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Establishing race-, gender- and age-specific reference intervals for pyridoxal 5′-phosphate in the NHANES population to better identify adult hypophosphatasia

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ABSTRACT

Introduction: Bisphosphonate treatment in adults with hypophosphatasia (HPP) may increase fracture risk. PLP is a useful marker in biochemically differentiating HPP from osteoporosis in adults. In order to identify elevated PLP, robust reference intervals are needed which are calculated in a large, representative sample population.

Methods: Complete data from 9069 individuals (ages 20–80, 50.6% female) from two years of the NHANES Survey (2007–2008 and 2009–2010) were investigated. Differences in PLP in the presence of four factors; inflammation (CRP ≥5.0 mg/L), low ALP (< 36 IU/L), chronic kidney disease (eGFR < 60 mL/min/1.73^2), and daily vitamin B6 supplementation, were investigated. Race, gender and age differences in PLP were then investigated; 95% reference intervals were calculated that reflected these differences.

Results: Inflammation and chronic kidney disease were associated with lower PLP ($p < .0001$ and $p = .0005$ respectively), while low ALP and vitamin B6 supplementation were associated with higher PLP (both $p < .0001$). Individuals were excluded based on the presence of these factors; a reference interval population ($n = 4463$) was established. There were significant differences in PLP depending on race and gender ($p < .0001$) Increasing age was correlated with decreasing PLP (spearman's rho −0.204, $p < .0001$). Race- and gender-specific 95% reference intervals were calculated. In male patients, these were also calculated according to age groups: young and older adults (ages 20–49 years and ≥50 years respectively).

Conclusions: In order to identify adult hypophosphatasia based on elevated PLP, considerations must be made depending on the race, gender and age of the individual. Factors associated with significant differences in PLP must also be considered when assessing biochemical measurements.

1. Introduction

Hypophosphatasia (HPP) is a rare genetic disorder characterised by mutations of the tissue non-specific alkaline phosphatase (TNSALP) gene. There is a high heterogeneity in the clinical presentation of HPP, with complications mainly originating from the skeleton. Adults with HPP typically present in middle age and their signs and symptoms include recurrent non-healing stress fractures (typically of the metatarsals), history of early shedding of deciduous teeth, thigh pain which can indicate femoral pseudofractures, chondrocalcinosis, osteoarthropathy and muscle weakness [1,2].

TNSALP is expressed in the skeleton, liver and kidney. Its main natural substrates are inorganic pyrophosphate (PPi), pyridoxal 5′ phosphate (PLP) and phosphoethanolamine (PEA). The bone-specific isoform of alkaline phosphatase (ALP) is required for the normal mineralisation of bone and the development of teeth. In HPP, the lack of the TNSALP activity is expressed by accumulation of PPi, a potent inhibitor of mineralisation [3].

Due to the wide range and severity of HPP symptoms and the low prevalence of the disease, diagnosis of adult HPP may prove difficult and it is often overlooked. Patients with fractures can be mistaken for having osteoporosis, a more common disease. Bisphosphonates, which are analogues of pyrophosphate, are the main drugs used in the treatment of osteoporosis. However, there have been several studies which suggest that treatment with bisphosphonate in HPP patients may result in atypical, usually non-traumatic subtrochanteric and femoral shaft fractures [4–6]. Therefore, it is critical to be able to suspect and diagnose HPP accurately.

The biochemical findings of HPP are persistently low serum ALP levels [2]. An inverse relationship has been described between

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circulating PLP levels and ALP. Patients with hepatic diseases and malignancy have been found to have reduced PLP levels [7–9]. On the contrary, patients with HPP have been found to have elevated PLP levels and its measurement has been described as a more sensitive and specific marker than a low ALP level for diagnosing HPP [2,9]. PLP is the biologically active and most abundant form of vitamin B6 (70–90%), a term that refers to six interconvertible compounds: pyridoxine (PN), pyridoxamine (PM), and pyridoxal (PL), and their phosphorylated derivatives pyridoxine 5-phosphate (PNP), pyridoxamine 5-phosphate (PMP), and pyridoxal 5-phosphate (PLP) [10,11]. PLP assessment has some challenges, as PLP decreases in samples stored at room temperature due to dephosphorylation to PL. It has been previously recommended that when sample handling is not optional, summarising the results of PLP as B6-aldehyde (PLP + PL) should be performed but this has not been validated. PLP is light sensitive so it is recommended that serum/plasma sample handling is carried out under non-UV lighting. [11–13]. It can decrease after high carbohydrate intake so it is best to obtain fasting samples. PLP is bound to albumin and several factors can affect its level like alcohol consumption, albumin concentration, smoking, inflammation and drugs (antiepileptics, non-steroidal anti-inflammatory drugs, hydralazine, isoniazid etc.) [11].

In order to reliably utilise PLP as a marker of HPP, robust PLP reference intervals are needed to reflect the population-based differences in PLP. Currently reported reference intervals for PLP show poor agreement, possibly as a result of the unrepresentative populations from which they are derived. There is an indication from collected laboratory reported 95% reference intervals (RIs) that gender differences exist in PLP levels [14].

Factors which may be associated with significant differences in PLP should be identified and excluded where present from the reference interval population. A previous investigation using data from the 2005–2006 National Health and Nutrition Examination Survey showed significant differences in PLP based on the presence of inflammation (defined as C-reactive protein, CRP $\geq$5.0 mg/L), but no difference in PLP in individuals with chronic kidney disease (CKD) stage 3–5 [defined as estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73^2] when compared to participants with normal kidney function (eGFR $\geq$60 mL/min/1.73 m^2 and absence of albuminuria) [15]. These findings need to be confirmed. Moreover, other factors like the association between low ALP levels and the levels of PLP have to be investigated. PLP is elevated in response to high dietary vitamin B6 intake, so associations between the daily supplement intake and differences in circulatory PLP should also be studied.

We used a combined dataset encompassing the 2007–2008 and 2009–2010 NHANES collections to investigate the associated differences in PLP in the presence of four factors of interest: inflammation, chronic kidney disease, low ALP, and vitamin B6 supplement intake [16].

The aims of our study were:

1. To examine factors associated with significant differences in PLP in a representative United States (US) population sample.
2. To test for the presence of race, gender and age differences in PLP
3. To calculate 95% RIs for PLP that reflect these differences.

2. Materials and methods

2.1. Study population

All study data was drawn from The National Health and Nutrition Examination Survey (NHANES). NHANES is an annual, cross-sectional collection of health and nutritional data in the general, non-institutionalised US population. Data from 20,015 subjects collected during two years of the survey were collated (2007–2008, n = 9762, 2009–2010, n = 10,253).

This study only included data from adults (ages 20–80) from three pre-defined race/ethnicity groups, Hispanic-Mexican American, Non-Hispanic Black and Non-Hispanic White. Cases with ambiguous race/ethnicity identification (Hispanic-Other Hispanic, Other) were excluded based on the difficulty of applying the Modification of Diet in Renal Disease Study (MDRD) equation which requires the specification of race to calculate eGFR [17]. Cases were omitted where data was incomplete for any variable of interest. Prior to examination of potential confounders of PLP, we did not exclude any subjects on the basis of any health conditions in order to best represent the general population. Overall, the total study population was n = 9069. Young adults were defined as 20–49 years old, and older adults ages 50 and older.

Further exclusions were made based on the presence of one or more known confounders of PLP (inflammation, chronic kidney disease, low ALP, and vitamin B6 supplement intake irrespective of the dose of vitamin B6), providing a final reference interval population of n = 4463.

2.2. Biochemical measurements

All biochemical measurements were completed as part of the NHANES programme.

The 2007–2008 and 2009–2010 datasets were selected as both years included measurement of serum PLP by the same reversed-phase high performance liquid chromatography (HPLC) method with post-column derivatisation and fluorometric detection. This is the method used in everyday practice to measure PLP [18,19]. The laboratory procedure manual used by NHANES recommended fasting samples, shipped on dry ice by an overnight carrier and then stored at −70 °C. If refrigerated samples were sent, they were only used if delivered within 2 h of collection [13].

The estimated glomerular filtration rate was calculated from serum creatinine [15] using the MDRD equation [17]. Chronic kidney disease (CKD) stage 3–5 was defined as eGFR < 60 mL/min/1.73^2 [20].

Inflammation was defined as CRP $\geq$5.0 mg/L, while low ALP as < 36 IU/L [15].

2.3. Statistical analyses

Statistical analyses were performed using MedCalc version 16.8.4 (MedCalc Software, Ostend, Belgium) and R studio statistical software, version 1.1.442 (RStudio, Inc., Boston, Massachusetts).

PLP values for the whole population were not normally distributed and so non-parametric tests were used. The Mann-Whitney U test was used to identify differences in PLP in the presence and absence of the four variables of interest.

A multiple linear regression was performed to first explore whether gender, race and age had an effect on PLP levels. Later, the Mann-Whitney U test was used to check for gender and age group differences in PLP. Differences in PLP associated with race were assessed using the Kruskal-Wallis test. A correlation analysis using Spearman's rho was also used for age. Parametric tests (student's t-test and analysis of variance) were used to compare differences in daily dietary B6.

For the alcohol consumption analysis, we have used the following definition for higher alcohol consumption: $\geq$12 units in women and $\geq$14 units in men. Smokers were defined as the participants who responded to the question “Do you now smoke cigarettes” with either “everyday” or “some days”. The Mann-Whitney U test was used to check for differences in PLP in smokers vs non-smokers and participants with higher alcohol consumption vs the rest. A multiple linear regression was then performed to explore whether gender had an interaction between alcohol or smoking. The explanatory variables used were gender, smoking, alcohol and the interaction between gender and alcohol and gender and smoking. For all tests significance was defined as p < .05.

For the calculation of reference intervals, the relevant function on MedCalc was used, following using a non-parametrical percentile method as described by the Clinical and Laboratory Standards Institute

Table 1
Baseline population characteristics before exclusions (total population) and after exclusions to establish the reference interval population.

Populations	Total population (n = 9069)	Reference interval population (n = 4463)
Female (%)	50.6	44.4
Age (years)[a]	50 (36 to 65)	45 (32 to 60)
PLP (nmol/L)[a]	42.9 (25.4 to 74.9)	38.6 (25.3 to 58.8)
CRP (mg/L)[a]	2.0 (0.8 to 4.7)	1.4 (0.6 to 2.6)
eGFR (mL/min/1.73^2)[a]	92.8 (77.1 to 110.0)	97.9 (83.5 to 114.3)
ALP (IU/L)[a]	67 (54 to 81)	66 (54 to 79)
Race categories; n (%):		
Mexican American	1892 (20.9)	1129 (25.3)
Non-Hispanic White	5220 (57.5)	2345 (52.4)
Non-Hispanic Black	1957 (21.6)	989 (22.2)

[a] Data are presented in median (interquartile range). PLP: pyridoxal 5′ phosphate.

(CLSI). Reed's criterion was used to look for outliers [21].

3. Results

The general characteristics of the study population before and following exclusions are presented in Table 1. Overall, the total study population was 9069 (50.6% female; n = 4458 from 2007 to 2008; n = 4611 from 2009 to 2010). Further exclusions were made based on the presence of one or more known confounders of PLP [inflammation (n = 2108); chronic kidney disease (n = 741); low ALP (n = 199); vitamin B6 supplementation (n = 2633)], providing a final reference interval population of n = 4463, 44.4% female (n = 2188 from 2007 to 2008; n = 2275 from 2009 to 2010). The mean intake of vitamin B6 in diet in the reference population was 2.0 mg (SD 1.3).

3.1. Confounder analysis

All four tested variables were associated with significant differences in PLP (Table 2). PLP was significantly lower in individuals with inflammation (p < .0001), and those with chronic kidney disease (p = .0005). Conversely PLP was shown to be significantly higher in subjects with low ALP (p < .0001), and those receiving a vitamin B6 supplement (p < .0001).

Table 2
Pyridoxal 5′ phosphate (PLP) in subjects according to the presence and absence of tested confounders.

		PLP (nmol/L), median (IQR)	Sig.
Inflammation (CRP ≥5.0 mg/L)	No n = 6961	47.4 (28.7 to 82.8)	p < .0001
	Yes n = 2108	29.3 (18.3 to 52.6)	
CKD (eGFR < 60 mL/min/1.73^2)	No n = 8328	43.2 (25.8 to 75.0)	p = .0005
	Yes n = 741	37.5 (21.4 to 74.2)	
Low ALP (< 36 IU/L)	No n = 8870	42.4 (25.1 to 73.5)	p < .0001
	Yes n = 199	83.3 (50.0 to 137.5)	
Vitamin B6 supplementation	No n = 6436	34.5 (22.2 to 55.1)	p < .0001
	Yes n = 2633	81.3 (48.2 to 143.0)	

CRP: C-reactive protein; CKD: chronic kidney disease; eGFR: estimated glomerular filtration rate; ALP: alkaline phosphatase; IQR: interquartile range.

Table 3
Comparison of gender and race differences in pyridoxal 5′ phosphate (PLP) in the reference interval population (n = 4463).

		PLP (nmol/L), median (IQR)	Sig.
Gender[a]	Male (n = 2483)	43.3 (28.7 to 64.9)	p < .0001
	Female (n = 1980)	33.2 (22.4 to 51.6)	
Race/ethnicity[b]	Mexican American[c] (n = 1129)	44.6 (30.0 to 63.9)	p < .0001
	Non-Hispanic White[c] (n = 2345)	37.8 (24.6 to 58.1)	
	Non-Hispanic Black[c] (n = 989)	34.3 (22.8 to 55.0)	
Age[a]	Young (n = 2641)	42.4 (28.0 to 63.6)	p < .0001
	Old (n = 1822)	33.3 (22.0 to 52.6)	
Age and gender	Young male (n = 1439)	50.4 (34.3 to 72.6)	p < .0001
	Old male (n = 1044)	34.2 (22.7 to 53.8)	
	Young female (n = 1202)	33.6 (23.0 to 51.8)	p = .136
	Old female (n = 778)	32.3 (21.3 to 51.3)	

[a] Mann-Whitney U Test was used for gender and age group comparisons.
[b] Kruskal Wallis Test was used for race comparisons.
[c] Post-hoc analysis showed each race was significantly different from the two other races (p < .05). Young: age < 50 years; old: age ≥ 50 years; IQR: interquartile range.

3.2. Gender, race and age differences in PLP

In the multiple linear regression model, age, gender and race were independent predictors of PLP levels (all p values < .0001). Gender and race differences in PLP were found in the reference interval population (Table 3). Female patients and black race had lower levels. There was a gradual decrease of PLP associated with increasing age [Spearman's rho −0.204 (95% confidence intervals −0.232; −0.176), p < .0001]. When testing for differences between the two age groups (young and old adults), a significant difference was found (the young adults had higher PLP levels, p < .0001). However, when testing for differences in age groups within females and males, young males had significantly higher PLP values than old males (p < .001), whereas female participants had similar PLP values independent of their age group (p = .136) (Table 3).

A further analysis was performed to check whether the differences in PLP could be explained by different dietary intake of vitamin B6. Male participants had a higher mean consumption of dietary vitamin B6 (2.3 and 1.7 mg respectively, p < .001). Young participants had a higher consumption when compared to older ones (2.1 and 1.9 respectively, p < .001). The difference remained significant when comparing younger and older males (p < .001) and females (p 0.018). There was no difference in the amount of dietary vitamin B6 according to race (p 0.654).

3.3. Smoking and alcohol consumption

Smokers had lower levels of PLP than non-smokers [median (interquartile range) in nmol/L: 32.1 (20.8; 51.3), 39.2 (25.9; 58.1) respectively, p < .0001]. PLP did not differ between participants with higher alcohol consumption compared to the rest (p = .210). The multiple linear regression showed no interaction of gender with smoking or alcohol [variables studied: gender (p = .002), alcohol (p = .595), smoking (p = .609), gender-alcohol interaction (p = .367), gender-smoking interaction (p 0.739)].

3.4. 95% reference intervals

Due to the differences in PLP relative to gender, race and age (in

Table 4
Race and gender-specific 95% reference intervals (RI) for pyridoxal 5′ phosphate (PLP) in adults.

Race/ethnicity	Age group	PLP (nmol/L), median (95% RI)	90% CI of lower limit	90% CI of upper limit
Male				
Mexican American	Young, n = 389	54.8 (21.8 to 170.5)	18.0 to 26.9	147.0 to 237.0
	Old, n = 216	39.0 (15.8 to 146.8)	12.1 to 16.6	131.0 to 178.0
Non-Hispanic White	Young, n = 737	48.1 (16.7 to 180.0)	14.8 to 18.0	149.0 to 210.0
	Old, n = 595	34.3 (11.0 to 195.1)	9.7 to 12.0	138.0 to 222.0
Non-Hispanic Black	Young, n = 313	46.9 (16.0 to 220.3)	13.5 to 18.8	170.0 to 278.0
	Old, n = 233	30.0 (9.1 to 131.2)	8.2 to 10.8	108.0 to 277.0
Female				
Mexican American	All, n = 524	36.7 (13.0 to 167.3)	12.0 to 14.0	138.0 to 242.0
Non-Hispanic White	All, n = 1013	32.2 (10.8 to 176.7)	9.6 to 11.6	141.0 to 219.0
Non-Hispanic Black	All, n = 443	29.5 (9.0 to 110.5)	8.0 to 10.1	92.7 to 130.0

In male participants, the results are presented according to two different age groups: young (< 50 years) and old (≥50 years). In female participants, PLP levels did not differ between the two age groups so the reference interval is given for the whole population. CI: confidence interval.

males), the 95% RIs were stratified to reflect these differences (Table 4).

4. Discussion

The standardised protocol of NHANES allowed us to combine data from two years of the programme, providing an increased sample representative of the general US population. PLP was found to be higher in males than in females and different across all race/ethnicity groups (highest in Mexican Americans). In males, there was a significant difference between young and older adults. Due to this large sample we have been able to calculate robust 95% reference intervals for PLP. To the best of our knowledge this is the first report of gender-, race-, and age-specific 95% reference intervals of PLP where proven confounders of PLP have been excluded from the study population.

We have observed a lot of variation around the upper limit of the reference intervals; black women have the lowest upper limit (92.7 nmol/L) compared to black men who have the highest (170.0 nmol/L). The differences in the upper limit of the RIs show the importance of gender/race/age stratification for the diagnosis of HPP.

The decrease of PLP with age has been described in study of men who were not taking vitamin B6 supplements [22]. It was also described in a previous study using NHANES data from 2003 to 2004. As this decrease could be theoretically explained by poor intake or changes caused by illness in older participants, the researchers controlled for diet, diabetes, creatinine, smoking, and alcohol intake and excluded participants with renal dysfunction, heart attack or stroke history. Possible explanations for this finding have been suggested to be decreased absorption, accelerated catabolism, and decreased phosphorylation of B6 [23]. In this study, amongst participants not on B6 supplements, PLP decreased significantly with increasing age in males after controlling for creatinine; with male teens having the highest plasma PLP levels. Female participants, both using and not using B6 supplements, had lower PLP levels than males, however, there was a pattern to this gender-difference. Differences started in menarche, with teen girls having similar levels of PLP to teen boys before, and differences disappearing again as more women progressed to menopause. This pattern suggests an association between the levels of estrogen and PLP [23]. It has been previously reported that oral contraceptive (OC) use can decrease PLP, but the mechanism is not well-understood. Possible explanations include the redistribution of PLP in the tissues, the induction of PLP-dependent enzymes involved in the tryptophan pathway by the use of OC, increasing the need for vitamin B6 and increased CRP caused by the use of OC [24]. However, the decreased PLP levels in females, are not completely explained by estrogen use, with both teenage girls and adult women never on OC use, having significantly lower PLP levels than similar-aged men [23]. We have found higher dietary consumption of vitamin B6 in males and younger participants. This could explain the differences by gender and age in PLP. Another explanation of the higher levels of PLP in men, is that levels increase with alcohol consumption and cigarette smoking, habits more common in men [25]. Once these are adjusted for, there is no longer any gender difference [25]. However, in previous studies, current smokers were found to have lower PLP levels compared to non-smokers [23,26]. We also found an effect of lifestyle on PLP levels, with smokers having lower PLP levels than non-smokers. However, we did not find an interaction between smoking or alcohol consumption with gender.

We found differences in PLP levels in the different races included in the data. Lower PLP levels in non-Hispanic blacks compared with non-Hispanic whites were previously reported [23]. These differences could not be explained by differences in dietary vitamin B6.

Of the four tested physiological variables (inflammation, chronic kidney disease, low ALP and vitamin B6 supplement intake), all were associated with significant differences in PLP compared to individuals without these factors. PLP was lower in people with inflammation defined by high CRP; this effect has been shown previously [27,28], including an investigation of data from the 2005–2006 NHANES database [15]. Most of the serum PLP (approximately 90%) is albumin bound [9]; during periods of inflammation increased capillary permeability leads to movement of plasma albumin into the extravascular space, while pro-inflammatory cytokines suppress hepatic production of albumin, leading to reduced serum levels of PLP [27].

Chronic kidney disease was associated with lower PLP in our study. This does not align with a previous investigation that reported no difference in PLP between NHANES participants with normal kidney function (eGFR ≥60 mL/min/1.73 m^2 and absence of albuminuria) and those with stage 3–5 CKD (eGFR < 60 mL/min/1.73^2). However a third group was included in that analysis (CKD stage 1 or 2, defined as eGFR ≥60 mL/min/1.73 m^2 and presence of albuminuria) [15] which was not represented in our study, as our investigation did not evaluate the presence of albuminuria and only included two groups (normal kidney function and CKD stage 3–5).

PLP was higher in individuals with low ALP (< 36 IU/L), than those with normal or high values. This negative correlation has been previously documented [29]. Higher PLP in cases of low ALP may be a result of inadvertent identification of HPP within the population, thus PLP is higher in these individuals.

PLP was also higher in individuals taking vitamin B6 supplementation, compared with those who were not. This was reported before, using data from the NHANES study 2003–2004; PLP increased by about 12 nmol/L per 1 mg increase in vitamin B6 intake [23]. Therefore, blood tests in clinical investigation of PLP should be taken at least a few weeks after the cessation of supplementary vitamin B6 intake although the exact timing is not clear from the literature [30–34].

The strengths of this study include the use of a large sample representative of the non-institutionalised US population. The major limitation of this analysis relates to the cross-sectional design of the

NHANES programme. This prohibits exploration of cause and effect relationships between PLP and other biomarkers. Blood samples are collected only once from each participant and so fluctuations in biomarkers over time cannot be understood. This is relevant in the interpretation of low ALP which may be transiently low in a range of scenarios without an effect on PLP levels, whereas persistently low ALP is often suggestive of HPP. The presence of albuminuria was not studied when evaluating the effect of kidney function. Dietary sources and intake of vitamin B6 was not considered for this study in order to reflect normal dietary differences in the population. Differences in dietary intake of vitamin B6 could provide insight into the observed differences in PLP.

In conclusion, four factors (inflammation, chronic kidney disease, low ALP and vitamin B6 supplement intake), have been shown to be associated with differences in PLP and should be considered in the interpretation of clinical PLP measurements. We have reported PLP gender-, race-, and age-specific 95% reference intervals for use in everyday clinical practice.

Disclosure summary

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Summary

In order for serum pyridoxal 5′-phosphate (PLP) to be used as a marker of hypophosphatasia (HPP) in adults, robust reference intervals are needed which reflect population differences in PLP. We found race, gender and age differences in PLP in a representative US population and calculated PLP reference intervals that reflect these differences. Factors proven to be associated with significant differences in PLP were excluded from the population.

CRediT authorship contribution statement

Marian Schini: Formal analysis, Writing - original draft, Writing - review & editing.**Phil Nicklin:**Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing.**Richard Eastell:**Methodology, Formal analysis, Writing - original draft, Writing - review & editing.

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