inheritance may be autosomal dominant or recessive, and multiple modes of inheritance may be demonstrated in individual families [4–7]. HPP is most severe when it presents in the perinatal and infantile period and may be lethal; however, significant disease burden may exist at any age, and disease presentation spans a large phenotypic spectrum [8,9]. This phenotypic spectrum, including age of onset, varies widely which can make appropriate and timely diagnoses a challenge, especially those with late onset or milder forms of the disease.

The underlying molecular and biochemical basis of HPP is due to the decreased activity of TNSALP and the accumulation of TNSALP substrates, inorganic pyrophosphate (PPi), pyridoxal 5′-phosphate (PLP), which is the major circulating form of vitamin B6, and urine phosphoethanolamine (PEA) [2,5,10,11]. The reduction in the TNSALP-mediated dephosphorylation of PPi to inorganic phosphate (P_i) leads to skeletal abnormalities and bone softening as the accumulation of PPi and the decreased concentration of P_i inhibits the development of hydroxyapatite crystals needed for bone mineralization [4]. TNSALP also mediates vitamin B6 metabolism and the dephosphorylation of PLP (the active form of vitamin B6) to pyridoxal (PL), which crosses into the blood brain barrier. Insufficient dephosphorylation can manifest in infantile HPP as vitamin B6 dependent seizures [2].

The diagnosis of HPP, particularly in adults, is challenging given the clinical heterogeneity, even within kindreds, the progressivity of disease burden over time, and the current lack of uniform guidelines on appropriate diagnosis in the literature [8,12–14]. Current standard practice for diagnosis is to utilize a combination of clinical, radiological, laboratory, and, if available, genetic findings [15]. Reduced serum or plasma ALP activity in conjunction with elevated levels of the natural substrates of TNSALP have been used to support the diagnosis of HPP; however, only PLP and urine PEA are currently commercially available in the United States [2,5,8,10,11].

Treatment of HPP involves targeted alkaline phosphatase enzyme replacement therapy (ERT; asfotase alfa). ERT treatment causes subsequent elevations in serum and plasma ALP activity levels which

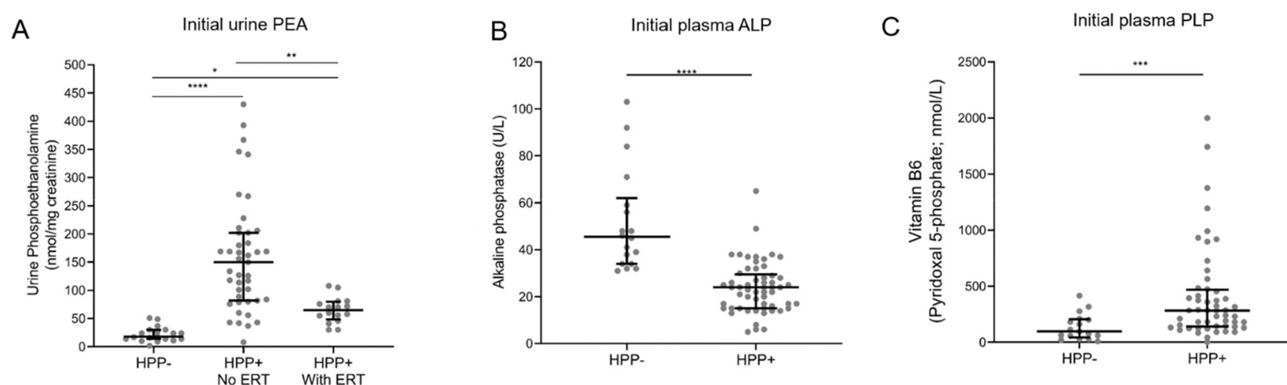

Fig. 1. Urine PEA, plasma ALP and plasma PLP between patients characterized as HPP positive and HPP negative. A) Initial urine PEA levels in patients negative for HPP, positive for HPP, and positive for HPP and on ERT. Each dot represents one patient's initial urine PEA value. Middle bars = median, vertical bars = the interquartile range. *$P < 0.05$ HPP negative vs. HPP positive on ERT, **$P < 0.01$ HPP positive vs. HPP positive on ERT, and ****$P < 0.0001$ HPP negative vs. HPP positive by the Kruskal-Wallis test. B) Initial ALP levels in patients negative for HPP, positive for HPP. Patients on ERT at their initial visit excluded. Each dot represents one patient's initial ALP value. Middle bars = median, vertical bars = the interquartile range. ****$P < 0.0001$ HPP negative vs. HPP positive by the Mann-Whitney test. C) Initial B6 levels in patients negative for HPP, positive for HPP. Each dot represents one patient's initial B6 value. Middle bars = median, vertical bars = the interquartile range. *$P < 0.05$ HPP negative vs. HPP positive on ERT, ***$P < 0.001$ HPP positive vs. HPP positive by the Mann-Whitney test.

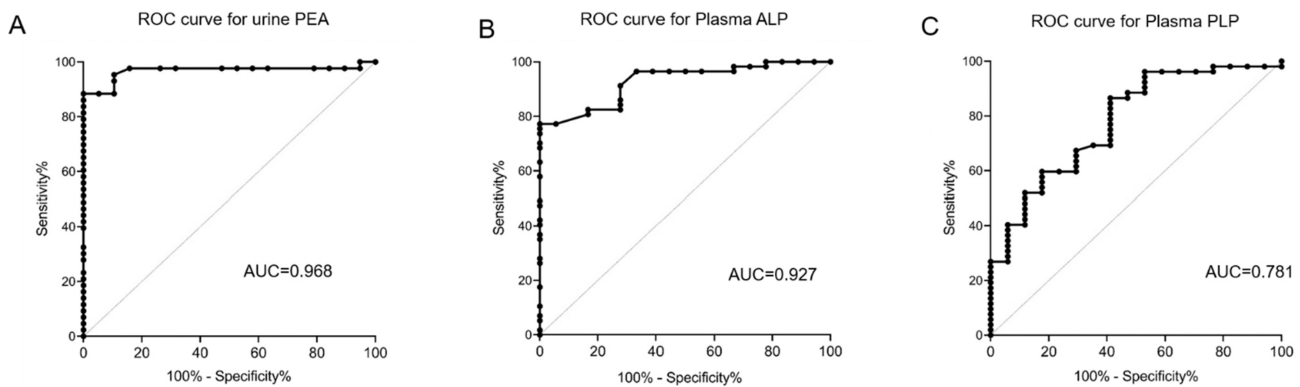

Fig. 2. ROC analysis for urine PEA, plasma ALP and plasma PLP. Patients on ERT at their initial visit were excluded. (A) For urine PEA, the area under the curve (AUC) is 0.968 with a standard error of 0.0234 and a 95 % confidence interval of 0.922–1.00 ****P-value <0.0001. (B) For Plasma ALP, the area under the curve (AUC) is 0.927 with a standard error of 0.0292 and a 95 % confidence interval of 0.870–0.985 ****P-value <0.0001. (C) For plasma PLP, the area under the curve (AUC) is 0.781 with a standard error of 0.0.0637 and a 95 % confidence interval of 0.656–0.906. ****P-value <0.0001.

complicates monitoring of HPP. Currently there are no reliable biomarkers to monitor ERT in HPP due to in-vitro metabolism from ERT.

Here we investigated the utility of urine PEA, PLP and ALP as a marker for diagnosing HPP by way of a retrospective analysis of patients with suspected HPP. The urine PEA results of those patients with a confirmed diagnosis of HPP and those patients with a diagnosis of HPP excluded were captured and compared to other laboratory and clinical parameters consistent with HPP, including ALP activity, plasma PLP, the presence of musculoskeletal abnormalities, and genetic testing when available. We also investigated if urine PEA can be used as a marker to monitor compliance and response to enzyme replacement therapy (ERT) by measuring urine PEA in patients diagnosed with HPP both before and during ERT. The ability to use urine PEA results to rule in HPP, and/or track response to therapy would be useful in assisting with clinical decision-making and management.

2. Materials and methods

2.1. Study design

This is a longitudinal, retrospective, analysis of adult patients referred to the Vanderbilt Program for Metabolic Bone Disease for evaluation of possible HPP or follow-up of HPP during a 5½ year period. This study was approved by the Vanderbilt University Medical Center Institutional Review Board, IRB, #192158.

2.2. Patient inclusion-exclusion criteria and Hypophosphatasia diagnostic criteria

Patients were included in this study if they were referred to the Vanderbilt Program for Metabolic Bone Disease for evaluation of possible or a confirmatory diagnosis of HPP or follow-up of HPP from July 2014 through December 2019 and if they were ≥18 years of age with a urine PEA in the medical record. The diagnosis of HPP required all of the following: (1) genetic testing consistent with a known HPP-causing mutation in the *ALPL* gene or Variants of Uncertain Significance (VUS) in the *ALPL* gene, (2) two or more plasma ALP levels below the age- and sex-adjusted reference range, and (3) musculoskeletal symptoms consistent with diagnosis including muscle weakness, pain, abnormal gait, and/or history of low-trauma fractures. Three patients were also included in the HPP positive cohort without confirmatory genetics due to historical confirmation of an *ALPL* mutation from a prior pediatric center of excellence and known family members with genetic adjudication of their *ALPL* mutation. Those considered to be HPP negative would be those without persistent suppression of plasma ALP levels below the age- and sex-adjusted reference range, and/or no variant in *ALPL* detected, or positive genetic testing for *Osteogenesis Imperfecta*.

2.3. Analysis of urine phosphoethanolamine (PEA), ALP, and pyridoxal 5-phosphate (PLP)

At each follow-up visit to the endocrinology clinic, urine samples were collected and sent to the Children's Hospital Colorado for PEA testing by ion exchange chromatography, reference interval 0–27 nmol/mg creatinine. ALP activity testing was performed at the Vanderbilt University Medical Center Clinical Core Laboratory using the Abbott Architect Alkaline Phosphatase assay (7D55, Abbott Laboratories, Chicago, Illinois, US), reference intervals 17–<19 yrs.: 48–95 U/L (female), 59–164 U/L (male); 19–150 yrs. (male and female): 40–150 U/L. Patient plasma samples were sent to ARUP (Salt Lake City, UT) for PLP testing by HPLC-MS, reference intervals 20–125 nmol/L.

2.4. ALPL genetics

Genetic testing was performed as part of routine clinical testing and sent to one of the following CLIA and CAP accredited laboratories: Ambry, Baylor Miraca Genetics, Centogene, CTGT, Gene DX, Invitae or Prevention Genetics. Variants were annotated using Variant Effect Predictor (VEP) [16] and frequencies were from gnomAD when available. Variant classification was from the ClinVar database. If variant classification in the ClinVar database was not available (NA) or a VUS, the result from the clinical laboratory report is also listed.

2.5. Retrospective analysis of collected data

Urine PEA results, plasma ALP, plasma PLP, molecular genetics, clinical symptoms, and ERT status were recorded from the electronic health record for analysis after the study collection period by two investigators. A random selection of 25 % of records were cross-checked by both investigators to verify accuracy.

2.6. Statistics and ROC analysis

Two groups were compared by non-parametric approaches using a Mann-Whitney test; multiple group comparison was compared using a Kruskal-Wallis test. A p-value <0.05 was considered statistically significant. Graphs scatterplots and statistics were rendered and performed in GraphPad Prism 8.4.2 (San Diego, CA). Sensitivity and specificity of urine PEA, plasma ALP and plasma PLP for diagnosing HPP were calculated using receiver operator characteristic curves and 95 % confidence intervals were analyzed by the Wilson/Brown method in

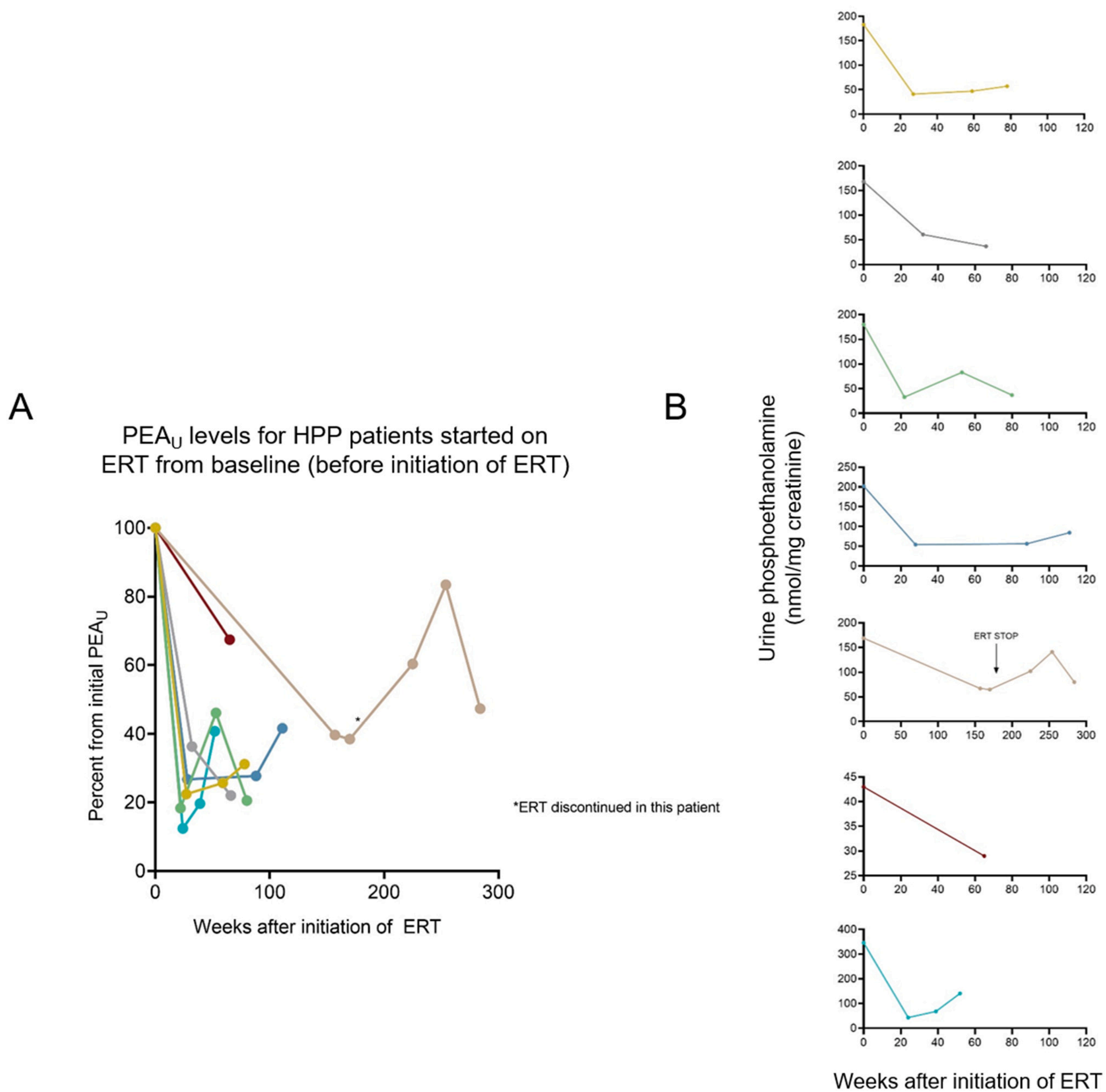

Fig. 3. Urine PEA concentration for HPP patients started on ERT from baseline. A) All patients who began ERT had a mean decline in urine PEA levels after treatment of 68.1 % (SD = 18.4, $N = 7$). Each color represents a different patient. X-axis represents each urine PEA measurement done per patient at each follow-up visit. B) Absolute urine PEA levels for each individual patient at week 0 (initiation of ERT) and at the indicated weeks on the x-axis.

GraphPad Prism 8.4.2 (San Diego, CA).

3. Results

3.1. Characteristics of patients in the HPP positive and HPP negative study groups

Baseline patient demographics and clinical and molecular characteristics are shown in Table 1. A total of 78 patients ≥18 years old were included in this study, age range 19–81, ($n = 59$ or 75.6 % female), with 59 patients in the HPP positive group and 19 patients in the HPP negative group. Median ages were similar in both the HPP negative and HPP positive groups (47 vs. 46, respectively). The distribution of females to males was also similar between the two groups (73.7 % in the HPP negative and 76.2 % in the HPP positive). A comparison of the signs of symptoms associated with hypophosphatasia revealed that in the HPP positive group, 78 % had a reported family history of bone disease (osteoporosis, osteopenia, fractures and deformities) compared to 47.4 % in the HPP negative group. The HPP patient group had a higher frequency of significant bone pain (84.7 % vs. 66.2 %), dental issues (80.0 % vs. 52.6 %), muscle pain (69.5 % vs. 52.6 %), and fatigue (72.9 % vs. 47.4 %). The HPP positive group consisted of 56/59 patients (94.9 %) who had genetic testing of *ALPL* performed. The HPP negative group consisted of 19 patients, 15 (78.9 %) of whom who had genetic testing of *ALPL* performed, all of whom had no detectable mutation in *ALPL*. Three additional patients declined genetic testing and one patient had genetic testing results consistent with *Osteogenesis Imperfecta* (Table 1).

Table 2
Initial urine PEA results and *ALPL* mutations or clinical data in the HPP positive patients.

ID	Age	Sex	Initial PEA	Variant	Rs. Number	Frequency	Classification**	Supporting Clinical Data for VUS or Patients without Genetic Report
2	28	F	70*	c.1133A>T (p.Asp378Val), heterozygous	rs121918008	0.000003976	Pathogenic	
4	68	F	42	c.648 + 1G>A, splicing, heterozygous	rs749544042	0.000003999	Pathogenic	
6	30	F	58*	c.1034C>T (p.Ala345Val), heterozygous	NA	NA	VUS	Persistently low ALP, high B6, short stature, bowing deformities, CPPD, mother, daughter +mutation and phenotype.
7	62	F	30*	c.1171C>T (p.Arg391Cys), heterozygous	rs371243939	0.00001193	Pathogenic/Likely pathogenic	
9	81	F	84	c.881A>C (p.Asp294Ala), heterozygous	rs121918002	0.00004251	Pathogenic	
18	49	F	30*	c.1171C>T (p.Arg391Cys), heterozygous	rs371243939	0.00001193	Pathogenic/Likely pathogenic	
19	25	F	102	c.1171C>T (p.Arg391Cys), heterozygous	rs371243939	0.00001193	Pathogenic/Likely pathogenic	
22	49	F	76	c.130C>T (p.GIn44*), heterozygous	rs1057516293	NA	Likely pathogenic	
24	41	F	118	c.1231A>G (p.Thr411Ala), heterozygous	NA	NA	Likely Pathogenic[a]	
25	28	M	101	c.1364G>A (p.Gly455Asp), heterozygous	rs1289406215	0.000	VUS[b,c]	Persistently low ALP, high B6, skull deformities, scoliosis, early loss of primary dentition, bone, muscle pain, fatigue. Foot fractures complicated by nonunion. Use of mobility devices since the age of 28. Mother +mutation and phenotype.
26	60	F	367	c.1364G>A (p.Gly455Asp), heterozygous	rs1289406215	0.000	VUS[b,c]	Persistently low ALP, high B6, atraumatic fractures in feet, wrists, and fibula. Early loss of secondary dentition due to abscesses requiring implants.
27	43	F	168	c.346G>A (p.116Thr), heterozygous	rs121918013	NA	Pathogenic/Likely pathogenic	
28	65	F	70*	c.571G>A (p.Glu191Lys), heterozygous	rs121918007	0.000003981	Pathogenic/Likely pathogenic	
				c.344C>T (p.Thr115lle), heterozygous	NA	NA	VUS[b,d,e]	
30	49	F	76*	c.340G>A (p.Ala114Thr), heterozygous	rs1320839573	0.000	Pathogenic[a]	
				c1415A>G (p.His472Arg), heterozygous	NA	NA	VUS[b]	
32	51	F	41*	c.1133A>T (p.Asp378Val), heterozygous	rs121918008	0.000003976	Pathogenic	
34	60	F	126	c.346G>A (p.Ala116Thr), heterozygous	rs121918013	NA	Pathogenic/Likely pathogenic	
36	32	M	81*	c.881A>C (p.Asp294Ala), heterozygous;	rs121918002	0.00004251	Pathogenic	
				c.571G>A (pGlu191Lys)[b], heterozygous	rs121918007	0.000003981	Pathogenic/Likely pathogenic	
37	31	F	60*	c.1133A>T (p.Asp378Val), heterozygous	rs121918008	0.000003976	Pathogenic	
41	33	F	180	c.1250A>G (p.Asn417Ser), heterozygous	rs121918014	0.00002829	Pathogenic/Likely pathogenic	
42	70	F	55*	c.1250A>G (p.Asn417Ser), heterozygous	rs121918014	0.00002829	Pathogenic/Likely pathogenic	
46	54	F	202	c.1133A>T (p.Asp378Val), heterozygous	rs121918008	0.000003976	Pathogenic	
48	47	F	46*	c.1133A>T (p.Asp378Val), heterozygous	rs121918008	0.000003976	Pathogenic	
49	23	F	184	c.1133A>T (p.Asp378Val), heterozygous	rs121918008	0.000003976	Pathogenic	
50	64	F	393	c.211C>T (p.Arg71Cys), heterozygous	rs121918001	NA	Likely pathogenic	
53	25	M	56*	c.1250A>G (p.Asn417Ser), heterozygous	rs121918014	0.00002829	Pathogenic/Likely pathogenic	
54	50	F	105*	c.1250A>G (p.Asn417Ser), heterozygous	rs121918014	0.00002829	Pathogenic/Likely pathogenic	
56	46	M	72*	c.1133A>T (p.Asp378Val), heterozygous	rs121918008	0.000003976	Pathogenic	
57	73	F	169	c.571G>A (p.Glu191Lys), heterozygous	rs121918007	0.000003981	Pathogenic/Likely pathogenic	
60	27	F	202	c.1250A>G (p.Asn417Ser), heterozygous,	rs121918014	0.00002829	Pathogenic/Likely pathogenic	
62	68	M	228	c.1250A>G (p.Asn417Ser), heterozygous	rs121918014	0.00002829	Pathogenic/Likely pathogenic	
64	70	F	127		rs121918002	0.00004251	Pathogenic	

(*continued on next page*)

Table 2 (*continued*)

ID	Age	Sex	Initial PEA	Variant	Rs. Number	Frequency	Classification**	Supporting Clinical Data for VUS or Patients without Genetic Report
				c.881A>C (p.Asp294Ala) heterozygous				
67	42	M	60	c.1250A>G (p.Asn417Ser), heterozygous	rs121918014	0.00002829	Pathogenic/Likely pathogenic	
74	19	F	80	c.1171C>T (p.Arg391Cys), Heterozygous	rs371243939	0.00001193	Pathogenic	
88	65	F	167	c.340G>A (p.Ala114Thr), heterozygous	rs1320839573	0.000	Pathogenic[a]	
89	27	F	162	c.1133A>T (p.Asp378Val), heterozygous	rs121918008	0.000003976	Pathogenic	
91	62	F	206	c.1156G>T (p.Gly386Cys), heterozygous	rs1057519962	NA	VUS[b]	Persistently low ALP, high B6. Atraumatic humerus fx, chronic metatarsal fractures, chronic metacarpal fractures, fibular fracture, wrist fracture. Abnormal gait. Mother, 3 sisters and son +mutation and phenotype.
92	81	F	150	c.1156G>T (p.Gly386Cys), heterozygous	rs1057519962	NA	VUS[b]	Persistently low ALP, high B6, early loss of primary dentition, wrist fracture, ankle fractures multiple, tibia fractures. History of dental implants due to early loss of secondary dentition. Four daughters and a grandson +mutation and phenotype.
95	72	M	211	c.1250A>G (p.Asn417Ser), heterozygous	rs121918014	0.00002829	Pathogenic/Likely pathogenic	
96	27	M	79	c.346 G>A p.Ala116Thr (A116T), heterozygous	rs121918013	NA	Pathogenic/Likely pathogenic	
97	33	F	430	c.881A>C (p.Asp294Ala), heterozygous;	rs121918002	0.00004251	Pathogenic	
				c.571G>A (pGlu191Lys), heterozygous.	rs121918007	0.000003981	Pathogenic/Likely pathogenic	
98	42	F	134	c.881A>C (p.Asp294Ala), heterozygous	rs121918002	0.00004251	Pathogenic	
99	40	F	43	Heterozygous for a ~1.46 kb deletion. *Deletion of entire Exon 2 known pathogenicity*			Pathogenic[a]	
100	62	F	169	c.1156G>T (p.Gly386Cys), heterozygous	rs1057519962	NA	VUS[b]	Persistently low ALP, high B6, Atraumatic foot fracture and shoulder fractures. Bone pain since age 20. Early loss of primary dentition, multiple abscesses and extractions of secondary dentition, mother, 3 sisters and nephew +mutation and phenotype.
101	63	F	267	c.1250A>G (p.Asn417Ser), heterozygous	rs121918014	0.00002829	Pathogenic/Likely pathogenic	
102	54	M	43	c.896T>C (p.Leu299Pro), heterozygous	NA	NA	Pathogenic[a]	
103	45	F	82	c.746G>T (p.Gly249Val), heterozygous	rs121918018	0.000003978	Conflicting interpretations: Pathogenic; VUS	
104	35	F	8	c.1133A>T (p.Asp378Val), heterozygous	rs121918008	0.000003976	Pathogenic	
105	46	M	169	c.340 G>A (p.Ala114Thr), heterozygous	rs1320839573	0.00	Pathogenic[a]	
106	30	F	346	c.1133A>T (p.Asp378Val). heterozygous	rs121918008	0.000003976	Pathogenic	
107	64	F	56	c.1171C>T (p.Arg391Cys), heterozygous	rs371243939	0.00001193	Pathogenic/Likely pathogenic	
108	55	F	89	c.881A>C (p.Asp294Ala), heterozygous	rs121918002	0.00004251	Pathogenic	
110	34	M	114	c.662del (pGly221Valfs*56), heterozygous	rs769948289 (multiallelic)	0.000007954	Likely Pathogenic[a]	
111	32	M	117	c.1250A>G (p.Asn417Ser), heterozygous	rs121918014	0.00002829	Pathogenic/Likely pathogenic	
113	40	F	341	c.1250A > G (p.Asn417Ser), heterozygous	rs121918014	0.00002829	Pathogenic/Likely pathogenic	
114	64	M	37	c.1000 G>A (p.Gly344Asp), heterozygous	rs769955594	NA	Pathogenic[a]	
115	31	F	81*	c.346G>A (p. Ala116Thr), heterozygous	rs121918013	NA	Pathogenic/Likely pathogenic	
14	81	F	108	No genetic results	NA	NA	NA	ALP 25 and PLP 454. Fractures consistent with HPP diagnosis and + FH of HPP with an ALPL mutation documented.
51	27	M	270	Genetic results not available	NA	NA	NA	Prior diagnosis of infantile HPP, ALP 7 and PLP >2000 at presentation. History of early loss of primary dentition (age 2) and recurrent metatarsal fractures.

(*continued on next page*)

Table 2 (*continued*)

ID	Age	Sex	Initial PEA	Variant	Rs. Number	Frequency	Classification**	Supporting Clinical Data for VUS or Patients without Genetic Report
66	30	F	156	Genetic results not available	NA	NA	NA	Diagnosis of juvenile HPP, genetics report not available. ALP 17 and PLP 234 at presentation. History of early loss of primary dentition (age 2) recurrent metatarsal and ankle fractures.

Initial Urine PEA results and *ALPL* mutations or clinical data in the HPP positive patients.
Genetic testing (Sequencing and Del/Dup) was performed at the following laboratories: Baylor Miraca Genetics (Patient 48), Centogene (Patient 56), CTGT (Patients 31,50, 64, 74, 96), Invitae (Patients 2,7,9,19,37 and 53), Gene, DX (Patient 105,114). Patient 30 has a mention of genetics in notes, but the original testing laboratory is unknown. The remaining 41 patients with genetic results were tested at Prevention Genetics.
For rs.Number, NA means no rsID available.
For Frequency, NA means the variant is not in gnomAD.
* test performed while patient was on ERT.
** Classification from clinVar database unless specified.
[a] Classified as pathogenic or likely pathogenic from clinical laboratory report and listed as VUS or NA in ClinVar.
[b] Classified as VUS from clinical laboratory report and listed as NA or VUS in ClinVar.
[c] Reported as adult hypophosphatasia in the JKU database https://alplmutationdatabase.jku.at/table/
[d] Reported as Odonothyphosphatasia in JKU database: https://alplmutationdatabase.jku.at/table/
[e] Reported to have very low in vitro residual activity and a small DNE effect. [43].

3.2. Laboratory analysis

Patients in our HPP positive cohort had initial urine PEA levels (median = 150.0 nmol/mg creatinine, IQR = 82.0–202.0) that were greater than eight times the median urine PEA levels of patients in our HPP negative group (median 18.0 nmol/mg creatinine, IQR = 14.0–30.0, $p < 0.0001$). ERT of HPP patients more than halved the median urine PEA values in patients with HPP (median = 65.0 nmol/mg creatinine, IQR = 48.25–79.75); however, ERT did not completely normalize the PEA values in HPP (Fig. 1). Plasma ALP levels were significantly lower in the HPP group (median = 24.0 U/L, IQR = 15.0–29.50) compared to the patients without HPP (median = 45.50 U/L, IQR = 34.0–62.0) (Fig. 1). PLP levels were higher in the HPP group (median = 284.0 nmol/L, IQR = 141.0–469.4) compared to the patients without HPP (median = 97.5 nmol/L, IQR = 43.7–206.0) (Fig. 1).

3.3. ROC analysis

Results of the ROC curves are shown in Fig. 2. The area under the curve (AUC) of urine PEA is 0.968 (standard error = 0.0234; 95 % confidence interval of 0.922 to 1.00, $p < 0.0001$). Urine PEA levels had 100 % specificity (95 % CI of 83.2 % to 100.0 %) for diagnosing HPP at a value >53.50 nmol/mg creatinine with a sensitivity of 88.4 %; 95 % CI 75.5 to 94.9 %. The AUC of ALP to distinguish between HPP and non-HPP disease is 0.927. ALP levels had a 100 % specificity (95 % CI of 82.4 % to 100.0 %) for diagnosing HPP at a value <30.5 U/L with a sensitivity of 77.2 %; (95 % CI 64.8 to 86.2 %). The AUC of PLP to distinguish between HPP and non-HPP disease is 0.781. PLP levels had a 100 % specificity (95 % CI of 81.6 % to 100.0 %) for diagnosing HPP at a value >436 nmol/L with a sensitivity of 26.9 %; (95 % CI 16.8 to 40.3 %).

3.4. Urine PEA concentrations in patients during ERT

All patients who began ERT had a decline in urine PEA levels after treatment (mean = 68.1 %, SD = 18.4). Individual declines in urine PEA changes from their baseline prior to treatment ranged from 14 to 303 nmol/mg creatinine. Termination of ERT in one of the patients preceded a rise in urine PEA of 39 nmol/mg creatinine (65 nmol/mg creatinine pre-ERT and 102 nmol/mg creatinine on ERT), before decreasing to 80 nmol/mg creatinine on the final visit (Fig. 3).

3.5. Molecular genetics in patients with HPP

In our cohort, 71/79 (89.9 %) patients had genetic testing reports available of the *ALPL* gene.

For the HPP positive group, genetic testing results of *ALPL* were available for 56/59 patients (94.9 %). Of these, forty-nine patients (83.1 %) had a pathogenic or likely pathogenic mutation in the *ALPL* gene; six patients (10.2 %) had a mutation categorized as a VUS in the *ALPL* gene; and one patient (1.7 %) had an *ALPL* mutation with conflicting interpretations of likely pathogenic mutation and VUS. (Table 2). The most common pathogenic/likely pathogenic mutations were c.1250A>G, p.Asn417Ser, (11 patients), c.1133A>T, p.Asp378Val (10 patients), c.881A>C, p.Asp294Ala, (6 patients) c.1171C>T, p.Arg391-Cys, (5 patients) and c.571G>A, p.Glu191Lys (4 patients). The most common VUS was c.1156G>T, p.Gly386Cys, (3 patients). Four patients (#28, 30,36 and 97) exhibited more than one mutation. The HPP negative group consisted of nineteen patients, fifteen (78.9 %) of whom who had genetic testing of *ALPL* performed; all fifteen patients had no detectable mutations in *ALPL* (Table 3).

4. Discussion

Adults with HPP can present with a wide range of clinical manifestations that overlap with other metabolic bone disorders, leading to misdiagnosis or a delay in diagnosis. The diagnosis of HPP is made upon the aggregate sum of features including clinical and radiographic manifestations, biochemical results, and, if available, molecular analysis confirming the presence of a disease-causing variant in *ALPL*.

The hallmark biochemical defect in this disease is ALP activity below the age-adjusted and gender-adjusted reference interval; however, the differential for low ALP activity is broad and ALP as a standalone marker of HPP is unreliable [17–19]. In adults, low ALP activity has been reported in patients who are post-cardiac surgery and post-cardiopulmonary bypass, in malnutrition, magnesium deficiency, anemia, massive blood transfusion, with steroid use, Wilson's Disease and Cleidocranial Dysplasia [20–27]. In children and infants, low ALP activity has been reported in patients with a diagnosis of hypothyroidism, steroid use, malnutrition, post-cardiopulmonary bypass, Wilson's Disease, Cleidocranial Dysplasia, Osteochondrodysplasia, and osteogenesis imperfecta IIC [22,28–31]. In our cohort, the majority of referred patients with low ALP values who were subsequently ruled out for HPP were taking high doses of steroids.

ALP requires magnesium and zinc cofactors for full in vivo and in vitro activity and deficiencies of magnesium and zinc were previously sources of falsely low ALP activity. Current ALP assay formulations use the International Federation of Clinical Chemistry method, which should include sufficient concentrations of magnesium and zinc to circumvent this issue [32]. Co-existing medical conditions (liver disease,

Table 3
Initial urine PEA results and clinical data in the HPP negative patients.

ID	Age	Sex	Initial urine PEA	*ALPL* genetic testing result	Supporting clinical data
3	46	F	37	Negative	
10	32	F	18	N/A	Two ALP activity levels below the RI during the first trimester of pregnancy. All other ALP activity levels were within normal range.
15	65	M	51	Negative	One isolated ALP activity level while taking high dose steroids.
16	34	F	27	Negative	Patient taking prednisone and history of hypothyroidism.
38	54	F	<2	Negative	One isolated ALP activity level while taking denosumab.
44	54	F	30	Negative	
63	20	F	24	Negative	Two low ALP activity levels in the setting of high Vitamin D. Subsequent normalizing of vitamin D resulted in ALP within the RI.
65	65	M	11	Negative	Low ALP activity levels while taking high dose steroids. ALP activity levels subsequently normalized.
116	68	F	30	Negative	Low ALP activity levels while taking denosumab. Previously, ALP activity levels were within normal range.
117	60	M	24	Negative	Low ALP activity levels while taking high dose steroids. ALP activity level subsequently normalized.
118	65	F	14	Negative	One low ALP activity level while taking denosumab. ALP activity level subsequently normalized after discontinuation.
119	47	F	16	Negative	Historically normal ALP activity level. Low ALP activity levels while on steroids.
120	32	M	17	N/A	No history of low ALP activity level.
121	25	M	9	Negative	No history of low ALP activity level. One result on lower end of reference range with documented history of IBD and malabsorption.
122	65	F	49	N/A	No history of low ALP activity level.
123	49	F	10	Negative	Low ALP activity level due to EDTA interference.
124	24	F	14	Negative	No history of low ALP activity level.
125	45	F	14	Negative	No history of low ALP activity level.
126	27	F	24	N/A	Positive for COL1A1 c.2299 g>A

Initial Urine PEA results and ALPL variant and/or clinical data in the HPP negative patients. Genetic testing (Sequencing and Del/Dup) of *ALPL* was performed at the following laboratories: Ambry (Patient 119), CTGT (Patients 16, 63,125) Invitae (Patient 3) and Gene, DX (Patient 105,114). Genetic testing of *ALPL* for the remaining 10 patients was at Prevention Genetics. Patient 126 had genetic testing performed at CTGT and was positive for a mutation in COLA1A1.

pregnancy, recent fractures and hyperparathyroidism) in patients with HPP, may cause elevation in ALP to within the normal range [33]. In addition, ALP activity levels may vary from patient to patient further adding to the diagnostic conundrum. Differentiation of HPP from other causes of metabolic bone disorders is particularly important as the prescribed treatments for these conditions (bisphosphonates and vitamin D) may be contraindicated for patients with HPP [21,34,35].

The present study describes and quantifies the diagnostic performance of urine PEA, plasma PLP and plasma ALP biomarkers for ruling in HPP. The strengths of our study include that this is the largest cohort to date of patients referred for evaluation of HPP, with molecular testing for ALPL in over 90 % of patients and systematic collection of urine PEA levels with the most common pathogenic/likely pathogenic mutations were c.1250A>G (p.Asn417Ser), c.1133A>T (p.Asp378Val), c.881A>C (p.Asp294Ala), c.1171C>T (p.Arg391Cys), and c.571G>A, (p.Glu191Lys). In addition, a subset of patients on ERT were consistently followed for up to 5 years.

Retrospective analysis of our data demonstrated that, in our cohort, urine PEA performed better as a biomarker for ruling in HPP than plasma ALP and significantly better than plasma PLP. PLP is the biologically active and most abundant form of vitamin B6 and accumulates in HPP. Measurement of PLP can be challenging due to pre-analytic factors: PLP is also light-sensitive and must be appropriately protected from light. PLP also decreases in samples stored at room temperature due to dephosphorylation to PL. PLP is bound to albumin and therefore may be affected by nutritional state, smoking, inflammation, and drugs. In addition, PLP can decrease after high carbohydrate intake and should be collected in the fasting state. Elevated concentrations of serum PLP have been observed in adult patients with HPP [8,36–39] and children with HPP [36]; however, a low or normal PLP does not rule out the presence of HPP in those with underlying deficiencies [40].

Urine PEA is also a natural substrate for TNSALP, but currently the effects of PEA on the clinical manifestations of HPP are not clear. Additional studies have reported elevated concentrations of urine PEA in adult patients with HPP [8,21,36] and children [36]. Previous studies have shown the utility of urinary PEA as both a biomarker for disease diagnosis and an indicator of disease burden [8,41]. Lefever et al. describe cohort of 19 patients where higher PEA levels were associated with biallelic mutations, earlier onset of first disease manifestation and overall disease burden severity. In this study not all patients described had urine PEA values above the reference range. Those within the normal range tended to have later onset and milder disease. Two patients with PEA within the normal reference range did not have detectable ALPL variants and one patient was described as a carrier [41].

To date, urine PEA has been used as a supportive test for the diagnosis of HPP [42]; however, our study demonstrates that urine PEA may also have utility when considering the differential diagnosis of other causes of low ALP. In our cohort, the most common association of low ALP in the HPP negative group was high dose steroids followed by use of antireosorption medications for osteoporosis treatment. Interestingly one patient was confirmed to have Osteogenesis Imperfecta with a COL1A1 mutation highlighting that there may be considerable phenotypic and biochemical overlap in skeletal dysplasias.

In this study, longitudinal monitoring of a subset of patients on ERT demonstrated that urine PEA values decreased upon ERT initiation and increased when ERT was interrupted. These results indicate that urine PEA may have substantial utility as a marker for monitoring response to ERT treatment. Other biomarkers for HPP present substantial challenges for specifically monitoring HPP patients on ERT. Measurement of ALP activity represents drug levels of asfotase alfa and does not correlate with clinical outcomes. In clinical studies, administration of ERT resulted in measurements of serum ALP activity well above the normal range [42]. Measurement of plasma PLP to monitor patients on ERT is also challenging as asfotase alfa hydrolyzes PLP, resulting in undetectable levels unless an ALP inhibitor, such as levamisole, is added. Careful and consistent addition of an ALP inhibitor, would be needed for PLP to have utility for monitoring ERT [42]. Additionally, urine samples are less of a patient burden than phlebotomy.

Further investigation is required to determine if PEA has utility beyond compliance as this may potentially be useful to determine immunogenicity and/or clinical outcomes with ERT. Although immunogencity was not associated with lack of efficacy in the clinical trials, postmarketing reports have described how some patients on ERT, despite an initial theurapeutic response, developed recurrence and

worsening in both radiographic and laboratory biomarkers (reference https://alexion.com/documents/strensiq_uspi.pdf) In this scenario, testing for anti-drug antibodies and neutralizing antibodies would potentially provide insight on immune responses; however, this testing is not available outside of the HPP registry (www.hppregistry.com) and immunogenicity testing has not yet correlated with clinical outcomes in the real-world setting [42]. Urine PEA is more readily available and may prove to be useful for evaluating patients with perceived treatment failure. Future studies are required to further evaluate this potential.

Limitations of this study are that it was performed at a single site and selection bias may impact our findings, as all participants were established patients at an academic medical center and may not reflect the general population of individuals with HPP. In addition, the cut-off values we established for urine PEA, plasma PLP and plasma ALP are method specific and may not be applicable to assays developed by other laboratories (PLP and urine PEA) or manufacturers (ALP). Thresholds for these assays would need to be established for wide-spread use. Future directions for additional study of the utility of urine PEA for diagnosing HPP and managing patients on ERT include establishing clinical correlates of disease severity, response to progression of disease burden and therapy, evaluation of immunogenicity for drug non-responders and establishing any genotype-phenotype correlates.

5. Conclusion

In this study, we evaluated the diagnostic performance of urine PEA in a large cohort of adults suspected of HPP. Retrospective analysis of the results demonstrated that, in our cohort, urine PEA performed better as a sensitive and highly specific diagnostic and confirmatory marker for HPP in patients suspected for HPP that plasma ALP or plasma PLP. In addition, urine PEA levels in HPP patients on ERT declined by their next visit, suggesting that urine PEA has potential use as a marker for monitoring ERT compliance to aid in the management of HPP. Future studies are necessary to evaluate the association between urine PEA levels and clinical outcomes.

CRediT authorship contribution statement

Zahra Shajani-Yi: Conceptualization, Methodology, Data curation, Investigation, Writing – original draft, Writing – review & editing. **Nadia Ayala-Lopez:** Investigation, Formal analysis, Data curation, Writing – review & editing. **Margo Black:** Investigation, Data curation, Writing – review & editing. **Kathryn McCrystal Dahir:** Conceptualization, Investigation, Writing – review & editing, Supervision.

Declaration of competing interest

ZSY is currently an employee of Labcorp. NAL is currently an employee of Labcorp Drug Development. KMD is a clinical trial investigator and has received consultancy fees and/or institutional research funding from Alexion Pharmaceuticals, Inc.

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